1   **Effects of Functional Electrical Stimulation on Denervated Laryngeal Muscle in a**

2   **Large Animal Model**

3

4   Cheetham J., Perkins J.D., Jarvis J.C., Cercone M., Maw M., Hermanson J.W., Mitchell

5   L.M., Piercy R.J., Ducharme, N.G.

6   From the Department of Clinical Sciences (Cheetham, Cercone, Mitchell, Ducharme),

7   Department of Biomedical Sciences (Hermanson), College of Veterinary Medicine,

8   Cornell University, Ithaca, NY, USA 14853, Comparative Neuromuscular Disease

9   Laboratory, Royal Veterinary College London, UK (Perkins and Piercy), Med-el,

10   Innsbruck, Austria (Maw) and Liverpool John Moores University , UK, L3 3AF (Jarvis)

11

14

15   Word Count:

16

18   *Author to whom correspondence should be addressed: Jon Cheetham, (607) 253-3100,

19   Fax (607) 253-3271, E-mail: jc485@cornell.edu

20

21 **Abstract**

22 Bilateral Vocal Fold Paralysis (BVCP) is a life threatening condition that follows injury

23 to the Recurrent Laryngeal nerve (RLn) and denervation of the intrinsic laryngeal

24 musculature. Functional electrical stimulation (FES) enables restoration and control of a

25 wide variety of motor functions impaired by lower motor neuron lesions. Here we

26 evaluate the effects of FES on the sole arytenoid abductor, the posterior cricoarytenoid

27 muscle (PCA) in a large animal model of RLn injury. Ten horse were instrumented with

28 two quadripolar intra-muscular electrodes in the left PCA muscle. Following a twelve

29 week denervation period, the PCA was stimulated using a once daily training session for

30 eight weeks in seven animals. Three animals were used as unstimulated controls.

31 Denervation produced a significant increase in rheobase ($p<0.001$). Electrical stimulation

32 produced a 30% increase in fiber diameter in comparison with the unstimulated control

33 group (33.9 ±2.6um FES+ , 23.6 ±4.2um FES -, $p=0.04$). A trend towards a decrease in

34 the proportion of type 1 (slow) fibers and an increase in type 2a (fast) fibers was also

35 observed. Despite these changes improvement in PCA function at rest was not observed.

36 These data suggest that electrical stimulation using a relatively conservative set of

37 stimulation parameters can reverse the muscle fiber atrophy produced by complete

38 denervation while avoiding a shift to a slow (type 1) fiber type.

39

40 **Introduction**

41 Bilateral Vocal Fold Paralysis (BVCP) is a life threatening condition that follows injury

42 to the Recurrent Laryngeal nerve (RLn), most commonly from trauma during thyroid

43 surgery or anterior cervical approaches to the spine [1-4]. In the case of complete RLn

44 transection without spontaneous recovery, the intrinsic laryngeal musculature is

45 chronically denervated and the vocal folds remain immobile in a closed position

46 producing dysphonia, stridor, aspiration and dyspnoea [5]. Functional electrical stimulation

47 (FES) is a well-established treatment option for lower motor neuron deficits that enables

48 restoration and control of a wide variety of motor functions impaired by lower motor

49 neuron lesions, from locomotion to breathing. Despite the technical limitation

50 represented by low excitability of muscle undergoing atrophy and fibrotic degeneration

51 following permanent motor neuron damage, FES has also been used clinically to provide

52 direct electrical activation of long term denervated muscle [6,7]. Animal models of RLn

53 injury have been previously used to evaluate the use of FES to stimulate a denervated

54 laryngeal musculature. These models have predominantly focused on the dog [8-10], sheep

55 [11,12], cat [13], pig [14-16], and more recently, horse [17,18]. Confounding features of these animal

56 studies have been variation in the parameters used for stimulation including number of

57 pulses, daily activation rate and rest periods between stimulation, in addition to varying

58 rates of denervation across species and both positive and negative effects of stimulation

59 on muscle integrity and the process of reinnervation have been reported [19-22]

60 . In this study we evaluate the effects on muscle size and function of direct intramuscular

61 stimulation in denervated laryngeal muscle using relatively long stimulation pulses at a

62 low daily activation frequency. We use an equine model as the large size of the posterior

63 cricoarytenoid muscle (PCA) reduces some of the technical difficulties associated with

64 electrode placement in other species [23]. We focus on the PCA as the sole arytenoid

65 abductor and because restored or returning PCA function is a requirement for

66 tracheostomy removal in patients with BVCP [9]

67

68

69 **Material and Methods**

70 This study was performed in accordance with the PHS Policy on Humane Care and Use

71 of Laboratory Animals, federal and state regulations, and was approved by the local

72 Institutional Animal Care and Use Committee (IACUC). Animals were brought into the

73 research unit and given a 7-day acclimatization period prior to any procedure. Daily

74 record logs of medical procedures were maintained.

75 *Experimental design*: Ten adult horses (Age range 5-7years, weight range 440-550kg)

76 with normal arytenoid function (Havemeyer grade 1,[24]) were instrumented with two

77 quadripolar intra-muscular electrodes (5 French) in the left posterior cricoarytenoid

78 (PCA) muscle. Implanted electrodes were connected to a custom connector placed

79 subcutaneously on the left side of the neck that facilitated FES using an external

80 multichannel stimulating system (Master-8, AMPI, Israel).

81 *Surgical procedure*: Electrodes were implanted as previously described under general

82 anesthesia [18]. Briefly, an open approach to the lateral compartment of the left PCA

83 muscle was made and electrodes (quadripolar, K5-P4, Osypka AG, Rheinfelden,

84 Germany, figure 1) with a spiraling cathode at their tip were inserted using an insertion

85 cannula (Pajunk 18G needle, Pajunk GmbH, Germany). The lateral neuromuscular

86   compartment was selected as stimulation in this location produces the greatest arytenoid

87   abduction [25]. Following a three week recovery period, and collection of baseline data,

88   each horse was anesthetized again and the left RLn was approached through a 10cm mid

89   cervical incision ventral to the jugular vein. Correct identification of the RLn was

90   confirmed using direct stimulation with an insulated needle (Stimuplex Insulated Needle;

91   Braun Medical, Bethlehem, PA) and 1 mA, 2 Hz, 0.15 ms duration pulses (Innervator

92   232; Fisher & Paykel Healthcare, Auckland, New Zealand): arytenoid abduction twitches

93   were verified endoscopically. The RLn was transected and the proximal and distal ends

94   ligated with 2/0 Ethilon. All horses received broad-spectrum antibiotics (trimethoprim-

95   sulfadiazine 30mg/kg, PO, BID) and Phenylbutazone (1mg/kg, PO, BID) for 5 to 7 days.

