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EFEITOS DE DIFERENTES PROTOCOLOS DE TREINAMENTO FÍSICO SOBRE A FUNÇÃO E MORFOLOGIA DO NERVO MEDIANO DE RATOS APÓS PROTOCOLO DE LESÃO POR ESMAGAMENTO

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Neurociências da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Mestre em Neurociências.

Orientadora: Prof[a]. Dr[a]. Maria Cristina Faccioni-Heuser

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RESUMO

A maioria das lesões nervosas periféricas em humanos, afeta a extremidade superior e o maior aspecto incapacitante dessa lesão é a perda dos movimentos da mão. As lesões do plexo braquial apresentam um índice de morbidade elevado que é representado por graves sequelas sensorio-motoras devido à fibrose que se desenvolve ao longo do tempo após a lesão. Evidências indicam que o tipo e a intensidade da atividade física induzem o remodelamento morfológico e eletrofisiológico da junção neuromuscular influenciando no reparo do nervo. No presente trabalho, um programa de treinamento de equilíbrio e coordenação, de repetição na esteira e uma associação desses treinamentos foram utilizados, por 4 semanas, após a lesão por esmagamento do nervo mediano em ratos para verificar a influência dessas atividades sobre os parâmetros morfométricos do nervo lesionado (área axonal, densidade axonal, diâmetro das fibras mielinizadas, diâmetro axonal e espessura da bainha de mielina da porção distal do nervo mediano), além de analisar a recuperação funcional dos membros anteriores lesados. Análises histológicas e morfométricas do nervo mediano foram utilizadas para avaliar a regeneração do nervo no final do tratamento. Os resultados do teste de motricidade sobre grade revelaram que houve uma recuperação funcional acelerada em todos os grupos lesionados após lesão do plexo braquial. No teste de suspensão no arame e no teste do cilindro, entretanto, os grupos tratados não apresentaram diferença significativa comparada ao grupo controle. O treinamento de equilíbrio e coordenação mostrou melhores resultados comparado ao treinamento de repetição e a associação dos treinamentos para a densidade axonal e o diâmetro axonal igualando-se estatisticamente aos resultados do grupo sham sedentário. Esses dados fornecem evidências de que o treinamento de equilíbrio e coordenação acelerou a regeneração do nervo mediano após lesão traumática experimental, apesar dos testes funcionais não demonstrarem diferenças entre os tratamentos.

LISTA DE ILUSTRAÇÕES

ARTIGO

LISTA DE ABREVIATURAS

SNC – Sistema nervoso central

SNP – Sistema nervoso periférico

FRC – Flexor radial do carpo

ARTIGO

LBC – Lesio balance and coordination

LR – Lesion repetition

LBCR – Lesion repetition + balance and coordination

LSE – Lesion sedentary

Sh-s – Sham sedentary

CNS – Central nervous system

FT – Footfault test

HW – Hanging wire

CT – Cylinder test

SUMÁRIO

1 INTRODUÇÃO

No Brasil, os dados epidemiológicos demonstram que as lesões traumáticas são as causas mais comuns de lesão do plexo braquial (Flores, 2006). Na América do Norte e Europa, 10 a 20% das lesões do SNP envolvem o plexo braquial. E dentre essas lesões, 80 a 90% são causados por traumatismos decorrentes de acidentes automobilísticos com vários mecanismos de tração das raízes nervosas cervicais (Ferreira, 1999).

A incidência, no mundo, de lesões traumáticas é estimada em mais de 500.000 novos pacientes a cada ano. Essas lesões levam a perda parcial ou total da função motora, sensorial e autonômica no segmento corporal envolvido (Rodríguez et al., 2004; Valero-Cabré e Navarro, 2002). Periférica e centralmente, os alvos musculares e os neurônios motores, respectivamente, perdem sua função (Johnson et al., 2005). No entanto, estas perdas podem ser recompensadas graças à recuperação dos neurônios lesionados, fazendo com que os axônios seccionados enviem novos prolongamentos ao coto distal e restabeleçam novas conexões funcionais com os órgãos periféricos apropriados (Valero-Cabré e Navarro, 2002).

Lesões do plexo braquial ainda constituem um desafio clínico e um problema cirúrgico, apesar do uso de técnicas microcirúrgicas especializadas. A maior parte das lesões de nervo periférico, em humanos afeta a extremidade superior (Bertelli et al., 1995). Essas lesões são responsáveis pela perda ou restrição da capacidade funcional de alcance, preensão e manipulação de objetos (Duff, 2005). Em função da perda evidente na qualidade de movimento após lesões nervosas periféricas, a potencialização da regeneração nervosa e a recuperação da função têm sido alvo de diversos estudos (Van Meeteren et al., 1998; Bontioti et al., 2003; Gordon et al., 2003; Bontioti et al., 2005; Ilha et al., 2008; Sebatier et al., 2008).

1.1 PLEXO BRAQUIAL

O plexo braquial em humanos é formado pela união de quatro raízes baixas cervicais (C5, C6, C7 e C8) e a primeira raiz torácica (T1). Essas raízes são formadas pela união das raízes dorsal sensorial e ventral motora. Cada raiz é formada pela união de 2 ou 3 raízes dorsais e ventrais. A união da raiz de C5 e C6 forma o tronco superior. A raiz de C7 sozinha forma o tronco médio, e as raízes de C7 e C8 formam o tronco inferior. As divisões anteriores do tronco superior e médio unem-se para formar o fascículo lateral, a divisão anterior do tronco inferior forma o fascículo medial e as divisões posteriores dos troncos se unem formando o fascículo posterior (Gray, 1995). Os nervos peitoral lateral, musculocutâneo e a raiz lateral do nervo mediano originam-se do fascículo lateral; os nervos axilar, radial, toracodorsal e o nervo subescapular originam-se do fascículo posterior; os nervos peitoral medial, raiz medial do nervo mediano, ulnar, cutâneo medial do braço e o nervo cutâneo medial do antebraço originam-se do fascículo medial (Netter, 2008) (Fig. 1).

O nervo mediano em humanos origina-se das raízes de C5 a T1 e é formado pela fusão dos ramos vindos dos fasciculos lateral e medial do plexo braquial. A raiz lateral do nervo mediano, derivado dos ramos ventrais do quinto ao sétimo nervos cervicais (C5, C6 e C7), inerva os músculos da região anterior do antebraço e curtos do polegar, assim como a pele do lado lateral da mão. A raiz medial do nervo mediano, originada dos ramos ventrais do oitavo nervo cervical e primeiro torácico (C8 e T1), inerva os músculos da região anterior do antebraço e curtos do polegar, assim como a pele do lado medial da mão (Netter, 2008).

No rato, o nervo mediano é formado pela fusão de 3 ramos vindos dos fasciculos lateral, posterior e medial do plexo braquial, respectivamente. Os ramos do fascículo posterior e medial, entretanto, são mais desenvolvidos que o ramo do fascículo lateral (C5 e C6). No pata anterior, o nervo mediano não se ramifica, mas perto da articulação do cotovelo ele

desprende-se de um ramo ao redor do músculo pronador que recebe um ramo anastomótico do nervo musculocutâneo. Alguns milímetros distalmente, um largo ramo parte do nervo mediano, o equivalente ao nervo interósseo anterior em humanos. Esse nervo inerva o flexor radial do carpo (FRC) e o flexor dos dedos. O nervo mediano continua distalmente entre o FRC e o flexor dos dedos. No terço distal do antebraço, ele dá origem a um ramo recorrente à metade medial do flexor profundo dos dígitos. E então é dividido em ramo lateral e medial. O ramo lateral inerva os músculos tenares e lumbricais antes de terminar como nervo colateral no segundo e no terceiro digitos (Bertelli et al., 1995).

