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EFEITOS DO TREINAMENTO DE EQUILÍBRIO E COORDENAÇÃO E DO TREINAMENTO AERÓBICO SOBRE A RECUPERAÇÃO FUNCIONAL E PLASTICIDADE NEUROMUSCULAR APÓS LESÃO NERVOSA PERIFÉRICA POR ESMAGAMENTO

TESE DE DOUTORADO

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Efeitos do treinamento de equilíbrio e coordenação e do treinamento aeróbico sobre a recuperação funcional e plasticidade neuromuscular após lesão nervosa periférica por esmagamento

Tese entregue ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Neurociências, da Universidade Federal do Rio Grande do Sul (UFRGS, RS), como requisito parcial para obtenção do grau de Doutor em Neurociências.

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RESUMO

O objetivo desta Tese foi analisar os efeitos do treinamento de equilíbrio e coordenação e do treinamento aeróbico sobre variáveis funcionais, morfológicas do nervo isquiático e do músculo sóleo, bem como a imunorreatividade para sinaptofisina e da neurotrofina-3 na medula espinal após uma lesão por esmagamento do nervo isquiático. Para isso, ratos Wistar adultos foram divididos de maneira aleatória em 4 grupos experimentais: Sham-operado (SH), Não-treinados (NT), Treinamento Aeróbico (*Endurance Training* – ET) e Treinamento de Equilíbrio e Coordenação (*Balance and Coordination Training* – BCT), sendo que os três últimos grupos experimentais foram submetidos à lesão por esmagamento do nervo isquiático, que teve como consequência uma axonotmese. Os protocolos de treinamento tiveram início na fase aguda, 48 horas após o procedimento cirúrgico para o esmagamento do nervo e tiveram a duração de 4 semanas. Nossos resultados demonstraram que na análise funcional sensoriomotora (Teste da Escada Horizontal e Teste da Barra Estreita) os animais do grupo BCT apresentaram um melhor desempenho que os animais dos outros grupos. Entretanto, na análise funcional locomotora (Índice de Funcionalidade do Nervo Isquiático e Comprimento da Passada do Membro Lesionado), os resultados do Índice de Funcionalidade do Nervo Isquiático foram similares entre os grupos lesionados (NT, ET e BCT) enquanto no Comprimento da Passada do Membro Lesionado, os animais do ET apresentaram resultados mais satisfatórios que os demais grupos. No estudo morfológico quantitativo do músculo sóleo, a análise morfométrica muscular demonstrou que os grupos ET e BCT apresentaram resultados semelhantes entre si e melhores que o grupo NT nos parâmetros de área de tecido muscular, de tecido conjuntivo, de vasos sanguíneos, de densidade de fibras musculares e da área de secção transversal das fibras musculares. Na análise morfométrica da porção distal do nervo lesionado os resultados dos dois grupos treinados foram similares entre si em todos os parâmetros analisados. Esses animais treinados apresentaram melhores resultados quando comparado ao grupo NT para a variável diâmetro médio das fibras nervosas, enquanto o grupo ET na área total dos vasos sanguíneos e na densidade das fibras mielínicas. A análise qualitativa, tanto do músculo sóleo como do nervo isquiático, mostrou que os grupos lesionados apresentaram características de nervo e de músculo em regeneração, não sendo possível verificar diferenças entre os grupos treinados e o grupo não treinado. A imunorreação para sinaptofisina na medula espinal, por meio da análise da densitometria óptica no corno dorsal dos níveis L4-L6 da medula espinal revelou-se significativamente maior no grupo BCT quando comparado ao grupo NT. Na análise da neurotrofina-3 na mesma região da medula espinal, o grupo BCT apresentou resultados significativamente superiores aos demais grupos. Hipotetizamos que essa imunorreação específica do grupo BCT está diretamente relacionada com os melhores resultados deste grupo nos testes sensoriomotores, uma vez que nas análises morfológicas quantitativas e qualitativas os grupos ET e BCT apresentaram resultados similares.

Palavras-chaves: Lesão nervosa periférica; Treino aeróbico; Treino de equilíbrio e coordenação; Recuperação sensoriomotora, Plasticidade neuromuscular.

