ABSTRACT: Introduction: It is not clear if electrical stimulation (ES) can affect muscle reinnervation. This study aimed to verify if ES affects neuromuscular recovery after nerve crush injury in rats. Methods: Denervated muscles were electrically stimulated daily for 6 or 14 days. Neuromuscular performance and excitability, and muscle morphology were determined. Muscle trophism markers (atrogin-1, MuRF-1, and myoD), as well as neuromuscular junction (NMJ) organization (muscle-specific receptor tyrosine kinase [MuSK], cytoplasmic protein downstream of kinase-7 [Dok-7], nicotinic ACh receptor [nAChR], and neural cell adhesion molecule [N-CAM]) were assessed. Results: ES impaired neuromuscular recovery at day 14 postdenervation. Muscle hypoexcitability was accentuated by ES at 6 and 14 days postdenervation. Although ES reduced the accumulation of atrogin-1, MuRF-1, and myoD mRNAs, it increased muscle atrophy. Gene expression of MuSK, Dok-7, nAChR, and the content of N-CAM protein were altered by ES. Conclusions: ES can delay the reinnervation process by modulating factors related to NMJ stability and organization, and inducing dysfunction, hypoexcitability, and muscle atrophy.

Peripheral nerve injuries (PNIs) can cause disability and reduced quality of life. Muscle denervation post-PNI is a devastating condition, which produces atrophy and paralysis. The innervation and reinnervation of skeletal muscle leads to the formation/reorganization of neuromuscular junctions (NMJs). Morphologically, 3 compartments form the NMJ: (1) the presynaptic compartment in the nerve ending, where the vesicles containing acetylcholine (ACh) and the Schwann cells are found; (2) the extracellular compartment, filled with basal lamina; (3) the postsynaptic compartment, composed of the muscle fiber sarcolemma, the junctional folds, and the sarcoplasm. There is controversy about using ES in treatment of denervated muscle. ES protocols are very diverse, and their effects are a consequence of many variables, including the type of electrical current, electrode position, electrode type, frequency, pulse width, amplitude, and duration of stimulation. In addition, most of these parameters are not reported in the literature, which compromises data interpretation and comparisons between studies. Some investigators have shown that 200 daily contractions are effective in preventing mass and strength loss in denervated muscles. Others report that 600 contractions per day, 5 days per week result in a higher number of functional motor units and enhanced functional recovery, however, muscle mass and force remain unchanged. Therefore, the amount of contractions is an important parameter for the success of ES. However, it should be observed that these studies used implanted electrodes, which differs from the clinical practice. Furthermore, these reports contradict studies that have shown that muscle ES provides no functional benefit when stimulation is applied infrequently or with the use of subthreshold amplitudes that could not elicit strong contractions.

Additional supporting information may be found in the online version of this article.

Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; CSA, cross-sectional area; D, denervated; DES, denervated electrically stimulated; Dok-7, cytoplasmic protein downstream of kinase-7; E, experimental; ES, electrical stimulation; ITS, intermediate toe spread; MuSK, muscle-specific receptor tyrosine kinase; N, normal; nAChR, nicotinic ACh receptor; N-CAM, neural cell adhesion molecule; NMJ, neuromuscular junction; PD, predenervation; PL, print length; PNI, peripheral nerve injury; SFI, sciatic functional index; TA, tibialis anterior; TENS, transcutaneous electrical nerve stimulation; TS, toe spread

Funding: This study was supported by FAPESP (2013/21321-6) and CAPES.

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

Correspondence to: T. L. Russo; e-mail: russo@ufscar.br

© 2017 Wiley Periodicals, Inc.
Published online 00 Month 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mus.25589
On the other hand, Tam and Gordon\textsuperscript{12} and Salmons et al.\textsuperscript{13} suggested that perhaps a more moderate activity protocol is needed to obtain optimal results and concluded that denervated muscles, due to their reduced excitability, require larger amplitude and pulse duration to obtain contractions. Therefore, ES protocols should be designed that take into account the number of contractions per day and pulse duration, to investigate its effectiveness to treat denervated muscles.

