Universidade Federal de São Carlos
Centro de Ciências Biológicas e da Saúde (CCBS)
Departamento de Fisioterapia

Clara Maria Pinheiro

Efeito da estimulação elétrica sob a reinervação de músculos desnervados em ratos

SÃO CARLOS
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Tese apresentada ao Programa de Pós-Graduação em Fisioterapia da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de Doutor em Fisioterapia.

Orientador: **Prof. Dr. Thiago Luiz de Russo**

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PRINCIPAIS ABREVIATURAS

CSA = cross-sectional area

Dok-7 = cytoplasmic protein Downstream of kinase-7

ECM / MEC = extracelular matrix/ matriz extracelular

EE = avaliações elétricas

ES / EE = electrical stimulation / estimulação elétrica

Fn14 = receptor fibroblast growth factor inducible 14

MAFBx (atrogin-1) = muscle-specific E3-ubiquitin ligases

MuRF1 = muscle-specific E3-ubiquitin ligases

MuSK = muscle-specific receptor tyrosine kinase

MMP = metaloproteinase

MMP-2 = metaloproteinase 2

MMP- 9 = metaloproteinase 9

NMJ = junção neuromuscular

N-CAM = neural cell adhesion molecule

PD = pré-desnervação

PNI / LNP = Peripheral nerve injury /lesão nervosa periférica

SFI / IFC = sciatic functional index / índice functional do ciático

TA = tibial anterior

TGF-β = cytokine transforming growth factor beta

TNF-α = tumor necrosis factor-alpha

TWEAK = tumor necrosis factor-like weak inducer of apoptosis

RESUMO

A lesão nervosa periférica interrompe as funções normais dos neurônios e leva a alterações rápidas e progressivas no músculo esquelético, tais como a atrofia muscular e a fibrose, causando perdas funcionais. Para o tratamento dos músculos desnervados a estimulação elétrica (EE) tem sido utilizada. A escolha dos melhores parâmetros de EE para minimizar a atrofia muscular devido a desnervação, é controversa. Além disso, não está claro se a EE pode afetar, de fato, a reinervação de músculos desnervados. Assim, esta tese tem dois objetivos principais: 1) verificar se a EE, aplicada aos músculos desnervados com eletrodos de superfície, pode afetar a recuperação neuromuscular após axonotmese do nervo ciático em ratos; e 2) avaliar o impacto da EE no estabelecimento da fibrose do músculo desnervado. Dois manuscritos foram produzidos e contavam com os mesmos grupos experimentais. Trinta e cinco ratos Wistar foram divididos em 5 grupos: (1) Normal (N); (2) Desnervado 7 dias (D7d); (3) Desnervado e ES 7 (DES7d); (4) Desnervado 15 dias (D15d); e (5) Desnervado e ES 15 dias (DES15d). Foi realizada a axonotmese no nervo isquiático. O protocolo de EE do músculo tibial anterior consistiu em: 200 contrações por dia, divididas em 4 séries consecutivas de 50 contrações, com 10 minutos de descanso entre cada série. Foram utilizados os seguintes parâmetros: pulso monofásico exponencial; tempo: 2 vezes cronaxia; amplitude: nível motor; TON: 3s e TOFF: 6s. O índice funcional do ciático foi calculado. A excitabilidade muscular foi avaliada considerando a reobase, cronaxia e acomodação. Análises morfométricas, tais como área de secção transversa da fibra muscular e a porcentagem de proliferação do tecido conjuntivo foram utilizados para caracterizar a morfologia. Marcadores moleculares relacionados à reinervação como a N-CAM (molécula de adesão celular neural), organização e manutenção da junção neuromuscular (JNM) (MuSK, Dok-7 e receptores de acetilcolina), controle de massa muscular (atrogin-1, MuRF1, myoD e miostatina), fibrose (TGF-β e miostatina), remodelação da matriz extracelular (metaloproteinases) e, inflamação (TWEAK / Fn14) foram investigados por técnicas de biologia molecular como western-blot, qPCR ou zimografia. Os principais resultados mostraram que a EE provocou perda da recuperação natural dos músculos desnervados acentuando a perda funcional, a atrofia muscular e a fibrose, assim como, reduzindo a excitabilidade muscular. Estas alterações morfofuncionais e eletrofisiológicas foram relacionadas à diferentes modulações de todos os marcadores moleculares, no decorrer do tempo estudado. De modo geral, a presente tese forneceu provas de que a EE pode atrasar o processo de reinervação por fatores relacionados com a estabilidade e a organização da JNM, bem como a induzir à incapacidade e à atrofia muscular, com diminuição da excitabilidade muscular. Além disso, a EE aplicada aos músculos desnervados induziu fibrose através da modulação da via inflamatória e também pela produção e remodelamento da matriz extracelular. Cuidados devem ser tomadas pelas equipes de reabilitação ao utilizar a EE no tratamento de músculos desnervados.

Palavras-chave: estimulação elétrica, metaloproteinase, fibrose e inflamação.

ABSTRACT

Peripheral nerve injury disrupts the normal functions of neurons and leads to rapid and progressive alterations in structural skeletal muscle, such as muscle atrophy and fibrosis, causing functional deficits. Electrical stimulation (ES) has been recommended to treat denervated muscles. The best parameters of ES to minimize muscle atrophy due to denervation is controversial. Furthermore, it is not clear if ES can, in fact, affect reinnervation of denervated muscles. Thus, this thesis has two main objectives: 1) to verify if ES, applied to denervated muscles by surface electrodes, can affect neuromuscular recovery after nerve crush injury in rats; and 2) to assess the impact of ES on fibrosis establishment in denervated muscles. Two manuscripts were produced and used the same experimental groups. Thirty-five Wistar rats were divided into 5 groups: (1) Normal (N); (2) 7-day denervation (D7d); (3) 7-day denervation and ES (DES7d); (4) 15-day denervation (D15d); and (5) 15-day denervation and ES (DES15d). *Tibialis anterior* (TA) muscle denervation was induced by crushing the sciatic nerve. The ES protocol to stimulate TA muscles consisted of: 200 contractions per day divided into 4 consecutive series of 50 contractions, with 10-minute rests between each set. The following parameters were used: exponential monophasic pulse; width time: 2x chronaxie; amplitude: motor level; time On: 3s and Off: 6s. The sciatic functional index was calculated. Muscle excitability was assessed considering the rheobasis, chonaxie and accommodation. Morphometric analyses, such as the muscle fiber cross-sectional area and percentage of connective tissue proliferation were used to characterize muscle morphology. Molecular markers related to reinnervation (neural cell adhesion molecule, N-CAM), neuromuscular junction organization and maintenance (MuSK, Dok-7 and nicotinic Acetylcholine Receptors (nAChR) subunits), muscle mass control (atrogin-1, MuRF1, myoD and myostatin), fibrosis (TGF-β and myostatin), extracellular matrix remodeling (metaloproteinases, MMPs) and inflammation (TWEAK/Fn14) were investigated by molecular biology techniques such as western-blot, qPCR or zymography. The main results showed ES impaired natural recovery of denervated muscles accentuating disability, muscle atrophy and fibrosis, as well as reducing muscle excitability. These morphofunctional and electrophysiological changes were related to different modulations of all molecular markers investigated in a timely manner. Overall, this thesis provides evidence that ES can delay the reinnervation process by modulating factors related to NMJ stability and organization, as well as induced disability and muscle atrophy, and decreased muscle excitability. In addition, ES applied to denervated muscles induced muscle fibrosis by modulating inflammatory pathways and also extracellular matrix production and remodeling. Warnings should be given to rehabilitation teams when recommending ES to treat denervated muscles.

Key-words: electrical stimulation, metalloproteinase, fibrosis e inflammation.

SUMÁRIO

APRESENTAÇÃO

Esta tese foi organizada segundo as normas do Programa de Pós-Graduação em Fisioterapia da UFSCar e faz parte de uma linha de pesquisa desenvolvida no Laboratório de Pesquisa em Fisioterapia Neurológica (LaFiN), do Departamento de Fisioterapia da UFSCar. Esta linha abrange estudos na área básica de recursos físicos utilizados na fisioterapia para tratamento de músculos desnervados.

Uma contextualização será apresentada inicialmente, com detalhamento da linha de pesquisa na qual o estudo está inserido. Em seguida, dois manuscritos vinculados a esta tese serão apresentados. O primeiro, intitulado "*Electrical stimulation delays reinnervation in denervated rat muscle*", foi submetido à revista *"Journal of Peripheral Nervous System"* (JCR 2016: 2.758). Já o segundo manuscrito foi intitulado de *"Electrical stimulation, based on chronaxie, increases fibrosis and modulates TWEAK/Fn14, TGF-β/myostatin, and MMP pathways in denervated muscles"* e submetido à revista *"American Journal of Physical Medicine and Rehabilitation"* (JCR2016: 2.202; Qualis A1 na área de Educação Física). Por fim, conclusões gerais da tese e a descrição de atividades desenvolvidas pela doutoranda no período serão apresentadas.

CONTEXTUALIZAÇÃO

Lesão nervosa periférica (LNP) é um trauma muito frequente que ocorre nos nervos periféricos, podendo resultar em parcial ou total perda de funções motoras, sensoriais e autonômicas, levando muitas vezes à incapacidade com redução de qualidade de vida. Essas lesões podem ocorrer por vários motivos, entre eles, acidentes de trabalho e automobilísticos, representando alto custo para a sociedade (Raducan et al., 2013; Schaakxs et al., 2013). A gravidade do dano nervoso e o tempo de recuperação pode variar de acordo com a lesão nervosa sofrida (Sunderland, 1991). As lesões podem ser classificadas como: neuropraxia, axonotmese ou neurotmese. A neuropraxia é sua forma mais branda, quando há perda momentânea da condução nervosa. A axonotmese é caracterizada como uma perda da continuidade das fibras axonais, mas com preservação do perineuro; apresenta uma recuperação espontânea e é classificada como uma lesão moderada. Já na neurotmese, há perda da continuidade do epineuro, portanto uma lesão severa, sendo necessária intervenção cirúrgica (Wood et al., 2011).

Os danos nos nervos periféricos são seguidos por degeneração Walleriana, que é a morte das fibras axonais seguida por degradação da bainha de mielina. Fatores inflamatórios e neurotróficos são liberados no local da lesão, para que ocorra a degradação dos debris celulares. Além disso, é iniciada a reestruturação da matriz extracelular, para que o cone de crescimento neural avance, ocorrendo a regeneração do nervo e reinervação de tecidos alvo, como fibras musculares, receptores sensoriais, entre outros (Misgeld, 2005; Chen et al., 2007; Madduri et al., 2010).

Além das alterações ocorridas no nervo, outro tecido que é bastante afetado após uma lesão nervosa periférica é o músculo esquelético. A inervação é um fator crítico para a integridade funcional e estrutural do músculo esquelético (Kostrominova, 2005). Na lesão nervosa ocorre a interrupção da transmissão do estímulo elétrico para os músculos, causando profundas alterações degenerativas nas fibras musculares (Ishido et al., 2004; Hyatt et al., 2005). Dentre as alterações musculares podemos destacar a ausência de contração muscular que provoca uma intensa atrofia muscular (Lieber, 2002; Ishido et al., 2004).

A atrofia muscular ocorre quando há diminuição da síntese e/ou aumento da degradação proteica, através de uma atividade coordenada de vias de sinalização que regulam o tamanho das miofibras a performance contrátil do músculo, como demonstrado na Figura 1.

A principal via de degradação proteica em músculos é a via ubiquitina-proteassoma. Duas E3 ligases chamadas atrogina-1/MAFbx e MuRF-1 foram descritas como atrogenes, ou seja, o aumento da sua expressão estava relacionado ao aumento da atrofia muscular em diferentes modelos, como imobilização, redução da gravidade e desnervação (Bodine et al, 2001). Trabalhos do nosso grupo mostraram que o aumento da expressão gênica de atrogina-1 e MuRF-1 podem ser observados em músculos já após 7 dias de desnervação. Este aumento da expressão é acompanhado por uma drástica perda de massa muscular (Russo et al., 2010).

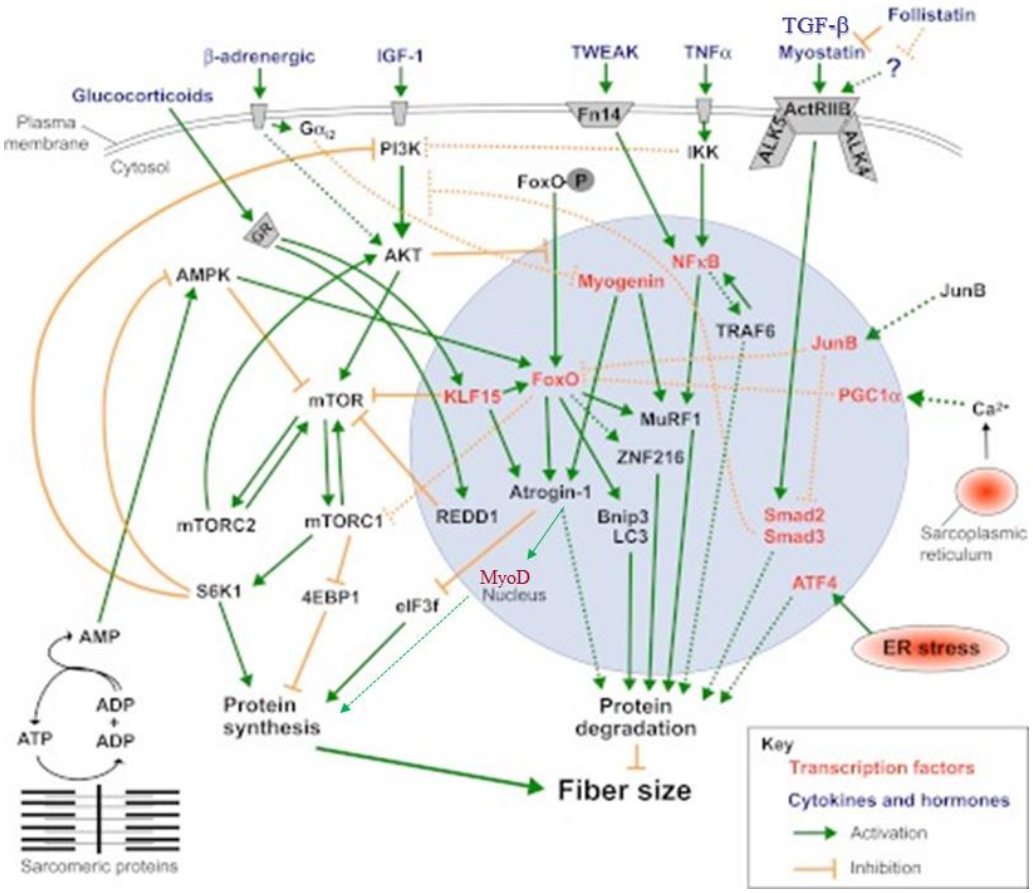

Figura 1. Principais vias sinalização de síntese e degradação de proteínas musculares. Síntese e degradação de proteína são reguladas por diferentes estímulos, na qual ativa múltiplas vias de sinalização, na qual convergem para um intermediário comum e/ou através de um outro mediador. Fonte: adaptado de Bonaldo and Sandri (2013).

Outra importante via de regulação da massa muscular é a via da miostatina. Ela é também conhecida como fator de diferenciação e crescimento-8 (GDF-8), sendo um membro da superfamília do fator transformante do crescimento β (TGF-β) (McPherron et al., 1997). Tem como função conhecida regular negativamente o crescimento do músculo esquelético e

inibir a proliferação de mioblastos e células satélites, sem causar aparente diferenciação e aumento de apoptose (Thomas et al., 2000). Vários mecanismos já foram identificados, na qual a miostatina é capaz de regular o crescimento do músculo esquelético. Ela induz a perda de massa muscular, agindo sobre o sistema proteolítico ubiquitina-proteassomo, evidenciando a regulação positiva de genes como atrogina-1, FOXO1, e MuRF-1; e afeta a massa muscular com a regulação da miogênese, através da diminuição da MyoD (para revisão ver Snijders et al., 2015). Sua avaliação pode ser considerada como um bom indicador da atrofia muscular (Zhang et al., 2006).

Além da atrofia muscular, outra alteração importante é a fibrose que se instala em músculos desnervados. É caracterizada pelo acúmulo anormal de matriz extracelular (MEC), de modo a interferir com a função muscular (Lieber e Ward, 2013). O TGF-β é considerado o principal indutor de processos fibróticos (Verrecchia, Mauviel, 2007), pois o aumento da expressão gênica de TGF-β1 é seguido pelo acúmulo de colágeno em músculos desnervados (Zhang et al., 2008). Além de atuar na atrofia muscular, a miostatina atua na fibrose (Zhu et al., 2007), provavelmente, através da estimulação e da proliferação de proteínas da MEC e dos fibroblastos (Li et al., 2008), como também aumento da resistência dos fibroblastos à apoptose (Bo et al., 2012).

Os processos de remodelamento e reorganização da MEC ocorrem ao redor das fibras musculares atrofiadas (Peviani et al, 2010; Silva-Couto et al, 2012) e, requerem a ação de enzimas proteolíticas, as metaloproteinases (MMPs), que regulam a taxa de produção e degradação de conteúdo de colágeno (Ozawa et al., 2013). As MMPs são uma família de enzimas proteolíticas dependentes de zinco, sintetizadas e secretadas no músculo esquelético pelas células de Schwann, células satélites, e fibroblastos nas junções neuromusculares e ao redor das fibras musculares (Kherif et al., 1998; Carmeli et al., 2004). As MMP-2 e 9 (gelatinases A e B, respectivamente) são as principais metaloproteinases envolvidas na remodelação do músculo esquelético, sendo que mudanças na demanda muscular (hipertrofia/atrofia) provocam alterações em sua atividade proteolítica (Carmeli et al., 2004). Há pouca informação sobre o papel das MMPs durante reinervação muscular, especialmente nas fases iniciais (Ozawa et al., 2013).

As MMPs têm uma interação complexa com citocinas pró-inflamatórias, como fator de necrose tumoral-alfa (TNF-α), que regula a expressão de MMP-2 e MMP-9 (Shubayev et al., 2002). Citocinas pró-inflamatórias são aumentadas quando ocorre perda de massa

muscular, como por exemplo nas doenças crônicas (Costamagna et al., 2015). Um potente indutor de perda de massa muscular esquelética é o sistema TWEAK / Fn14. TWEAK (fator de necrose tumoral indutor de apoptose) é um membro da superfamília TNF-α e parece ser a primeira citocina envolvida na perda de massa muscular em condições de desuso. Ele ativa várias vias proteolíticas e estimula a degradação da proteína miofibrilar in vitro e in vivo (Bhatnagar e Kumar, 2012). Esta citocina atua preferencialmente no seu receptor, o receptor do fator de crescimento de fibroblastos 14 (Fn14).