96   The effects of denervation were allowed to proceed without further intervention for

97   twelve weeks and then the left PCA muscle was stimulated using a once daily training

98   session for eight weeks (50Hz, 2ms pulse width, 2 s on 2s off 10 volts, for 48 min/day) to

99   produce a total of 72,000 impulses/day (daily frequency equivalent 0.83 Hz). Daily

100   activation below 1 Hz was used to minimize any shift to type I (slow) fibers [26]. All

101   stimulation pulses used were biphasic with the negative (cathodic) phase occurring before

102   the positive phase. Seven animals were stimulated using these parameters (referred to as

103   the FES+ group) and three were implanted but not stimulated (controls, referred to as the

104   FES- group)). The other intrinsic laryngeal muscles were not stimulated and served as

105   internal controls.

106

107   Outcome measures:

108 *Overview*: The effects of denervation and stimulation were determined using quantitative

109 assessments of muscle excitation (strength-duration curve), muscle contraction judged by

110 arytenoid abduction response to increasing stimulation, computed-tomography based

111 reconstruction of PCA volume and, standardized exercise tests to determine laryngeal

112 function under increasing inspiratory loads. These observations were made immediately

113 after surgery, after the 12 week period of denervation and after a subsequent eight weeks

114 of FES. At the end of the study, immunohistochemistry of the left and right PCA and

115 Lateral Cricoarytenoid muscles (LCA) was performed to determine fiber type

116 distributions and minimum fiber (Feret's) diameters (MFD).

117 *Strength-duration trials*: To assess the excitation characteristics of the PCA muscle

118 following denervation, rheobase - the minimum amplitude needed to elicit a threshold

119 response at infinitely long pulse duration of the electrical stimulation and chronaxie -the

120 pulse duration at twice the rheobase were determined [27]. The procedure was performed

121 under light sedation with videoendoscopic observation of the left arytenoid cartilage [18].

122 Single biphasic balanced pulses were applied to the left PCA muscle with pulse durations

123 from 0.5 ms to 8ms. Pulse amplitude was increased from zero in 0.1mA steps until a

124 perceptible abduction twitch of the left arytenoid cartilage was observed via the

125 endoscope (threshold). The test stimuli were applied during expiration hold (the short

126 hesitation at the end of expiration) and the sequence of pulse durations was randomized.

127 Threshold was readily observed endoscopically.

128 The PCA response to increasing levels of electrical stimulation was determined at rest by

129 increasing the stimulation voltage (0, 0.5,2,..10 volts) of a short 10 second burst using

130 fixed pulse duration (2ms) and frequency (50Hz). Thirty seconds rest was allowed

131   between bursts and the sequence of stimulation was randomized. These parameters were

132   chosen as 50Hz is above the fusion frequency for the equine PCA [18]. The PCA response,

133   measured by the degree of arytenoid abduction, was determined at each stimulation level

134   using a flexible videoendoscope (Olympus GIF-140) passed into the nasopharynx via the

135   right ventral nasal meatus (figure 2). Briefly, a line was drawn to connect the dorsal and

136   ventral-most points of the rima glottidis and extending dorsally for a distance of one third

137   of the dorsoventral height of the rima glottidis. Lines were then drawn from the dorsal

138   most end of this line, tangential to each of the arytenoid cartilages. Finally, the angles

139   between the dorsoventral line and the tangents were measured[28,29]

140

141   *Determination of PCA volume using Computed Tomography*: PCA volume was

142   estimated in vivo using a validated quantitative Computed Tomography (QCT) technique

143   [*WM, this paper is currently in review how would you like me to reference it?*]. Briefly,

144   under general anesthesia, QCT (Aquilion LB16, Toshiba) with reconstruction was used to

145   determine volume of the left and right PCA muscles. The cricoid cartilage was used as a

146   volume standard throughout the study. To improve spatial resolution, scan spacing and

147   thickness were set to minimum levels to minimize in-plane pixel size (0.25-0.50 mm),

148   scan spacing (1 mm), and scan thickness (1 mm). DICOM sets were imported into CT

149   reconstructive software (Mimics ™, Materialise, Belgium) using Lossless compression.

150   A semi-automated segmentation tool, 3D LiveWire™, was used to outline the muscle of

151   interest [30]. In-plane segmentation was repeated along the entire length of each PCA

152   muscle, and the tool was used to segment each portion. A gradient magnitude of 60%

153   with 0 attraction was used to best outline the muscle body. Mimics settings were further

154   adjusted to optimize smoothness without shrinkage (100 iterations, 1.00 smooth factor,

155   shrinkage compensation *on*). Triangle reduction using an advanced edge reducing mode

156   (0.0370mm tolerance, 15º edge angle, and 100 iterations) was also applied. Other

157   parameters remained at their default settings. From these operations, a custom three-

158   dimensional (3D) reconstruction of each muscle was generated with associated

159   geometrical properties. The volume of the cricoid cartilage was obtained at each time

160   point to provide a known standard.

161

162   *Exercise Protocol and Instrumentation*: All horses were exercised on a treadmill (0º

163   incline) under control conditions to verify normal upper respiratory tract at exercise

164   (laryngeal grade IA) [24] prior to denervation. PCA function under increasing levels of

165   inspiratory load was determined using a standardized exercise test [18,31]. Each horse was

166   subsequently evaluated at an exercise intensity corresponding to 50,80,90 and 100% of

167   their maximum heart rate (HRmax) [32] to standardize the load experienced by the PCA

168   muscle at increasing levels of exercise intensity. After a warm up period, one minute was

169   spent at each level before increasing to the next. After the period of denervation, during

170   each one minute interval, the PCA was unstimulated in the first 30 seconds to determine

171   native function and the PCA was transiently stimulated in the second 30 seconds (50Hz,

172   2ms, 10V) to determine the response to stimulation. Horses were evaluated under these

173   conditions before RLn transection (baseline), after the period of denervation and after the