Much attention has been given to the effects of ES to recover muscle trophism; however, little information is available about how ES can affect the muscle fiber reinnervation process. Molecular modifications in the NMJ are important to reestablish innervation. The surface of the postsynaptic sarcolemma of the NMJ contains a high density of nicotinic ACh receptors (nAChRs). The nAChRs are integral membrane proteins composed of 5 subunits $\alpha$, $\beta$, $\delta$, $\gamma$, $\epsilon$ (alpha, beta, delta, gamma, and epsilon) arranged in rosette form.\textsuperscript{14–16} These subunits are present in the NMJ postsynaptic membrane of innervated muscle, but they are up-regulated in response to peripheral nerve lesions. In addition, there are NMJ proteins that stimulate nAChR clustering and anchoring in the postsynaptic membrane. This is done by coordinated activity, such as muscle-specific receptor tyrosine kinase (MuSK) and cytoplasmic protein downstream of kinase-7 (Dok-7).\textsuperscript{17,18} To promote synapse stability, the neural cell adhesion molecule (N-CAM) is a potent regulator of synaptic stability and strongly influences neurotransmission.\textsuperscript{19–21} N-CAM is also a potent regulator of synaptic stability and strongly influences neurotransmission.\textsuperscript{22,23}

Furthermore, with regard to muscle protein synthesis and degradation, there is a reduction in the synthesis and increase in protein degradation following PNI, leading to muscle mass loss.\textsuperscript{24} The expression of muscle-specific genes involved in the ubiquitin-proteasome pathway, such as MuRF1 and MAFBx (atrogin-1), is increased in denervated muscles. MuRF1 and atrogin-1 are important markers of muscle atrophy and could be considered the master genes for muscle wasting and are associated with loss of muscle mass.\textsuperscript{25–27} Atrogin-1 is also related to protein synthesis and control of transcription by ubiquitination of myoD (a regulatory myogenic factor).\textsuperscript{27}

Due to controversy about using ES to treat denervated muscles, more studies are needed to characterize the effects, identify more efficient and safe parameters, and show effectiveness of ES protocols to prevent muscle atrophy after denervation. Thus, the aim of this study was to assess early changes induced by ES in neuromuscular, neurophysiological, and muscle morphology performance, as well as molecular biomarkers of denervated muscles. ES was conducted using muscle electrical excitability evaluation using surface electrodes, as is normally done in rehabilitation programs to treat denervated muscles. Furthermore, axonotmesis was chosen for this study to verify the effects of ES during the natural reinnervation process. Based on previous results, the hypothesis of this study was that ES sessions might interfere negatively in the muscle reinnervation process, and thus, impair muscle recovery processes that modulate muscle atrophy (atrogin-1 and MuRF1) and NMJ maintenance (MuSK, Dok-7, N-CAM, and nAChRs) in denervated muscles after axonotmesis.

**MATERIAL AND METHODS**

**Animal Care and Experimental Groups.** Thirty-five male 3-month-old Wistar rats weighing $230 \pm 3$ g were housed in plastic cages in a room with controlled environmental conditions and had free access to water and standard food. The experimental procedures were approved by the Ethics Committee of the Federal University of São Carlos (033/2014) and were conducted in accordance with the Guide for Care and Use of Laboratory Animals. The animals were randomly (by body mass using 1-way analysis of variance [ANOVA]) divided into 5 groups ($n = 7$); the sample calculation was made by GPower software and was based on previous studies of our laboratory using cross-sectional area (CSA) as a main variable, considering a power of 80%. The groups are shown in Figure 1. They consisted of (1) normal (N) control animals without PNI that were euthanized 15 days after the beginning of the experiment. (2) 7-day denervation (D7d) animals underwent PNI by axonotmesis of the right sciatic nerve. They were anesthetized daily. In addition, they were submitted to 2 electrical excitability evaluations and 2 motor function evaluations (predenervation and the other on day 6). (3) 7-day denervation and ES (DES7d) animals underwent axonotmesis of the right sciatic nerve, 2 electrical excitability evaluations, 2 motor function evaluations (1 predenervation [PD] and the other on day 6), and...
daily anesthesia, as well as ES. They were euthanized 7 days after nerve injury. (4) 15-day denervation (D15d) animals underwent axonotmesis of the right sciatic nerve, 3 electrical excitability evaluations, and 3 motor function evaluations (1 predenervation, 1 on day 6, and the last on day 14). They also received anesthesia daily and were euthanized 15 days after injury. (5) 15-day denervation and ES (DES15d) animals underwent axonotmesis of the right sciatic nerve, 3 electrical excitability evaluations, and 3 motor function evaluations (1 PD, 1 on day 6, and the last on day 14). They also received anesthesia and ES daily. They were euthanized 15 days after injury. For more procedure details carried out in the experimental groups, see Figure 1.