O papel do sistema TWEAK-Fn14 na atrofia por desuso foi confirmada pelas observações de que a perda da massa muscular esquelética, induzida por desnervação, foi significativamente inibida em ratos knockout para TWEAK (Mittal et al., 2010). Músculos desnervados de ratos que hiperexpressam TWEAK apresentam uma profunda perda de massa (Hindi et al., 2014). Recentemente, Tajrishi e colaboradores (2014) mostraram que o aumento da expressão do gene Fn14 após a desnervação, induziu a atrofia muscular, confirmando o envolvimento deste sistema, em resposta a desnervação. Chen e colaboradores (2012) sugeriram que o sistema de TWEAK / Fn14 aumenta a proliferação celular e a síntese de colágeno através da ativação da via NFκB e aumento da atividade de MMP-9 na fibrose do miocárdio.

Apesar de marcantes, a atrofia muscular e a fibrose não são os únicos processos ativados em músculos desnervados. Há uma tentativa de sinalização das fibras musculares para que novos axônios as reinervem. Este processo dependerá de fatores produzidos tanto pelo nervo como pelo músculo, procurando se reestabelecer a interação neuromuscular. Uma importante mudança que ocorre no músculo após um LNP, visando o reestabelecimento da inervação, é o aumento dos receptores de acetilcolina (RACh) no sarcolema. Os receptores de acetilcolina são normalmente expressos na junção neuromuscular (JNM), em músculos inervados. Contudo, quando a fibra muscular se torna desnervada, estes receptores passam a ser expressos ao longo de toda a fibra. Esta estratégia permite que o axônio possa estabelecer uma nova placa motora em qualquer ponto da fibra muscular e não apenas na placa motora degenerada (Lieber, 2002).

O estabelecimento de conexões funcionais, organizando e controlando o conteúdo de receptores na JNM ocorre em decorrência da modulação de MuSK (receptor tirosina quinase). MuSK como representado na Figura 2, é uma proteína transmembrana que desenvolve um papel crítico na sinalização entre o neurônio motor e o músculo esquelético.

Esse gene é expresso nas células musculares e, uma vez ativada, MuSK estimula vias para o agrupamento e ancoramento dos receptores de acetilcolina e proteínas musculares essenciais para a contração muscular (DeChiara et al., 1996). A diminuição da expressão desse gene leva a desestabilização da JNM e da contração muscular (Kong et al., 2004; Hesser et al., 2006).

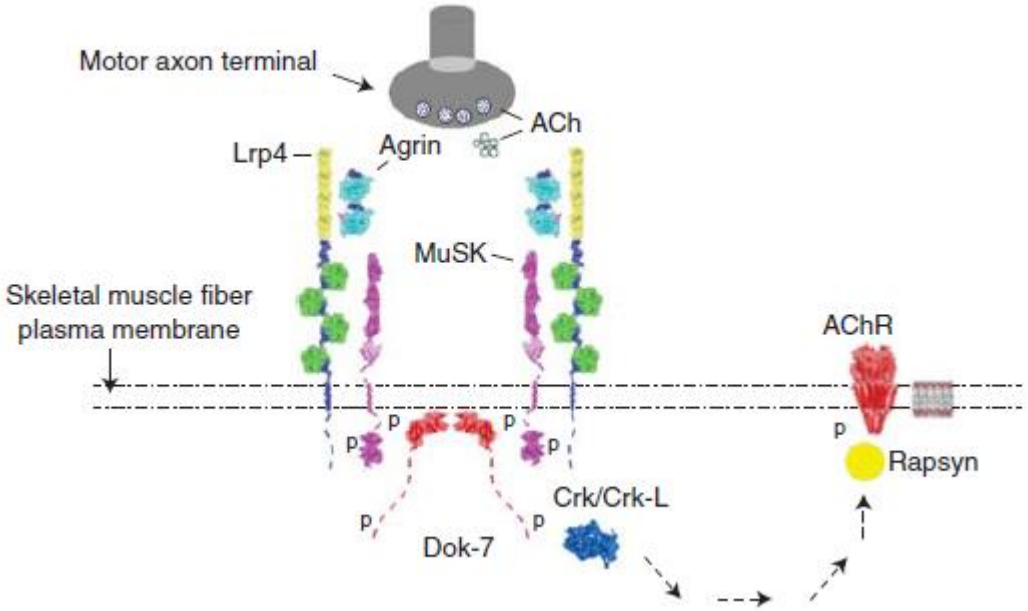

Figura 2. Axônio motor terminal liberando agrina e acetilcolina. Fonte: Burden et al., 2013.

Para que MuSK seja ativada, é necessário que ocorra uma fosforilação induzida por agrina, uma proteoglicana produzida pelos motoneurônios. Parece que é justamente esta ligação entre agrina e MuSK que estabiliza a JNM e aciona a agregação de receptores de acetilcolina (Burden et al., 2011; 2014). Contudo, para que esta agregação ocorra de forma adequada é necessária a atuação de uma proteína de membrana chamada de Dok-7 (Okada et al., 2006). Animais knockout para Dok-7 apresentaram problemas na JNM e declínio na expressão de MuSK. Desse modo, MuSK, Dok-7 e RACh estão envolvidos em uma cascata de regulação e, são indispensáveis para a transmissão do impulso nervoso e o trofismo muscular.

A complexidade dos processos moleculares que regem as adaptações morfofuncionais de músculos esqueléticos após as lesões nervosas periféricas evidencia a dificuldade dos

profissionais da área de saúde para atuar na recuperação destes músculos e facilitar os processos de reinervação. Grande parte das pesquisas desenvolvidas sobre esta temática estão relacionadas a técnicas cirúrgicas de reparo nervoso, contudo, nas últimas décadas, um interesse constante sob o efeito de recursos físicos usados na reabilitação de músculos desnervados pode ser observado.

A reabilitação do indivíduo com músculos desnervados tem como objetivos terapêuticos centrais amenizar a atrofia muscular e a fibrose, bem como estimular o crescimento axonal e reinervação das fibras musculares, receptores e glândulas. Contudo, a literatura científica evidencia uma falta de consenso sobre a eficácia e a segurança da recomendação de recursos físicos para a recuperação de músculos desnervados. Por exemplo, trabalhos experimentais mostraram que o alongamento intermitente de músculos desnervados pode induzir o aumento da fibrose e acentuar a atrofia muscular (Faturi et al., 2015), mas também pode amenizar as alterações musculares deletérias decorrentes da desnervação (Agata et al., 2009) de acordo com os parâmetros de tratamento escolhidos, como número de alongamentos, tempo de descanso entre cada alongamento, etc.

Outro recurso amplamente utilizado na prática clínica para a reabilitação de músculos desnervados é a estimulação elétrica (EE). Geralmente aplicada com eletrodos de superfície, é proposto que a EE seria capaz de induzir contrações musculares, na ausência do estímulo nervoso, e assim deter ou amenizar a atrofia. Também se espera que os estímulos elétricos pudessem guiar o crescimento axonal. Contudo, alguns trabalhos do nosso grupo mostraram que a EE, apesar de modular uma série de genes musculares, não é capaz de deter a atrofia ou a proliferação de tecido conjuntivo de músculos desnervados (Russo et al., 2007; 2008 e 2010), ao contrário, ela pode induzir alterações deletérias, como atrapalhar a recuperação da função e acentuar a atrofia (Gigo-Benato et al., 2010). Estudos de outros grupos mostraram que 200 contrações diárias seriam suficientes para deter a atrofia muscular e a diminuição da força (Dow et al., 2008). Contudo, não há informação sobre a segurança da EE sobre o processo de reinervação muscular. Desta forma, avaliar protocolos clínicos de EE em modelo animal são relevantes para a prática baseada em evidências, garantindo sua eficiência e segurança.

A presente tese tem como principal inovação a investigação, em modelo experimental de desnervação/reinervação de músculos esqueléticos, de um protocolo de EE, semelhante como prescrito na prática clínica do fisioterapeuta. Análises funcionais, morfológicas e

moleculares integradas evidenciaram que a EE, de acordo com os parâmetros utilizados, pode atrapalhar o processo de reinervação, acentuar a atrofia muscular, induzir fibrose e atrapalhar a recuperação funcional em ratos.

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MANUSCRITO 1

Title:

Electrical stimulation delays reinnervation in denervated rat muscle

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Running title: Electrical stimulation and muscle reinnervation

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Abstract

Peripheral nerve injury disrupts the normal functions of neurons and leads to rapid and progressive alterations in structural skeletal muscle causing functional deficits and electrical stimulation (ES) has been recommended to treat denervated muscles. It is not clear if ES can in fact affect reinnervation of denervated muscles. Thus, this study aimed to verify if ES, applied to denervated muscles by surface electrodes, can affect neuromuscular recovery after nerve crush injury in rats. Functional recovery at day 14 post-denervation was significantly lower in DES15d (15-day denervation and ES) compared to D15d (15-day denervation. The DES15d had chronaxie values significantly higher on days 6 and 14 compared to D15d, which indicates a decreased in muscle excitability in the DES15d. ES reduce the accumulation of mRNA of atrogenes, but further increased muscle fiber atrophy in DES15d than other denervated groups. N-CAM content decreased in DES7d compared to other denervated groups. ES altered gene expression of important markers of NMJ organization and maintenance as MuSK, Dok-7, nAChR. All together, these results provide evidence that ES can delay the reinnervation process by modulating factors related to NMJ stability and organization, as well as induced disability and muscle atrophy, and decreased muscle excitability.

Key words: Physiotherapy, neurorehabilitation, skeletal muscle, muscle atrophy, neuromuscular junction.

Introduction

Peripheral nerve injuries (PNI) are usually related to disability and reduced quality of life (Schaakxs, et al., 2013). Muscle denervation post-PNI is a devastating condition, which produces atrophy (Russo, et al., 2010) and paralysis (Wood, et al., 2011). According to severity, PNI can be classified into three main types: neuropraxis (momentary loss of nerve impulse, nerve compression slight), axonotmesis (axon transection with both perineurium and epineurium remaining intact with spontaneous recovery, moderate injury), and neurotmesis (nerve transection where the continuity of the epineurium is disrupted requiring surgical intervention, severe injury) (Wood, et al., 2011).

Regenerative process after moderate and severe PNI includes Wallerian degeneration with distal fragmentation of axon and myelin sheaths made through macrophages and Schwann cells which phagocyte degraded materials. Fine sprouts the proximal axonal end occurs through restructuring the extracellular matrix, allowing axonal reconnection with target cells, maturation of the nerve fiber, reinnervation of muscle fibers, glandules, and sensorial receptors (Navarro, et al., 2007; Misgeld, 2005; Madduri, et al., 2010).

The innervation and reinnervation of skeletal muscle leads to the formation/reorganization of neuromuscular junctions (NMJ). Morphologically, three compartments form the NMJ. The presynaptic compartment where the vesicles containing acetylcholine (ACh) and Schwann cell are found in the nerve endings; the extracellular compartment filled with basal lamina; and the postsynaptic compartment composed of muscle fibers' sarcolemma and junctional folds, as well as the sarcoplasm that provides structural and metabolic support to the postsynaptic region (Engel, 2003; Malomouzh, 2012). The postsynaptic surface sarcolemma contains a high density of nicotinic ACh receptors (nAChRs). When presynaptically released, the ACh activates those receptors, cations are permitted to flow through the nAChR pore, leading to depolarization of the muscle membrane and creating an end-plate potential. If the end-plate potential is large enough, an action potential is reached and ultimately causes muscle contraction (Wang, et al., 2010).

The presence of nAChR in NMJ is critical for muscle function as it determines robustness and reliability of NMJ (Strack, et al., 2015). The nAChR are integral membrane proteins composed of five subunits α,β, δ, γ, ε (alpha, beta, delta, gamma, and epsilon) arranged in rosette form or ion channels (Changeux; Edelstein, 1998; Lindstrom, 2000; Ma,

et al., 2007). Those subunits are presented in the NMJs postsynaptic membrane of the innervated muscle, which are up regulated in response to peripheral nerve lesions.

In addition, there are NMJs proteins to stimulate the pathway of the nAChR clustering and anchoring in the postsynaptic membrane. That is accomplished through coordinated activity, such as muscle-specific receptor tyrosine kinase (MuSK) and cytoplasmic protein Downstream of kinase-7 (Dok-7) (DeChiara, et al., 1996; Okada, et al., 2006). In order to promote synapse stability, the neural cell adhesion molecule (N-CAM) is up-expressed (Polo-Parada, et al., 2001; Vitureir,a et al, 2012 in Chipman, et al, 2014).

For promoting muscle fiber reinnervation, N-CAM expression and protein content around and inside the muscle fiber is increased as well as there is an increase of the nAChR along all the muscle fiber (Wang, et al., 2010; Burden et al., 2011, 2013). N-CAM is a potent regulator of synaptic stability and strongly influences neurotransmission (Polo-Parada et al., 2001; Vitureira, et al, 2012 in Chipman, et al, 2014) (Chipman, et al., 2010; Enriquez-Barreto, et al, 2012; Chattopadhyay,a et al., 2013). Therefore, N-CAM is an important molecule to study the interaction between muscle and nerve.

Furthermore, the post-PNI causes muscle fibers denervation experiencing a complex process of unbalance between anabolic and catabolic processes (Bodine, et al., 2014). That process reflects in both the reduction of the synthesis and the increase of the protein degradation, leading to a muscle mass loss (Jackman and Kandarian, 2004). The expression of muscle-specific genes, involved the ubiquitin-proteasome pathway, such MuRF1 and MAFBx (atrogin-1) is increased in denervated muscles (Bodine, et al., 2001, 2013, 2014).

MuRF1 and MAFBx are muscle-specific E3-ubiquitin ligases that play an important role in the ubiquitin-proteasome system, since those enzymes mark selective substrates to ubiquitination and subsequent degradation by the 26S proteasome. Those two genes are actually the best markers of muscle atrophy and could be considered the master genes to muscle wasting, associated the muscle mass loss (Bodine, et al., 2014).

Due to many neuromuscular disorders that occur in PNI, the research on therapeutic resources that can favor the process of muscle reinnervation and atrophy attenuation are important to clinical practice. Among the physical resources used on those injuries' rehabilitation programs, there is the electrical stimulation (ES). Usually applied with surface electrodes, ES is able to induce muscle contractions in the absence of nerve stimulation. In addition, the ES could also stop or ameliorate atrophy as well as guide axonal growth.

However, some studies from our group showed that the ES, although modulating a variety of muscle genes, is not able to hold atrophy or connective tissue proliferation of denervated muscles (Russo, et al, 2007; 2008 and 2010). On the other hand, it can induce deleterious changes, as impairment of the function recovery; and accentuated muscle atrophy (Gigo-Benato, et al., 2010).

There is controversy about the use of the ES in denervated muscle treatment, some investigators showed 200 daily contractions are effective to prevent mass and strength loss in denervated muscles (Dow, et al., 2007). Others, showed 600 contractions per day, 5 days a week, resulting a higher number of functional motor units and enhanced functional recovery; however, muscle mass and force remained unchanged (Willand, et al., 2015). These studies used implanted electrodes, differing from the clinical practice, and contradicting studies that have shown the muscle electrical stimulation provides no functional benefit with stimulation being applied infrequently or with subthreshold amplitudes being used that could not elicit strong contractions (Sinis, et al., 2009; Gigo-Benato, et al., 2010). On the other hand, Tam and Gordon (2003) and Salmons and cols (2004) suggested that perhaps a more moderate activity protocol is needed to obtain optimal results and concluded that denervated muscles, due to their little excitability, require larger amplitude and pulse duration to obtain contraction.

Because of that, more studies are needed to characterize the effects, to identify more efficient and safe parameters, and to show an effective protocol to prevent muscular atrophy after denervation. Based on previous results (Gigo-Benato, et al, 2010), the hypothesis of this study was that ES sessions may interfere negatively in the muscle reinnervation process and thus impair muscle recovery and functionality, through modulating muscular atrophy pathways (atrogin-1 and MuRF1) as well as maintaining the NMJ (MuSK, Dok-7, N-CAM and nAChRs) in denervated muscles after axonotmesis.

Thus, this study investigated the early functional, neurophysiological, morphological, and molecular changes induced by ES in denervated muscles. The ES was conducted through muscle electrical excitability evaluation and the application through surface electrodes as it is normally used in the rehabilitation of denervated muscles in axonotmesis controlled models. Considering the adaptation of the NMJs after denervation, the results of this study should provide new information to denervated muscle treatment and should help explain the mechanism involved for the electrical stimulation therapeutic effects after nerve injuries.

Material and Methods

Animal care and experimental groups

Thirty-five male 3-month-old Wistar rats weigthing 230 ± 3g 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 Sao Carlos Federal University (033/2014) and conducted in accordance with the *Guide for Care and Use of Laboratory Animals*.

The animals were randomly (by body mass through anova one way) divided into 5 groups (n = 7; the sample calculation was made by GPower software and based on previous studies of our laboratory using CSA with main variable. The groups are shown at the Figure 1: (1) normal (N), control animals without PNI and euthanized 15 days after the beginning of the experiment; (2) 7-day denervation (D7d): animals submitted to PNI by axonotmesis at the right sciatic nerve. They were anesthetized daily. In addition, they were submitted to two electrical excitability evaluations and two motor function evaluations (pre-denervation and the other one on the sixth day); (3) 7-day denervation and electrical stimulation (ES) (DES7d); animals submitted to an injury by axonotmesis at the right sciatic nerve, two electrical excitability evaluations, two motor function evaluations (one pre-denervation and the other one on the sixth day), and daily anesthesia, as well as electrical stimulation (ES). They were euthanized 7 days after nerve injury; (4) 15-day denervation (D15d): animals were submitted to an injury by axonotmesis at the right sciatic nerve; three electrical excitability evaluations and three motor function evaluations (one pre-denervation, one on the sixth day, and the other one on the fourteenth day); they also received anesthesia daily, and were euthanized 15 days after injury; and (5) 15-day denervation and electrical stimulation (DES15d): animals submitted to an injury by axonotmesis at the right sciatic nerve; three electrical excitability evaluations and three motor function evaluations (one pre-denervation, one on the sixth day, and the other one on the fourteenth day); they also received anesthesia and electrical stimulation (ES) daily. They were euthanized 15 days after injury. For more procedure details carried out in the experimental groups see Figure 1.