174   period of stimulation. No stimulation was applied at the baseline timepoint. Data

175   recorded during exercise trials included heart rate, electrocardiogram and accelerometer

176   measurements. Nasopharyngeal and laryngeal movements were recorded using a flexible

177   videoendoscope (Olympus GIF-140) passed into the nasopharynx via the right ventral

178   nasal meatus and secured. Heart rate was measured by an on-board monitor (Hippocard

179   Systems, Lexington, KY). Data were recorded onto DVD disks for subsequent analysis.

180

181   Immunohistochemistry:

182   Following euthanasia, the left and right PCA and LCA muscles were isolated and mid-

183   body samples ($6mm^3$ approximately) embedded in cutting medium (Tissue-Tek OCT

184   Compound, Sakura Finetek, Netherlands) so that the fibers were orientated vertically and

185   frozen in melting isopentane precooled in liquid nitrogen (-156°C). Thereafter, 7µm

186   cryosections were air-dried onto glass slides (Superfrost plus microscope slides, VWR

187   International Ltd, Lutterworth, UK) and stored at -80°C. Routine Hematoxylin and Eosin

188   staining was performed on two sections form each muscle.

189   All mature skeletal muscle fiber types (1, 2a and 2x), hybrid fibers and collagen V were

190   identified in single equine muscle cryosections using a multiple immunofluorescence

191   labelling technique with 4 different primary antibodies. [33]

192   Images were obtained using a Leica DMRA2, or DM4000 florescent microscope (Leica

193   Microsystems (UK) Ltd, Buckinghamshire, U.K.) via the x10 objective and LEICA A4,

194   L5, TX2, Y5 filters. The fluorescent camera used was a Zeiss MRm camera (ZEISS

195   microscopy), with a dedicated software program (Axiovision 4.8, www.zeiss.co.uk).

196   Background correction was performed in Volocity (Version 6, PerkinElmer,

197   Massachusetts 02451, USA) and was performed by imaging the even field in all filters

198   using Fluor-Ref (www. MicroscopyEducation.com). For determination of MFD by

199   individual fiber type or hybrid fiber designations, merged images were imported into

200   Image J (www.imagej.nih.gov) and fibers (n >400) were manually measured and

201   subjectively assigned a fiber type or hybrid fiber designation by relative fluorescence.

202

203   Separate 7 μm muscle sections were used to determine the percentage collagen V

204   (severity of fibrosis) per unit area. A goat anti-collagen V antibody (1:20 diluted in

205   phosphate buffered saline) (Goat anti-type V collagen, Southern Biotechnology,

206   Birmingham, Alabama, USA) was used with a fluorescent secondary antibody (1:1000

207   diluted in phosphate buffered saline, Alexafluor 488 rabbit antigoat IgG,

208   www.invitrogen.com). Two images were obtained from each muscle sample using the

209   x10 objective and background corrected as described. Images were thresholded and

210   analysed in Volocity (Version 6, PerkinElmer, Massachusetts 02451, USA) for

211   objective quantification of collagen V expression per unit area.

212

213   Data Analysis

214   Strength duration data were subjected to curve fitting with the exponential function $V =$

215    $a * e^{b+c*\tau} + d$ whereas V=Threshold voltage, τ = Stimulus pulse width and a, b, c, d

216   being constants that are evaluated for every SDC Curve, using custom software (Matlab

217   Curve-Fitting toolbox). Robust bisquare fitting and the trust region algorithm were used

218   to optimize curve fit with a maximum of 500 iterations and 750 evaluations. Lower

219   boundaries were 0.1, -0.1, -100 and 0, upper boundaries were 100, 100, -0.15 and 100

220   and starting points were 1.5, 1, -1.7 and 1 for a, b, c and d respectively. A logarithmically

221   spaced weighing vector was used to assign the first and last measured values the weight

222   1. The minimum weight was 0.3 for the middle value.

223

224 Endoscopic images of the *rima glottidis* were captured from the DVD recordings using

225 editing software (Video Wizard, Womble Multimedia, CA, USA). The degree of

226 arytenoid abduction was measured using a previously validated technique [34]

227   with commercially available software (Able Image Analyser). Briefly a line is drawn

228 connecting the dorsal- and ventral- most points of the *rima glottidis.* This line was then

229 extended dorsally for a distance of one third of the dorsoventral height of the *rima*

230 *glottidis*. A tangential line to the arytenoid cartilages was drawn, and the angle between

231 the dorsoventral line and the tangential line measured [18]

232 . Minimum fiber diameters obtained for each fiber type within each muscle were

233 allocated into 5um bins and plotted as histogram envelopes.

234

235 For continuous outcome measures (arytenoid abduction response, rheobase, chronaxie,

236 PCA volume, fiber type proportion) a mixed effect model was fitted to the data to

237 determine the relationship between the outcome variable and relevant fixed effects using

238 horse as a random effect; interaction terms were included as appropriate. Minimum fiber

239 diameter data was fitted with a mixed effect model with muscle nested within horse

240 identity. To determine PCA response at increasing levels of inspiratory load, separate

241 models were fitted to the data for each time period (baseline, post denervation, post FES)

242 and each condition during testing under load (native function or FES applied

243 (stimulated)). Tukey's *post hoc* tests and linear contrasts were used as appropriate.

244 Statistical analysis was performed using JMP (SAS Institute, Cary, North Carolina,

245 USA). Significance was set at $p < 0.05$ throughout.

246

247 **Results**

248 All horses recovered uneventfully from surgery. Postoperative swelling and discomfort

249 were minimal and resolved within 10-14 days. Appropriate healing was confirmed in all

250 horses at suture removal. Horses remained comfortable throughout the study.

251

252 *Muscle Excitation characteristics*: The algorithm used to fit the strength duration curves

253 fit the raw data well with an R^2 (mean ± sd) of 0.97 ± 0.022.   Evaluating changes in

254 rheobase, there was no significant interaction between time point and animal group (FES

255 + or control, p=0.11). Controlling for animal group (FES+ or FES-) there was a

256 significant increase in rheobase following denervation (baseline 0.91± 0.44 mA, post

257 denervation 4.96± 0.54 mA and post FES 3.64 ± 0.79 mA, p<0.001). There were no

258 differences between the FES + and FES – groups. Controlling for animal group, mean

259 chronaxie also slightly increased following denervation (1.01 ±0.54ms, 1.42 ±0.44ms,

260 1.32 ±0.60ms), although these changes were not significant (p=0.24).