Intraperitoneal injections of xylazine (12 mg/kg) and ketamine (95 mg/kg) anesthetized the rats for the surgical and denervation processes, electrical excitability evaluations, ES treatment, and muscle removal. Two animals from the D15d and DES15d groups died during the trial period, probably due to daily exposure to anesthesia. Then, an overdose of the anesthetics on days 7 or 15 of the experimental procedures was used to euthanize the rats. These periods of denervation were chosen, because muscle fiber reinnervation processes happen during the first weeks after nerve crush injuries.15,17

**Denervation Process.** The denervation process consisted of sciatic nerve axonotmesis conducted as previously done by Russo et al.2 and Gigo-Benato et al.8 Detailed information is described in the Supplementary Materials, which are available online.

**Motor Function Evaluation.** The assessment of nerve function recovery was carried out by calculating the sciatic functional index (SFI). Animals were tested on a confined walkway that was 42 cm long and 8.2 cm wide, with a dark shelter at the end. A piece of white paper was placed on the floor of the walkway. The hind paws of the rats were pressed down onto an ink-soaked sponge, and they were then allowed to traverse the walkway, leaving hind-paw prints on the paper. Three measurements were taken from the hind-paw prints: (1) the print length (PL), which is the distance from the heel to the third toe; (2) the toe spread (TS), which is the distance from the first to the fifth toe; (3) the intermediate toe spread (ITS), which is the distance from the second to the fourth toe. These 3 measurements were obtained from the experimental (E) and normal (N) sides. The SFI was calculated according to the following equation:

\[
\text{SFI} = -38.3 \left( \frac{EPL - NPL}{NPL} \right) + 109.5 \left( \frac{ETS - NTS}{NTS} \right) + 13.3 \left( \frac{EITS - NITS}{NITS} \right) - 8.8
\]

**Electrical Excitability Evaluation and ES Procedures.** Rats from the DES groups received ES treatment as previously described by Russo et al.2 and Gigo-Benato et al.2,7,0,28 In brief, the skin was shaved, cleaned, and covered with a layer of conductive gel, where 2 electrodes were positioned, an indifferent electrode (5 × 5 cm self-adhesive electrode) on the animal’s back and an active electrode (3 mm round metallic electrode) small enough to stimulate only the tibialis anterior (TA) muscle. During the ES procedure, the metallic electrode was in contact with the skin overlying the TA muscle and perpendicular to the muscle fibers. Surface electrodes were used because they are more commonly used for therapeutic applications, and their position does not require procedures that are invasive. ES equipment (Nemsys 941, Quark, Brazil), which allows changes in the electrical parameters was used to assess muscle excitability and for the ES treatment.

Before each evaluation of electrical parameters, we identified the site over the TA where the lowest stimulus amplitude fully activated the muscle. The electrical parameters were evaluated before each ES treatment to provide rheobase, chronaxie, and muscle accommodation values. Afterward, the chronaxie values were used to determine the ES parameters applied to the TA muscle. These electrical indices were previously reported.2,8 Briefly, the DES7d and DES15d groups underwent muscle excitability evaluations, 1 predenervation, and the other on the day before euthanasia (day 6); the same evaluations were conducted for D15d and DES 15d, and another on the day before euthanasia (day 14).

After the muscle excitability evaluation, the amplitude necessary to induce a contraction of the TA muscle was identified visually. The current amplitude was increased until full dorsiflexion of the ankle was observed. An exponential phasic current was used (frequency, 20 Hz; pulse duration, the chronaxie value times 2; time on: 3 s; and time off: 6 s). The pulse amplitude necessary to induce a visible contraction was selected. The ES sessions were conducted daily for 6 (DES 7d) or 14 (DES 15d) days, beginning 24 h after denervation, and producing 200 TA muscle contractions. These contractions were divided into 4 sets of 50 contractions, with a 7.5-min per set duration and a 10-min rest (without ES) between sets to minimize muscle fatigue.29 Dow et al.3 previously demonstrated that 200 muscle contractions are effective to maintain muscle mass and strength, and Kostrominova et al.30 demonstrated that to reduce the gene expression generally increased during denervation, 200 muscle contractions are also effective.