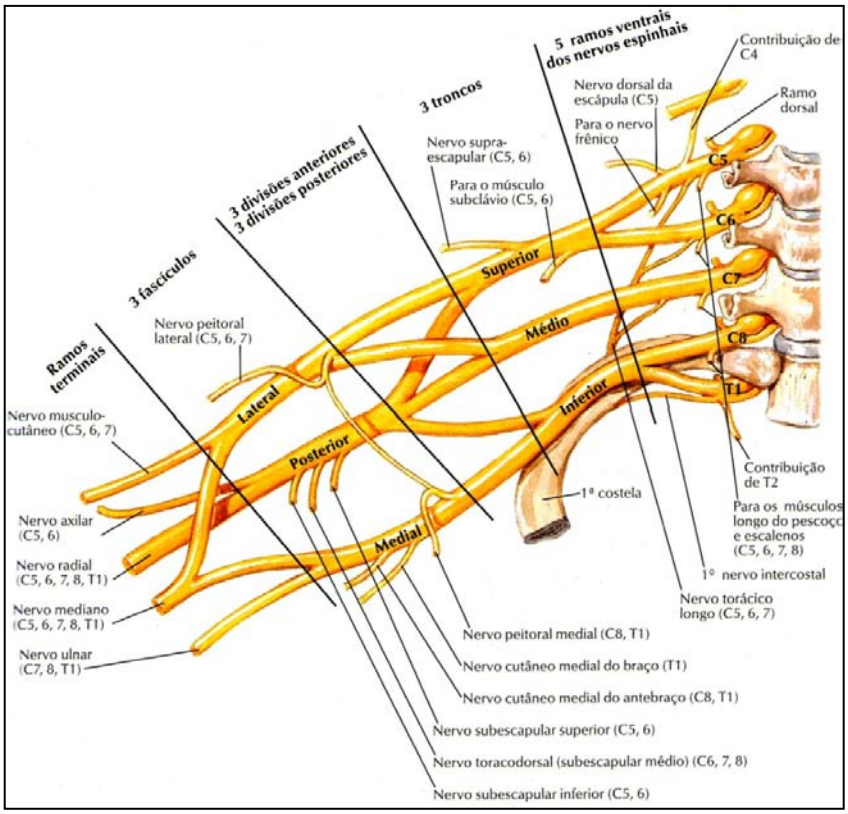

Figura 1 – Desenho esquemático do plexo braquial humano
Fonte: (Netter, 2000)

1.2 MODELO DE LESÃO DO PLEXO BRAQUIAL

São poucos os estudos usando o modelo experimental para o estudo da regeneração nervosa nos membros anteriores do rato (Galtrey e Fawcett, 2007; Bontioti et al., 2003; Bertelli et al., 1995). Entretanto, a maioria das lesões de nervo periférico em humanos afeta a extremidade superior, e por essa razão, um modelo experimental de lesão nervosa na extremidade superior é de grande importância (Bontioti et al., 2003). A distância para os órgãos-alvo é pequena nos membros anteriores do rato (músculo e pele), a reinervação é rápida, e o tempo requerido para a recuperação funcional é menor do que a dos membros posteriores (Santos et al., 2007; Bertelli e Mira, 1993; Bontioti et al., 2003). Assim, supõe-se que a regeneração nervosa e a recuperação funcional sejam obtidas mais rapidamente do que os modelos de lesão do nervo ciático.

1.3 ESTRUTURA NORMAL DOS NERVOS PERIFÉRICOS

O axônio é uma extensão longa e delgada do corpo celular, que possui uma estrutura arborescente em sua região distal - terminação axonal. É por meio dela que os axônios realizam contatos sinápticos com os órgãos alvo. Em um nervo existem axônios mielinizados e não mielinizados. No primeiro tipo, as células de Schwann se organizam ao redor do axônio formando a bainha de mielina, que é interrompida em intervalos regulares, pelos nodos de Ranvier (Figs. 2 e 3). A função normal dessas fibras depende da integridade da bainha de mielina, a qual isola e protege o axônio, além de aumentar a velocidade de condução dos impulsos nervosos (Fredericks, 1996). Os axônios amielínicos, embora não possuam bainha de mielina e nodos de Ranvier, também estão em contato íntimo com as células de Schwann (Peters et al., 1976). As fibras somáticas e proprioceptivas são as fibras mielínicas de maior

diâmetro, enquanto que as fibras sensoriais que medeiam a dor são as menores (Fredericks, 1996).

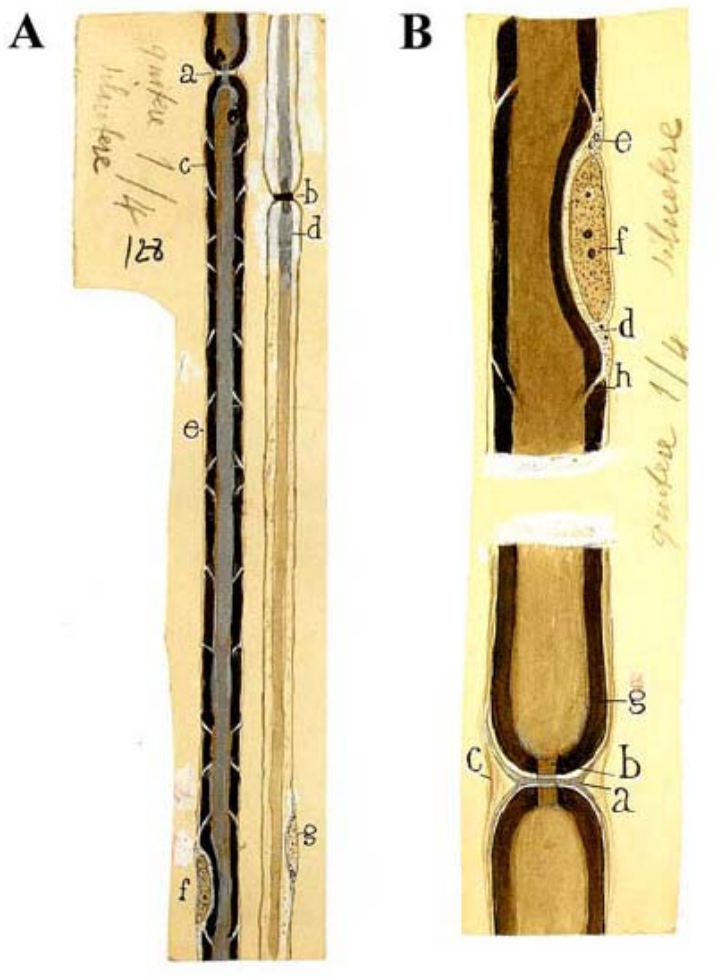

Figura 2 - Desenho esquemático das fibras nervosas mielínicas do SNP. (A) a, tecido conjuntivo; b, nodo de Ranvier; c, bainha de mielina; d, axônio; e, região internodal; e – f, núcleo da célula de Schwann. (B) a, nodo de Ranvier; b, região paranodal; c, tudo de tecido conjuntivo; d – e, citoplasma da célula de Schwann; f, núcleo da célula de Schwann; g, bainha de mielina; h, incisura de Lantermann
Fonte: Modificado de (Rámon Y Cajal, 2003)

Os nervos periféricos englobam, de uma maneira geral, os axônios dos neurônios motores e sensoriais, que constituem os nervos espinais e cranianos, os plexos e os troncos nervosos do sistema nervoso vegetativo (Vallat e Magy, 2005).

Os nervos espinais são estruturas formadas por axônios associados a células de Schwann, que são envoltos por 3 camadas de tecido conjuntivo: endoneuro, perineuro e

epineuro. (Fig. 3). Essas sucessivas camadas de tecido conjuntivo servem para proteger e sustentar as fibras nervosas, auxiliando-as durante o processo de regeneração. O perineuro também fornece uma grande força mecânica e serve como uma barreira de difusão perivascular. Ele isola quimicamente os feixes de fibras preservando um ambiente fluido no interior dos fascículos, muito similar à proteção exercida pela barreira hemato-encefálica no SNC. O perineuro atua como uma barreira para macromoléculas, podendo proteger as fibras nervosas de várias substâncias danosas, como certas toxinas, antígenos e vírus (Fredericks, 1996).

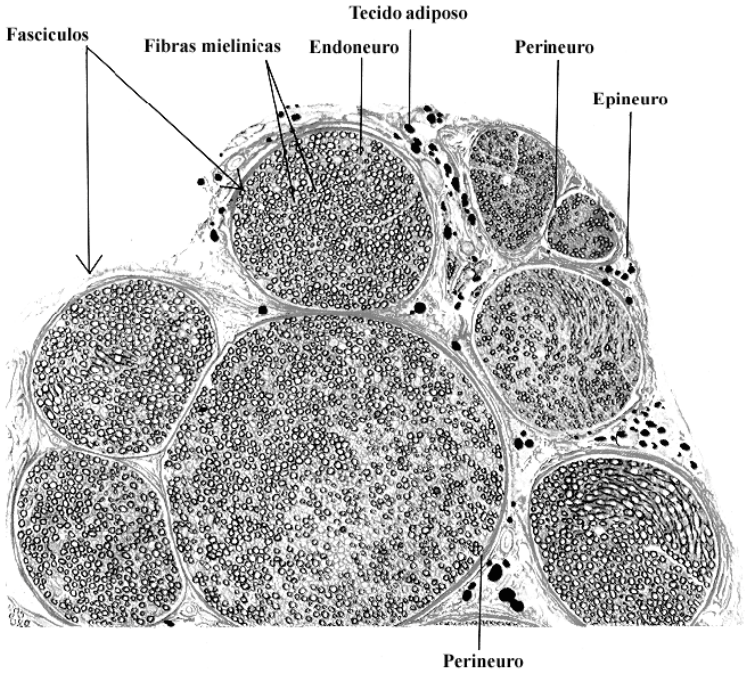

Figura 3 - Corte transversal de um nervo periférico
Fonte: Modificado de (Williams, 1995)

1.4 FISIOPATOLOGIA DO TRAUMA AXONAL & REGENERAÇÃO NERVOSA PERIFÉRICA

Com base nas lesões do sistema nervoso periférico, diversos sistemas de graduação foram desenvolvidos a fim de permitir a correlação entre as alterações microscópicas e a

sintomatologia clínica. Dentre as mais amplamente aceitas esta a que divide as lesões em três categorias, de acordo com a severidade da lesão: neuropraxia, axonotmese e neurotmese (Seddon, 1943). E dentre esses tipos de lesão, a axonotmese é o tipo de lesão mais amplamente utilizado para o estudo da regeneração nervosa periférica.