ABSTRACT

The aim of this thesis is to analyze the effects of balance and coordination training, and aerobic training on functional variables, morphological variables of the sciatic nerve and soleus muscle, as well as synaptophysin and neurotrophin-3 immureactivity in the spinal cord after a crush injury of the sciatic nerve. Therefore, adult Wistar rats were randomly divided into 4 groups: Sham-operated (SH); Non-trained (NT); Endurance Training (ET), and Balance and Coordination Training (BCT). The last three experimental groups were submitted to sciatic nerve crush injury, which resulted in an axonotmesis. The training protocols, which lasted for four weeks, were initiated early during the acute phase - 48 hours after the surgical procedures. Our results showed that for the sensorimotor functional analysis (Horizontal Ladder Rung Walking Test and Narrow Bar Test) the BCT group animals presented better performance than the other group's animals. However, for the locomotor functional analysis (Sciatic Functional Index and Right Hindlimb Paw Stride Length), the Sciatic Functional Index presented similar results between the injured groups (NT, ET, and BCT) while the Right Hindlimb Paw Stride Length, the animals from the ET group showed better results than those from the other groups. In the quantitative morphological study of the soleus muscle, the muscular morphometric analysis showed that ET and BCT groups presented similar results in comparison to each other, and better results than NT group regarding the parameters of muscle tissue areas, connective tissue, blood vessels, muscle fiber density, and the muscle fibers cross-sectional area. In the morphometric analysis of the distal portion of the damaged nerve, the results from both trained groups were similar in comparison to each other in all analyzed parameters as well. This trained animals presented better results when compared to the NT group to the nerve fiber diameter, while the ET group presented better results regarding the blood vessel total area and the myelinated fiber density. The qualitative analysis - both of the soleus muscle and the sciatic nerve - showed that the injured groups had characteristics of nerve and muscle regeneration, not being possible to verify differences between the trained and untrained groups. The spinal cord synaptophysin immureactivity, through optical densitometry at spinal cord L4-L6 levels of the dorsal horn revealed to be significantly in the BCT in comparison to the NT group. In the same region of the spinal cord neurotrophin-3 analysis, the BCT group showed significantly better results than the other groups. We hypothesized that this specific immureactivity in group BCT is directly related to the best results from this group in the sensorimotor tests, once the quantitative and qualitative morphological analyzes of the ET and BCT groups showed similar results.

Keywords: Peripheral nerve injury; Endurance training; Balance and coordination training; Sensorimotor recovery, neuromuscular plasticity.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

BCT ..... *Balance and coordination training*

BDNF ..... *Brain-derived neurotrophic factor*

ET ..... *Endurance training*

HLRWT ..... *Horizontal ladder rung walking test*

ICBS ..... Instituto de Ciências Básicas da Saúde

IFNI ..... Índice de funcionalidade do nervo isquiático

L4 ..... Quarta vértebra lombar

L5 ..... Quinta vértebra lombar

L6 ..... Sexta vértebra lombar

LNP ..... Lesão nervosa periférica

NBT ..... *Narrow beam test*

NGF ..... *Nerve growth factor*

NT ..... Não-treinado

NT-3 ..... Neurotrofina-3

NT-4/5 ..... Neurotrofina-4/5

NT-6 ..... Neurotrofina-6

NT-7 ..... Neurotrofina-7

S1 ..... Primeira vértebra sacral

S2 ..... Segunda vértebra sacral

SH ..... Sham-operados

SNC ..... Sistema nervoso central

SNP ..... Sistema nervoso periférico

UFRGS ..... Universidade Federal do Rio Grande do Sul

SUMÁRIO

1 INTRODUÇÃO DA TESE

1.1. Lesões nervosas periféricas

As lesões nervosas periféricas (LNPs) são um problema clínico comum, ocorrem após eventos traumáticos e acabam impactando negativamente na vida cotidiana das pessoas (IJKEMA-PAASSEN et al., 2004). Apesar de escassos, estudos epidemiológicos mostram que após uma lesão traumática, 1,3 a 3% dessa população tem como consequência uma LNP (NOBLE et al., 1998; KOUYOUMDJIAN et al., 2006; TAYLOR et al., 2008; SAADAT, ESLAMI & RAHIMI-MOVAGHAR, 2011). Estas lesões acometem principalmente jovens do sexo masculino, representando mais de 71% da população acometida (ROBINSON, 2004; KOUYOUMDJIAN et al., 2006; UZUM et al., 2006; ESER et al., 2009; SAADAT, ESLAMI & RAHIMI-MOVAGHAR, 2011). As LNPs ocasionam problemas de ordem social e econômica, uma vez que a maioria dos indivíduos acometidos estão em idade adulta produtiva e esse fato acaba impactando sobre a economia do estado e do sistema de saúde (WALDRAM, 2003; ESER et al., 2009).