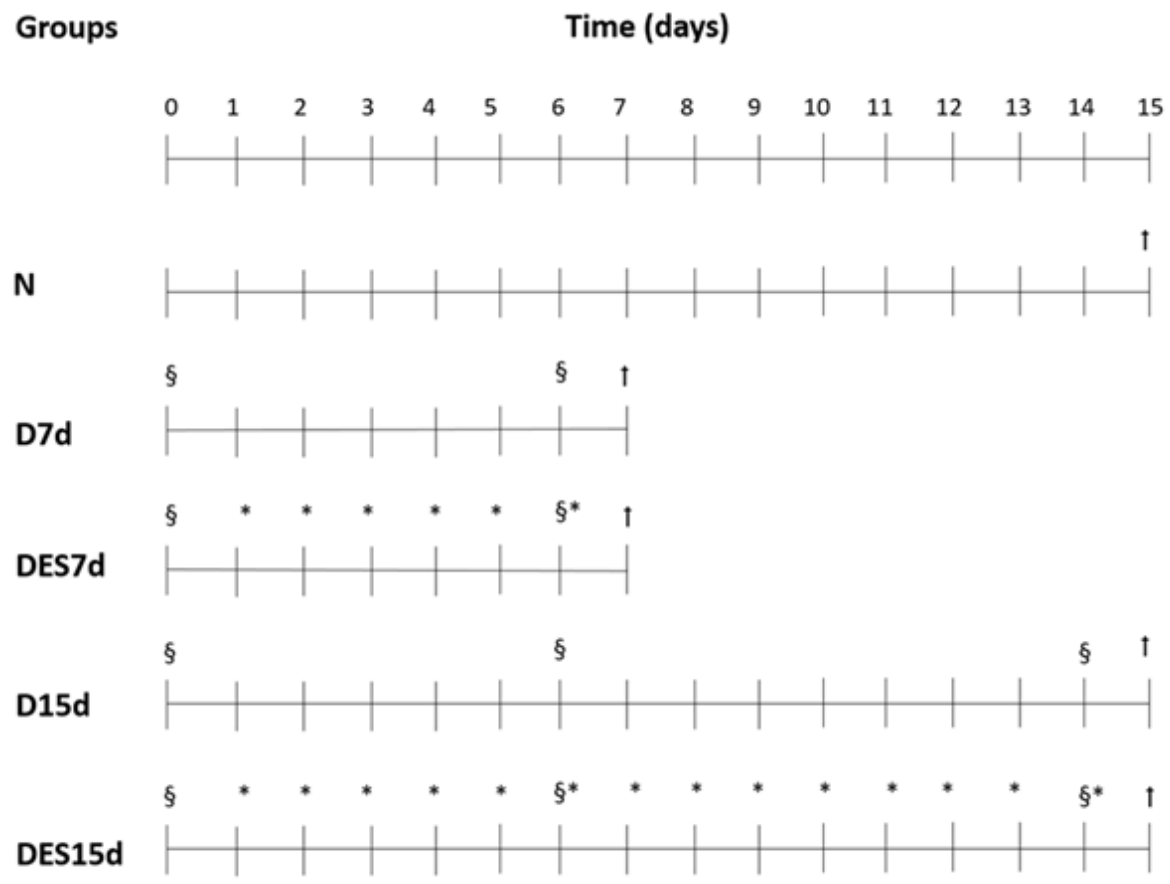


Figure 1. Diagram of the experimental groups. In the upper part of the figure, a time line is show; in the middle and lower portions of the figure, the experimental groups and the procedures are show. At the right, the subtitle. At the end the ES protocol: electrical stimulation schematic representation of the TA muscle including electrical evaluation (EE). EE was performed at the initial moment followed by 4-sets of ES lasting 7.5 min each with a 10 min rest interval between sets. Legend: †: euthanized; §: electrical excitability evaluations; *: electrical stimulation.

Intraperitoneal injections of xylazine (12 mg/kg) and ketamine (95 mg/kg) anaesthetized the rats to the surgical and denervation processes, electrical excitability evaluations, ES treatment, and muscle removal. Two animals of D15d and DES15d groups died during the trial period, probably due to daily exposure to anesthesia. Then, an overdose of the anaesthetic on the 7^{th} or 15^{th} day of the experimental procedures euthanized the rats. Those periods of denervation were chosen because muscle fiber reinnervation processes happen during the first weeks after nerve crush injuries (Bodine et al., 2001 and 2014; Espielhoz, 2001).

Denervation process

The denervation process consisted of a sciatic nerve axonotmesis conducted as previously done by Russo and cols (2010) and Gigo-Benato and cols (2010). Shortly after the rat was anaesthetized, a small incision was made through the skin and fascia (cleaned with 10% povidine iodine) near the right trochanter between the Gluteus maximus and the Femoris biceps muscles to expose the sciatic nerve. Next, a flat-tipped watchmaker´s tweezer number five (D.L. Micof) exerting a 54 N-force (Beer et al, 2011) was used to crush the sciatic nerve for a 30-second period. The nerves were kept moist at 37°C in sterile saline solution throughout the surgical intervention. After that, the fascia and skin were sutured from distal to proximal, using silk thread. That procedure promoted distal hind-limb muscle denervation, including the TA muscle. The same researcher performed the crush to ensure that the damage was inflicted on the same area and with comparable duration and pressure in all denervated animal groups. After surgery, animals were housed in single cages and fed with rat chow and water *ad libitum*. For the first 3 days, paracetamol was added to the animals' drinking water for pain reduction. That model of nerve injury is characterized by axonal degeneration and no sign of muscle reinnervation during the first 10 days after the nerve injury were seen (Carmignoto et al., 1983; Carter et al., 1998).

Motor Function Evaluation

The assessment of nerve function recovery was carried out by calculating the sciatic functional index (SFI), as described by Brain et al. Animals were tested in a confined walkway that was 42 cm long and 8.2 cm wide, with a dark shelter at the end. A 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 the animals were then allowed to walk down 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. The experimental (E) and normal (N) sides got those three measurements. The SFI was calculated according to the following equation:

$$SFI = -38.3(EPL-NPL/NPL) + 109.5\ (ETS-NTS/NTS)+13.3(EITS-NITS/NITS)-8.8$$

Two weeks after surgery, all animals were euthanized with anesthesia overdose; the sciatic nerves and the TA muscles were carefully dissected and immediately weighed at a precision balance (Model 100a; Denver Instruments, Denver, Colorado).

Electrical Excitability Evaluation and Electrical Stimulation Procedures

Rats from the DES groups received ES treatment as previously described by Russo et al. (2004, 2007, 2008 e 2010; Gigo-Benato et al., 2010). ES equipment which allows changes in the electrical parameters was used to assess muscle excitability and for the ES treatment (NeMESys 941, Quark, Brazil). Briefly, before fixing the electrodes, the skin was shaved, cleaned, and covered with a layer of conductive gel, where two electrodes were positioned: an indifferent electrode (5 cm-round self-adhesive electrode) was positioned on the animal´s back, and an active electrode (3 mm-round metallic electrode) small enough to stimulate only the TA muscle. There was no hyperemia observed after the electrical procedures. During the ES procedure, the metallic electrode was in contact with the skin overlying the TA muscle and perpendicular to the muscle fibers. Furthermore, surface electrodes are more commonly used for therapeutic applications, and their position does not require procedures that are invasive to ES (Sheffler, 2007).

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. Afterwards, the chronaxie values were used to determine the ES parameters applied to the TA muscle. These electrical indexes were previously reported (Russo et al., 2007 and 2010; Gigo-Benato et al., 2010).

Briefly, rheobase is the minimal electrical stimulus intensity necessary to produce a muscle contraction (rectangular monophasic current; pulse interval, 1s; interpulse interval, 2s). Chronaxie is the minimal pulse duration necessary to induce a muscle contraction (rectangular monophasic current; interpulse interval, 2s; amplitude, twice the rheobase value). Finally, accommodation is the muscle capacity not to respond to slowly incrementing electrical pulses (exponential monophasic current; pulse interval, 1s; interpulse interval, 2s). The values identified during the electrical evaluation were used to determine the ES parameters for each session, as previously described (Russo et al., 2004, 2007, 2010). An exponential phasic current was used (frequency, 20 Hz; pulse duration, the chronaxie value times two; time On: 3 s; and time off: 6 s). Pulse amplitude necessary to induce a visible

contraction was selected. The selected stimulation frequency granted strong muscle contraction using low current amplitudes, as previously described (Cummings, 1990). Briefly, the D7d and DES7d groups were submitted to muscular excitability evaluations, one pre-denervation, and the other one on the day before euthanasia (sixth day); the same evaluations were conducted to D15d and DES 15d, and another one on the day before euthanasia (fourteen day).

After the muscle excitability evaluation, the amplitude necessary to induce a maximal contraction of the TA muscle (right ankle maximal full flexion) was identified. The ES sessions were applied daily for 6 (DES 7d) or 14 (DES 15d) days, beginning at 24h after denervation, and producing 200 TA muscle maximal contractions. Those maximal contractions were divided into four 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 (Cummings, 1990). Dow et al., 2004 has previously demonstrated that 200 muscle contractions are effective to maintain muscle mass and strength, and Kostrominova et al., 2005 has demonstrated that, to reduce the gene expression generally increased during denervation, 200 muscle contractions are also effective.

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

Sciatic nerve histology

To confirm the injury, nerve histology was performed. The sciatic nerve stump was prepared to a light microscopy. Samples were fixed in 10% formalin for 3h and then washed in phosphate buffered saline (PBS) 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´s Trichrome, following the manufacturer instructions, and observed under a light microscope (Axiolab, Carl Zeiss, Jena, Germany) equipped with a Carl Zeiss AxioCam HRc camera. Whether no nerve injury was detected, the animal was not considered to analyses.

Muscle evaluation

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

Morphometric analysis

Histological cross-sections (10 μm) were obtained in a cryostat microtome (Microm HE 505, Jena, Germany), along the TA muscles middle belly. For morphological evaluation by a light microscope (Axiolab, Carl Zeiss, Germany), tissue sections were stained using Toluidine Blue/1% Borax (TB). One histological cross-section of each TA muscle located in the central region of muscle injury was chosen to measure the cross-sectional area, using a light microscope and a software to evaluate general muscle morphology and morphometry (Axiovision 3.0.6 SP4, Carl Zeiss, Germany). One image central region of all muscle cross-sections was done at 20x low 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 fibers was measured using the Axiovision 4.7.1.0 software (Carl Zeiss, Jena, Germany), with 100-muscle fibers per animal.

Immunofluorescence Analysis

The primary antibody Rabbit anti-N-CAM affinity-purified (1:100 diluition; Catalog No. 5032; Merck Millipore, Bioscence) and the secondary antibody rhodamine red goat anti-rabbit IgG (1:150 dilution; Catalog No. Rb394; Molecular Probes, Eugene; Oregon) were used for immunofluorescence. The muscle cross-sections used for immunostaining were fixed at 4% paraformaldehyde (Sigma P6148) in 0.2 M phosphate buffer (PB) for 10 min in room temperature. Then, they were blocked using 0.1 M glycine in PBS for 5 min, and permeated in 0.2% Triton X-100-PB for 10 min. Subsequently, the sections were incubated at 1% bovine serum albumin (BSA) for 20 min in room temperature to block no specified binding. After that, they were incubated at the primary antibody (diluted in 1% BSA), at 4°C, overnight. After the slides were washed with 0.1 M PB (3 times for 10 min each), they were incubated at the secondary antibody (diluted in 1% BSA) for 2 h in a dark room. Slides were washed in 0.1 M PB (3 times for 10 min each) and mounted with glycerol. Negative control

sections were not incubated at the primary antibody and experimental results were considered only if those controls did not show immunoreactivity.

For qualitative measurements of immunoreactivity, images of five different regions from the mid-belly of the TA muscles were captured using a fluorescence microscope (Axiocam, Carl Zeiss, Jena, Germany) equipped with a rhodamine filter at a final magnification of 40x, with the microscopic setting kept the same for all slides.

RNA Isolation and Analysis

RNA was isolated from one frozen fragment of each TA muscle using 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer instructions. The extracted RNA was dissolved in tris-HCl and ethylenediaminetetracetic acid (TE) pH 7.6 as well as quantified by spectrophotometry. The purity was assessed by determining the absorbance ratio at 260 nm and 280 nm. All samples had 260/280 nm ratios above 2.0. The RNA integrity was confirmed by the ethidium bromide inspection (Invitrogen, Carlsbad, CA) which stained the 18S and 28S ribosomal RNA under ultra-violet light.

Total RNA (1µg) 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^{TM} cDNA Synthesis Kit, Bio-Rad, CA). The reaction contained 4µl of 5x iScript reaction mix, 1 µl iScript reverse transcriptase, and 1µg RNA. The final volume was adjusted to 20 µl with nuclease-free water. The reaction was incubated for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, and hold at 4°C. The resulting cDNA samples were aliquoted and stored at -20°C. The RNA transcript levels for the different experimental and control muscles were analyzed simultaneously, and the reactions were carried out in duplicate in the real time PCR (CFX 96 Touch^{TM} Real Time PCR Detection System, Version 3.0, Bio-Rad, CA), using fluorescent dye SYBR green detection (Thermo Scientific, US) and 180 nM of each primer in a final volume of 40µl. The thermal cycling conditions were 95°C for 10 min and 40 94°C-cycles for 15 s, 60°C for 1 min, and 72°C for 1 min. For each gene, all samples were amplified simultaneously in duplicate using a final volume of 20 µL. Melting dissociation curves were plotted to confirm that only a single product was amplified. Control reactions omitting cDNA template were run to check for reagent contamination. Gene expression was compared among individual samples using the ΔΔCq method. The reference genes for the normalization was chosen using Normfinder. Three genes were used: beta cytoskeletal actin (ACTB), hypoxanthine–guanine

phosphoribosyltransferase (HPRT), and peptidylprolyl isomerase A (PpIa). The sequences used were in table 1.

Table 1: Oligonucleotide primers used for real-time PCR amplification of reverse transcribed RNA

Gene	Sequency of primer (5' – 3')
ACTB	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG
HPRT	F: CTCATGGACTGATTATGGACAGGA R: GCAGGTCAGCAAAGAACTTATAGC
PpIa	F: TGGCAAATGCTGGACCAAAC; R: TGCCTTCTTTCACCTTCCCAA
atrogin-1	F: TACTAAGGAGC-GCCATGGATACT R: GTTGAATCTTCTGGATC-CAGGAT
MuRF1	F: TGTCTGGAGGTCGTTTCCG R: ATGCCGGTC-CATGATCACTT
MyoD	F: GGAGACATCCTCAAGCGATGC R: AGCAC-CTGGTAAATCGGATTG
Dok-7	F: CTTCATCTCTGCAATGACATC R: TGAGGACAAGAAGAAGACTC
MuSK	F: TAATGTGCAAAAGGAAGACG R: TTACAAAGGAACCAAAGGTG
βAChR	F: GGCAACGTCAAAG-TTTCCAT R: CATCGAGTCTCTCCGTGTCA
αAChR	F: AGCCGTCA-TAGGTCCAAGTG R: TCCCTTCGATGAGCAGAACT
δAChR	F: CCGAGGTCTTCTCTCCACAG R: ACCACCAAGACGTCACCTTC
εAChR	F: AGCCATACATGTTCGGAAGG R: GGCTCAACTTCAGCAAGGAC

N-CAM content assessed by Western blot

Protein content of N-CAM was quantified in TA muscle extracts through Western blot assays. Muscle samples were homogenized in lysis buffer (clapboard buffer: 10 mM Tris- HCl, pH 7.4; 150 mM NaCl; 1% Nonideto P-40, 1 % sodium deoxycholate , 0.1 % SDS , 10 % glycerol) with protease inhibitors (10 mM sodiopirofosfato; 10 mM NaF; 1 mM SoV4, 2 mM PMSF, 10 ml leupeptin inhibitor, trypsin, aprotinin and antipain). The samples were shaken and centrifuged for 30 min each at 4°C. The total proteins were quantified using the Bradford method in the spectrophotometer at 550 nm and compared to a BCA concentration curve.Eighty micrograms of total protein were separated through one-dimensional SDS-PAGE and they were stained with Ponceau S red (Sigma Chemical) to confirm the equal loading of each sample. As a second approach to verify similar binding sites loads between the lines lanes, gels were loaded done in duplicate, and one of the gels was stained with Coomassie blue. Proteins were transferred from gel to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific binding sites were blocked with a 3% bovine serum albumin (BSA) solution in phosphate-saline buffer (PBS-T: 0.1 M NaH2PO4·H2O, 0.1 M Na2HPO4·7H2O, 0.15 M NaCl, 0.1% Tween-20, pH 7.4) for 10 min. Following blocking, the membranes were incubated overnight with specific primary antibodies against N-CAM (1:250, Millipore, Temecula, USA; #AB5032), in a 1% BSA solution. After four washing steps with PBS-T, membranes were incubated in a 1:5000 dilution of specific secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which were conjugated with horseradish peroxidase for 10 min. Finally, immunoreactivity protein signals were detected using Chemic DocTM MP Imaging System (Bio-Rad, CA) according to the manufacturer's instructions. The chemiluminescent signal was visualized and quantified by densitometry using the Gene Tools software, version 3.06 (Syngene, Cambridge, UK). The values were normalized by the values obtained through quantifying the alpha-tubulin protein.

Statistical Analysis

The Shapiro-Wilk test and the Levene's test were used to investigate whether the data was normally distributed. One-way ANOVA was used to identify possible differences among groups. The electrical parameters and the motor function evaluation, presented normal distribution, after including variables, through repeated measures (evaluation time) analyses of variance two way ANOVA (group x evaluation time), followed by the Tukey HSD post

hoc test. Differences were considered significant when $p<0.05$. Statistical analysis was performed using the IBM SPSS Statistics 20.

Results

Sciatic Functional Index (SFI)

Table 2 shows the functional assessment results of the posttraumatic sciatic nerve recovery. Interaction (time and group) was observed in SFI ($F_{(1,8)} = 21.792$, $p = 0.001$). The pre-denervation (PD) moment showed no difference among the experimental groups ($p>0.05$). On the 6th day post-PNI, both D15d and DES15d reduced SFI compared to PD values ($p<0.05$), indicating complete absence of sciatic nerve function, with no difference between them (D15d vs DES15d: $p = 0.473$). On the other hand, on the 14th day, D15 started to recover functionality, increasing SFI, while DES15d remained similar to its values observed on the 6th day (Table 2). Furthermore, on the 14th day post-PNI, there was a significant ($p = 0.0001$) difference between DES15d and D15d animals.

Table 2. Functional deficit in the D15d and DES15d groups assessed by sciatic functional index (SFI) calculation.