A axonotmese ocorre quando há completa interrupção da continuidade de fibras axonais e prejuízo da camada de mielina circundante, mas com manutenção da integridade do tecido conjuntivo que envolve os feixes de fibras (perineuro) e o nervo (epineuro). Ocorre degeneração axonal e mielínica distal ao foco da lesão, causando completa denervação. O prognóstico de regeneração é favorável, uma vez que a preservação do tecido conjuntivo provê orientação para o crescimento axonal e reinervação (Burnett e Zager, 2004).

Após a injúria nervosa periférica, entretanto, a axotomia das fibras nervosas determina uma série de reações induzidas pela lesão axonal (reação axonal) que começam a ocorrer nos neurônios sensoriais e motores, principalmente no soma celular, no local e distalmente à lesão. O axônio desconectado do soma pela injúria tem seu segmento axonal distal gradualmente degenerado, sendo chamado de degeneração Walleriana (Fig. 4). Os principais alvos celulares da degeneração Walleriana são o axônio, as células de Schwann e a bainha de mielina por ela formada (Schröder, 1975; Ide, 1996; Dahlin e Brandt, 2004).

Os macrófagos e as células de Schwann, todavia, mantém uma íntima interatividade após a lesão nervosa periférica. Ao mesmo tempo em que as células de Schwann auxiliam os macrófagos na remoção do axônio degenerado e dos resíduos de mielina, os macrófagos estão envolvidos na produção de fatores que estimulam a mitose das células de Schwann (Baichwal et al., 1988) e a regulação da síntese de fatores de crescimento por essas células (Lindholm et al., 1987). A presença de moléculas tróficas no microambiente neural periférico, como o fator de crescimento do nervo (NGF) e o fator de crescimento derivado do encéfalo (BDNF), são alguns dos fatores responsáveis pela maior capacidade de regeneração em lesões do sistema

nervoso periférico quando comparado a lesões do sistema nervoso central (David e Aguayo, 1981; Yan et al., 1992; Yin et al., 1998; Burnett e Zager, 2004).

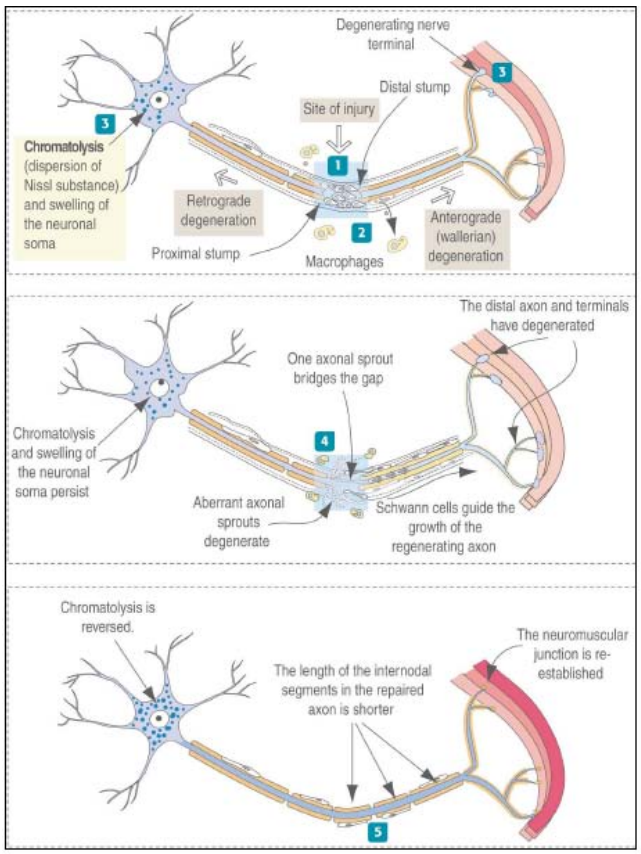

Figura 4 - Figura esquemática mostrando resumidamente o processo de degeneração e regeneração no SNP. Após uma lesão axonal por esmagamento, as células de Schwann sofrem divisão mitótica e preenchem os espaços entre os cotos proximais e distais do axônios (1). Essas células fagocitam a mielina. Gotículas de mielina são excretadas por essas células de Schwann e, em seguida, fagocitadas por macrófagos (2). Ocorre cromatólise (3) e é observada a degeneração dos segmentos distal e proximal do axônio (degeneração anterógrada e retrógrada, respectivamente). O coto proximal do axônio gera múltiplos brotamentos que avançam por entre as células de Schwann, e estes brotamentos persistem e crescem distalmente para reinervar o músculo (4). Uma vez que o axônio regenerado atinge o órgão-alvo, as células de Schwann começam a produzir mielina (5)
Fonte: Modificado por (Kierszenbaum, 2008).

As células de Schwann, além disso, promovem o crescimento de neurônios e regulam a ação local intercelular envolvida na orientação da extensão e direção de axônios durante a reinervação muscular após a lesão. Entretanto, a denervação parcial do músculo esquelético é seguida pelo crescimento de finos processos (brotamento) da fibra nervosa intramuscular remanescente, levando eventualmente a reinervação das fibras musculares denervadas. Esses brotamentos podem surgir tanto de segmentos pré-terminais não-mielínicos do axônio

(brotamento terminal) quanto de nódulos de Ranvier (brotamento nodal ou colateral) (Wang et al., 2007).

As modificações no corpo neuronal e no segmento proximal das fibras dependem da severidade da lesão, assim como da proximidade entre o segmento lesado e o corpo do neurônio (Cullheim et al., 2002). As células de Schwann inevitavelmente degradam no segmento próximo à lesão e os axônios e a mielina tornam-se visivelmente reduzidos em diâmetro. Essa degradação proximal pode ser mínima (até o nodo de Ranvier mais próximo) ou estender-se até o corpo do neurônio. Se o corpo do neurônio degenera, o que ocorre em casos de trauma moderado a severo, todo o segmento proximal sofre degeneração e é fagocitado (Lundborg, 2000).

Mesmo em lesões brandas, o corpo neuronal passa por modificações comparáveis após a lesão. O núcleo migra para a periferia da célula e ocorre desmembramento dos corpúsculos de Nissl e do retículo endoplasmático rugoso, processo denominado de cromatólise. Simultaneamente, ocorre rápida resposta proliferativa das células gliais, de certa forma sinalizada pela cromatólise. As células gliais, então, se estendem pelo neurônio afetado e interrompem as conexões sinápticas, possivelmente para isolar o neurônio durante a fase de recuperação (Burnett e Zager, 2004).

1.5 EXERCÍCIO FÍSICO E NEUROPATIAS PERIFÉRICAS

Terapias empregando programas de exercícios físicos são freqüentemente utilizados na reabilitação de pacientes com neuropatias periféricas. Os exercícios físicos diminuem as complicações comuns às patologias do SNP e promovem a recuperação funcional e o aumento da capacidade aeróbica de pacientes com doenças neuromusculares (Herbison et al., 1983; Linderman et al., 1995; Wright et al., 1996). Alguns resultados sustentam também que

a velocidade do crescimento axonal após lesão é aumentada por protocolos de treinamento específicos (Van Meeteren et al., 1997).

Estudos experimentais têm empregado exercícios físicos na reabilitação de lesões traumáticas do nervo ciático em modelos animais para estimular a regeneração nervosa e melhorar a recuperação funcional, porém com resultados conflitantes. O treinamento de endurance, por exemplo, promove a normalização da função motora dos membros posteriores após uma semana de exercício de endurance (Ilha et al., 2008). Em um estudo realizado em nosso laboratório, comparando-se exercícios acrobáticos e caminhada livre, observou-se uma significativa melhora funcional e uma regeneração nervosa periférica satisfatória no grupo tratado com o protocolo de equilíbrio e coordenação (Bonetti et al, 2011). Em outro estudo, entretanto, ratos com esmagamento de nervo mediano e ulnar foram submetidos aos protocolos de treinamento de habilidade, que consistia em alcançar alimento de dentro de uma caixa de acrílico e um protocolo de treinamento de repetição, que consistia em caminhar em uma esteira. Nesse estudo, evidenciou-se que ambos os protocolos de treinamento foram suficientes para acelerar a recuperação funcional, porém o treinamento de repetição produziu um maior grau de regeneração nervosa periférica do que o treinamento de habilidade (Pagnussat et al., 2009). Por outro lado, outro estudo demonstrou que a natação não interfere com a recuperação sensório-motora após lesão do nervo ciático e que um programa intermediário de caminhada em esteira retarda a recuperação em ratos (Van Meeteren et al., 1998). Também foi observado que a atividade motora intensa, realizada diariamente (natação) em ratos com esmagamento do nervo ciático leva a deficiências na diferenciação das fibras em regeneração (Gutmann e Jakoubek, 1963). Todos esses achados indicam que o tipo e a intensidade do exercício físico podem exercer diferentes conseqüências na regeneração nervosa periférica.