In this study, a small number of muscle contractions was used to reproduce what is usually applied during a single rehabilitation session for recovery in human denervated muscles. Normally, in a single treatment session, electrical treatment of denervated muscle is associated with other interventions such as physical exercise, muscle stretching, and range of motion. Therefore, we decided on 200 contractions using on-time 3 s and off-time 6 s. More than that could cause muscle fatigue, as all contractions were applied in a short time period during a single treatment session.

**Muscle Evaluation.** Two weeks after surgery, all animals were euthanized with an anesthetic overdose. The sciatic nerves and the TA muscles were carefully dissected and immediately weighed on a precision balance (Model 100a; Denver Instruments, Denver, Colorado).

The muscles were split in half in the middle of the muscle belly. The distal fragment was used for the histological and morphometric measurements. The proximal fragment was divided into 2, immediately frozen in liquid nitrogen, and stored at -80°C (Forma Scientific, Marietta, Ohio) for the mRNA analysis, as well as the western blotting analysis.

**Nerve Morphology.** To confirm the injury, nerve histology was performed. The sciatic nerve stump was prepared for a light microscopy. Samples were fixed in 10% formalin for 3 h and then washed in phosphate buffered saline until embedded. The samples were dehydrated, embedded in paraffin, and cut at 7 µm perpendicular to the main nerve axis. Sections were stained with Masson trichrome, following...
manufacturer’s instructions and observed under a light microscope (Axioskop, Carl Zeiss, Jena, Germany) equipped with a Carl Zeiss AxioCam HRc camera. If no nerve injury was detected, the animal was not included in the analyses.

Muscle Morphometric Analysis. Morphometric analysis was performed according to Pinheiro-Dardis and Russo. Histological cross-sections (10 μm) were obtained with a cryostat microtome (Microm, Heidelberg, Germany), through the middle belly of the TA muscles. Muscle sections were stained using toluidine blue/1% Borax. A light microscope (Axioskop, Carl Zeiss, Germany) was used to evaluate general muscle morphology. One histological cross-section of each TA muscle located in the central region, with contiguous muscle fibers, was chosen to measure the muscle fiber CSA. One image from this area was taken at 20× magnification; the image was obtained using a light microscope equipped with a digital camera (Carl Zeiss AxioCam HRc). From each picture, the CSA of 100 contiguous muscle fibers in the central region was measured using Axiovision 4.7.1.0 software (Carl Zeiss, Jena, Germany).

N-CAM Content Assessed by Western Blot. Protein content of N-CAM was quantified in TA muscle extracts using Western blot assays as described before. A specific primary antibody (N-CAM, 1:250, Millipore, Temecula, California #AB3052) and a secondary antibody conjugated with horseradish peroxidase (1:5000, Santa Cruz Biotechnology, Santa Cruz, California) were used. Immunoreactive protein signals were detected using a Chemi DocTM MP Imaging System (Bio-Rad, Hercules, California) according to the manufacturer’s instructions. The chemiluminescent signal was visualized and quantified by densitometry using Gene Tools software, version 3.06 (Syngene, Cambridge, United Kingdom). The values were normalized by the values obtained from quantifying the alpha-tubulin protein.

RNA Isolation and Analysis. RNA was isolated from a frozen fragment of each TA muscle using 1 ml of Trizol reagent (Invitrogen, Carlsbad, California), according to the manufacturer’s instructions. The total RNA (1 μg) from each sample was treated with DNase I (Sigma, AMP-D1) to remove contaminating genomic DNA, then the total RNA was reverse transcribed to synthesize cDNA using synthesis Kit (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, California). The RNA transcript levels for the different experimental and control muscles were analyzed simultaneously, and the reactions were carried out in duplicate in real time polymerase chain reaction (PCR) (CFX 96 Touch Real Time PCR Detection System, Version 3.0, Bio-Rad, Hercules, California), using fluorescent dye SYBR green detection (Thermo Scientific, Waltham, Massachusetts). For each gene, all samples were amplified simultaneously in duplicate using a final volume of 20 μl. Gene expression was compared among individual samples using the ΔΔCq method. The reference genes for normalization were chosen using Normfinder. Three genes were used: beta cytoskeletal actin, hypoxanthine-guanine phosphoribosyltransferase, and peptidylprolyl isomerase A. The primer sequences used are listed in Supplementary Table S1, which is available online. Detailed information about Western blot and quantitative PCR analyses is listed in the Supplementary file.