A etiologia das LNPs abrange principalmente lesões por laceração, penetração, esmagamento, tração e isquemia do nervo envolvido (ROBINSON, 2004) e a grande maioria dessas lesões ocorrem em consequência de acidentes automobilísticos (NOBLE et al., 1998; UZUM et al., 2006; ESER et al., 2009; SAADAT, ESLAMI & RAHIMI-MOVAGHAR, 2011). Outras causas incluem traumas por objetos afiados, causas iatrogênicas (ROBINSON, 2004; DANEYEMEZ et al., 2005; UZUM et al., 2006), acidentes com arma de fogo e alongamento ou esmagamento nervoso após quedas (ROBINSON, 2004, DANEYEMEZ et al., 2005).

Os membros superiores são a região anatômica mais acometida pelas LNPs, representando mais de 70% dos casos (KOUYOUMDJIAN et al., 2006; ESER et al., 2009). A maioria dos estudos também demonstrou que o nervo ulnar é o mais acometido, seguido pelo

nervo mediano e o radial (UZUM et al., 2006; ESER et al., 2009; SAADAT, ESLAMI & RAHIMI-MOVAGHAR, 2011). No membro inferior, o nervo mais acometido é o isquiático, representando aproximadamente 10% de todas as lesões nervosas periféricas (DANEYEMEZ et al., 2005; UZUM et al., 2006; KOUYOUMDJIAN et al., 2006; ESER et al., 2009). O nervo isquiático apresenta uma localização superficial e um longo percurso, tem origem nas raízes nervosas L4, L5, S1 e S2, formando o plexo sacral e se bifurca na região da coxa em nervo tibial e nervo peroneal (IJKEMA-PAASSEN et al., 2004; ERGUN & LAKADAMYALI, 2010; DE SOUZA & CHOI, 2012). Devido a isto, traumas na região pélvica e na região da articulação do quadril, ocorridos em acidentes automobilísticos, quedas ou devido a causas iatrogênicas, são as principais causas das lesões do nervo isquiático (INTISO et al., 2010; SAADAT, ESLAMI & RAHMIMI-MOVAGHAR, 2011; DE SOUZA & CHOI, 2012; ANTONIADIS et al., 2014).

Com relação à magnitude da lesão nervosa, duas principais classificações foram propostas e são amplamente aceitas na literatura. A primeira, proposta por Seddon (1942, 1943), considera que as LNPs podem ser qualificadas em três tipos: (i) as neuropraxias, quando ocorre a interrupção temporária da condução sem perda de continuidade axonal; (ii) as axonotmeses, quando ocorre uma perda de continuidade relativa do axônio e de seu revestimento mielínico, mas a estrutura do tecido conjuntivo do nervo é preservada; e, (iii) as neurotmeses, quando ocorre uma ruptura completa, tanto da estrutura axonal, da mielina e do tecido conjuntivo, promovendo uma perda completa da continuidade estrutural da fibra nervosa. Sunderland (1951) reclassificou em cinco categorias as LNPs, com base na histopatologia, ao invés do grau de lesão, e adicionou critérios clínicos e eletrodiagnósticos. O primeiro grau de lesão corresponde à neuropraxia de Seddon, onde ocorre o bloqueio da condução nervosa com a estrutura axonal intacta e devido a isto, o prognóstico é bom. O segundo grau de lesão envolve transecção do axônio, mas com o endoneuro completamente

intacto e a recuperação ocorrendo por meio da regeneração axonal através dos tubos endoneurais. No terceiro grau de lesão ocorre a transecção axonal e dos tubos endoneurais, mas o epineuro permanece intacto, no entanto, a recuperação depende da capacidade dos axônios atravessarem o local da lesão e se conectarem aos tubos neurais. No quarto grau de lesão, há perda da continuidade axonal, dos tubos endoneurais e do perineuro, mas o epineuro ainda se apresenta intacto. O prognóstico geralmente é ruim sem uma intervenção cirúrgica. Já o quinto grau de lesão nervosa periférica, segundo a classificação de Sunderland (1951) representa a neurotmese da classificação de Seddon, onde ocorre a perda da continuidade do tecido nervoso. A Tabela 1 apresenta as classificações de Seddon e Sunderland.

Tabela 1 - Classificação das lesões nervosas periféricas de acordo com a classificação de Seddon e Sunderland

Seddon	Processo	Sunderland
Neuropraxia	Desmielinização segmentar	Primeiro grau
Axonotmese	Axônio lesionado, mas endoneuro intacto (ótima circunstância para regeneração)	Segundo grau
Axonotmese	Axônio descontinuado, tubo endoneural descontinuado, mas perineuro e fascículos preservados	Terceiro grau
Axonotmese	Perda da continuidade axonal, tubo endoneural descontinuado, e perineuro e fascículos preservados	Quarto grau
Neurotmese	Perda da continuidade do nervo	Quinto grau

Sunderland subdivide a axonotmese em três tipos, com diferentes graus de rompimento do nervo e diferentes capacidades para regeneração espontânea.