O treinamento de habilidade com tarefas acrobáticas induz à sinaptogênese,

potenciação sináptica, e reorganização da representação dos movimentos do córtex motor. O treinamento de endurance, entretanto, induz á angiogênese no córtex motor, mas não altera a organização do mapa motor cortical ou o número de sinapses. Além disso, o treinamento de força altera a excitabilidade dos motoneurônios espinais e induz à sinaptogênese na medula espinhal, mas não altera a organização do mapa motor cortical. Esses achados suportam a idéia de que a natureza específica da reorganização é dependente da demanda comportamental de cada treinamento (Adkins et al., 2006).

Há muito se sabe que o SNC depende de fontes de feedback sensorial para assegurar um ótimo desempenho dos movimentos e a performance motora é comprometida quando esse feedback é abolido ou quando ocorre algum distúrbio desta informação (Jones et al., 1999; Kleim et al., 1996; VandenBerg et al., 2004). Entretanto, além das discrepâncias nos resultados obtidos por esses prévios estudos, eles não especificam o tipo de treinamento empregado, nem discutem se as suas respectivas ações no processo de regeneração poderiam ser diferentes.

No sistema neuromuscular intacto de modelos de lesão em animais, diferentes protocolos de exercícios têm demonstrado exercer distintas ações, o que remete a possibilidade de que estes diversos efeitos dependentes do tipo de treinamento também ocorram quando o exercício é aplicado após lesões do sistema nervoso. Por exemplo, treinamentos aeróbicos não causam uma significante hipertrofia muscular, embora aumentem a atividade colinesterásica (Ach) e resultem em significante expansão dos componentes pré e pós sinápticos da junção neuromuscular em ratos (Crockett et al., 1976; Tomas et al., 1997). Por outro lado, treinamentos de resistência muscular com altas cargas resultam em adaptações neurais e hipertrofia muscular, as quais são responsáveis pelo aumento da força dos músculos treinados (Deschenes et al., 2000; Lee et al., 2004). Desta forma, a especificidade do treinamento é um fator importante e deve ser levado em consideração dentro do contexto da

regeneração nervosa quando se busca estudar os efeitos do exercício físico após lesões do sistema nervoso. O tratamento de lesões de nervo periférico em humanos visa abordar a combinação de diferentes estratégias de tratamento, por isso se faz necessário analisar o efeito de diferentes protocolos de exercícios e suas associações.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Analisar o efeito de seis semanas de treinamento de repetição, treinamento de equilíbrio e coordenação e treinamento de repetição associado ao treinamento de equilíbrio e coordenação na regeneração nervosa periférica em ratos machos adultos após lesão por esmagamento do nervo mediano.

2.2 OBJETIVOS ESPECÍFICOS

- Analisar, morfometricamente, o nervo mediano (área axonal, densidade axonal, espessura da bainha de mielina, diâmetro axonal e diâmetro das fibras mielínicas) dos ratos dos grupos sham sedentário e lesão por esmagamento do nervo mediano submetidos aos treinamentos de equilíbrio e coordenação, repetição e a associação desses treinamentos;

- Avaliar melhoras funcionais dos ratos dos grupos sham sedentário e lesão por esmagamento do nervo mediano submetidos aos treinamentos de equilíbrio e coordenação, repetição e a associação desses treinamentos através do teste do cilindro, teste de motricidade sobre grade (footfault test) e teste de suspensão no arame (hanging wire).

3 MÉTODOS E RESULTADOS

3.1 ARTIGO

Artigo – Juliana D. Neves, Fernando Soares Camelier, Sandro Antunes da Silva, Nígia Ramalho Arsego, Jocemar Ilha, Simone Marcuzzo, Léder Leal Xavier, Maria Cristina Faccioni-Heuser. **Effects of different protocols of physical training on the median nerve funtion and morphology of rats after crush injury.**

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Effects of different protocols of physical training on the median nerve funtion and morphology of rats after crush injury

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ABSTRACT

Introduction: Numerous therapeutic interventions have been tested to enhance functional recovery after crush lesion, but these studies did not examine the effects of specific types of exercise. *Methods:* After median nerve crush injury we tested the effect of balance and coordination, repetition training and the combination of both types of training program (for 6 weeks) on nerve regeneration in Wistar rats using functional tests and nerve morphometric analysis. *Results:* Functional recovery was similarly accelerated in lesioned groups in the footfault test, but in the hanging wire and in the cylinder test, the injured groups showed no differences compared to control group. Balance and coordination training caused significantly better values than repetition training alone, the association of the two protocols training, or control for the axonal density and axonal diameter. *Discussion:* These data provide evidence that balance and coordination training had greater potential for enhancing median nerve histological regeneration after experimental traumatic injury, spite functional tests not show differences between the treatments.

Keywords: Median nerve crush, physical training, peripheral nerve regeneration.

INTRODUCTION

Peripheral nerves are damaged by different factors such as acute trauma, chronic repetitive insults, and inherit or acquired metabolic disorders[1,2]. The incidence of traumatic injuries is estimated as >500.000 new patients annually [3]. Peripheral nerve injuries reduce muscular recruitment and sensation and can disrupt coordination through the changes that occur in the peripheral and the central nervous system (CNS)[4,5].

The majority of human peripheral nerve injuries affect the upper limbs and the most disabling aspect of this injury is the loss of skilled hand movements[6]. Exercise training improves motor function after experimental and clinical peripheral nervous lesion, and it can be considered as an effective treatment of sensorial deficit[7].

There are evidences that the type and intensity of activity induces morphological and electrophysiological remodeling of neuromuscular junction and the motor nerve endings are continuously changing and can suffer influence of modifications in functional demands[8,9,10]. These adaptations of synapses can exert influence in nerve orientation and repair since one of the mechanisms governing the regeneration is the secretion of growth factors by target muscles during the period of denervation[10,11].

A range of forms of exercise training has shown beneficial effects in various muscle and nerve function related parameters in animal models of nerve injury[12,13,14,15]. Experimental and clinical studies have employed physical exercise in the rehabilitation of traumatic injury of the sciatic nerve with the purpose of stimulating nerve regeneration and improving functional recovery, however conflicting results have been obtained. For example, the endurance training promotes the normalization of hindlimb motor function after one week of exercise[15]. In another study, the acrobatic exercise demonstrates significant functional improvement and satisfactory nerve regeneration[16]. However, there is a few data about

forelimb exercise training after peripheral nerve injury.

Consequently, different training programs have diverse effects on peripheral nerve regeneration. The results of specific treatments, as well as the combination of treatments are necessary for the knowledge of mechanisms of recovery, and so for the application in humans rehabilitation. Therefore, the present study was designed to compare the effects of balance and coordination, repetition and the combination of these two types of training protocols on functional recovery, and to carry out a morphometric analysis of the median nerve after crush injury.

MATERIALS AND METHODS

Experimental design and surgical procedures

The experiment was performed on 46 three-month-old, male Wistar rats weighing 280-330 g (initial age and weight) from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul, Brazil). Rats were housed in standard Plexiglass boxes, under a 12:12 h light/dark cycle in a temperature-controlled environment (20 ± 1 °C) with food and water *ad libitum*. All the procedures were approved by the Ethical Committee at the Federal University of Rio Grande do Sul (nº 2008194) and all the animals were handled in accordance with the Brazilian laws. Animals were randomly divided in five groups: (1) sham-operated rats, without median nerve crush and unexercised, sham sedentary (Sh-s, n = 8); (2) rats with median nerve crush and unexercised, lesion sedentary (LSE, n = 9); (3) rats with median nerve crush and repetition training (LR, n = 9); (4) rats with median nerve crush and balance and coordination training (LBC, n = 10); and (5) rats with median nerve crush and repetition + balance and coordination training (LBCR, n = 10) (Table I). Before the surgical procedures, animals were adapted for 5 days in each training program protocol. For the surgical

procedures, animals were anesthetized using ketamine and xylazine (90 and 15 mg/kg, i.p., respectively; Vetbrands, Brazil) and the right median nerve was exposed through a skin incision in the axillopectoral region and the right pectoralis major muscle was removed with preservation of the cephalic vein on the same side. The nerve crush injury was performed with 1 mm hemostatic forceps for 30 seconds (as previously described by Bridge and coworkers (1994). The muscle and skin were then closed with 4-0 nylon sutures (Somerville, Brazil), and the animals were put in their cages to rest. Four days after the surgery, the animals from the LR, LBC and LBCR groups began specific training for 6 weeks, while Sh-s and LSE animals were put in the same location as the training animals for a few minutes in order to equal as much as possible the handling of all groups, but they did not perform any kind of motor activity.