	Group	Median	Minimum	Maximum
PD	**D15d**	-14.88	-19.05	-8.80
	DES15d	-13.43	-16.39	-8.80
day-6	**D15d**	-60,15	-78.00	-33.46
	DES15d	-57,16	-60.95	-21.31
day-14	**D15d**	-30.66	-40.61	-16.28
	DES15d	-63.73	-65.71	-37.31

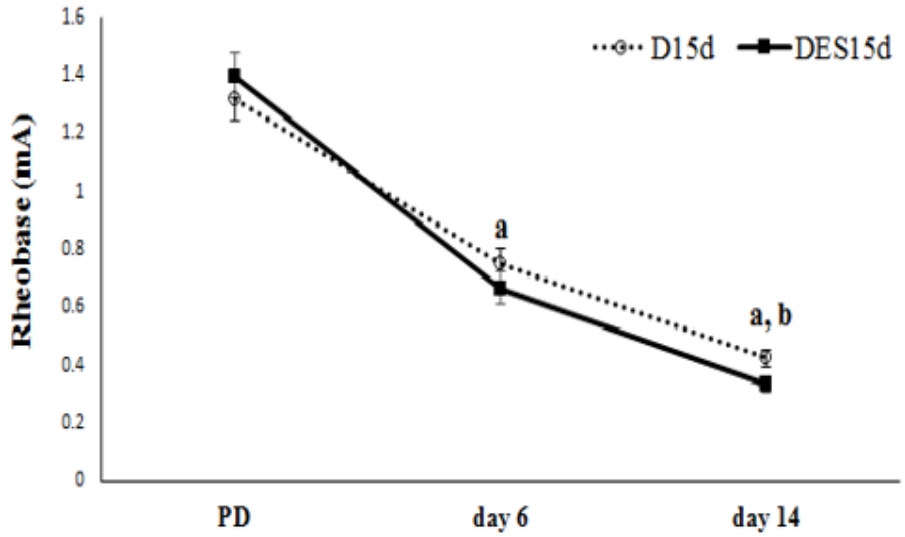
Neuromuscular electrical excitability evaluation

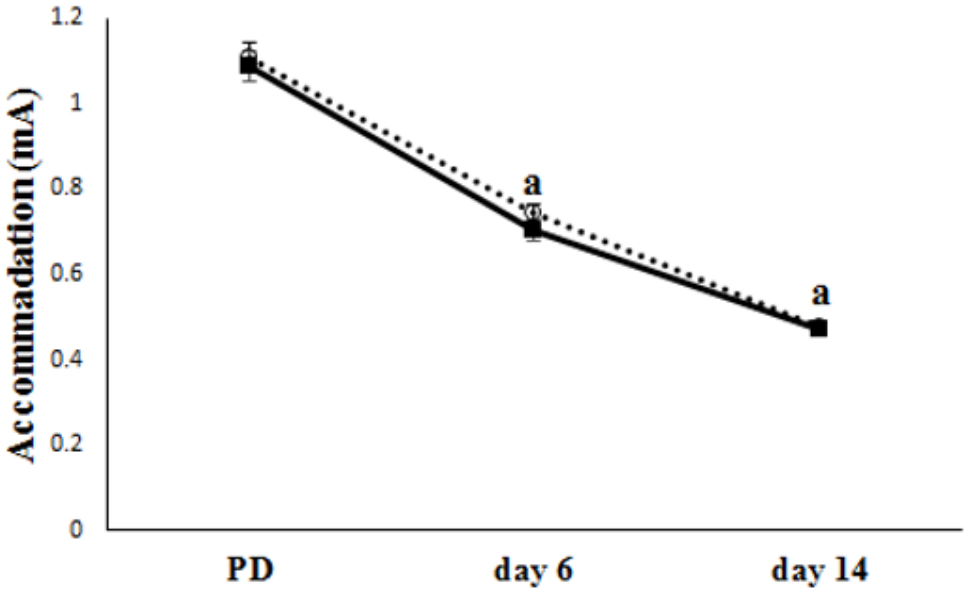
Pre-denervation (PD) measurements of the D and DES groups obtained from the right TA muscles immediately before denervation were considered as normal muscle excitability values for rheobase, chronaxie and accommodation (Figure 2A-C). Interaction between time and group, was observed in chronaxie ($F_{(1,8)} = 42.904$, $p = 0.0001$); 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 day-6 and -14 in both groups (D15d and DES15d), compared to PD values ($p = 0.0001$). Reduction was more pronounced 14 days after denervation, compared to 6 days ($p = 0.0001$; Fig. 2A). Both groups showed statistical difference ($p=0.0001$) in day 6 compared to day 14.

The chronaxie presented an important increase in both D and DES groups throughout 14 days ($p<0.05$) compared to PD values. Denervated electrical stimulated group presented higher chronaxie values on 6^{th} and 14^{th} days compared to denervated not stimulated one (Fig. 2B).

Similar to rheobase, accommodation values decreased after denervation in both denervated groups (submitted or not to ES) compared with PD levels ($p<0.05$). There was no difference between the D and DES groups on the 6^{th} and 14^{th} days post nerve injury ($p>0.05$; Fig. 2C).


D15d
DES15d
Rheobase (mA)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a, b
PD
day 6
day 14


Accommadation (mA)
1.2
1
0.8
0.6
0.4
0.2
0
a
a
PD
day 6
day 14

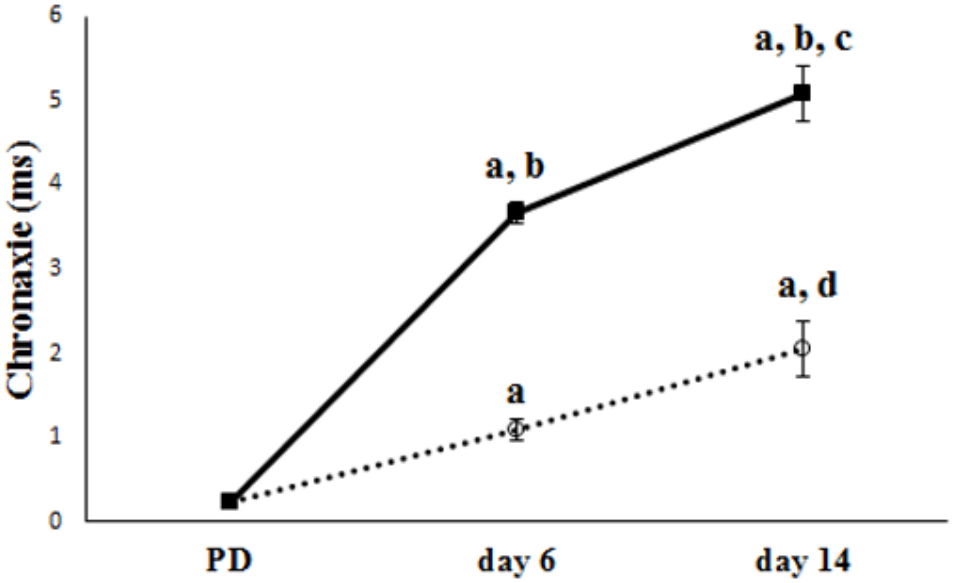

Chronaxie (ms)
6
5
4
3
2
1
0
a, b, c
a, b
a, d
a
PD
day 6
day 14

Figure 2. Electrical variables of the *tibialis anterior* (TA) muscles obtained from electrical evaluations (EE) throughout 15 days of denervation. Pre-denervation data obtained from all denervated groups were considered as normal values of rheobase, chronaxie and accommodation. Data are the mean ± mean standard error (A) Rheobase, (B) Chronaxie, (C) Accommodation: a: represents statistical differences ($p < 0.05$) when day-6 and day-14 was compared to PD state b: represents statistical differences ($p<0.001$) when D15d was compared to DES15d at 6 and 14 days; c: represents statistical differences ($p<0.05$) between group D15d at 6 and 14 days; and d: represents statistical difference ($p<0.01$) between groups DES15d at 6 and 14 days. Chronaxie increased significantly in denervated muscles submitted or not to ES, and DES15d presented higher chronaxie values compared with D15d at days 6 and 14 days. Note that both rheobase and accommodation decreased after denervation.

Body and TA Muscle Weights

All groups increased body weight during the experimental period, nevertheless no difference was noted in the final body weight between D and DES groups (D7d: 258±12.1g; DES7d: 260±16.6g; D15d: 252.9±16.8g; DES15d: 254±13.6g; $p>0.05$); however they were different from the N group (N: 321±32.2g; $p<0.05$). Both D and DES groups decreased their muscle weights compared to the N group ($p<0.05$). The muscle weights presented differences when comparing the DES15d to the other groups ($p<0.05$).

Muscle Morphology and Muscle Fiber CSA

Figure 3 shows intense atrophy in the denervated TA muscle fibers in both the D (7 and 15 days) and DES (7 and 15 days) groups when compared to normal groups. The DES groups also showed central nuclei, and degenerated/regenerated muscle fibers. The CSA of the muscle fiber in DES15d animals was significantly smaller than the one in of denervated animals ($p<0.05$; Fig. 3). Due to e differences in body weight among groups, CSA data was normalized to the body weight of each animal in all groups and expressed as μm^2 per gram of body weight (Fig 4 B).

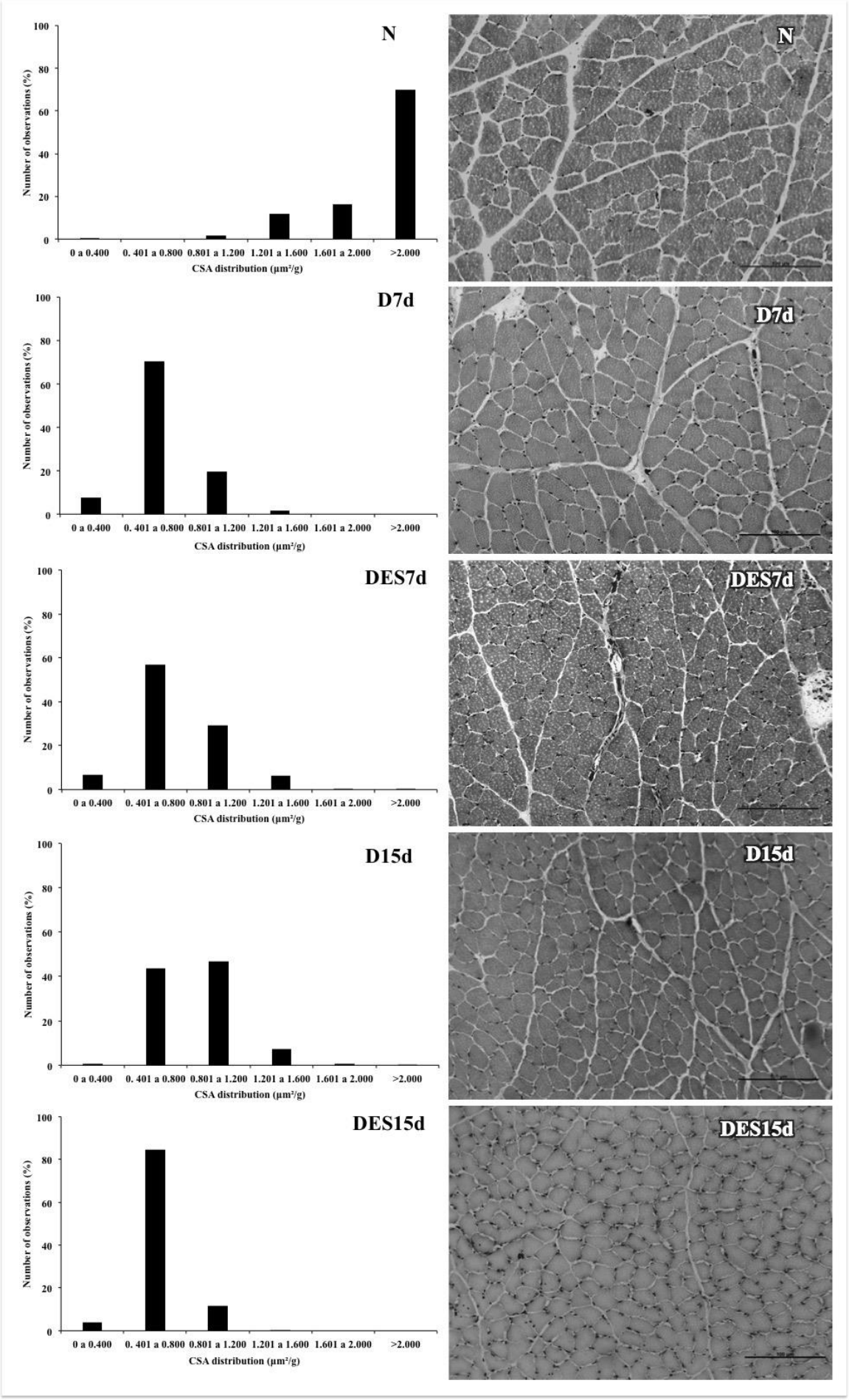

N
D7d
DES7d
D15d
DES15d
Number of observations (%)
0
20
40
60
80
100
0 a 0.400
0. 401 a 0.800
0.801 a 1.200
1.201 a 1.600
1.601 a 2.000
>2.000
CSA distribution (µm²/g)

Figure 3. Muscle fiber cross-sectional area, and muscle morphology of the *tibialis anterior* (TA) muscle. The CSA decreased in all denervated groups, with an important statistical difference between groups D15d and DES15d ($p < 0.05$). Muscle fiber atrophy was observed in all denervated groups when compared to the normal group. However, the muscle fiber CSA distribution levels of denervated muscles were significantly smaller than normal group. Bar: 100 µm.

Nerve morphology

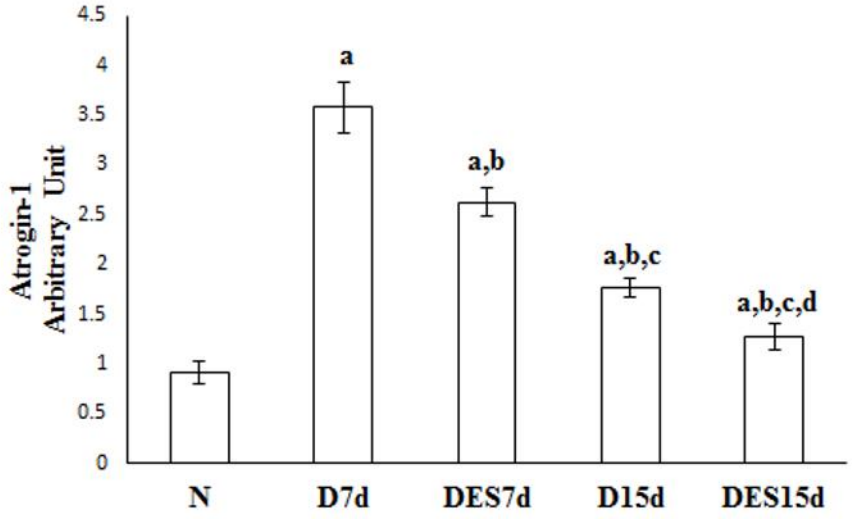
Crush procedure induced the nerve injury. Nerves presented degenerative/regenerative morphology (data not showed).

Atrogin-1, myoD, MuRF1 gene expression by real-time RT-PCR

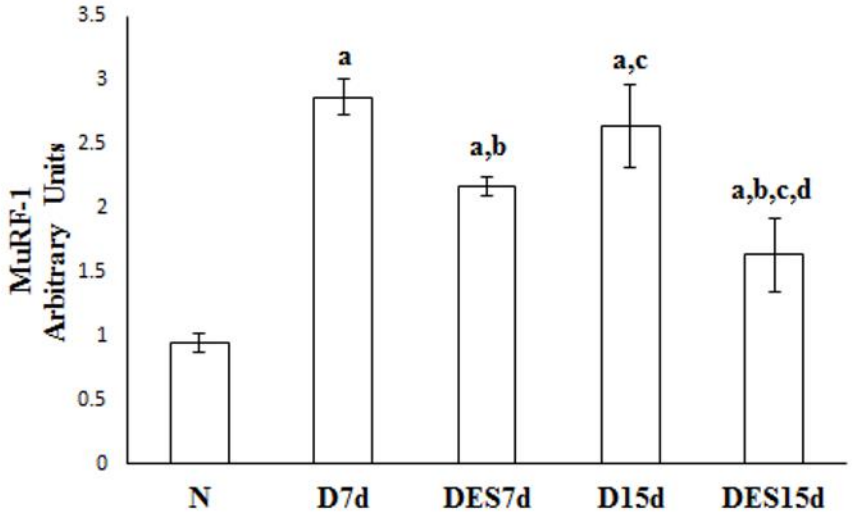
Denervation increased both atrogin-1 (Fig. 4A) and myoD (Fig. 5C) gene expressions compared to N. Electrical stimulation reduced atrogin-1 and myoD the gene expression accumulation compared to denervated non-stimulated groups (Fig. 4A and C). The D7d presented the highest levels of atrogin-1 and myoD compared to the other groups (Fig. 4A and C).

MuRF1 was up regulated in all denervated groups on the 7^{th} and 14^{th} days compared to N (Fig 4 B, $p<0.05$). This increment remained after 15 days of denervation, differently than atrogin-1 and myoD (Fig. 4A and C). The ES decreased the gene expression of both DES7d and DES15d compared to D7d and D15d ($p<0.05$). Interestingly, that DES15d showed the lowest MuRF1 gene expression values compared to D7d and D15d ($p<0.05$, Fig. 4B).

A


Atrogin-1
Arbitrary Unit
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a,b
a,b,c
a,b,c,d
N
D7d
DES7d
D15d
DES15d

B


MuRF-1
Arbitrary Units
3.5
3
2.5
2
1.5
1
0.5
0
a
a,b
a,c
a,b,c,d
N
D7d
DES7d
D15d
DES15d

C

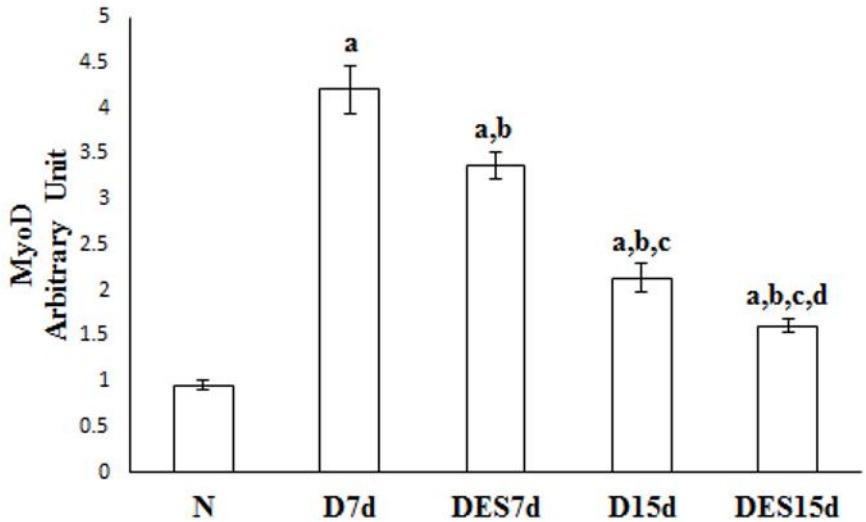

MyoD
Arbitrary Unit
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a,b
a,b,c
a,b,c,d
N
D7d
DES7d
D15d
DES15d

Figure 4. Atrogin-1(A), MuRF1(B), and MyoD (C) mRNA levels in *tibialis anterior* (TA) muscle. Data is the mean ± standard deviation: a: represents a statistical differences compared to N; b: represents statistical differences compared to D7d; c: represents statistical differences compared to DES7d; d: represents statistical differences compared to D15d. Note that the denervated muscle submitted to ES (DES7d and DES15d) showed a decline in the gene expression of atrogin-1, myoD, and MuRF1 when compared to D7d and D15d. The DES15d presented decreased values compared to the D15d ($p<0.05$).