Statistical Analysis. The Shapiro-Wilk and Levene tests were used to investigate whether the data were parametric. One-way ANOVA followed by Tukey test were used to identify possible differences among groups. For the electrical (rheobase, chronaxie and accommodation) and functional variables, the 2-way ANOVA (groups×time) with repeated measures followed by Tukey test were used. Differences were considered significant when P<0.05. Statistical analysis was performed using the IBM SPSS Statistics 20.

RESULTS

SFI. Table 1 shows the neuromuscular walking performance test pre- and postnerve injury. No difference between the experimental groups was observed PD (P=0.627). Decreased neuromuscular performance was observed on the day 6 post-PNI in both D15d and DES15d compared with PD values (D15d, P=0.001; DES15d, P=0.005), with no difference between them (D15d vs. DES15d: P=0.597). Nevertheless, on the 14th day, whilst D15 improved the neuromuscular performance, increasing SFI, DES15d remained similar to its values observed on the 6th day (Table 1), with a difference between groups (P=0.001). Data are also published elsewhere (Pinheiro-Dardis and Russo, ahead of print).

Neuromuscular Electrical Excitability Evaluation. PD measurements of the denervated (D) and denervated electrically stimulated (DES) groups obtained from the right TA muscles immediately before denervation were considered to be normal muscle excitability values for rheobase, chronaxie, and accommodation (Fig. 2A–C). Interaction between time and group was observed in chronaxie [F(1,8) = 42.904; P=0.0001], but not in rheobase [F(1,8) = 1.732; P=0.218] and accommodation [F(1,8) = 0.072; P=0.796].

The rheobase values declined at days 6 and 14 in both groups (D15d and DES15d), compared with the PD values (P=0.0001). Reduction was more significant 14 days after denervation, compared with 6 days (P=0.0001; Fig. 2A). Both groups showed a statistical difference on day 6 compared with day 14.

Chronaxie increased significantly in both D and DES groups throughout 14 days (P=0.002) compared with PD values. The DES group had the
highest chronaxie values on days 6 and 14 compared with the nonstimulated D group (Fig. 2B).

Similar to rheobase, accommodation values decreased after denervation in both D and DES groups (submitted or not to ES) compared with PD levels ($P = 0.0001$). There was no difference between the D and DES groups on days 6 and 14 postinjury ($P = 0.210$; Fig. 2C).

**Body and TA Muscle Mass.** Body weight increased in all groups during the experimental period. Although no difference was observed in the final body weight between D and DES groups at 7 or 15 days postdenervation ($P = 0.663$), they were around 25% less than N group ($P = 0.0001$). Both D and DES groups had decreased muscle mass compared with the N group ($P = 0.0001$). In addition, the muscle masses differed when comparing the DES15d with the other groups (N, D7d, DES7d, $P = 0.0001$; D15d, $P = 0.004$).

**Muscle Morphology and Muscle Fiber CSA.** Figure 3 shows the muscle fiber CSA distribution. There was intense atrophy in the denervated TA muscle fibers in the D7d ($607.26 \pm 49.38 \mu m^2$, $P = 0.0001$), D15d ($470.36 \pm 68.00 \mu m^2$, $P = 0.0001$), DES7d ($641.20 \pm 128.76 \mu m^2$, $P = 0.0001$), and DES15d ($375.84 \pm 30.42 \mu m^2$, $P = 0.0001$) groups when compared with the normal group ($947.42 \pm 35.49 \mu m^2$). At 7 and 15 days, no difference between groups was observed (D7d vs. DES7d: $P = 0.943$; D15d vs. DES15d: $P = 0.267$). However, both D15d and DES15d had reduced muscle fiber CSA compared with D7d ($P = 0.0001$; vs. DES15d, $P = 0.0001$) and DES7d (vs. D15d, $P = 0.01$; vs. DES15d, $P = 0.0001$). Morphology of DES groups also showed a few central nuclei and degenerating/regenerating muscle fibers.

**Atrogin-1, MuRF1, and myoD Gene Expression by Real-Time RT-PCR.** Denervation increased both atrogin-1 (Fig. 4A) and myoD (Fig. 4C) gene expressions compared with N. ES reduced atrogin-1 and myoD the RNAm accumulation compared with the non-stimulated groups (Fig. 4A and C). The D7d had the highest levels of atrogin-1 and myoD compared with the other groups (Fig. 4A and C).