Rehabilitation protocols

After four days of brachial plexus crush animals received one of the following treatments. Both protocols were performed along 6 weeks, 5 days per week (Fig. 1).

Repetition training

The repetition training program was performed in a treadmill for human (Runner, Brazil) modified for use in rats[15]. This task consisted in walking to an adapted motorized rodent treadmill during 20 min (0.03 m/s - 3 initial min; 0.05 m/s - 14 min; 0.03 m/s - last 3 min). The grade of the treadmill remained at 0% and no aversive stimulus was used. This velocity was chosen in order to avoid possible effects of aerobic treadmill exercise. Since this type of training used in this work is a simulation of a very low intensity exercise.

Balance and Coordination training

The balance and coordination training program was adapted from the acrobatic training used by Black et al. (1990), Anderson, Alcantara and Greenough (1996) and Kleim et al. (1996)[17,18,19]. Animals were required to traverse 5 different elevated obstacles per day, such as suspension bridges, rope bridges, parallel bars, etc. (each 100 cm long) ending in dark box. Each rat of this group crossed these obstacles 25 times, walking 2.500 cm each day of training[16]. These obstacles require motor learning, balance and coordination from these animals.

Repetition + Balance and coordination training

This group was daily subjected to the LR protocol followed to the protocol of LBC.

Functional tests

During training, the animals underwent the following behavioral assessments: footfault test (FT), hanging wire (HW) and cylinder test (CT). For FT, the animals were filmed 3 times from a lateral view and for CT, however, the animals were filmed during 4 minutes from an inferior view, and for HW they were recorded only once.

Footfault test

The footfault test was performed to assess whether training enhanced coordinated placement of the forelimbs[20]. Rats were placed on an elevated grid platform (75X20 cm) for 3 trials. Rats moved across the platform by placing their paws on the rungs of the grid. Errors were measured as slips with right forelimb through the grid openings[21]. In this study the animals carry out the test on the 3rd postoperative day and after, weekly, and 24 hours after completion of training.

Hanging Wire

This task was used to measure the ability to grasp and the strength of the forelimbs. The animals used their forelimbs to suspend their body weight on a wire stretched between two bars, 60 cm above the ground and the time (in seconds) before the animal fell was recorded. A score of zero was used if the rat fell down immediately and 120 seconds was the time limit of the test. The animals carry out the test on the ninth postoperative day and after, weekly, and 24 hours after completion of training. A single trial was conducted for every rat on each test day[22].

Cylinder test

To examine the effect of brachial plexus crush and treatment on spontaneous forelimb use during exploratory activity movements, animals were individually placed into a transparent cylinder (20 cm diameter and 40 cm high) on a glass tabletop and video recorded from below through an angled mirror for 4 min during each test session. The cylindrical shape encouraged rearing and vertical exploration of the walls with the forelimbs. The number of forepaws wall contacts used for postural support was counted and the percentage of asymmetry of single-limb wall contacts [(contralateral/contralateral + ipsilateral) x 100] was calculated. A single cylinder test session was performed 3 days after the surgery, 21 days after the surgery and one day before perfusion[23].

Histological and morphometric studies

Two days after the period of training the animals were anesthetized with sodium thiopental (50 mg/kg, i.p.; Cristália, Brazil), injected with 1000 IU heparin (Cristália, Brazil) and were transcardially perfused with 300 ml of saline solution, followed by 0.5% glutaraldehyde (Sigma Chemicals Co., St Louis, MO) and 4% paraformaldehyde (Reagen,

Brazil) in a 0.1 M phosphate buffer (pH 7.4, PB) at room temperature. One short segment of the right median nerve was rapidly excised, 5 mm after the crush injury site, in the distal portion[15]. This region was chosen in order to show major alterations after injury, since the changes are most pronounced in the distal portion of the nerve[5]. The specimens were postfixed by immersion in a fixative solution of glutaraldehyde 2.5% and paraformaldehyde 2% at 4 °C until processed; after that, the samples were washed in 0.1 M PB and postfixed in 1% OsO_4 (Sigma Chemicals Co., St Louis, MO) in 0.1 M PB for 30 min.; washed again in 0.1 M PB; dehydrated in a graded series of acetone, embedded in resin (Durcupan, ACM-Fluka, Switzerlend) and polymerized at 60° C. Cross-semithin sections (1 µm) were obtained using an ultramicrotome (MT 6000-XL, RMC, Tucson, USA) and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil)[15,24].

Afterwards, images of the distal portion of the right median nerve was captured and digitalized (initially 1000x and further amplified 200% for analysis) using a Nikon Eclipse E-600 microscope (Japan) coupled to a Pro-Series High Performance CCD camera and processed with Image Pro Plus Software 6.0 (Media Cybernetics, USA)[15,24].

For morphometric evaluation of the nerve, distal portion of the right median nerve was analyzed; a set of 5 images was chosen using random sampling of one slice. Morphometric measurements of the median nerve included the (1) myelinated fiber density (number of fibers/mm^2); (2) average myelinated fiber area (μm^2); (3) average myelin sheath thickness (µm); (4) average myelinated fiber diameter (µm); (5) average axon diameter (µm) of the myelinated fiber). These morphological parameters were chosen to assess the differentiation of regenerating median nerve[15].

The average myelin sheath thickness was estimated using the measurement tools of Image Pro Plus software. The measurements of areas were estimated with a point-counting

technique[15,24] using grids with point density of 1 point per 1.30 μm^2 and the following equation:

Â=Σp.a/p, where Â is area, Σp the sum of points, and a/p the area/point value. To estimate the axon and neural fiber diameters, the area of each individual fiber was converted to the diameter of a circle having an equivalent area.

Statistical analysis

Behavioral assessment were analyzed using one-way repeated measures analysis of variance (ANOVA) and morphometric measurements of the median nerve were analyzed using one way analysis of variance (ANOVA). All analyses were followed by post hoc Duncan's. Data were expressed as means ± standard error of the mean (SEM). The significance level was $p<0.05$. Statistical analysis was performed using the Statistical software package.

RESULTS

Behavioral Study

Footfault Test

Repeated measures analysis of variance of footfault test, showed time ($F_{(6.198)}$= 34.16863, $p<0.001$), group ($F_{(1.33)}$ = 5.47632, $P<0.01$) and a significant interaction between time and group ($F_{(24.198)}$ = 4.34956, $p<0.001$). As shown in Fig. 2, Duncan's post hoc analysis revealed that the number of right forelimb errors was significantly decreased in the Sh-s group (0.58 ± 0.36) 3 days postlesion (pre-training), compared with all the lesioned groups: LSE (2.59 ± 0.54, $p<0.05$), LBC (4.43 ± 0.80, $p<0.05$), LR (2.36 ± 0.35, $p<0.05$) and LBCR (2.43

± 0.28, $p<0.05$). After 9 days postlesion (one week of training), the LBCR (1.46 ± 0.22) showed significantly more right forelimb errors than the Sh-s (0.33 ± 0.16; $p<0.05$). Differently, the LBC (0.79 ± 0.25), LR (0.73 ± 0.23) and LSE (1.10 ± 0.23) groups showed no signicant differences between the Sh-s group, after 9 days postlesion. Afterward, from day 15 poslesion, the injured groups revealed no signicant differences between the Sh-s group on other days of testing.

Hanging Wire

Repeated measures analysis of variance of the hanging wire showed effect for time factor ($F_{(5.205)} = 3.9437$, $P<0.01$) and an significant interaction between time and group effects ($F_{(20.205)} = 1.9323$, $P<0.05$), but no group effect ($F_{(1.41)} = 2.2340$, $P>0.05$) (Fig. 3).

Cylinder Test

Repeated measures analysis of variance for the cylinder test showed effect for time factor ($F_{(2.82)} = 4.8422$, $P<0.05$), but no group effect ($F_{(1.41)} = 1.2943$, $p>0.05$) and no significant interaction between time and group ($F_{(8.82)} = 0.7112$, $P>0.05$) (Fig. 4).

Histological analysis

The structural analysis of regenerating nerves (Fig. 5) showed important qualitative differences between the Sh-s and injured groups (LSE, LBC, LR and LBCR). The histological characteristics of distal portion of median nerve in the crush groups comprise enlargement of endoneurial connective tissue between the nerve fibers and reduction of myelinated fiber diameter. In LBC group these pathological features were apparently reduced and less endoneurial connective tissue was observed.