Contents and localization of the N-CAM protein

N-CAM expression in normal muscles was slight and restricted to nerve terminations, vessels, and periphery of a few muscle fibers (Fig 5A). D7d, D15d, and DES15d increased expression of N-CAM, which was observed around atrophied muscle fibers. Quantitative analysis of the N-CAM protein confirmed that increase in D7d, D15d, and DES15d groups compared to N (Fig. 5 B and C), whereas DES7d showed a decrease when compared to D7d, D15d, and DES15d ($p<0.05$). The content of NCAM high maintained after 15 days after denervation.

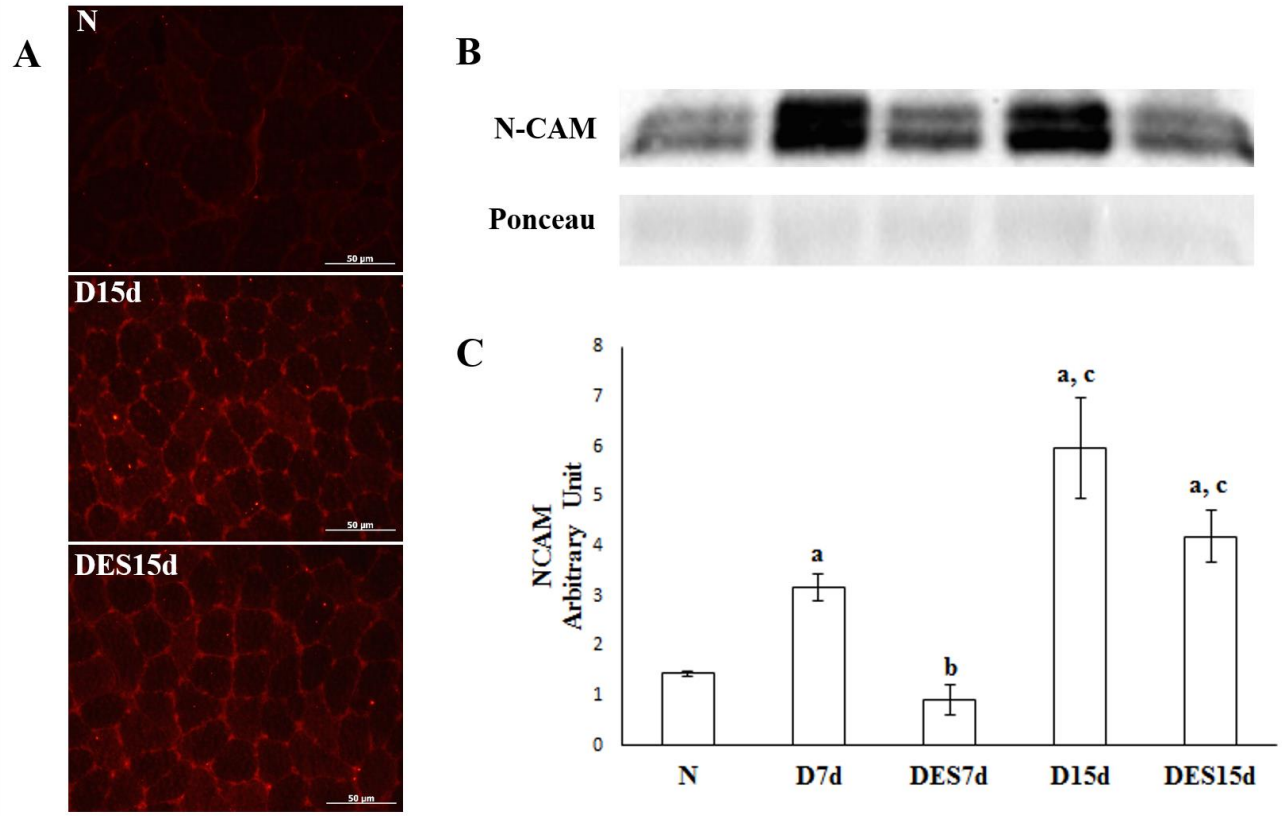


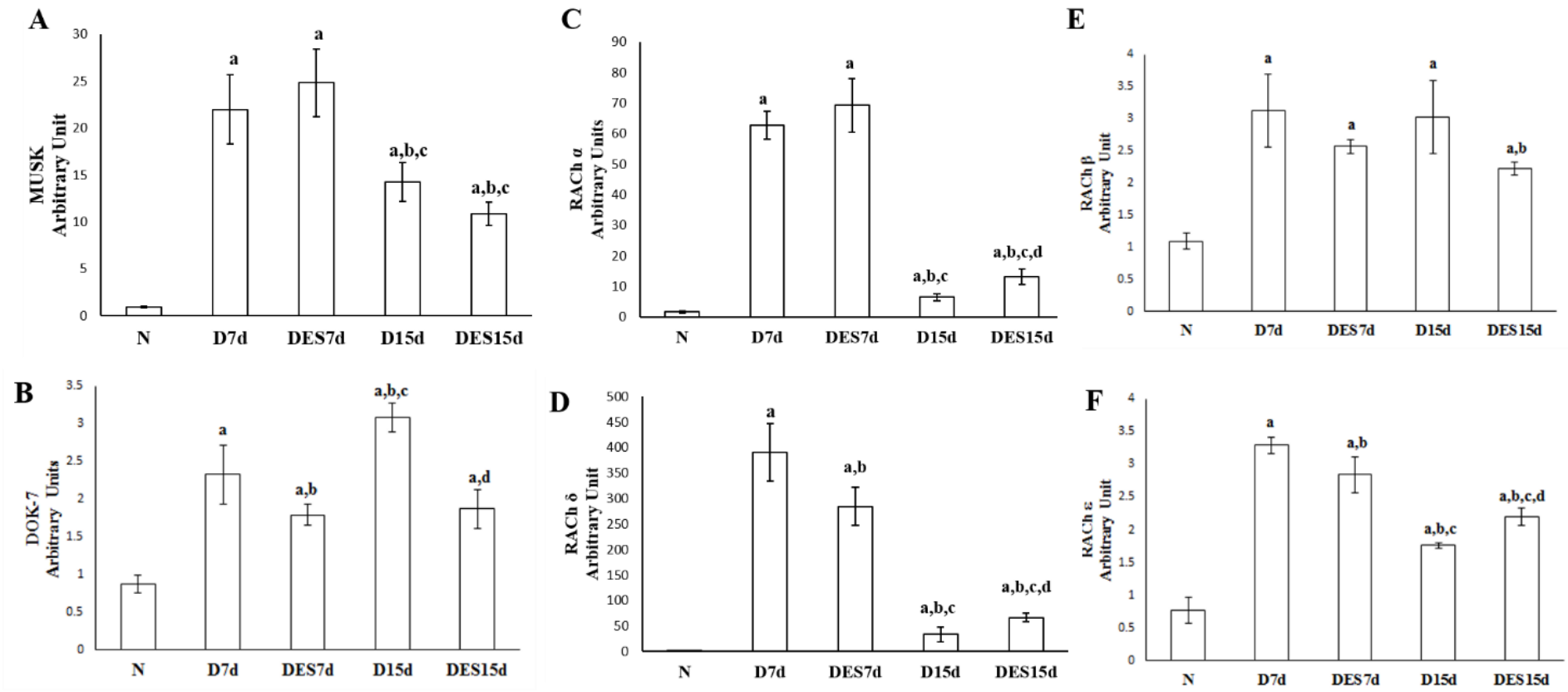
Figure 5. Contents and localization of the N-CAM protein. (A) N-CAM expression and localization in normal muscles (N), in denervated muscles (D15d), and muscles submitted to electrical stimulation (DES15d). (B) Representative blots of N-CAM. (C) Quantitative analysis of N-CAM. D7d, D15d and DES15d groups increased N-CAM contents compared to N; DES7d showed a decrease when compared to D7d, D15d, and DES15d (B and C) ($p<0.05$). Data is the mean ± standard error: a: represents statistical differences compared to N; b: represents statistical differences compared to D7d; c: represents statistical differences compared to DES7d; d: represents statistical differences compared to D15d. Bar: 50μm.

MUSK, DOK-7 and Acetylcholine Nicotinic Receptors gene expression

MUSK, αAChR, and δAChR followed the same pattern, with an increase in all denervated groups (D7d, DES7d, D15d and DES15d) compared to N (Fig 6; $p<0.05$), and a decrease on the 15th day, compared to the 7th day in D7d, DES7d, D15d and DES15d ($p<0.05$). The DES15d showed an increase in αAchR and δAChR expressions, compared to D15d ($p<0.05$).

The Dok-7 and βAchR levels increased in all denervated groups compared to N (Fig 6; $p<0.05$). The ES decreased the levels of Dok-7 on the 7th and 15th days compared to D7d and D15d, respectively, whereas βAchR was down-regulated through ES on the 15th day compared to D15d ($p<0.05$; Fig. 6). No differences were found between DES7d and DES15d ($p>0.05$; Fig. 6).

εAchR showed an increase in all denervated groups compared to N (Fig 6); $p<0.05$). The DES15d increased εAchR levels compared to D15d.


A
MUSK Arbitrary Unit
30
25
20
15
10
5
0
a
a
a,b,c
a,b,c
N
D7d
DES7d
D15d
DES15d
C
RACh α Arbitrary Units
90
80
70
60
50
40
30
20
10
0
a
a
a,b,c
a,b,c,d
N
D7d
DES7d
D15d
DES15d
E
RACh β Arbitrary Unit
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
a
a,b
N
D7d
DES7d
D15d
DES15d
B
DOK-7 Arbitrary Units
3.5
3
2.5
2
1.5
1
0.5
0
a
a,b
a,b,c
a,d
N
D7d
DES7d
D15d
DES15d
D
RACh δ Arbitrary Unit
500
450
400
350
300
250
200
150
100
50
0
a
a,b
a,b,c
a,b,c,d
N
D7d
DES7d
D15d
DES15d
F
RACh ε Arbitrary Unit
4
3.5
3
2.5
2
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1
0.5
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a
a,b
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a,b,c,d
N
D7d
DES7d
D15d
DES15d

Figure 6. The expression levels of MUSK (A), DOK-7 (B), and Acetylcholine Nicotinic Receptors (C,D,E,F) in *tibialis anterior* (TA) muscle. Data is the mean ± standard deviation. a: represents statistical differences compared to N; b: represents statistical differences compared to D7d; c: represents statistical differences compared to DES7d; d: represents statistical differences compared to D15d. Note that the MUSK gene expression was not affected by ES, whereas nAChR and Dok-7 are modulated differently.

Discussion

The present study supported the premise that daily sessions of ES, applied to denervated muscles by surface electrodes, affect muscle reinnervation, compromising muscle recovery by impaired functionality and excitability, accentuated muscle fiber atrophy and altered gene expression and protein content of important markers of muscle tropism, and NMJ organization and maintenance. Briefly, results showed early signs of functional recovery could be observed in the D15d group, but not in electrical stimulated animals (DES15d). Furthermore, ES increased chronaxie values indicating it could interfere with muscular excitability after denervation. Although, ES was also able to reduce 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 reinnervation process. These results corroborated to previous data (Gigo-Benato et al, 2010).

Electrical stimulation has been widely applied clinically as a countermeasure to attenuate muscle atrophy (Adams et al., 2011). Some authors reported that electrical stimulation was effective in ameliorating the muscle atrophy (Fujita et al., 2011). Dow and collaborators (2004 and 2005) showed that the ES could be effective to recover muscle force and mass of denervated muscle. In addition, other recent study showed that 600 contractions per day 5 days a week, immediately following nerve transection and repair, enhance electrophysiological and behavioral recovery (Willand et al., 2015). Authors argue there is a relationship between the daily number of contractions and their distribution to induce trophic effects on denervated muscles, indicating the 200 to 800 contractions per day, could be a good parameter to consider (Dow et al., 2004). However, although in the present study we have used 200 contractions, and it seems that this parameter (number of contractions) is not enough to ameliorate muscle atrophy.

Tomori and collaborators (2010) demonstrated that relatively low intensities of ES (4 mA or 8 mA) are able to improve muscle membrane disruptions, and the 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 the current intensity is also an important aspect. The current intensity used in the present 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 in the present study. Recently, Pieber and collabors (2015) recommend parameters of ES, similar to the present study, to stimulate denervated *tibialis anterior* muscles in humans. They supported the use of monophasic triangular pulses current with width pulse of 200 ms.

Denervated muscles are different from innervated muscles in their response to electrical stimulations. Rheobase and chronaxie values provide efficient muscle excitability parameters, and are consistently elevated in denervated muscle, indicating lower excitability compared to normal muscle (Geuna et al., 2009). Because of this, the denervated muscles request higher pulse duration (chronaxie) to contract (Salmons et al., 2004; Russo et al., 2007). The increase in chronaxie values confirmed that all muscles were denervated on the sixth and fourteenth day post-denervation; however, ES accentuated the chronaxie increment, indicating that these muscles are hypoexcited; so there is a decreased velocity of electrical conduction. (Salmons et al, 2005).

Distinct results between studies and the present one could also be related to other parameter differences of ES protocols. For example, previous reports from Willand and cols (2013; 2015) showed that ES ameliorated muscle atrophy, force loss, and did not affect muscle reinnervation. The ES protocol consisted of biphasic train of 400 ms duration (40 pulses at 100 Hz) with a pulse width of 200 s per phase. They induced 600 muscle contractions using implanted electrodes in a 1-hour session, considering 6 s off between each contraction. Daily sessions were performed 5 times week. Comparing the present parameters to the Willand's ones, some aspects should be considered: type of current (monophasic x biphasic and burst), type of electrode (surface x implanted), relationship between time On and Off (1:2 x 1:6), and number of sessions (daily sessions x 5 sessions/week). Thus, these differences should be considered in future studies to provide the best parameters to electrical stimulate denervated muscles using surface electrodes.

Nevertheless, caution is necessary when comparing ES of denervated muscles to ES of injured nerves. A randomized controlled clinical trial verified brief ES applied to crushed median nerves in humans is able to accelerate nerve regeneration and target reinnervation (Gordon et al., 2010). Elzinga and colls (2015) investigated the effect of brief ES (1h, 20 Hz, 0.1 ms, supra-maximal stimulation) on nerve regeneration after delayed nerve repair in rats. The results indicated 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 and colleagues (2014) showed in mice that two hours of high-TENS frequency (100 Hz), after nerve crush injury, is deleterious for regeneration, whereas low-TENS frequency (10 Hz) improved nerve regeneration. All together, these studies showed that brief ES of injured nerves could be beneficial to recovery according to ES frequency used. Low frequencies ($\leq$ 20 Hz) seem to be safe and efficient to improve nerve regeneration, rather than high frequencies (100 Hz).

Regarding functional data, a previous report (Gigo-Benato et al., 2010) 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 to choose the best electrical parameters of stimulation. The TA denervated muscle was electrically stimulated in alternated days during 15 days. They showed twenty contractions induced by ES were able to impair functional recovery. The results herein corroborated with Gigo-Benato and collaborators (2010) data. It is possible to suppose that both muscle hypoexcitability (higher levels of chronaxie) and accentuated muscle atrophy are related to disability of walking in DES15d.

The present study also provided interesting data about molecular regulation of denervated muscles due to ES. Corroborated to previous reports, which showed that ES can down-regulate the atrogenes atrogin-1 and MuRF-1, and also myoD (a member of myogenic regulatory factors, MRFs) in denervated muscles (Russo et al, 2007, 2010). Kostrominova et al. (2005) also showed that denervated muscles submitted to ES down-regulated many different genes, including those related to hypertrophy and atrophy pathways, and also the MRFs, supporting the present results. Because gene expression can be post-transcriptionally regulated, it is possible the amount of mRNA of atrogenes is enough to maintain atrophy. Furthermore, the effects of ES in different pathways such as inflammatory, lysosomal, and synthesis control of denervated muscles must be investigated to understand the biological

meaning of such modulation. Nevertheless, according to the presented data, it is possible to conclude that down-regulation of atrogenes or myoD caused by ES is inefficient to ameliorate muscle mass, though accentuating the atrophy process and disability due to denervation.

One innovative aspect of the present study is that ES, applied by surface electrodes, can modulate gene expression and protein content of reinnervation and NMJ stability and maintenance related factors. About N-CAM protein localization and content, ES neither decreased N-CAM protein content nor changed its localization on denervated muscles; instead ES remained N-CAM content maintained at DES15d compared to D15d. This result should be interpreted in a scenario of accentuated atrophy, muscle hypoexcitability, and dysfunction. In innervated muscles, N-CAM is mainly expressed at the NMJ, however, after nerve injury, N-CAM is expressed around muscle fibers signalizing to the reinnervation process (Gigo-Benato et al., 2010). In addition, studies that investigated hindlimb muscle reinnervation after crush nerve injury showed muscle fibers are reinnervated 2 weeks after injury (Brown and Tronton, 1978), differing the present results of N-CAM maintained increased in D15d. On the other hand, Kostrominova el al. (2005) showed chronic ES reduced N-CAM in denervated muscles in the absence of reinnervation.

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 the 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 nAChRs clustering even in the absence of agrin (for revision see Tintignac et al., 2015). The present results showed denervation increase MuSK and Dok-7 gene expression. Previous study showed MuSK overexpression is sufficient to maintain neuromuscular synapses in amyotrophic lateral sclerosis model, improving muscle function (Péres-García & Burden, 2012). Thus, the increase of MuSK and Dok-7 expression in denervated muscles could be associated to the integrity of NMJ.

The expression of nAChRs in long-term denervated gastrocnemius muscles was described previously (Ma et al., 2007). They showed that alpha, gamma, delta and epsilon nAChR subunits are significantly upregulated in the first week post-denervation, but then returned to normal levels within the first month by affecting NMJ stabilization. The present results differed in part from Ma and collaborators ones. First, the increase of all nAChR

remained different from control until the 2nd week post-denervation, except by gamma subunit, which was not detected in the present study. Second, beta subunit expression remained elevated D15d compared to D7d, whereas other nAChR subunits started to decrease their expression at 15 days. The persistence of beta subunit expression could be related to the clustering of nAChR at the NMJ (Zhang & Peng, 2011). Finally, the absence of gamma subunit detection could be related to the type of injury (axonotmesis versus neurotmesis – Ma et al., 2007).