261

262 *Response to increasing stimulation*: Prior to denervation, the PCA response to increasing

263 stimulation, measured using left arytenoid abduction reached a plateau, with maximal

264 arytenoid abduction of 50 degrees, above 3 volts of stimulation (figure 3). The response

265 to increasing stimulation following denervation and FES was markedly damped. No

266 significant differences were observed between FES+ and control horses.

267

268   *Volumetric changes*: Denervation produced a profound and significant 30-40% decrease

269   in left PCA volume in both FES+ and control (FES-) groups over the twelve week

270   denervation period (figure 4). In the FES + group, this atrophy did not proceed after the

271   onset of stimulation, in contrast atrophy did proceed in the control group during this

272   period. Left PCA volume was not significantly different between control and FES +

273   groups at the post FES time point (linear contrast, p=0.22). CT determination of cricoid

274   volume remained very stable throughout the time course with estimated volumes of 31.9

275   ± 3.5 cm^3, 31.6 ± 4.0 cm^3 , 29.6 ± 3.4 cm^3 at each time point and no significant difference

276   between FES + and FES- groups.

277

278   *Function under load*: PCA function under increasing levels of inspiratory load (negative

279   inspiratory pressure) was markedly reduced after denervation (figure 5). No significant

280   differences were observed between FES + and control (FES -) groups either pre-

281   denervation (baseline) or post-denervation in native function or function during transient

282   electrical stimulation (stimulated). However, after the period during which the FES+

283   group received daily stimulation, native function was significantly improved at high

284   levels of inspiratory load (90 and 100 HRmax) in FES+ animals compared to controls

285   (p=0.05 and p<0.01 respectively, linear contrasts). No significant differences were

286   observed between FES + and control animals during transient stimulation at exercise at

287   this time point, although the means for function during transient stimulation showed the

288   same trend as the means for the native function.

289

290   *Immunohistochemistry*:

291   Controlling for other factors in the model, denervation of the left PCA produced a

292 37% reduction in minimum diameter (mean ± se) for all fibers (23.6 ± 4.2 um) compared

293 to the innervated right PCA muscle (37.4 ± 4.2 um, p<0.05). Denervation produced a

294 similar decrease in the mean minimum fiber diameter of the left LCA (23.9 ± 4.2 um)

295 compared to the innervated right LCA (40.4 ± 4.2 um, p<0.05).

296   Electrical stimulation of the left PCA muscle produced a 30% increase in fiber

297 diameter in comparison with the unstimulated control group (33.9 ±2.6um FES+ , 23.6

298 ±4.2um FES -, p=0.042, linear contrast). The modal fiber diameter remained at

299 approximately 25um, but a much higher proportion of fibers had hypertrophied to

300 between 40-60um in the stimulated left PCA compared to the control left PCA (figure 6).

301   Electrical stimulation in the left PCA muscle produced a trend towards a decrease

302 in the proportion of type 1 (slow) fibers (linear contrast, p=0.09) and a trend towards an

303 increase in type 2a (fast) fibers (linear contrast, p=0.052, figure 7). No other significant

304 differences in type 1 or type 2a fibers were observed. Overall there was a significant

305 increase in the proportion of hybrid fibers in the denervated (left PCA and LCA) muscles

306 compared to the innervated (right PCA and LCA) muscles (linear contrast, p=0.0004)

307 with no hybrid fibers detected in the innervated muscles. There was also a significant

308 decrease in the proportion of hybrid fibers in the stimulated PCA compared with the

309 unstimulated PCA (p=0.003). There were no significant differences in any fiber type

310 proportion between horses in the stimulated and unstimulated groups in any of the

311 muscles to which FES was not applied – for example left LCA (L LCA stimulated) in the

312 horses which received FES in the left PCA and the left LCA in the horses which did not

313 receive FES in the left PCA (L LCA control). Overall model fits were good for each

314   fiber type (Model: Proportion ~ Horse$_{random}$ + muscle+ group (stimulated or control) +

315   muscle*group; adjusted R^2 =0.63, 0.54, 0.98, 0.56 for types 1, 2a, 2x and hybrid fibers

316   respectively). Unlabeled fibers summed to 0.54% across all animals and muscles and

317   were excluded from subsequent analysis.

318   Denervation produced an increase in the proportion of collagen V expression per

319   unit area (figure 8). Controlling for other factors in the model the denervated left PCA

320   and LCA (56.4 ±3.6%, 59.9 ±5.6%) had significantly more collagen than the innervated

321   right PCA and LCA (23.6 ±3.6%, 21.6 ± 3.6%, p=0.001). There was no effect of FES in

322   the left PCA muscle (p=0.72, linear contrast) and no significant difference between other

323   unstimulated muscles in the FES + or FES − groups (p=0.73).

324

325   **Discussion**

326   These data demonstrate arrest of, and partial recovery from the consequences of complete

327   denervation using electrical stimulation in a large animal model. We identify increased

328   muscle volume and fiber cross-sectional area within the the posterior cricoarytenoid

329   muscle with stimulation We also identify a 30% increase in the muscle fiber diameter

330   with electrical stimulation and a trend towards a shift in an increasing proportion of type

331   2a fibers with electrical stimulation. These changes were produced using a relatively

332   conservative set of stimulation parameters [72,000 pulses/day, a daily activation rate of

333   1.83% (0.83 Hz) and a daily rest period of 23 hours]. These parameters were selected to

334   avoid a shift to increasing proportions of type 1 fibers seen at daily stimulation

335   frequencies above 1Hz [26]and achieved this goal.

336   The decrease in PCA volume of 30-40% over a 20 week denervation period,

337   suggests that slow rather than rapid atrophy occurs after denervation in this model. This

338   is consistent with slow atrophy and absence of necrosis found after denervation in other

339   large animal [14,22,35] rather than rodent models of denervation. [36,37] The equine model may

340   represent a good system in which to study the effects of electrical stimulation on

341   laryngeal muscle as the larger size of the equine PCA reduces some of the technical

342   considerations for electrode implantation, and endoscopic and volumetric reconstruction

343   techniques allow longitudinal assessment of structure and function.