MuRF1 was up-regulated in all denervated groups on days 7 and 14 compared with N ($D7d$, DES7d and D15d: $P = 0.0001$; DES15d: $P = 0.002$; Fig. 4B). This increment remained after 15 days of denervation, unlike atrogin-1 and myoD (Fig. 4A and C). ES decreased gene expression of both DES7d and DES15d compared with D7d and D15d (For D7d compared with DES7d: atrogin-1, $P = 0.0001$; MuRF-1, $P = 0.002$; and myoD, $P = 0.0001$; For D15d compared with DES15d: atrogin-1, $P = 0.003$; MuRF-1, $P = 0.0001$; and myoD, $P = 0.003$). Of interest, DES15d showed the lowest MuRF1 gene expression values compared with D7d and D15d ($P = 0.0001$, Fig. 4B).

**Content of N-CAM Protein.** Quantitative analysis of N-CAM protein detected an increased content in D7d, D15d, and DES15d groups compared with N (vs. D7d, $P = 0.0001$; vs. D15d, $P = 0.002$, and vs. DES15d, $P = 0.001$; and DES7d, $P = 0.001$; and DES15d, $P = 0.001$; and DES7d, $P = 0.001$; and DES15d, $P = 0.001$; and DES7d, $P = 0.001$).
FIGURE 3. Muscle fiber CSA, and muscle morphology of the TA muscle. CSA decreased in all denervated groups compared with the N group ($P = 0.0001$). D15d and DES15d had reduced muscle fiber CSA compared with D7d (vs. D15d, $P = 0.048$; vs. DES15d, $P = 0.0001$) and DES7d (vs. D15d, $P = 0.01$; vs. DES15d, $P = 0.0001$). Scale bar = 100 μm.
MuSK, Dok-7, and AChR Gene Expression. MuSK, αAChR, and δAChR followed the same pattern, with an increase in all denervated groups (D7d, DES7d, D15d, and DES15d) compared with N (Fig. 6; \(P=0.0001\)), and a decrease on day 15, compared with day 7 in D7d, DES7d, D15d, and DES15d (D7d compared with D15d: MuSK \(P=0.003\); αAChR and δAChR \(P=0.0001\); DES7d compared with DES15d: MuSK, αAChR, and δAChR \(P=0.0001\)). DES15d showed an increase in zAChR and δAChR expressions, compared with D15d (zAChR \(P=0.001\); δAChR \(P=0.023\)). Dok-7 and βAChR levels increased in all denervated groups compared with N (Fig. 6; \(P=0.0001\)). ES decreased the levels of Dok-7 on the days 7 and 15 compared with D7d (\(P=0.041\)) and D15d (\(P=0.0001\)), respectively, whereas βAChR was down-regulated through ES on the day 15 compared with D15d (\(P=0.049\); Fig. 6). No differences were found between DES7d and DES15d (\(P=0.633\); Fig. 6). ES showed an increase in all denervated groups compared with N (Fig. 6; \(P=0.0001\)). DES15d increased the εAChR levels compared with D15d (\(P=0.020\)).

DISCUSSION

This study showed that daily sessions of ES applied to denervated muscles by surface electrodes interfered with muscle reinnervation, affected muscle recovery by impaired neuromuscular performance and excitability, accentuated muscle atrophy, and altered gene expression and protein content of important markers of muscle tropism and NMJ organization and maintenance. The major findings were that early signs of functional recovery could be observed in the D15d group, but not in electrically stimulated animals (DES15d). Furthermore, ES increased chronaxie values in treated animals (DES15d), which indicated that it could interfere with muscle excitability after denervation. Although ES also reduced mRNA levels of atrogenes (atrogin-1 and MuRF-1), muscle atrophy
was accentuated. In fact, a recent study provided evidence that ES applied to denervated muscles could impair the reinnervation process. These results corroborated those data.

ES has been widely applied clinically as a countermeasure to attenuate muscle atrophy. Some authors have reported that ES was effective in decreasing muscle atrophy. Dow et al. showed that ES could be effective in recovery of muscle force and mass of denervated muscle. In addition, another recent study showed that 600 contractions per day, 5 days per week, immediately following nerve transection and repair, enhanced electrophysiological and behavioral recovery. The authors argue that there is a relationship between the daily number of contractions and their distribution to induce trophic effects on denervated muscles, indicating that 200 to 800 contractions per day could be a good parameter to consider. However, the protective effects of the number of muscle contractions per day should be combined with the amount of force generated by the muscles and the type of muscles investigated. As no muscle torque generation analysis was performed in our study, and muscle composition varied between studies, comparisons between the Dow et al. results and our results should be interpreted with caution.