On the other hand, ES changed MuSK, Dok-7 and nAChRs gene expressions. It is possible that muscle contractions or membrane depolarization caused by ES are mimicking 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 time investigated. Beta nAChR subunit was reduced at 15 days, whereas delta and epsilon subunits are downregulated by 7 days. The ES is delaying gene expression accumulation. It is possible to hypothesize ES is affecting deleterious NMJ organization, because functional, excitability, and tropism deficits were also observed. Zhang and Peng (2011) showed electrical field elicits molecular reactions in muscle cells to trigger AChR clustering via MuSK/rapysin pathway. However, the effects of these changes caused by ES on terminal nerve sprouting or in different factors related to reinnervation process, such as agrin, Lrp4 and rapsyn should be verified in future studies.

Briefly, ES is widely used and disseminated by physical therapists to treat denervated muscles, however, the present study showed ES applied similar to 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 to potential nerve regeneration. In conclusion, ES applied to muscles by surface electrodes delays reinnervation process by modulating factors related to NMJ stability and organization, as well as induced disability and muscle atrophy, and decreased muscle excitability.

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MANUSCRITO 2

Title:

Electrical stimulation, based on chronaxie, increases fibrosis and modulates TWEAK/Fn14, TGF-β/myostatin, and MMP pathways in denervated muscles.

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The study has not been submitted previously in any form.

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Running title: Electrical stimulation and connective tissue.

Abstract

Objective: To investigate the effects of electrical stimulation (ES) of rat denervated muscles in neuromuscular performance, muscle atrophy and fibrosis formation.

Design: Wister Rats were into divided Normal (N); 7 or 15-day denervation (D7d and D15d); D7d or D15d plus ES (DES7d and DES15d, respectively). Sciatic nerves were crushed causing muscle denervation. Two hundred muscle contractions were electrically induced daily by surface electrodes, considering muscle chronaxie. Sciatic functional index was used to determine neuromuscular performance during walking. The muscle fiber cross-sectional area and percentage of connective tissue were assessed by light microscopy. Molecular markers of extracellular matrix production and remodeling were evaluated. Metaloproteinase (MMP) activity was assessed by zymography, and TWEAK, Fn14, myostatin and TGF-β gene expressions were determined by qPCR.

Results: ES impaired natural recovery of walking at 15 days. In addition, ES induced fibrosis and accentuated muscle atrophy in denervated muscles. Although ES reduced the accumulation of TWEAK and myostatin expressions, it up-regulated Fn14 and TGF-β in a time dependent manner. ES also increased the activity of MMP-2 compared to the other groups ($p<0.05$).

Conclusions: ES applied to denervated muscles induced muscle fibrosis and atrophy, as well as loss performance. The TWEAK/Fn14 system, TGF-beta/myostatin pathway and MMP activity seems to be involved in these deleterious changes.

Key-words: electrical stimulation, metalloproteinase, fibrosis and inflammation.

Introduction

Peripheral nerve injuries (PNI) cause a serious loss of neuromuscular performance due to the establishment of severe muscle atrophy and fibrosis. Loss of muscle mass can prolong a patient´s recovery time and increase rehabilitation time[1]. In addition, fibrosis, characterized by abnormal accumulation of extracellular matrix (ECM) can also interfere with function[2]. Remodeling and reorganization processes in the ECM surrounding the atrophied muscle fibers[3,4] require the action of proteolytic enzymes, called metalloproteinases (MMPs), which regulate the rate of production and degradation to collagen content[5].

MMPs are a zinc-dependent proteolytic enzyme family, synthesized and secreted in the skeletal muscle by Schwann cells, satellite cells, and fibroblasts into the skeletal muscle at the neuromuscular junctions (NJM) and around the muscle fibers[6]. The MMP-2 and 9 (A and B gelatinases, respectively) are the main metalloproteinases involved in remodeling skeletal muscle, and changes in the muscular demand cause alterations to the proteolytic activity[6]. During muscle reinnervation, especially in the early phases, there is little information about the role of MMPs[5]. Thus, MMP activity can be considered a good marker for investigating peripheral nerve recovery.

MMPs have a complex interaction with pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), which regulate the expression of MMP-2 and MMP-9[7]. Pro-inflammatory cytokines are increased when there is a loss of muscle mass, such as in chronic diseases[8]. A potent inducer of skeletal muscle wasting is the TWEAK – Fn14 system. TWEAK (tumor necrosis factor-like weak inducer of apoptosis) is a member of the TNF-α superfamily and appears to be the first cytokine involved in muscle wasting in disuse conditions. The system activates various proteolytic pathways and stimulates the degradation of myofibril protein both in vitro and in vivo[9]. This cytokine acts preferentially on the receptor fibroblast growth factor inducible 14 (Fn14). The role of the TWEAK-Fn14 system in disuse atrophy was confirmed by observations that denervation-induced loss of skeletal muscle mass and function was significantly inhibited in TWEAK-KO mice[10]. However, on exposure to atrophy-triggering stimuli, such as denervation, TWEAK transgenic mice showed more profound loss of skeletal muscle mass[11]. Recently, Tajrishi and colleagues[12] (2014) showed an increase in Fn14 gene expression after denervation induces muscle atrophy, confirming the involvement of this system in response to denervation. Chen and colleagues[13]

(2012) suggested that the TWEAK/Fn14 system increases cell proliferation and collagen synthesis by activating the NFκB pathway and increasing MMP-9 activity in myocardial fibrosis.

The transforming growth factor-β (TGF-β) cytokine is considered the master switch for the induction of the fibrotic process. The increase in the TGF-β1 expression is followed by increased collagen deposition in long-term denervated posterior cricoarytenoid muscles[14]. A member of the TGF-β superfamily is myostatin [2,8,15]. Multiple mechanistic pathways through which myostatin is able to regulate skeletal muscle growth have already been identified. Various mechanisms have been identified, in which the myostatin is capable of regulating the growth of skeletal muscle. It induces the loss of muscle mass, acting on the ubiquitin-proteasome proteolytic system, and up-regulating atrogenes such as atrogin-1, FOXO1 and MuRF-1. In addition, it affects the muscle mass by regulating myogenesis and decreasing the MyoD (for review see Snijders et al., 2015).

Early studies on animal models verified the effects of physical interventions used during neurorehabilitation and showed that there was little evidence concerning the efficacy and safety of therapeutic interventions used in clinical practice for denervated muscle treatment. Pre-clinical studies of interventions that stimulate nerve growth and reduce or prevent muscle atrophy are still needed. For example, Gigo-Benato and colleagues[16] (2010) showed that electrical stimulation (ES), based on chronaxie and applied by surface electrodes in denervated muscles, seems to impair neuromuscular recovery after nerve crush injury. Furthermore, ES associated with or without stretching neither prevents muscle atrophy nor avoids connective tissue proliferation in denervated muscles, although it regulates muscle pathways, such as ubiquitin-proteasome, transcription factors (such as the myogenic regulatory factors), myostatin, and the MMPs[17]. On the other hand, ES seems to induce degeneration/regeneration cycles in denervated muscle, activating satellite cells[17,18] and probably the inflammatory signalization.

Studies in humans showed some beneficial effects of functional electrical stimulation (FES) when it is applied to denervated muscles. For example, home-based protocols of functional electrical stimulation (FES), applied to long-term paraplegic individuals, seem to improve partially quadriceps muscle mass and force. The authors discussed, as immediate benefits for the patients, the improved cosmetic appearance of lower extremities and the enhanced cushioning effect for seating[19]. Methodological differences in the type of ES

application, parameters used, type of PNI and time post-injury, making generalizations about ES effects precipitated. In order to investigate parameters for the success or failure of rehabilitation interventions, pre-clinical studies are necessary.

Considering the premises of clinical practice, this experimental study formulated the following hypothesis: ES can accelerate neuromuscular performance recovery, avoid or reduce muscle atrophy and fibrosis formation in denervated muscles. These morphofunctional changes in denervated muscles might be associated to the modulation of muscle mass control, inflammatory and ECM remodeling biomarkers. Therefore, the main aim of the present study was to investigate the effects of ES on neuromuscular performance, muscle morphology and molecular regulation in animals submitted to axonotmesis.

Material and Methods

The animals were randomly divided into 5 groups (n = 7, per group). The sample calculation based on previous studies of our laboratory, and considered as the main outcome the muscle fiber cross-sectional area. GPower software was used, considering a power of 0,8. Thirty five 3-month-old male Wistar rats weighing 230 ± 3g 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 at the "XXXXXX" (033/2014) and conducted in accordance with the *Guide for Care and Use of Laboratory Animals*. Functional, morphological and molecular analyses were performed in animals submitted to axonotmesis.

The animals were randomly divided into 5 groups (n = 7): (1) normal (N), control animals without peripheral nerve injuries (PNI) and without electrical stimulation (ES), euthanized 15 days after the beginning of the experiment; (2) 7-day denervation (D7d): animals submitted to PNI by axonotmesis at the right sciatic nerve and without ES. They were anesthetized daily. In addition, they were submitted to two motor function evaluations (pre-denervation and the other one on the sixth day); (3) 7-day denervation and ES (DES7d); animals submitted to an injury by axonotmesis at the right sciatic nerve, two motor function evaluations (one pre-denervation and the other one on the sixth day), daily anesthesia, and daily electrical stimulation (ES). They were euthanized 7 days after nerve injury; (4) 15-day denervation (D15d): the animals were submitted to an injury by axonotmesis at the right sciatic nerve and without ES.; three motor function evaluations (one pre-denervation, one on

the sixth day, and the other one on the fourteenth day); they also received anesthesia daily, and were euthanized 15 days after injury; and (5) 15-day denervation and electrical stimulation (DES15d): animals submitted to an injury by axonotmesis at the right sciatic nerve; three motor function evaluations (one pre-denervation, one on the sixth day, and the other one on the fourteenth day); they also received anesthesia and electrical stimulation (ES) daily. They were euthanized 15 days after injury.

Intraperitoneal injections of xylazine (12 mg/kg) and ketamine (95 mg/kg) anaesthetized the rats to the surgical and denervation processes, electrical excitability evaluations, ES treatment, and muscle removal. Then, an overdose of the anaesthetic on the 7^{th} or 15^{th} day of the experimental procedures euthanized the rats. These periods of denervation were chosen because muscle fiber reinnervation processes happen during the first weeks after nerve crush injuries.

Denervation process

The denervation process consisted of a sciatic nerve axonotmesis conducted as previously done by Russo and colleagues (2010) and Gigo-Benato and colleagues (2010). Briefly, a flat-tipped watchmaker´s tweezer number five (D.L. Micof) exerting a 54 N-force was used to crush the sciatic nerve for a 30-second period. This procedure promoted distal hind-limb muscle denervation, including the TA muscle. The same researcher also crushed it to ensure that the damage was inflicted on the same area and with comparable duration and pressure in all denervated animal groups. This model of nerve injury is characterized by axonal degeneration and no sign of muscle reinnervation during the first 10 days after the nerve injury was founded.

Motor Function Evaluation

The assessment of nerve function recovery was carried out by calculating the sciatic functional index (SFI), as previously described by Gigo-Benato and colleagues (2010). Briefly, the animals were tested in 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 the animals were then allowed to walk down 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. Three measurements were taken on the experimental (denervated limb) side (E) and the normal side (N). Measurements from D15d and DES15d in a pre-denervation moment were considered as control values. Previous studies reported that SFI is a very stable measure throughout time in control/non-denervated animals[4]. The SFI was calculated according to the following equation:

$$SFI = -38.3(EPL-NPL/NPL) + 109.5\ (ETS-NTS/NTS)+13.3(EITS-NITS/NITS)-8.8$$

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

Electrical Stimulation Procedures

Rats from the DES7d and DES15d groups received ES treatment as previously described by Russo et al. 2008 e 2010; Gigo-Benato et al., 2010. ES equipment which allows changes in the electrical parameters was used to assess muscle excitability and also for the ES treatment. During the ES procedure, the metallic electrode was in contact with the skin overlying the TA muscle and perpendicular to the muscle fibers. Furthermore, surface electrodes are more commonly used for therapeutic applications, and their position does not require procedures that are invasive to ES.

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. Afterwards, the chronaxie values were used to determine the ES parameters applied to the TA muscle. These electrical indexes were previously reported[16,20]. A monophasic current was used (frequency, 20 Hz; pulse duration, the chronaxie value times two; time On: 3 s; and time off: 6 s). A pulse amplitude necessary to induce a visible

contraction was selected. The selected stimulation frequency granted strong muscle contraction using low current amplitudes, as previously described[21]. Values of pulse duration used in this protocol are presented as supplemental digital content (Figure S1).

The amplitude necessary to induce a maximal contraction of the TA muscle (right ankle maximal full flexion) was identified. The ES sessions were applied daily for 6 (DES 7d) or 14 (DES 15d) days, beginning at 24h after denervation, and producing 200 TA muscle maximal contractions. These maximal contractions were divided into four 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[21]. Dow et al., 2004[22] previously demonstrated that 200 muscle contractions are effective to maintain muscle mass and strength, and Kostrominova et al., 2005 demonstrated that, to reduce the gene expression generally increased during denervation, 200 muscle contractions are also effective.

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

Muscle evaluation

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

Muscle morphology and muscle fiber cross-sectional area (CSA)

Histological cross-sections (10 μm) were obtained in a cryostat microtome (Microm HE 505, Jena, Germany), along the TA muscles of the middle belly. Muscle sections were stained using Toluidine Blue/1% Borax (TB). A light microscope (Axiolab, 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 20x low 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 muscle fibers side by side was measured using the Axiovision 4.7.1.0 software (Carl Zeiss, Jena, Germany). The same picture was used for the percentage of connective tissue assessment.

Percentage of connective tissue

For analysis of intramuscular connective tissue, a planimetry system was used with scoring points, and the quantification was accomplished by means of a reticulum, containing 182 straight-line intersections. The percentage connective tissue density of the connective tissue was calculated by dividing the sum of the number of coincident points in the straight-line intersections in the connective tissue endomysium and perimysium by the total number of points[20,23].

Zymography

Muscle samples were homogenized in a lysis buffer (clapboard buffer: 10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Nonideto P-40, 1 % sodium deoxycholate , 0.1 % SDS , 10 % glycerol) with protease inhibitors (10 mM sodiopirofosfato; 10 mM NaF; 1 mM SoV4, 2 mM PMSF, 10 ml leupeptin inhibitor, trypsin, aprotinin and antipain). The samples were shaken and centrifuged for 30 min each at 4°C. The total proteins were quantified using the Bradford method in the spectrophotometer at 550 nm and compared to a BCA concentration curve. Briefly, equal amounts of total protein (80 μg) per animal were subjected to electrophoresis in duplicate. Zymography analysis was performed according to the current methodology[3]. The molecular mass of gelatinolytic activities was determined by comparing it to the reference protein molecular mass marker Kaleidoscope Prestained Standards (Bio-Rad Laboratories, Hercules, CA). Activity bands were identified following a previous description according to their molecular weights (72 kDa: pro-MMP-2; 66 kDa: intermediary-MMP-2; 62 kDa: active-MMP-2). The bands found in all groups were 72– 62 kDa, suggesting the activation of MMP-2. Data are expressed as a concentration of MMP-2 (i.e. the totality of

integrated optical density for the MMP-2 pro-enzyme, intermediate and active forms) and MMP-2 active form. Densitometric quantitative analysis of the protein bands in the zymography gels were performed using Image Lab ™ Software of ChemiDoc XRS (Bio-Rad, Hercules, CA).

RNA Isolation and Analysis

RNA was isolated from one frozen fragment of each TA muscle using 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer´s instructions. The extracted RNA was dissolved in tris-HCl and ethylenediaminetetracetic acid (TE) pH 7.6, as well as quantified by spectrophotometry. The purity was assessed by determining the absorbance ratio at 260 nm and 280 nm. All samples had 260/280 nm ratios above 2.0. The RNA integrity was confirmed by the ethidium bromide inspection (Invitrogen, Carlsbad, CA) which stained the 18S and 28S ribosomal RNA under ultra-violet light.

Total RNA (1µg) 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, CA). The reaction contained 4µl of 5x iScript reaction mix, 1 µl iScript reverse transcriptase, and 1µg RNA. The final volume was adjusted to 20 µl with nuclease-free water. The reaction was incubated for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, and hold at 4°C. The resulting cDNA samples were aliquoted and stored at -20°C. 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 PCR (CFX 96 Touch™ Real Time PCR Detection System, Version 3.0, Bio-Rad, CA), using fluorescent dye SYBR green detection (Thermo Scientific, US) and 180 nM of each primer in a final volume of 40µl. The thermal cycling conditions were 95°C for 10 min and 40 94°C-cycles for 15 s, 60°C for 1 min, and 72°C for 1 min. For each gene, all samples were amplified simultaneously in duplicate using a final volume of 20 µL. Melting dissociation curves were plotted to confirm that only a single product was amplified. Control reactions omitting a cDNA template were run to check for reagent contamination. Gene expression was compared among individual samples using the ΔΔCq method. The reference genes for the normalization were chosen using the

Normfinder. Three genes were used: beta cytoskeletal actin (ACTB), hypoxanthine–guanine phosphoribosyltransferase (HPRT), and peptidylprolyl isomerase A (PpIa). The sequences used were: for rat ACTB (forward: GTAACCCGTTGAACCCCATT reverse: CCAT-CCAATCGGTAGTAGCG); HTPR (forward: CTCATGGACTGATTATGGACAGGA; reverse: GCAGGTCAGCAAAGAACTTATAGC); Ppla (forward: TGGCAAATGCTGGA-CCAAAC; reverse: TGCCTTCTTTCACCTTCCCAA); TWEAK (forward: GCTACGA-CCGCCAGATTGGG; reverse: GCCAGCACACCGTTCACCAG); Fn14 (forward: AAGTGCATGGACTGCGCTTCTT; reverse: GGAAACTAGAAACCAGCGCCAA); TGFβ (forward: CCCCTGGAAAGGGCTCAACAC; reverse: TCAACCCAGGTCCTTCCTAAA-GTC); Myostatin (forward: CTACCACGGAAACAATCATTACCA; reverse: AGCAACA-TTTGGGCTTTCCAT).

Statistical Analysis

The Shapiro-Wilk test and the Levene's test were used to investigate whether the data were normally distributed. One-way ANOVA was used to identify possible differences among the groups. In order to verify if there was a correlation between connective tissue with MMP-2 and, connective tissue with SFI, we used the Pearson correlation coefficient (r). The Spearman test was used to correlate connective tissue proliferation or the muscle fiber cross-sectional area to SFI. The motor function evaluation presented normal distribution, although repeated measures (evaluation time) analyses of variance two-way ANOVA (group x evaluation time), followed by the Tukey HSD post hoc test. Differences were considered significant when $p<0.05$. Statistical analysis was performed using the IBM SPSS Statistics 20.