Morphometric analysis of median nerve

For median nerve, one way ANOVA analyses evidenced effect of lesion for Myelinated fiber area ($F_{(1.23)}$ = 4.3358, P<0.01); Myelin sheath thickness ($F_{(1.23)}$ = 5.5932, P<0.01); Myelinated fiber diameter ($F_{(1.23)}$ = 6.0849, P<0.01); Axon diameter ($F_{(1.23)}$ = 5.4737, P<0.01) and Axonal density ($F_{(1.23)}$ = 3.7803, P<0.05). Analysis of morphometric data with Duncan's post-hoc test revealed that the LSE (24496 ± 2050.98 fibers/mm^2; P=0.007) and LR (25098 ± 3033.58 fibers/mm^2; P=0.005) groups showed a greater density of the myelinated fibers than the sham group (15004 ± 1831.82 fibers/mm^2; Figure 6A). Furthermore, the experimental groups (LSE: 5.5 ± 0.43 μm^2; P=0.001; LBC: 9.51 ± 0.93 μm^2; P=0.048; LR: 6.08 ± 0.99 μm^2; P=0.002; LBCR: 8.66 ± 1.78 μm^2; P=0.028) had smaller average myelinated fiber areas than the sham group (14.32 ± 3.13 μm^2; Figure 6B).

The average myelin sheath thickeness of the lesioned groups (LSE: 0.65 ± 0.02 μm; P=0.0008; LBC: 0.69 ± 0.06 μm; P=0.0016; LR: 0.65 ± 0.02 μm; P=0.0007; LBCR: 0.74 ± 0.06 μm; P=0.0058) were thinner than that of the sham group (1.00 ± 0.08 μm; Figure 6C).

The average myelinated fiber diameters in the lesioned groups (LSE: 3.95 ± 0.13 μm; P=0.0003; LBC: 4.84 ± 0.27 μm; P=0.012; LR: 4.04 ± 0.21 μm; P=0.0004; LBCR: 4.74 ± 0.43 μm; P=0.010) were different from the sham group (6.18 ± 0.56 μm; Figure 6E). In addition, the average axon diameter in the LSE (2.63 ± 0.10 μm; P=0.0007), LR (2.74 ± 0.23 μm; P=0.0011) and LBCR (3.25 ± 0.30 μm; P=0.0230) groups was different from the sham group (4.18 ± 0.41 μm; Figure 6D). However, the LBC group had the higher average myelinated axon diameter (3.46 ± 0.16 μm), approximating of the values of the sham group.

DISCUSSION

Experimental studies showed that exercise training improves motor function after clinical and experimental peripheral nervous lesion, and can be considered as an effective treatment of sensorial deficits[25,26,27,28]. However, only one study have used proprioceptive and repetition training as treatment for brachial plexus injury or tests of skilled forelimb function to assess recovery after peripheral nerve injury and repair[29].

In the present study we investigated the hypothesis that balance and coordination, repetition training and the association of this two protocols, started four days after the crush and performed for six weeks, could produce different effects on functional recovery and morphological changes in the regenerating nerve. We chose to begin the exercise program as early as four days after the crush in order to show that the training protocols (LBC and LR) can be used in the early phase of rehabilitation, as long as performed carefully and moderately.

Our results show that the injured groups (LSE, LBC, LR and LBCR) decreased forelimb errors after one week, as evaluated by means of FT. What may prove a spontaneous recovery by these groups or even suggest that the test was not sufficiently sensitive to demonstrate the differences between them, since this task is more used in brain lesions than peripheral ones[21,30,31]. Furthermore, 9 days postlesion the LBCR group showed more forelimb error than Sh-s group. Perhaps, the high demand imposed by the association of training could have a deleterious effect on functionality of the animals at earlier stages, and after, there is a normalization of function. In the same way, the HW and CT show no significance difference between the groups. The reason for this may be explained, as a hypothesis, since they evidently demonstrated impaired right forelimb movements, until at least two weeks after the injury. We think that the animals acquired compensatory strategies to perform the required

tasks and thus the deficits could not be quantified. More accurate analysis, which evaluates the quality of movement, may be more effective to show the possible functional differences of the treatments in the forelimbs. Moreover, in the HW, there was a learning curve in sham group, shown by less time spent in performing the task every day analyzed. This demonstrates that not only the motor skill training that is being evaluated in this test, but the ability to adapt to it, dificulting the conclusions about this data.

In a crush lesion, the axon undergoes alterations both in the distal and proximal portions to the injury, but such alterations occur mainly in the distal portion; thus, the morphometric parameters distal to the lesion are more important for the analysis of the influence of exercise in nerve regeneration[5]. In this portion of the nerve, the average myelinated fiber area, myelin sheath thickness and myelinated fiber diameter showed a significant difference between the lesioned groups and Sh-s. These results show that the injury caused a pathological modification in nerve. But, there were some levels of recovery, indicated by following morphological parameters: axonal density and axonal diameter. The balance and coordination training associated or not to repetition showed a decreased number of fibers/mm^2, approximating of the sham values. This reduction indicates an increase in myelinated fiber area caused by these training programs. Corroborating this data, the LBC group presented an improvement in axonal diameter, statistically equaling to sham group. In a previous study carried through our laboratory, the balance and coordination exercise training had also demonstrated changes in morphological parameters (average myelinated fiber area, average myelin sheath thickness, myelinated fiber diameter and axon diameter) of peripheral nerves in young rats compared to free walking training[16]. In other study realized in our laboratory, moderate endurance training showed a greater degree of the myelinated fiber maturation than the sedentary, resistance-trained and concurrent training groups. Furthermore, in this study, the endurance-trained group showed a smaller percentage area of endoneurial

connective tissue and a greater percentage area of myelinated fibers than the sedentary group after sciatic crush injury, promoting the normalization of hindlimb motor function after one week of exercise[15]. This study, thus provides evidence that resistance training or the combination of two strategies may delay functional recovery and do not alter nerve regeneration. All these findings indicate that the time and the type of exercise training can exert different consequences in peripheral nerve regeneration.

In humans, the major aim of physiotherapy after nervous system injury is to restore the patient's autonomy in activities of daily living and to avoid permanent disability. In this context, one of the most important tools in experimental studies is the functional assessment as they provide the validation of more effective therapies with respect to self-sufficiency of the patients. Unfortunately, in our study, the functional tests were not appropriate for this type of injury. Since the crushed group, even showing some degree of protection of the forelimbs visible until two weeks after lesion, demonstrated no deficit in the tests used. Perhaps this is because of compensation used by these animals to perform the tasks, since the lesion is unilateral. Also this may be due to the role of the forelimb, which is used more for skills tasks than weight bearing and locomotion, variables involved in the tests performed in the present work[32]. Tasks that assess the quality of movement can be more effective to show the functional recovery in this type of injury/training (such as electromyography, staircase test, pawprint analysis, kinematic analysis of movements).

In conclusion the present study showed that balance and coordination training can accelerate the median nerve regeneration after an experimental traumatic injury compared to sedentary and repetition groups and the association of treatments. The functional tests proved no effective in demonstrate the real deficits of the forelimb and thus is necessary better skill tests to show more subtle changes of movement. The need for efficiency in the recovery of hand and forelimb movements requires further studies to verify the success of different

methods of treatment in promoting neuronal reorganization and functional improvement after injury of the peripheral nervous system.

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LEGENDS

Figure 1. Time line of experimental procedures: Treadmill: Electrical Treadmill habituation; BC: habituation of apparatus of Balance and Coordination; FT: Footfault test; CT: Cylinder test; HW: Hanging Wire.

Figure 2. Footfault test: The footfault test was performed to assess whether acrobatic and repetition training or the association of these two protocols enhanced coordinated placement of the forelimbs. Each animal was recorded with a digital video camera crossing three times and the errors were measured as slips with right forelimb through the grid openings. All the damaged groups showed functional recovery over the weeks. At day 3 postlesion, all the injured groups showed a statistically significant difference compared to Sh-s group. Furthermore, in day 9 the LBCR group showed significant difference compared to Sh-s group. Values presented are mean ± SEM (* indicates that injured groups are different to Sh-s group and ** indicates that LBCR group are different to Sh-s group). Sh-s = sham; LSE = lesion + sedentary; LBC = lesion + balance and coordination trained; LR = lesion + repetition trained; LBCR = lesion+ repetition + balance and coordination trained.

Figure 3. Hanging Wire: This task was used as a measure of the ability of grasp and of strength of the forelimbs. Animals used their forelimbs to suspend their body weight on a wire stretched between two bars. In day 9 postlesion, the LBC group showed that after one week of training was unable to improve the strength of the forelimbs. Differently, the other injured groups were superior in the ability to grasp and in the strength of the forelimbs. Values presented are mean ± SEM. Sh-s = sham; LSE = lesion + sedentary; LBC = lesion + balance and coordination trained; LR = lesion + repetition trained; LBCR = lesion+ repetition + balance and coordination trained.

Figure 4. Cylinder test: Cylinder test measure the number of contralateral forelimb contacts compared to ipsilateral while the animal reared in a cylinder. All the injured groups revealed no difference between treatments and to Sh-s group. Values presented are mean ± SEM. Sh-s = sham; LSE = lesion + sedentary; LBC = lesion + balance and coordination trained; LR = lesion + repetition trained; LBCR = lesion+ repetition + balance and coordination trained.