Tomori et al. demonstrated that relatively low intensities of ES (4 mA or 8 mA) can reduce muscle membrane disruptions, and improve ultrastructural organization of t-tubules in denervated muscles. Higher intensities (16 mA) did not provide any additional effect from those observed with 4 or 8 mA. These results indicate that the current intensity is also an important aspect. The current intensity used in this study was enough to produce a tetanic contraction and a full flexion and inversion of the animal’s ankle, thus this parameter was not underestimated here. Recently, Pieber et al. recommended similar ES parameters to stimulate denervated TA muscles in humans. They supported the use of monophasic triangular pulse currents with a pulse width of 200 ms.

Denervated muscles are different from innervated muscles in their response to ES. Rheobase and chronaxie values provide efficient muscle excitability parameters. They are consistently elevated in denervated muscle, indicating lower excitability compared with normal muscle. Because of this, denervated muscles require higher pulse duration (chronaxie) to contract. The increase in chronaxie values confirmed that all muscles were denervated on days 6 and 14 postdenervation, however, ES accentuated the chronaxie increment, indicating that these muscles are less excited and there is a decreased velocity of electrical conduction.

Distinct differences between these other studies and ours could also be related to other differences in ES protocols. For example, previous reports from Willand et al. showed that ES exacerbated muscle atrophy and force loss, but did not affect muscle reinnervation. The ES protocol consisted of a biphasic train of 400 ms duration (40 pulses at 100 Hz) with a pulse width of 200 ms per phase. They induced 600 muscle contractions using implanted electrodes in a 1-h session, with 6 s off between each contraction. Daily sessions were

![Figure 6](image_url)

**FIGURE 6.** The expression levels of MuSK (A), Dok-7 (B), and nAChRs (RACh; C–F) in TA muscle. Data are means ± SD. *a*: Statistical differences compared with N. *b*: Statistical differences compared with D7d. *c*: Statistical differences compared with DES7d. *d*: Statistical differences compared with D15d. Note that MUSK gene expression was not affected by ES, whereas nAChR and Dok-7 are modulated differently.
performed 5 times per week. Comparing our parameters with Willand et al., some aspects should be considered, including type of current (monophasic vs. biphasic and burst), type of electrode (surface vs. implanted), relationship between time on and off (1:2 vs. 1:6), and number of sessions (daily sessions vs. 5 sessions/week). Thus, these differences should be considered in future studies to provide the best parameters to electrically stimulate denervated muscles using surface electrodes.

Nevertheless, caution is needed when comparing ES of denervated muscles to ES of injured nerves. A randomized controlled clinical trial verified that brief ES applied to crushed median nerves in humans accelerated nerve regeneration and target reinnervation. Elzinga et al. investigated the effect of brief ES (1 h, 20 Hz, 0.1 ms, supra-maximal stimulation) on nerve regeneration after delayed nerve repair in rats. They found that brief ES of chronically axotomized motor and sensory neurons was effective in accelerating axon outgrowth into chronically denervated nerve stumps and improving target reinnervation after delayed nerve repair. On the other hand, Assis et al. showed that 2 h of high-transcutaneous electrical nerve stimulation (TENS) frequency (100 Hz), after nerve crush injury, is deleterious for regeneration, whereas low-TENS frequency (10 Hz) facilitated nerve regeneration in mice. Overall, these studies showed that brief ES from injured nerves could be beneficial to recovery according to the ES frequency used. Low frequencies (≤20 Hz) seem to be safe and efficient to improve nerve regeneration, rather than high frequencies (100 Hz).

Regarding functional data, a previous report showed that ES impaired walking recovery in rats with sciatic nerve crush injury. They electrically stimulated denervated muscles using surface electrodes, and considered the muscle excitability in choosing the best ES parameters. The denervated TA muscle was electrically stimulated on alternate days for 15 days. They showed that 20 contractions induced by ES impaired functional recovery. Our results correlated with the Gigo-Benato et al. data. It can be supposed that both muscle hyperegibility (higher levels of chronaxie) and accentuated muscle atrophy are related to neuromuscular walking performance in DES15d.