344   Denervation produced a 37% reduction in fiber diameter as anticipated. The

345   relative proportion of collagen V also increased with denervation as fiber diameter

346   decreased. This increase in the proportion of collagen V is a consequence of both the

347   smaller fibers (producing an increase in circumference relative to cross-sectional area)

348   and an increased amount. The changes in fiber diameter were partially reversed with

349   electrical stimulation with a 30% increase in the shortest cross-sectional diameter

350   (minimum fiber diameter, MFD) when FES was applied to the left PCA muscle. These

351   changes are similar the 25% increase in MFD identified in other large animal models

352   evaluating the effects of FES [38] in completely denervated laryngeal muscle. One

353   limitation of this study is that no assessment of muscle recruitment by FES was made and

354   additional optimization of electrode placement may improve the observed response. In

355   addition, longer pulses [22] or a longer period of stimulation [39] may be required to optimize

356   direct intramuscular stimulation of the denervated PCA muscle

357   The trend towards a shift in an increasing proportion of type 2a fibers with

358   electrical stimulation suggests that the parameters used met the required goal of avoiding

359   a shift towards type 1 (slow) fibers which are characterized by reduced contraction force,

360   and time to peak contraction, characteristics that are particularly important in the PCA

361   which maintains airway patency during inspiration [11]

362   The increase in the proportion of type 1 fibers with denervation is anticipated as

363   these fibers are more resistant to denervation [36,40-42]

364

365

366   The response of the PCA muscle under load, imposed by increasing levels of

367   exercise intensity and so increasing the inspiratory pressure against which the PCA must

368   contract to maintain airway patency, is interesting. FES has been shown to improve

369   resting muscle tone even in the presence of complete denervation [43]

370   and increased tonicity in the PCA muscle will prevent complete collapse of the arytenoid

371   cartilage into the airway. This increase in tonicity is particularly important under high

372   inspiratory loads (90 and 100% HRmax) and may explain the significant differences in

373   native function at these levels, despite the absence of a detectable difference at lower

374   levels of exercise intensity (50 and 80% HRmax). Testing of intramuscular stimulation

375   during exercise subjects the arytenoid to high inspiratory negative pressures and may be a

376   more strenuous requirement than restoration of abduction in humans and dogs [10,38,44]

377   .

378   These data also demonstrate prevention of progression of the effects of

379   denervation in the stimulated animals (figure 5) with equivalent function in the stimulated

380   group after the denervation period and after FES. In contrast, the control group

381   demonstrated considerable worsening in native function after the additional denervation

382   period of eight weeks during which the FES+ animals were stimulated. This may be due

383   to increased resting muscle tone or shortened resting length in the stimulated CAD

384   muscles. An alternative explanation would be additional innervation provided by the

385   cranial laryngeal nerve. Function during stimulation was improved compared to native

386   function and so although a similar trend to prevent progression during stimulated function

387   was observed, this was less marked than the trend in changes in native function.

388   The prevention of progression identified by evaluating native function was

389   reflected in volumetric changes of the PCA muscle suggesting that FES at these relatively

390   low stimulation levels may reverse the ongoing atrophy produced by denervation. The

391   use of cricoid cartilage volume provided a useful known standard over time. This

392   technique has not been used in other animal models of laryngeal denervation will be used

393   in future experiments evaluating the effects of FES on the rate of reinnervation after

394   nerve injury. These techniques could also be applied in human patients to track the

395   response to electrical stimulation or reinnervation.

396   The increase in rheobase following denervation is consistent with ongoing

397   denervation and loss of neuromuscular junctions within the first 72 hours after

398   denervation. This algorithm was practical to fit to the data and provides an unbiased

399   method to obtain both chronaxie and rheobase without subjective curve fitting. It is

400   therefore recommended for future studies evaluating the effects of electrical stimulation

401   on reinnervation after nerve injury. The excitability of the PCA muscle improved only

402   slightly with once-daily activation. This demonstrates that there was no evidence of

403   reinnervation of the PCA.

404   In summary, these data suggest that electrical stimulation using a relatively

405   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.

Figure legends.

Figure 1. llustration of K5-P4 electrode. Note: three anodes each 1.5 mm long, screw length 2.5 mm with 2.5 turns; screw diameter 1.7 mm; screw surface area 8 mm2. Screw material is platinum-iridium (80%–20%). Total electrode length 300 mm. Insertion tool shown in place (left).
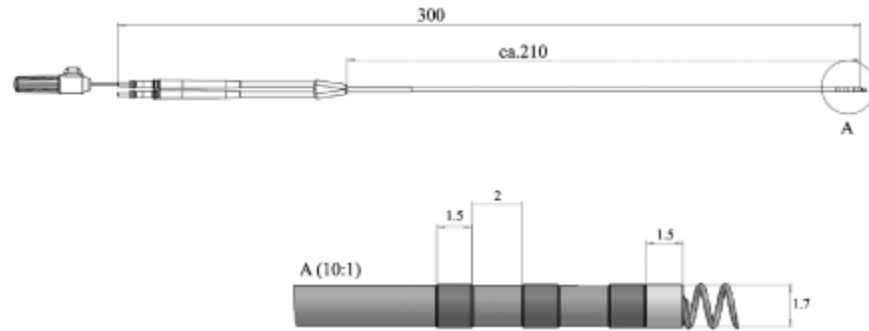
Figure 2. Quantification of left PCA muscle function using videoendoscopic assessment of arytenoid abduction. Left arytenoid abduction at rest (A); and during stimulation at rest (B). Images are obtained from the same horse at the same time point prior to denervation.
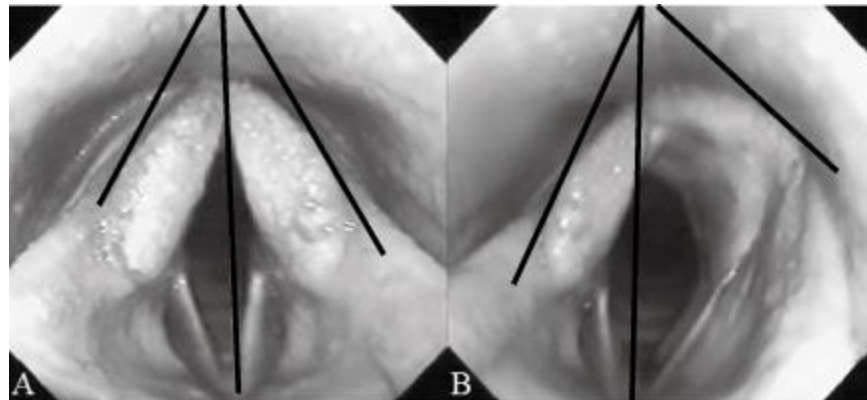
Figure 3. PCA response to increasing levels of stimulation at rest. Response determined as abduction of the left arytenoid in response to biphasic pulses with duration 2 ms, 50 Hz. Data shown are mean and standard deviation.
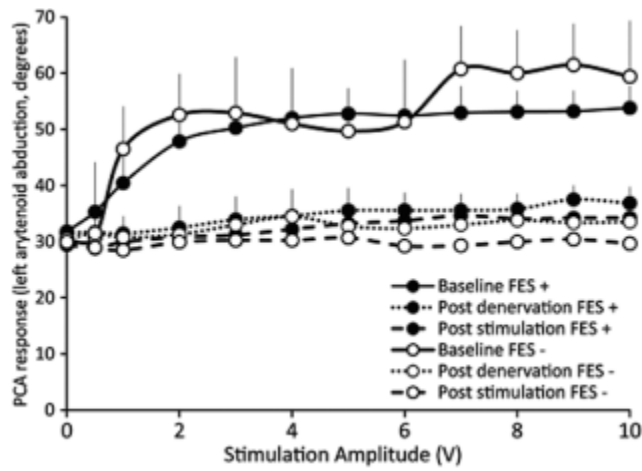