Figure 5. Digitized images of transverse-semithin sections (1 µm) of distal portion obtained from regenerating median nerves after 6 weeks of specific exercise training. Sham-sedentary group: Note the large size of the myelinated fibers and the large myelin sheath thickness. Lesion sedentary group: Note that there is a predominance of small-diameter thin myelin sheath fibers and increase in endoneurial connective tissue between the nerve fibers. Lesion group submitted to balance and coordination training: Note the endoneurial connective tissue between the nerve fibers and large myelin sheath thickness compared to LSE group. Lesion group submitted to repetition training: Observe the myelinated fibers that appear to be similar to the LSE, with predominance of small-diameter thin myelin sheath fibers and increase in endoneurial connective tissue between the nerve fibers. Lesion group submitted to balance and coordination + repetition training: Myelinated fibers appear to be similar to the LBC with large myelinated fibers, but with more endoneurial connective tissue between the nerve fibers. The arrow indicates myelin sheath; * (asterisk), myelinated nerve fiber; Ect, endoneurial connective tissue. Semithin sections were stained with toluidine blue. Scale bar = 20 µm.

Figure 6. Effects of specific physical exercise training on the morphometric parameters of regenerating right median nerve fibers after 6 weeks of training. Graphics show the mean area at distal portion of the nerve (A); myelinated fiber density (B); the average myelinated fiber area (C), average myelin sheath thickness (D), average myelinated fiber diameter (E) and average axon diamenter of the myelinated fiber (F). Data are expressed as means ± SEM (* indicates differences to Sh-s group). Sh-s = sham; LSE = lesion + sedentary; LBC = lesion +

balance and coordination trained; LR = lesion + repetition trained; LBCR = lesion+ repetition + balance and coordination trained.

Table 1: Experimental groups and number of animals used for each analyses.