Results

Sciatic Functional Index (SFI)

Figure 1 shows the functional assessment results of the posttraumatic sciatic nerve recovery. Interaction (time and group) was observed in SFI ($F_{(1,8)} = 21.792$, $p = 0.001$). The pre-denervation (PD) moment showed no difference among the experimental groups ($p>0.05$). On the 6th day post-PNI, both D15d and DES15d reduced SFI compared to PD values ($p<0.05$), indicating complete absence of sciatic nerve function, with no difference between them (D15d vs DES15d: $p = 0.473$). On the other hand, on the 14th day, D15 started to recover neuromuscular performance, increasing SFI, while DES15d remained similar to its values observed on the 6th day (Fig. 2). Furthermore, on the 14th day post-PNI, there was a significant ($p = 0.0001$) difference between DES15d and D15d animals.

FIGURE 1

Body Mass, TA Muscle Mass, Muscle Fiber CSA and Percentage of Connective Tissue

All groups increased body mass during the experimental period ($p < 0.05$). Nevertheless, the final body mass of the N group was around 20% heavier than the denervated ones (Table 1; $p < 0.05$). There was no difference in the final body mass among the denervated stimulated or non-stimulated groups (Table 1. $p > 0.05$). The D7d, DES7d, D15d and DES15d groups decreased their muscle masses compared to the N group ($p = 0.0001$). The muscle masses presented differences when comparing the DES15d to the other groups ($p<0.05$; Table 1). The CSA of the muscle fiber in DES15d animals was significantly smaller than other denervated animals (compared with D7d and DES7d: $p = 0.0001$; D15d: $p = 0.004$ Table 1). The CSA data was normalized to the body mass of each animal in all groups and expressed as μm^2 per gram of body weight (Table 1).

Connective tissue showed an increased in all groups compared to the N group ($p = 0.0001$). DES15d presented a considerable gain to connective tissue compared to the other groups ($p = 0.0001$ for to D7d, DES7d and D15d; Table 1).

TABLE 1

Qualitative morphology from DES15d is presented as supplemental digital content data (Figure 2S). Muscles showed increased of perimysium, degenerated muscle fibers and central nuclei.

TWEAK, TGF-β, Fn14 and myostatin gene expression by real-time RT-PCR

The TWEAK, Fn14, TGF-β and myostatin (Fig. 2) gene expressions are increased in denervated muscles (submitted or not to ES) compared to N ($p<0.05$). Denervation per se up-regulated TWEAK, Fn14 and TGF-β expressions at day-15 compared to day-7 (Fig.2; $p<0.05$), whilst myostatin presented the same expression at 7 and 15 days (Fig. 2; $p>0.05$).

Regarding the effects of ES, it down-regulated TWEAK and myostatin gene expressions in a similar way, in both DES7 and DES15d compared to their denervated non-stimulated controls (Fig. 2, $p<0.05$). On the other hand, Fn14 and TGF-β were up-regulated by ES at 7 days compared to D7d (Fig. 2, $p<0.05$). At 15 days, ES reduced Fn14 expression but not TGF-β when compared to D15d (Fig. 2).

FIGURE 2

MMP activity

MMP-9 was not detected by gelatin zymography, thus, only the total MMP-2 activity was accessed. Denervation increased MMP-2 activity at 15 days compared to N and D7d (Fig. 3, $p<0.05$). However, ES increased total MMP-2 activity at 7 days compared to N and D7d. In addition, ES at 15 days MMP-2 reached the highest activity and was different from all the groups ($p<0.05$, Fig. 3).

FIGURE 3

Correlation coefficient

No correlation was observed between connective tissue and MMP-2 ($r = - 0.169$; $p = 0.786$), connective tissue and SFI ($r = - 0.154$; $p = 0.804$) or muscle fiber cross-sectional area and SFI ($r = 0.205$ and $p = 0.741$).

Discussion

This study demonstrated that denervated muscles submitted to the ES protocol have impaired neuromuscular performance during walking, muscle atrophy and fibrosis probably associated to modulations of TWEAK/Fn14, TGF-beta/myostatin expressions and MMP-2 activity in rats. Although this is not a mechanistic study to establish cause-consequence relationships, these results supported the idea that, according to the parameters used, ES can impair muscle recovery/reinnervation after PNI. These results are not expected because, according to clinical practice, ES should reduce muscle atrophy and fibrosis formation in denervated muscles. As far as we know, this is the first demonstration that ES can increase fibrosis in denervated muscles.

Regarding neuromuscular performance, the present data indicated ES impaired natural walking recovery after axonotmesis. Because no correlations in walking performance/muscle mass/proliferation of connective tissue were observed, it can be supposed that the innervation process is affected by fibrosis or an inflammatory process. A previous report[16] also showed that ES impaired walking recovery in rats with sciatic nerve crush injury. They treated the denervated muscle with electrical stimulation using surface electrodes, and considered the muscle excitability to choose the best electric stimulation parameters The TA denervated muscle was electrically stimulated every other day for 15 days. They showed twenty contractions induced by ES and were able to impair functional recovery. This study corroborates with data from Gigo-Benato et al (2010). In addition, future studies should investigate the effects of ES on different muscles related to walking activity, such as soleus and grastrocnemius muscles, and verify correlations between muscle trophism and neuromuscular performance.

Electrical stimulation has been widely applied clinically as a countermeasure to attenuate muscle atrophy[24]. Some authors reported that electrical stimulation was effective in

attenuating the muscle atrophy[25]. Dow and colleagues (2004 and 2005) showed that the ES can be effective to recover muscle force and mass of denervated muscle. In addition, another recent study showed that 600 contractions per day 5 days a week, immediately following nerve transection and repair, enhances electrophysiological and behavioral recovery[26]. Authors argue there is a relationship between the daily number of contractions and their distribution to induce trophic effects on denervated muscles, indicating the 200 to 800 contractions per day, could be a good parameter to consider. The main idea of these studies above was to mimic the number of muscle contractions performed by animals during normal exploratory activities in the cage. However, this study considered this previous information[23] and induced 200 contractions, and it seems that it is not only this parameter (number of contractions) which is important to attenuate muscle atrophy. Rest times not longer than 8 hours between stimulation sets are important to prevent atrophy[27]. These aspects of the ES protocol, similar to this study, applied for short periods, can be harmful to denervated muscles.

The number of contractions can also be discussed based on clinical studies. Kern and cols[28] (2004) showed that progressive regime of FES can partially recover long-term denervated muscles in paraplegic individuals. They used a biphasic rectangular current, with 150 to 200 ms and up to 200 mA of amplitude, to elicit quadriceps muscle contractions. The progressive regime of ES was divided into 4 phases according to muscle excitability recovery. During the first months, 150 muscle contractions were induced following the protocol: frequency of 2 Hz, delivered for 15 min/day (time on: 4s; time off: 2s), 5 days/week. Muscle fatigue was considered to stop the therapy session. Our data also corroborated with a previous report[28], showing the duration of pulse was increased after denervation, confirming muscle hypoexcitability. In addition, Kern and colleagues (2004) also recommended for the ES progression to perform sets of muscle stimulation, rather than continuous periods of ES. On the other hand, it is also important to consider that the present study used a monophasic exponential polarized current to elicit muscle contractions whilst the previous study[28] used balanced non-polarized currents. Polarized currents provoke ionic changes below the electrodes and can be harmful to tissues (Cummings, 1990) and could explain fibrosis formation and degenerative/regenerative cycles in muscle fibers. Finally, the target tissue for ES in PNI does matter. For example, acute brief low-frequency ES after surgical nerve repair promoted axonal regeneration in diverse types of peripheral nerve injuries in humans[26,29].

Therefore, discrepancies either ES parameterization or target tissue (damaged nerve versus denervated muscle) might be related to the success or not among studies.

Much attention has been given to the TWEAK/Fn14 system recently because of its association to cancer and cachexia. However, Mittal and colleagues[10] (2010) described the effects of up-expression of TWEAK in skeletal muscle. They reported that the TWEAK FN14 receptor expression was up-regulated after casting or denervation. It was also observed that the mRNA level of Fn14 was increased approximately six-to sevenfold in denervated gastrocnemius muscle compared with contralateral innervated muscle, suggesting a potential role of the TWEAK-Fn14 pathway in skeletal muscle atrophy[10]. This study showed both TWEAK and Fn14 expressions were up-regulated by denervation. In addition, the reduction of TWEAK mRNA accumulation caused by ES was not followed by any protective effect. It seems either post-transcriptional regulation is involved or the up-regulation of Fn14 is enough to trigger atrophy cascades. Therefore, the accentuated muscle atrophy observed DES7d and DES15d group might be explained in part by the up-regulation of Fn14 gene expression at 7 days.

The contribution of denervated muscle fibrosis to contracture formation and the impact on functionality in humans is still uncertain[30]. It is postulated, after nerve injury occurs, increase in the collagen content surrounding atrophied muscles might contribute to muscle stiffness, reduced muscle force generation and disability[5,30]. The present study also corroborates with this idea because no correlation was detected between the neuromuscular performance and proliferation of connective tissue or muscle atrophy. It seems that the muscle fiber structure and composition may be involved in contracture formation and disability in humans[30]. Regarding molecular regulation of muscle fibrosis, one important biomarker related to this process is the TGF-β pathway. It is well documented the increase in TGF-β triggers the secretion of collagen type I and III[31,32], and fibronectin. In addition, TGF-β signaling suppresses miR-29, this then relieves the repression of HDAC4 (histone deacetylase 4), suppresion of myogenesis occurs, and the myoblasts transdifferentiate to myofibroblasts (for review see MacDonald & Cohn, 2012)[33].

The presence not only of atrophied fragmented muscle fibers, but also fibrosis in the groups submitted to ES can support the premise that inflammation is increased in these muscles. It has been demonstrated that the increase in TWEAK and Fn14 results in the activation of NFκB leading to an increase in the MuRF1 expression, an E3 ligase from the

ubiquitin-proteossome system, and reduces the MyoD, a myogenic regulatory factor[9]. It is well known that the TWEAK/Fn14 system can increase cell proliferation and collagen synthesis[10,13] via NFκB[13] corroborating with the present data. The increase in the percentage of connective tissue due to ES observed at 7 and 15 days, compared to their denervated non-stimulated controls, could also be involved in the overexpression of TGF-beta and Fn14 at 7 days. It can be hypothesized that denervated muscles with inherent weaknesses are prone to alternating cycles of degradation and self-renewal due to electrical stimulation. As a result, there is a persistent inflammation that promotes the formation of fibrosis (reviewed in MacDonald and Cohn, 2012). It is also relevant to discuss that different pathways could also be involved in this process, such as activins. According to Chen and colleagues (2014), activins A and B promote muscle atrophy and fibrosis. They showed that actin and myostatin are highly catabolic factors via ActRIIB receptor activation. They also discussed that activin and the TWEAK/ FN14 system together play an important role in the induction of muscle atrophy Future studies should verify if activins are up-regulated by ES in denervated muscles.

Regarding the increase in MMP-2 activity in denervated electrical stimulated muscles, it was already described that TGF-β is related to the formation of scar tissue during muscle repair and regulates the production of ECM-degrading enzymes, such as collagenases and gelatinases (MMP-2), as well as the synthesis of enzymes that inhibit the degradation of ECM, such as tissue inhibitors of metalloproteinases and plasminogen activator inhibitor[35]. Thus, the up-regulation of TGF-β observed at DES7d could be related to MMP-2 activity observed in DES7d and 15d, groups. Koskinen and colleagues (2000) reported that ES can elevate the gelatinolytic activity of pro-MMP-2, suggesting an increase in muscle type IV collagen degradation capacity in spinal cord injury individuals, which indicates an adaptive, remodeling process of intramuscular collagen response to ES of skeletal muscle. Peviani and colleagues 2010 showed an increased MMP-2 gene expression and activity protein in rat denervated muscle after concomitant stretching and ES procedures, suggesting ECM remodeling. On the other hand, Russo and colleagues (2008) reported similar increments of MMP-2 activity in stimulated and non-stimulated denervated muscles. Differences among ES protocols and types of PNI can explain these discrepancies, such as the number of contractions, time of stimulations, post-injury time and severity of neurological damage.

Myostatin inhibits myogenesis and promotes atrophy of adult skeletal muscle by suppressing the proliferation of satellite cells and turning them into myofibroblasts[37]. This study showed ES reduced myostatin mRNA accumulation in denervated muscles. Similar to

previous studies[17], reduced levels of myostatin expression did not reflect in protective effects on muscle mass and ECM proliferation in denervated stimulated muscles. Interestingly, a recent report showed intermittent stretching applied to denervated muscles was able to induce fibrosis 15 days post-axonotmesis. These deleterious changes were followed by increasing TGF-β and myostatin mRNA at 7 and 15 days post-denervations, respectively, corroborating to the idea of the mechanical susceptibility to injuries of denervated muscles.

Some limitations should be considered for the present study. The presence of sham surgery group could strength the results. However, previous studies, which used the same surgical procedures, did not report differences concerning neuromuscular excitability, morphology and molecular biomarkers of muscle trophism and ECM remodeling in rats when compared to the control group[20]. In addition, not only can muscle atrophy and fibrosis be considered as the only cause of limiting functional recovery after PNI, but also the rate of axons regrowing and chronicity of PNI[38]. Studies might consider the assessment of different protocols of nerve and muscle ES after PNI. A variety of ES regimes should also be tested, trying to minimize fatigue of denervated muscles. Sets of ES with fewer muscle contractions might be better for muscle plasticity than high number of muscle contractions in one set of ES.

This study clearly showed that ES, according to the parameters chosen, can impair neuromuscular recovery post-PNI, inducing impaired neuromuscular performance, accentuated atrophy and fibrosis. In addition, new information was provided about how the ES can modulate molecular pathways involved in mass muscular control and ECM production/turnover. The Fn14 and TGF-β genes seems to be more sensitive to ES/muscle contraction in denervated muscles and might be involved in deleterious findings. Finally, the increase in MMP-2 activity brings new insights into ECM remodeling. Despite the fact that this data cannot be generalized for all types of ES, warnings should be given to rehabilitation teams when recommending ES to treat denervated muscles.

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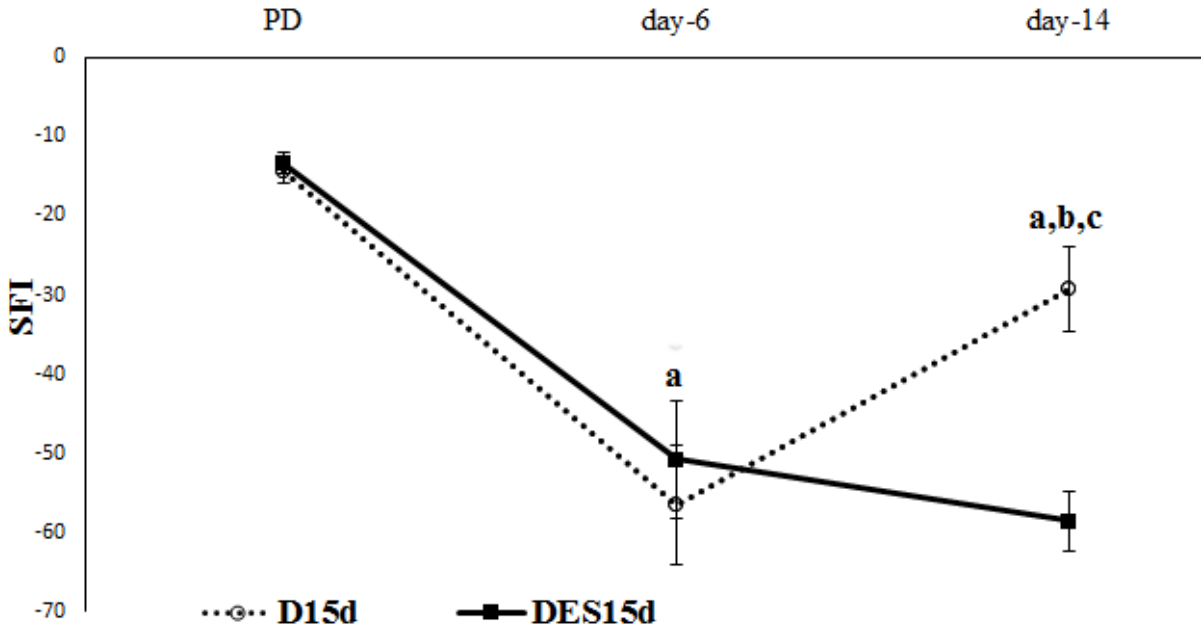

Figure 1. Functional deficit in the D15d (no-ES) and DES15d groups assessed by sciatic functional index (SFI) calculation. In the pre-denervation (PD) state, functional was considered normal in both groups. Data are the mean ± mean standard error; a: represents statistical differences ($p < 0.05$) when day-6 and day-14 was compared to the PD state; b: represents statistical differences ($p = 0.032$) when day-6 was compared to day-14 at D15d (no-ES) group; c: represents statistical differences ($p = 0.001$) when D15d (no-ES) was compared with DES15d. Note that DES15d did not recover the performance at 15 days of crush nerve injury.

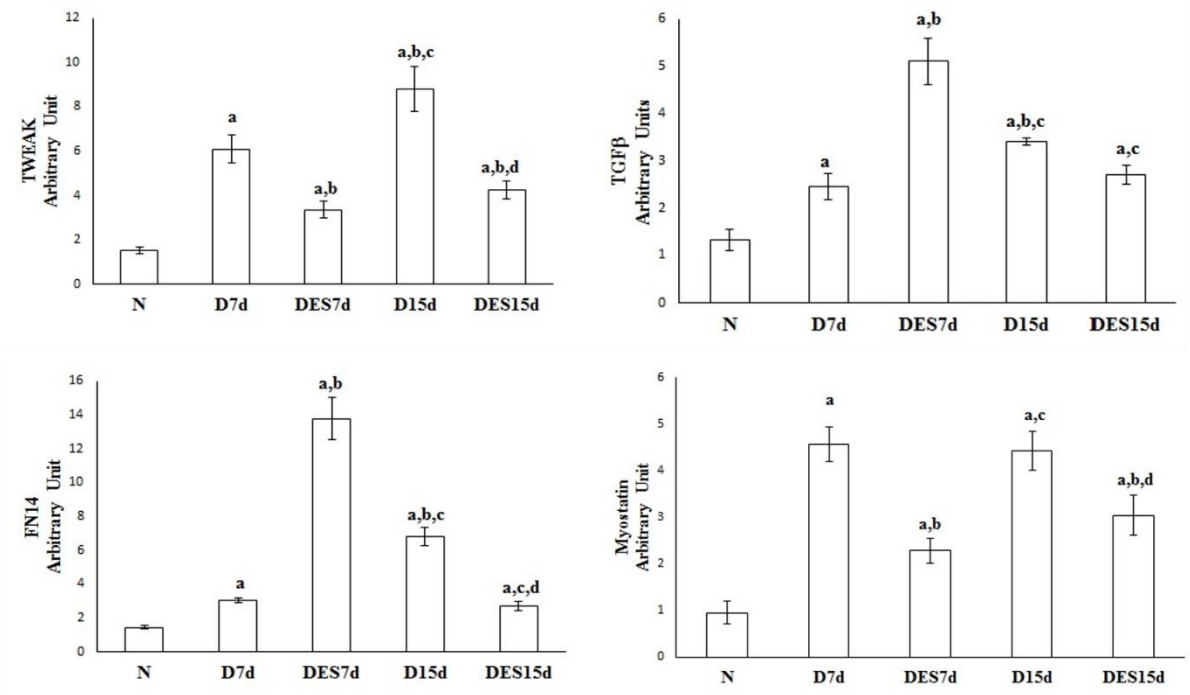

Figure 2. TWEAK, Fn14, TGF-β and myostatin mRNA levels in *tibialis anterior* (TA) muscle. Data is the mean ± standard deviation: a: represents statistical differences compared to N; b: represents statistical differences compared to D7d (no-ES); c: represents statistical differences compared to DES7d; d: represents statistical differences compared to D15d (no-ES). Note that the denervated muscle submitted to ES (DES7d and DES15d) showed a decline in the gene expression of TWEAK, and myostatin when compared to D7d (no-ES) and D15d (no-ES). The DES15d presented decreased values compared to the D15d (no-ES) ($p<0.05$). Fn14 and TGF-β presented a peak after 7 days in DES7d.