This study also provides interesting data about molecular regulation of denervated muscles due to ES. The findings correlate with previous reports which showed that ES can down-regulate the atrogens atrogin-1 and MuRF-1, and also myoD (a member of myogenic regulatory factors, MRFs) in denervated muscles. Kostrominova et al. also showed that denervated muscles submitted to ES down-regulated many genes, including those related to hypertrophy and atrophy pathways, and also the MRFs, which support our results. Because gene expression can be posttranscriptionally regulated, it is possible the amount of mRNA of atrogens is enough to maintain atrophy. Furthermore, the effects of ES in different pathways such as inflammatory, lysosomal, and synthetic control of denervated muscles must be investigated to understand the biological meaning of such modulation. Nevertheless, according to the data presented, it can be concluded that down-regulation of atrogens or myoD caused by ES is inefficient for protecting muscle mass from degradation.

An innovative aspect of this study is that ES applied by surface electrodes can modulate gene expression and protein content of reinnervation and NMJ stability and maintenance related factors. We found that innervated muscles expressed N-CAM mainly at the NMJ, but denervation increased N-CAM around denervated fibers in D7d and 15d compared with N. ES affected the increase of N-CAM at 1 week postdenervation, but not by week 2. These results correlate with previous studies. In addition, Kostrominova et al. showed that chronic ES reduced N-CAM in denervated muscles in the absence of reinnervation. It seems that ES could be harmful if applied very early after muscle denervation, because it reduced N-CAM production and accumulation. However, new studies with later time points are needed to know if these modifications in N-CAM content caused by ES will impair late recovery.

MuSK is the signaling component in the Lrp4-MuSK receptor complex necessary for triggering postsynaptic differentiation upon binding to neural agrin. MuSK is also required for formation of the postsynaptic apparatus, which clusters and anchors nAChRs in the adult postsynaptic membrane. However, MuSK function is dependent of Dok-7. MuSK and Dok-7 can induce nAChR clustering even in the absence of agrin (for a review see Tintignac et al. ). Our results showed a denervation increase in MuSK and Dok-7 gene expression. A previous study showed that MuSK overexpression is sufficient to maintain neuromuscular synapses in an amyotrophic lateral sclerosis model, improving muscle function. Thus, the increase in MuSK and Dok-7 expression in denervated muscles could be associated with the integrity of the NMJ.

The expression of nAChRs in long-term denervated gastrocnemius muscles was described previously. The studies showed that alpha, gamma, delta, and epsilon nAChR subunits are significantly up-regulated in the first week postdenervation but then returned to normal levels within the first
month by affecting NMJ stabilization. These results differ partially from those of Ma and collaborators. First, the increase of all nAChRs remained different from the control until the second week postdenervation, except for the gamma subunit, which was not detected in this study. Second, beta subunit expression remained elevated at D15d compared with D7d, whereas other nAChR subunits started to have decreased expression at 15 days. The persistence of beta subunit expression could be related to clustering of nAChRs at the NMJ. Finally, the absence of detection of the gamma subunit could be related to the type of injury (axonotmesis versus neurotmesis, see Ma et al.).

On the other hand, ES changed MuSK, Dok-7, and nAChR gene expressions. It is possible that muscle contractions or membrane depolarization caused by ES mimic reinnervation. ES reduced MuSK and Dok-7 gene expressions at 15, and 7 and 15 days, respectively. In addition, nAChR subunits were modulated according to the time investigated. The β nAChR subunit was reduced at 15 days, whereas the δ and ε subunits were downregulated by 7 days. ES delays accumulation of gene expression. It can be hypothesized that ES affects NMJ organization in a harmful way, because functional, excitability, and tropism deficits were also observed. Zhang and Peng showed that the electrical field elicits molecular reactions in muscle cells to trigger AChR clustering by means of the MuSK/rapsyn pathway. However, the effects of these changes caused by ES on terminal nerve sprouting or in different factors related to the reinnervation process, such as agrin, Lrp4, and rapsyn should be verified in future studies.

In summary, ES is widely used and recommended by physical therapists to treat denervated muscles, however, this study showed that ES applied in a manner that simulates clinical practice can affect reinnervation of denervated muscles. Evaluation of ES protocols similar to those applied in rehabilitation can bring relevant information to the development of effective and safe therapies for treating denervated or recovering muscle, with no harmful effects for potential nerve regeneration. In conclusion, ES applied to muscles by surface electrodes delays reinnervation processes by modulating factors related to NMJ stability and organization, as well as inducing neuromuscular performance and muscle atrophy, and decreasing muscle excitability.

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.

REFERENCES