FIGURES AND TABLE

Figure 1

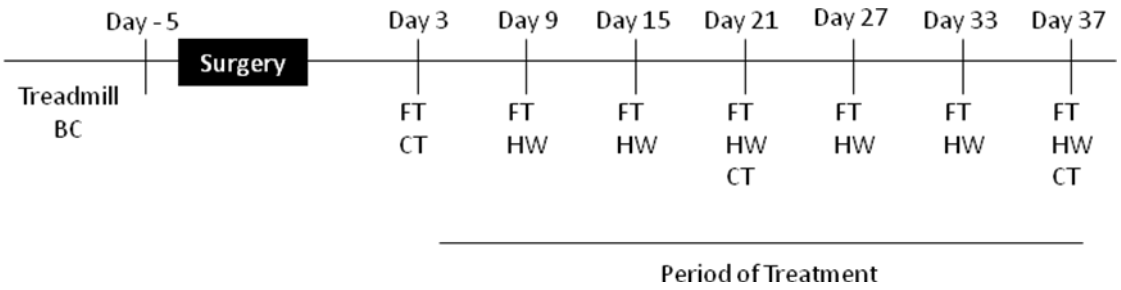

Figure 2

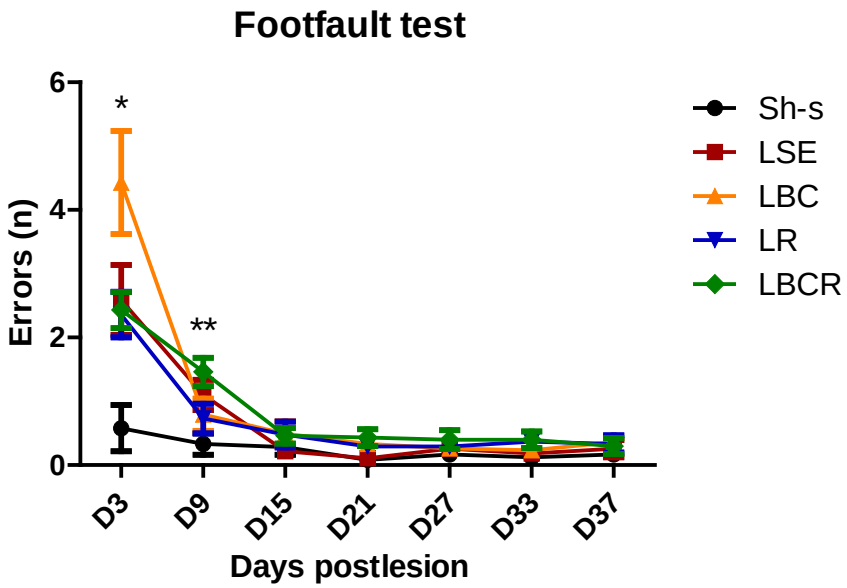

Figure 3

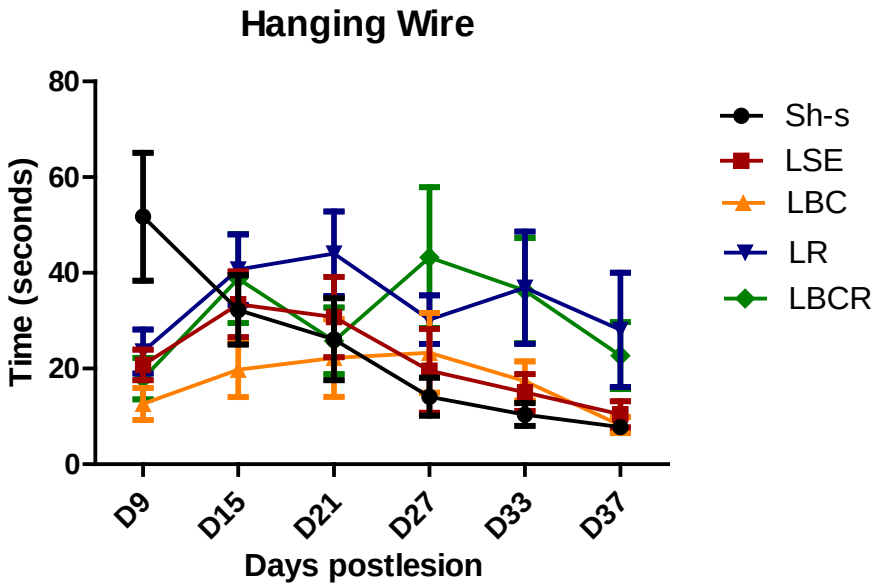

Figure 4

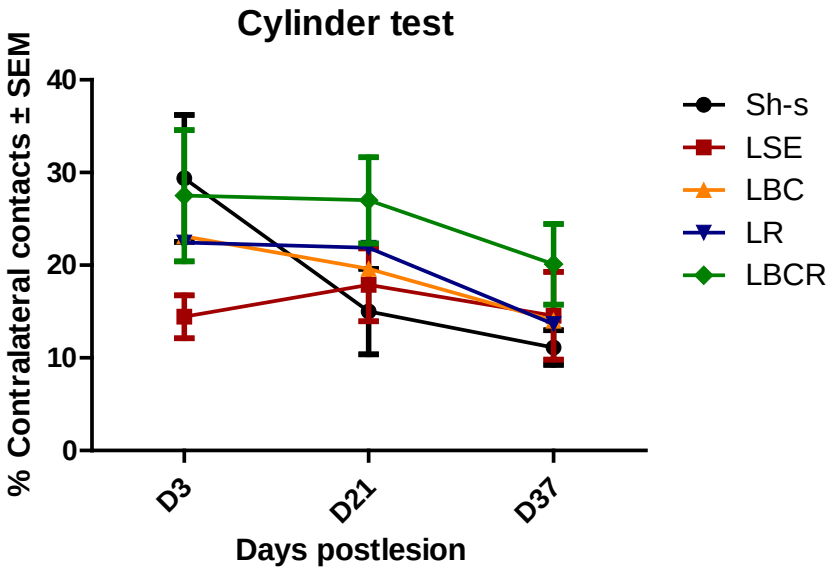

Figure 5

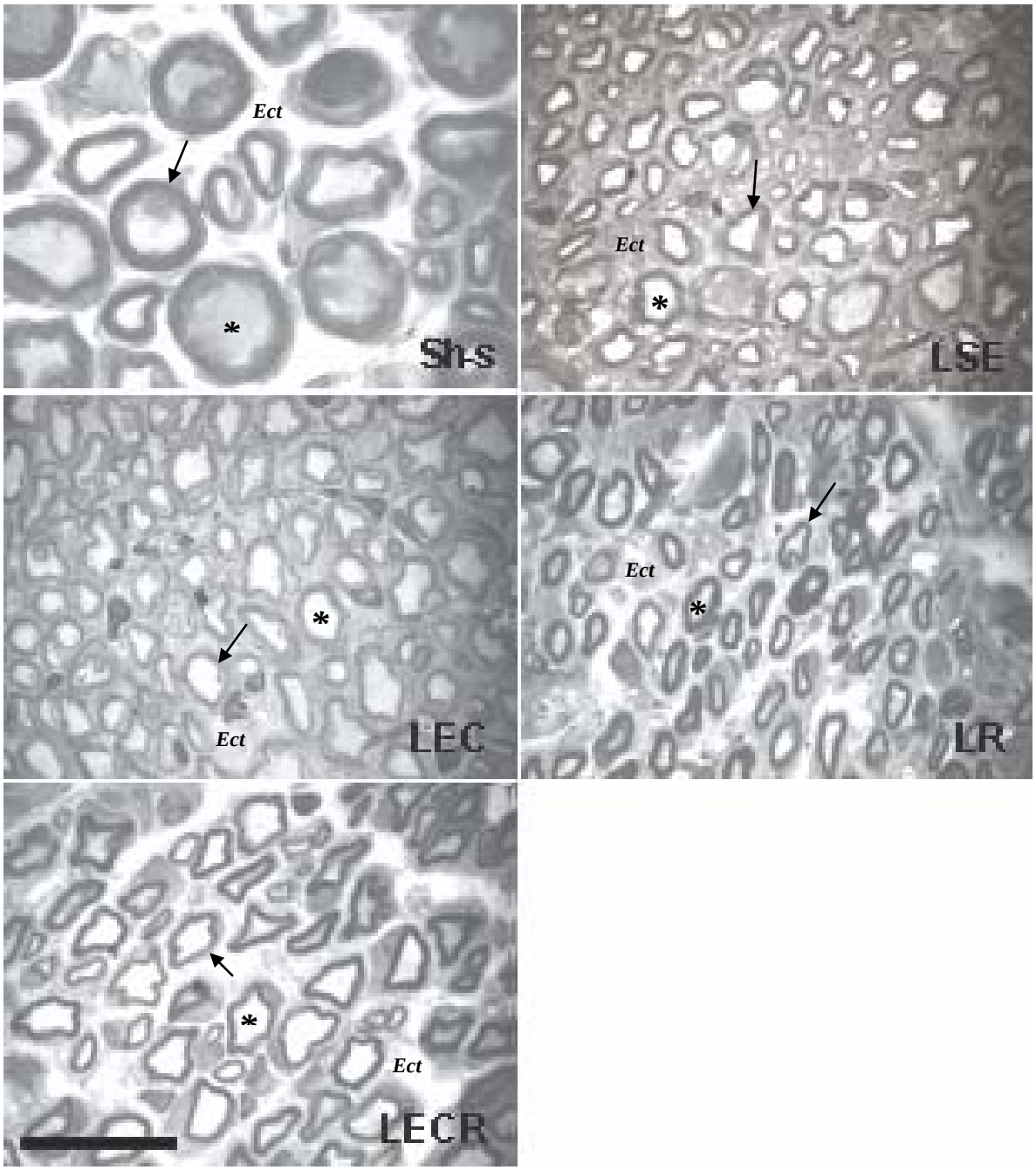

Figure 6

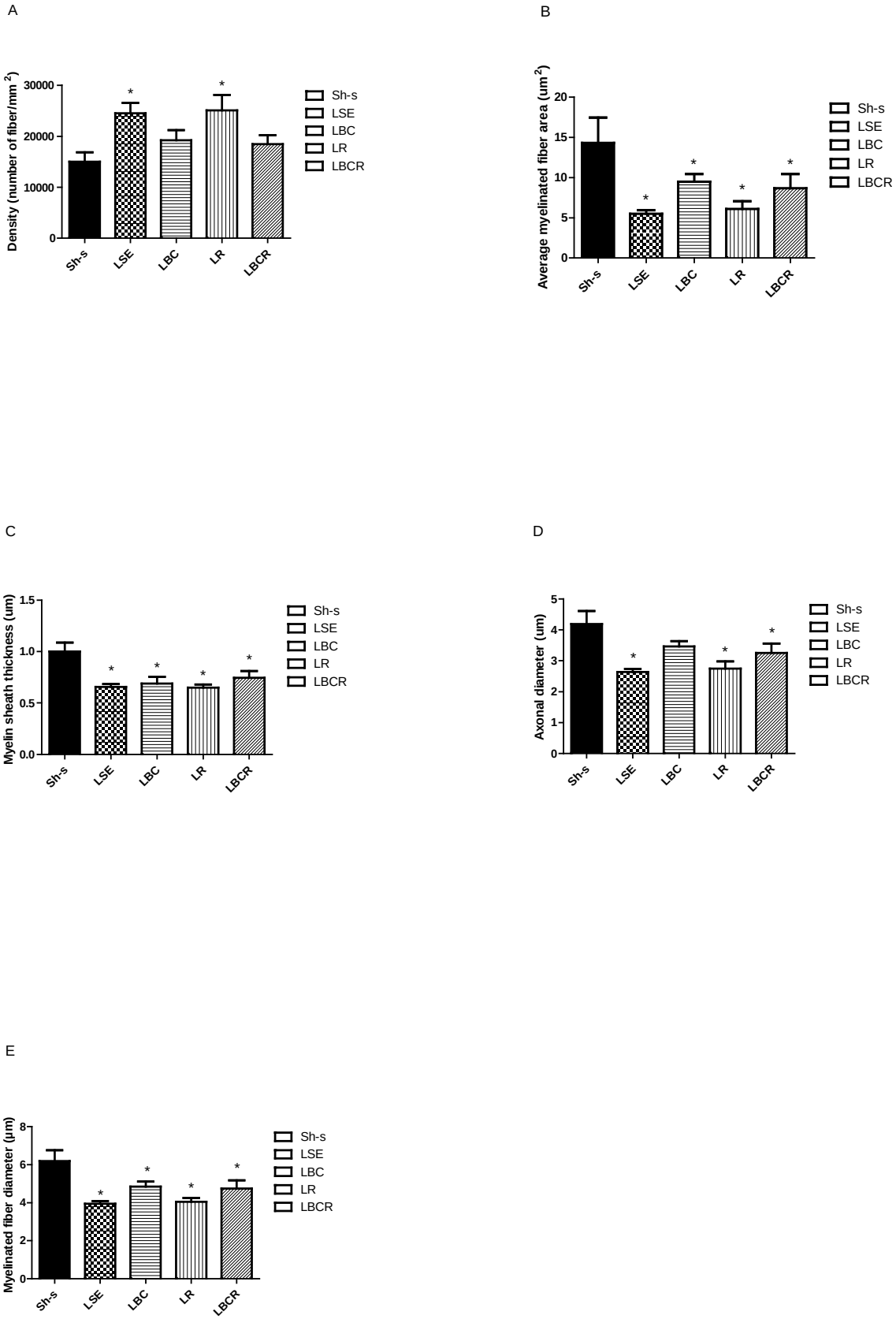
A
Density (number of fiber/mm 2)
30000
20000
10000
0
Sh-s
LSE
LBC
LR
LBCR
B
Average myelinated fiber area (um 2)
20
15
10
5
0
C
Myelin sheath thickness (um)
1.5
1.0
0.5
0.0
D
Axonal diameter (um)
5
4
3
2
1
0
E
Myelinated fiber diameter (µm)
8
6
4
2
0

Table I

Group Nº	Group Description	Designation (Abbreviation)	Nº of animals	Functional evaluation	Morphometric analysis
1	Sham animals (untouched - unlesioned)	Sham (Sh-s)	8	8	5
2	Animals submitted to median nerve crush and not treated	Sedentary (LSE)	9	9	6
3	Animals submitted to median nerve crush and treated with balance and coordination training	Balance and coodination (LBC)	10	10	6
4	Animals submitted to median nerve crush and treated with repetition training	Repetition (LR)	9	9	5
5	Animals submitted to median nerve crush and treated with repetition + balance and coordination training	Reptition + balance and coordination (LBCR)	10	10	6

4 CONCLUSÕES E PERSPECTIVAS

Os dados apresentados neste trabalho mostram evidências de que o treinamento de equillíbrio e coordenação acelerou a regeneração nervosa após lesão traumática esperimental, demonstrando que esse protocolo pode interferir no planejamento terapêutico dos profissionais da área da saúde que trabalham com lesões do SNP. Os testes funcionais, no entanto, não se mostraram efetivos em demonstrar os déficits reais dos membros anteriores e por isso se faz necessário testes mais acurados para a obtenção de tarefas de habilidade, enfocando as alterações mais sutis de movimento, como as causadas no presente trabalho.

Diante disso, os resultados obtidos nesse estudo servem como referência para futuras pesquisas que busquem elucidar os efeitos da variação da intensidade e dos tipos de protocolos utilizados e seus efeitos sobre a regeneração nervosa. Além disso, se faz necessário analisar os efeitos da lesão no SNP e as tarefas utilizadas para reabilitação em neurônios da medula espinhal e o papel de outras estruturas encefálicas relacionadas à atividade motora.

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