Figure 4. Changes in left PCA volume muscle in response to denervation and subsequent stimulation. Data shown are mean and standard deviation. Different letters denote significant differences between groups (Tukey's post hoc test, $P < 0.05$).
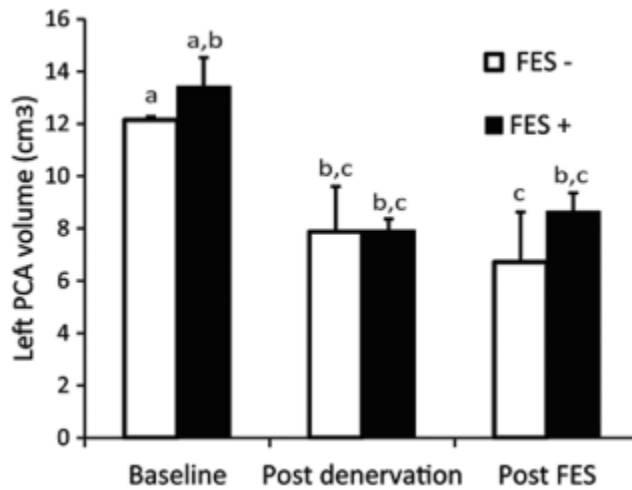
Figure 5. PCA response to increasing levels of inspiratory load and increasing exercise intensity (%HRmax). Native function significantly improved at high levels of inspiratory load (90 and 100 HRmax) in FES+ animals compared with controls (*$P = 0.05$ and **$P < 0.01$, linear contrasts).
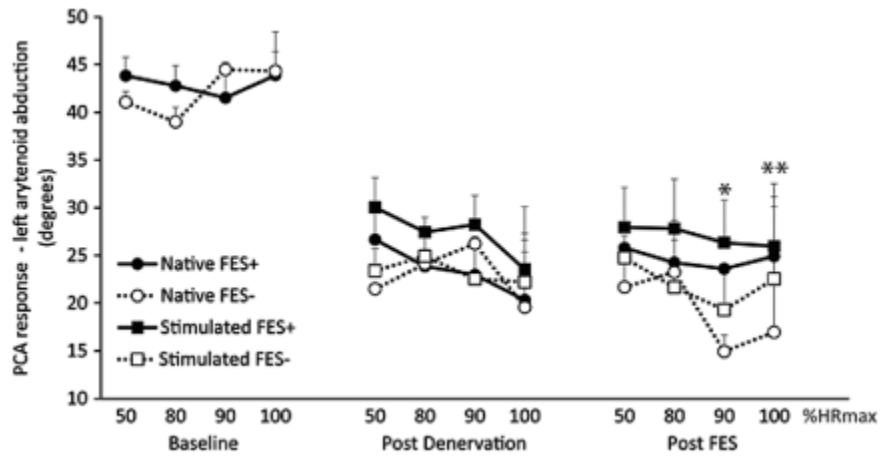