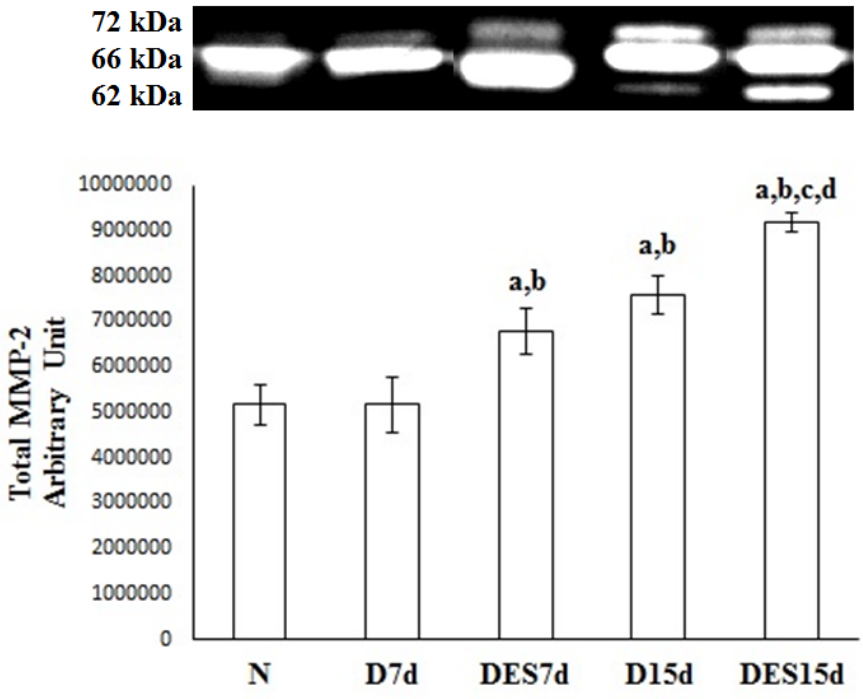

Figure 3. Quantitative densitometry of zymographic bands of total MMP-2 in arbitrary units in *tibialis anterior* (TA) muscle. Data is the mean ± standard deviation: a: represents a statistical differences compared to N; b: represents statistical differences compared to D7d (no-ES); c: represents statistical differences compared to DES7d; d: represents statistical differences compared to D15d (no-ES). Note that ES increased the MMP-2 activity on the 7th day post-denervation and ES, with peak in 15th day, compared to N ($p<0.05$).

Table

Table 1. Final body mass, muscle mass, muscle fiber cross-sectional area, and connective tissue of the tibialis anterior (TA) muscle.

Group	Body Mass (g)	Muscle mass (%/g of total body mass)	CSA (μm^2/g of body mass)	Connective tissue (%)
N	321 ± 32.2	0.189 ± 0.01	2.938 ± 0.23	20.33 ± 3.85
D7d	258 ± 12.1 [a]	0.127 ± 0.01 [a]	2.306 ± 0.20 [a]	34.16 ± 3.47 [a]
DES7d	260 ± 16.6 [a]	0.135 ± 0.01 [a]	1.956 ± 0.17 [a,b]	47.80 ± 2.67 [a,b]
D15d	252.9 ± 16.8 [a]	0.105 ± 0.01 [a,b,c]	1.750 ± 0.11 [a,b]	55.68 ± 4.77 [a,b,c]
DES15d	254 ± 13.6 [a]	0.085 ± 0.01 [a,b,c,d]	1.400 ± 0.06 [a,b,c,d]	72.80 ± 3.82 [a,b,c,d]

a: $p<0.05$, compared to N; b: $p<0.05$, compared to D7d (no-ES); c: $p<0.05$, compared to DES7d; and d: compared to D15d (no-ES).

SUPPLEMENTAL DIGITAL CONTENT

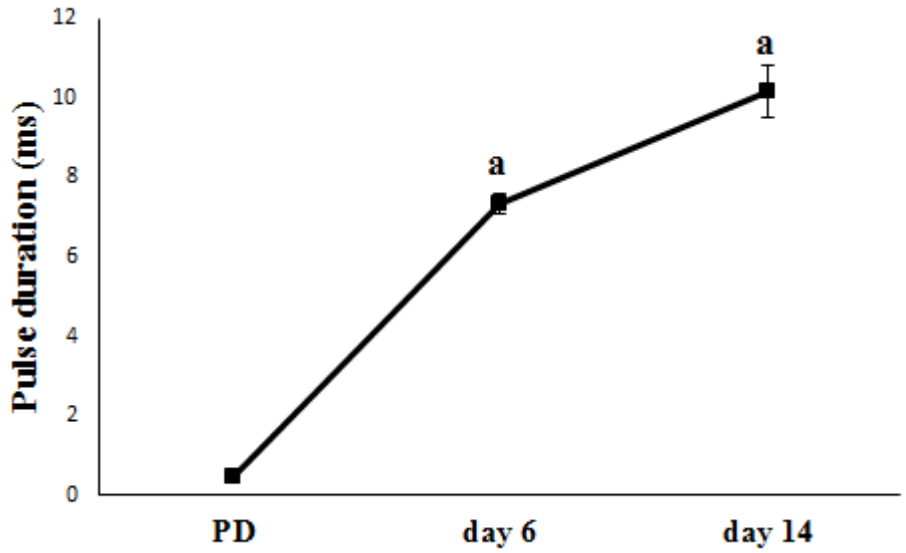

Figure 1S. Increase of pulse duration (ms) in DES15d group. PD: pre-denervation moment. Data are the mean ± standard deviation. a: $p<0.05$ indicated difference intragroup throughout time. Note an increase of pulse duration after 6 and 14 days of denervation.

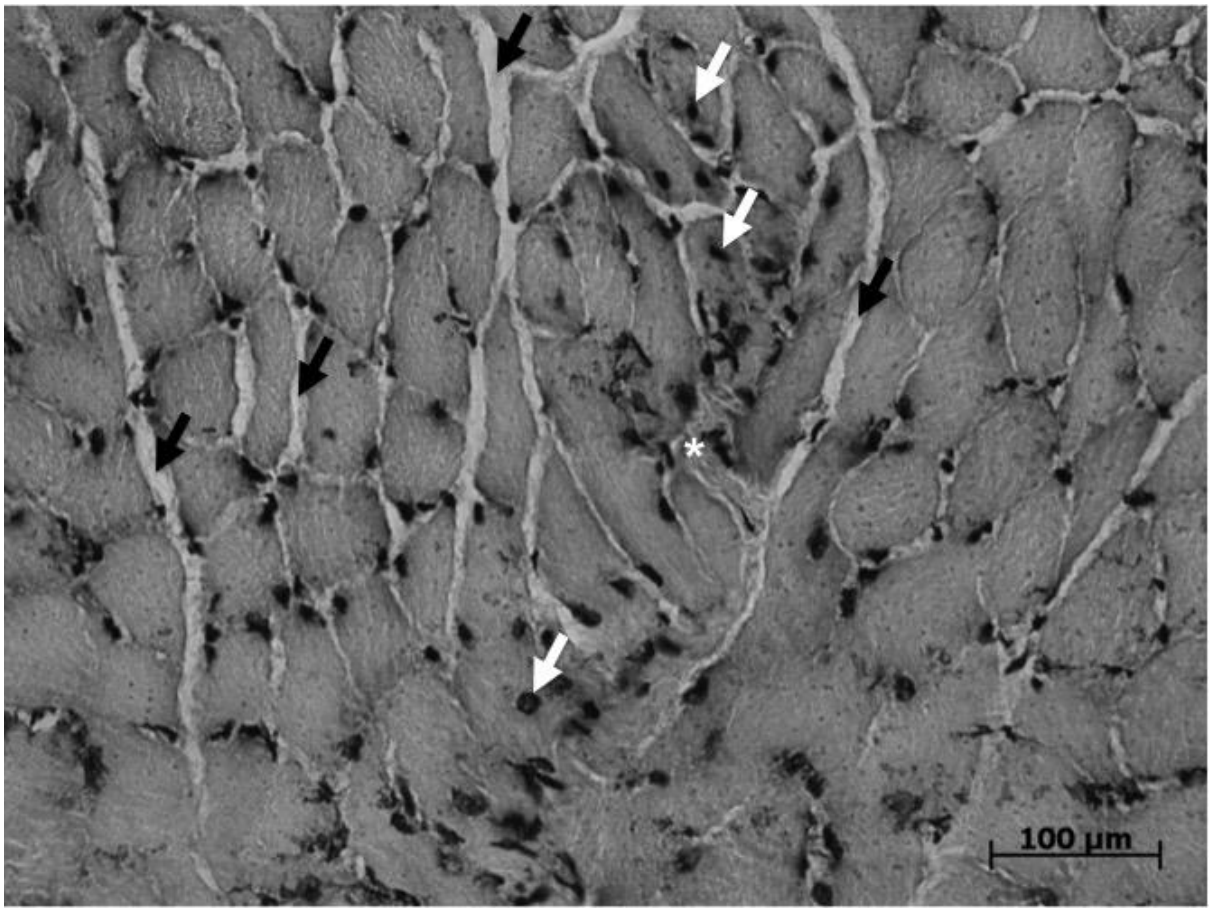

Figure 2S. Representative photomicrography of DES15d TA muscle. White arrows indicate central myonuclei; black arrows indicate increase of perimysium; and asterisk showed degenerated muscle fibers. At 15 days, denervated muscles submitted to ES showed increased of perimysium, degenerated muscle fibers and central nuclei, compared to denervated non-stimulated muscles.

ATIVIDADES DESENVOLVIDAS NO PERÍODO

Atividades de Pesquisa

Iniciei o doutorado com o projeto intitulado: "*Efeitos da infusão de células-tronco mesenquimais sobre a morfologia, morfometria e expressão gênica musculares em modelo de neuropatia diabética"*, no qual aprendi e desenvolvi, todo o processo que envolve a cultura de células-tronco mesenquimais, desde o flush de medula óssea de rato até sua infusão das células nos animais. Entretanto, em 2015 foi necessária a mudança de orientação, com mudança de projeto para o tema do projeto atual.

Como resultado deste projeto, foi submetido dois artigos científicos, como se encontra no corpo desta tese, intitulados: "*Electrical stimulation delas reinnervation in denervated rat muscle*" e "*Electrical stimulation increases fibrosis in denervated muscles via TWEAK/Fn14, TGF-β/myostatin, and MMP pathway*".

Durante o período de doutorado estive envolvida em outras atividades, além do desenvolvimento deste projeto, trabalhando em artigos como colaboradora.

Produções no período

Durante o período de doutorado participei de outra pesquisa, através da realização de experimentos, que resultaram em um artigo que está em fase final de preparação intitulado "*Physical training protects diaphragm muscle against atrophy through regulation of anti-oxidant genes and matrix metalloproteinases in mice exposed to chronic cigarette smoke"*(Gracielle Vieira Ramos, Alessandra Choqueta de Toledo, Clara Maria Pinheiro, Camila Liyoko Suehiro, Thiago Luiz de Russo, Rodolfo de Paula Vieira, Milton Arruda Martins, Tania de Fátima Salvini, João Luiz Quagliotti Durigan).

Outro artigo também se encontra na fase de final de preparação, resultado de uma colaboração, que teve como objetivo avaliar os colágenos tipo I, II e IV após sessões de alongamento em ratos idosos (Sabrina Peviani Messa, Clara Maria Pinheiro, Yara Pinter, ACR Fioravante, João Luiz Quagliotti Durigan, Adriana Sagawa, Fernanda Faturi, Tania de Fátima Salvini,).

No começo deste ano (2016), foi publicado um artigo, na qual participei como co-autora intitulado "*Cryotherapy reduces inflammatory response without altering muscle*

regeneration process and extracelular matrix remodeling of rat muscle" na Scientific Reports, 6: 18525; 2016. (Gracielle Vieira Ramos, Clara Maria Pinheiro, Sabrina Peviani Messa, Gabriel Borges Delfino, Rita de Cássia Marqueti, Tania de Fátima Salvini e João Luiz Quagliotti Durigan). Este estudo observou que a crioterapia reduziu os processos inflamatórios, sem alterar a área de lesão não alterando o processo de regeneração após lesão do músculo tibial anterior.

Um artigo de revisão está em fase de elaboração, com o objetivo de destacar os principais modelos utilizados para indução de isquemia cerebral e, a partir de suas características, propor recomendações para se trabalhar com tais modelos. Foi feita a análise de 1451 artigos científicos, resultados de uma busca realizada em quatro bases de dados: Cinahal, Pubmed, Scopus e Web of Scicence, através do software Start.

Atividades de ensino

Em 2013 participei do Programa de Educação pelo Trabalho para Saúde/Saúde da Família (PET-Saúde/SF) como tutora voluntária de fevereiro a julho 2013. Esse trabalho foi realizado em no Posto de Saúde do Jockey Clube, com atendimentos semanais, na qual acompanhei um aluno de estágio em fisioterapia nos atendimentos prestados à população deste bairro da cidade de São Carlos.

Atividade de Extensão

Participei da atividade de extensão intitulada: "Grupo terapêutico para indivíduos hemiparéticos crônicos" que fornece atendimento à um grupo de hemiparéticos crônicos, duas vezes por semana, totalizando 4 horas semanais. Nesse grupo, os integrantes participavam de protocolos de fortalecimentos e de atividades funcionais para membros superiores e inferiores, sendo avaliados antes e depois de um período de 16 atendimentos. O atendimento era voluntário e realizado na Unidade Saúde Escola, localizada na Universidade Federal de São Carlos.

Outras Atividades

No final do ano passado participei do I curso de Capacitação em Terapia por Contensão Induzida: Infantil e Adulto oferecido pela Universidade Federal de São Carlos, Departamento de Fisioterapia.

Por fim, os resultados parciais desse projeto foram aceitos para apresentação em forma de pôster no 9th World Congress for Neurorehabilitation (WCNR 2016), na Philadelphia, USA, 10 a 13 de maio de 2016.

Futuras análises poderão ser realizadas no nervo isquiático para melhor entendimento das alterações provocadas pela eletroestimulação durante o processo de reinervação após lesão nervosa. Os nervos encontram-se em cortes realizados em parafina histológica.

ANEXOS

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Parecer da Comissão de Ética no Uso de Animais

nº 033/2014

A Comissão de Ética no Uso de Animais da Universidade Federal de São Carlos - CEUA/UFSCar **APROVOU** o projeto de pesquisa *Efeito da estimulação elétrica sob a reinervação de músculos desnervados em ratos*, submetido pelo pesquisador **Clara Maria Pinheiro**.

São Carlos, 23 de Fevereiro de 2015

Azair Liane Matos do Canto de Souza

Profa. Dra. Azair Liane Matos do Canto de Souza

Presidente da Comissão de Ética no Uso de Animais

Journal of the Peripheral Nervous System

Electrical stimulation delays reinnervation in denervated rat muscle

Journal:	*Journal of the Peripheral Nervous System*
Manuscript ID	Draft
Manuscript Type:	Research Report
Date Submitted by the Author:	n/a
Complete List of Authors:	Pinheiro, Clara; Universidade Federal de Sao Carlos Centro de Ciencias Biologicas e da Saude, Physiotherapist Erbereli, Bruna; Universidade Federal de Sao Carlos Centro de Ciencias Biologicas e da Saude, Department of Physiotherapy Gigo-Benato, Davilene; Universidade Federal de Sao Carlos Centro de Ciencias Biologicas e da Saude, Department of Physiotherapy Castro, Paula; Universidade Federal de Sao Carlos Centro de Ciencias Biologicas e da Saude, Department of Physiotherapy Russo, Thiago; Universidade Federal de Sao Carlos Centro de Ciencias Biologicas e da Saude, Department of Physiotherapy
Key Words:	Physiotherapy, neurorehabilitation, skeletal muscle, muscle atrophy, neuromuscular junction

American Journal of Physical Medicine & Rehabilitation

Electrical stimulation increases fibrosis in denervated muscles via TWEAK/Fn14, TGF-β/myostatin, and MMP pathways.

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Keywords:	Electrical Stimulation; metalloproteinase; fibrosis and inflammation.
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Manuscript Region of Origin:	BRAZIL
Abstract:	Objective: To investigate the effects of electrical stimulation (ES) of rat denervated muscles in functionality, muscle atrophy and fibrosis formation. Design: Wister Rats were into Normal (N); 7 or 15-day denervation (D7d and D15d); D7d or D15d plus ES (DES7d and DES15d, respectively). Sciatic nerves were crushed causing muscle denervation. Two hundred muscle contractions were electrically induced daily by surface electrodes. Sciatic functional index was used to determine neuromuscular performance. Muscle fiber cross-sectional area and percentage of connective tissue was assessed by light microscopy. Molecular markers of extracellular matrix production and remodeling were evaluated. Metaloproteinase (MMP) activity was assessed by zymography, and TWEAK, Fn14, myostatin and TGF-gene expressions were determined by qPCR. Results: ES impaired natural recovery of walking at 15 days. In addition, ES induced fibrosis and accentuated muscle atrophy in denervated muscles. Although, ES reduced the accumulation of TWEAK and myostatin expressions, it up-regulated Fn14 and TGF-β in a time dependent manner. ES also increased the activity of MMP-2 compared to the other groups ($p<0.05$). Conclusions: ES applied to denervated muscles induced muscle fibrosis and atrophy, as well as disability. The TWEAK/Fn14 system, TGF-β/myostatin pathway, and MMP activity seems to be involved in these deleterious changes.