Figure 6. Fiber diameter distribution (%) in the denervated left PCA and LCA muscles compared with contralateral innervated controls (right PCA and LCA). Bin size is 5 µm. FES was only applied to the left PCA in stimulated animals (solid line). Other muscles served as internal controls and FES was not 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 (*$P = 0.042$).
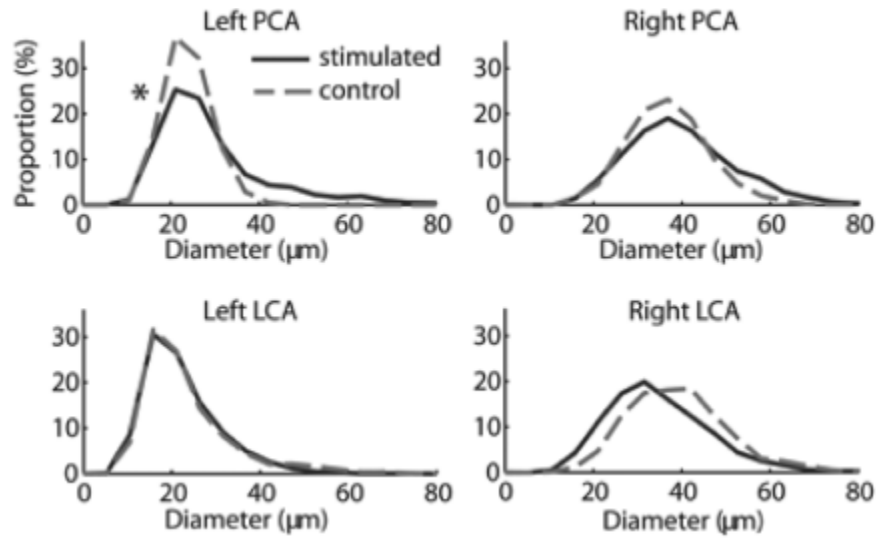
Figure 7. Proportion of fiber types 1, 2a, and 2x within each PCA and LCA muscle.
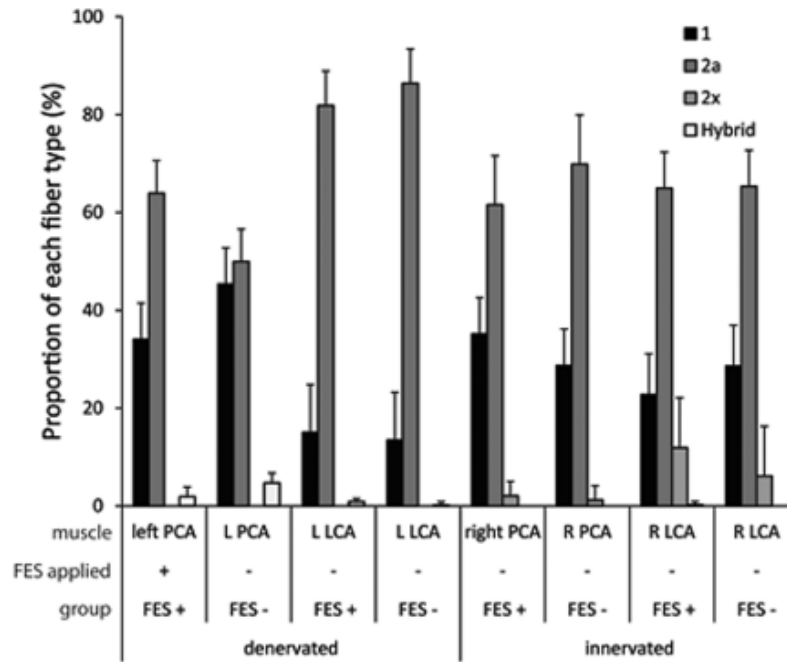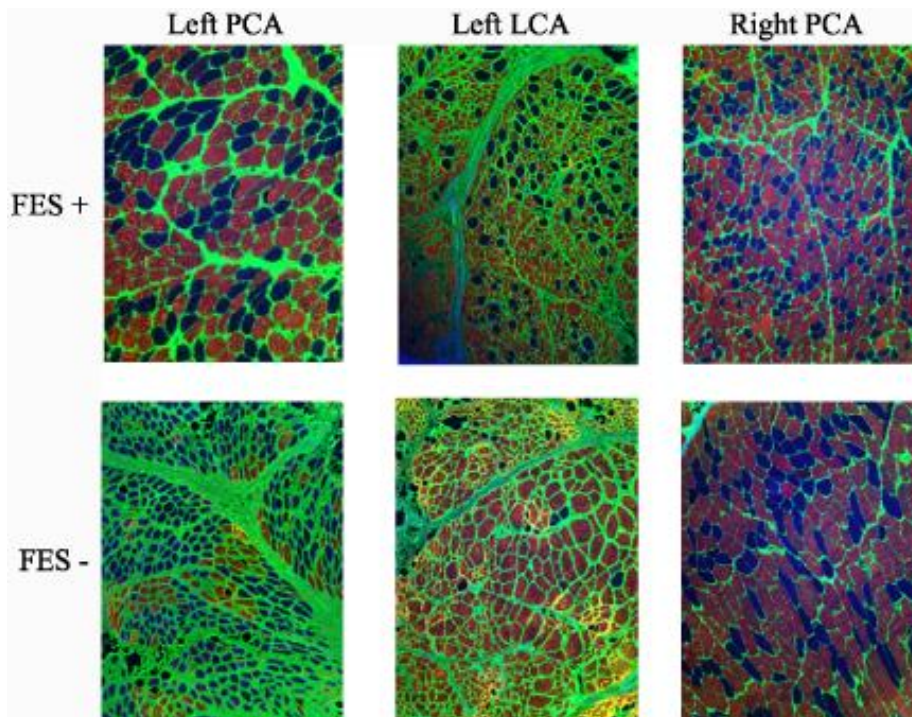

Figure 8. Representative immunohistochemistry images of intrinsic laryngeal musculature labeled simultaneously for type 1, 2a, and 2x fibers. Note increasing collagen V proportion with decreasing fiber diameter.

1. Rosenthal LH, Benninger MS, Deeb RH. Vocal fold immobility: A longitudinal analysis of etiology over 20 years. *Laryngoscope*. 2007;117(10):1864-1870.

2. Kern KA. Medicolegal analysis of errors in diagnosis and treatment of surgical endocrine disease. *Surgery*. 1993;114(6):1167-73; discussion 1173-4.

3. Chiang FY, Wang LF, Huang YF, Lee KW, Kuo WR. Recurrent laryngeal nerve palsy after thyroidectomy with routine identification of the recurrent laryngeal nerve. *Surgery*. 2005;137(3):342-347.

4. Chiang FY, Lee KW, Huang YF, Wang LF, Kuo WR. Risk of vocal palsy after thyroidecitomy with identification of the recurrent laryngeal nerve. *Kaohsiung J Med Sci*. 2004;20(9):431-436.

5. Cros AM, Pitti R, Conil C, Giraud D, Verhulst J. Severe dysphonia after use of a laryngeal mask airway. *Anesthesiology*. 1997;86(2):498-500.

6. 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*. 468 2007;104(49):19339-19344.

7. 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(1):98-101.

8. Sanders I. Electrical stimulation of laryngeal muscle. *Otolaryngol Clin North Am*. 1991;24(5):1253-1274.

9. Zealear DL, Billante CR, Courey MS, et al. Reanimation of the paralyzed human larynx with an implantable electrical stimulation device. *Laryngoscope*. 2003;113(7):1149-1156.

10. Zealear DL, Swelstad MR, Sant'Anna GD, et al. Determination of the optimal conditions for laryngeal pacing with the itrel II implantable stimulator. *Otolaryngol Head Neck Surg*. 2001;125(3):183-192.

11. Carraro U, Catani C, Saggin L, et al. Isomyosin changes after functional electrostimulation of denervated sheep muscle. *Muscle Nerve*. 1988;11(10):1016-1028.

12. 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(8):445-448.

13. Katada A, Nonaka S, Adachi M, et al. Functional electrical stimulation of laryngeal adductor muscle restores mobility of vocal fold and improves voice sounds in cats with unilateral laryngeal paralysis. *Neurosci Res*. 2004;50(2):153-159.

14. Birchall MA, Kingham PJ, Murison PJ, et al. Laryngeal transplantation in minipigs: Vascular, myologic and functional outcomes. *Eur Arch Otorhinolaryngol*. 2011;268(3):405-414.

15. Murison PJ, Jones A, Mitchard L, Burt R, Birchall MA. Development of perioperative care for pigs undergoing laryngeal transplantation: A case series. *Lab Anim*. 2009;43(4):338-343.

16. Barker E, Murison P, Macchiarini P, et al. Early immunological changes associated with laryngeal transplantation in a major histocompatibility complex-matched pig model. *Clin Exp Immunol*. 2006;146(3):503-508.

17. 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(6):534-540.

18. Cheetham J, Regner A, Jarvis JC, et al. Functional electrical stimulation of intrinsic laryngeal muscles under varying loads in exercising horses. *PLoS ONE*. 502 2011;6(8):e24258.

19. Salmons S, Jarvis JC. Functional electrical stimulation of denervated muscles: An experimental evaluation. *Artif Organs*. 2008;32(8):597-603.

20. Kern H, Boncompagni S, Rossini K, et al. Long-term denervation in humans causes degeneration of both contractile and excitation-contraction coupling apparatus, which is reversible by functional electrical stimulation (FES): A role for myofiber regeneration? *J Neuropathol Exp Neurol*. 2004;63(9):919-931.

21. Borisov AB, Dedkov EI, Carlson BM. Interrelations of myogenic response, progressive atrophy of muscle fibers, and cell death in denervated skeletal muscle. *Anat* 511 *Rec*. 2001;264(2):203-218.

22. Ashley Z, Sutherland H, Lanmuller H, et al. Atrophy, but not necrosis, in rabbit skeletal muscle denervated for periods up to one year. *Am J Physiol Cell Physiol*. 514 2007;292(1):C440-51.

23. Passman SN, Cheetham J, Bonassar LJ, Ducharme NG, Rawlinson J. Biomechanical characterisation of equine laryngeal cartilage. *Equine Vet J*. 2011;43:592-598.

24. Dixon P, Robinson E, Wade JF. Workshop summary. . 2004;11:93-97.

25. Cheetham J, Radcliffe CR, Ducharme NG, Sanders I, Mu L, Hermanson JW. Neuroanatomy of the equine dorsal cricoarytenoid muscle: Surgical implications. *Equine* 520 *Vet J*. 2008;40(1):70-75.

26. Ashley Z, Sutherland H, Russold MF, et al. Therapeutic stimulation of denervated muscles: The influence of pattern. *Muscle Nerve*. 2008;38(1):875-886.

27. Ashley Z, Sutherland H, Lanmuller H, et al. Determination of the chronaxie and rheobase of denervated limb muscles in conscious rabbits. *Artificial Organs*. 2005;29:212.

28. Cheetham J, Witte TH, Rawlinson JJ, Soderholm LV, Proudman HO, Ducharme NG. Intra-articular stabilisation of the equine cricoarytenoid joint. *Equine Vet J*. 2008.

29. Witte TH, Cheetham J, Soderholm LV, Mitchell LM, Ducharme NG. Equine laryngoplasty sutures undergo increased loading during coughing and swallowing. *Vet Surg*. 2010;39(8):949-956.

30. Poon M, Hamarneh G, Abugharbieh R. Efficient interactive 3D livewire segmentation of complex objects with arbitrary topology. *Comput Med Imaging Graph*. 2008;32(8):639-650.

31. Cheetham J, Pigott JH, Hermanson JW, et al. Role of the hypoglossal nerve in equine nasopharyngeal stability. *J Appl Physiol*. 2009;107(2):471-477.

32. Rose RJ, Hendrickson DK, Knight PK. Clinical exercise testing in the normal thoroughbred racehorse. *Aust Vet J*. 1990;67(10):345-348.

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(4):500-503.

34. Herholz C, Tschudi P, Gerber H, Moens Y, Straub R. Ultrasound spirometry in the horse: A preliminary report on the method and the effects of xylazine and lobeline hydrochloride medication. *Schweiz Arch Tierheilkd*. 1997;139(12):558-563.

35. Shindo ML, Herzon GD, Hanson DG, Cain DJ, Sahgal V. Effects of denervation on laryngeal muscles: A canine model. *Laryngoscope*. 1992;102(6):663-669.

36. Shiotani A, Flint PW. Myosin heavy chain composition in rat laryngeal muscles after denervation. *Laryngoscope*. 1998;108(8 Pt 1):1225-1229.

37. Rhee HS, Lucas CA, Hoh JF. Fiber types in rat laryngeal muscles and their transformations after denervation and reinnervation. *J Histochem Cytochem*. 2004;52(5):581-590.

38. Chi FL, Jing JH, Dai CF. Reanimation of paralyzed laryngeal muscles by electrical stimulation synchronized with inspiration. *Otolaryngol Head Neck Surg*. 2006;135(1):40-45.

39. Helgason T, Gargiulo P, Johannesdottir F, et al. Monitoring muscle growth and tissue changes induced by electrical stimulation of denervated degenerated muscles with CT 556 and stereolithographic 3D modeling. *Artif Organs*. 2005;29(6):440-443.

40. Dedkov EI, Borisov AB, Carlson BM. Dynamics of postdenervation atrophy of young and old skeletal muscles: Differential responses of fiber types and muscle types. *J Gerontol A Biol Sci Med Sci*. 2003;58(11):984-991.

41. 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(2):203-218.

42. Kingham PJ, Birchall MA, Burt R, Jones A, Terenghi G. Reinnervation of laryngeal muscles: A study of changes in myosin heavy chain expression. *Muscle Nerve*. 2005;32(6):761-766.

43. Douglas AJ, Walsh EG, Wright GW, Creasey GH, Edmond P. The effects of neuromuscular stimulation on muscle tone at the knee in paraplegia. *Exp Physiol*. 1991;76(3):357-367.

44. Zealear DL, Rainey CL, Herzon GD, Netterville JL, Ossoff RH. Electrical pacing of the paralyzed human larynx. *Ann Otol Rhinol Laryngol*. 1996;105(9):689-693.