Fonte: Adaptado de WALDRAN (2003)

Um estudo retrospectivo realizado no Brasil demonstrou que a maioria das LNPs se caracteriza como axonotmese (45% das LNPs) (KOUYOUMDJIAN, 2006), sendo as lesões por esmagamento a causa mais comumente registrada (ROBINSON, 2000; HÖHNE et al., 2011).

1.2. O modelo animal e as lesões por esmagamento do nervo isquiático

O modelo de lesão por esmagamento do nervo isquiático em ratos é o mais frequentemente utilizado no estudo da regeneração nervosa periférica (RODRIGUEZ, VALERO-CABRÉ & NAVARRO, 2004; MAZZER et al., 2008; WANG et al., 2008a; SAVASTANO et al., 2014). Os primeiros motivos da frequente utilização desse modelo estão relacionados à anatomia dos ratos, que apresentam a distribuição de seus troncos nervosos semelhante à distribuição dos troncos nervosos de humanos (RODRIGUEZ, VALERO-CABRÉ & NAVARRO, 2004). Além desse fato, esses animais têm um comprimento adequado para manipulações cirúrgicas e introdução de enxertos na porção média da coxa (VALERO-CABRÉ & NAVARRO, 2002; WANG et al., 2008b). Outro motivo pela frequente utilização deste modelo é que a lesão por esmagamento do nervo isquiático é extremamente útil na obtenção de informações significativas sobre a clínica das lesões por compressão, laceração e esmagamento do nervo periférico (VOGELAAR et al., 2004; DIAO et al., 2004; MAZZER et al., 2008; PENG et al., 2010). Além disso, pode-se se afirmar que esta é uma técnica confiável para a reprodução de uma axonotmese por esmagamento (RONCHI et al., 2009; RONCHI et al., 2010).

Na maioria dos estudos em laboratório com ratos, esta lesão é produzida pela compressão do nervo isquiático com força máxima durante trinta segundos utilizando-se uma pinça hemostática (BRIDGE et al., 1994). Como resultado, esse esmagamento provoca uma lesão nervosa de segundo grau, de acordo com a classificação de Sunderland (1951). Esta técnica ocasiona uma axonotmese com danos suficientes para promover a degeneração Walleriana, com um bom prognóstico de regeneração nervosa, reinervação muscular e recuperação funcional, o que o torna um modelo muito útil no estudo da regeneração nervosa

periférica (DE MEDINACELI, FREED & WYATT, 1982; STOLL, JANDER & MYERS, 2002; MAZZER et al., 2008).

A degeneração Walleriana é o termo usado para a degeneração dos axônios e de suas bainhas de mielina após uma lesão nervosa, que usualmente ocorre após uma lesão traumática do nervo. Essa degeneração ocorre a partir do local da lesão, tanto na porção proximal até o próximo nódulo de Ranvier, como na porção distal à lesão. As células de Schwann se diferenciam, sofrem mitose e preenchem o espaço entre os cotos proximal e distal. Estas células de Schwann e os macrófagos atuam na fagocitose das bainhas de mielina, que perdem sua integridade durante esta fase da degeneração Walleriana. No corpo celular, ocorre uma série de alterações em consequência desta lesão nervosa. Inicialmente ocorre o aumento do volume do corpo celular, um deslocamento excêntrico do nucléolo para a periferia do núcleo e em seguida uma dispersão da substância de Nissl – cromatólise. Como consequência da degeneração Walleriana, as células alvo também sofrem alterações. Se a célula alvo for uma célula muscular, esta célula sofrerá com uma atrofia progressiva das miofibrilas e substituição por tecido conjuntivo (Figura 1) (ROBINSON, 2000; STOLL, JANDER & MYERS, 2002; WALDRAN, 2003; KOEPPEN, 2004; RODRÍGUEZ et al., 2004; GORDON, TYREMAN & RAJI, 2011).

Durante o processo de regeneração celular, as células de Schwann também exercem uma importante função para que este processo ocorra de maneira adequada. O coto proximal do axônio lesionado irá gerar brotos que irão avançar pelas células de Schwann que após a fragmentação da mielina se desdiferenciam e formam um cordão preenchendo o espaço entre o coto proximal e distal, até o alvo de reinervação, dando suporte para estes brotos em regeneração crescerem. Os fatores de crescimento NGF (*nerve growth factor*), BDNF (*brain-derived neurotrophic factor*), NT-3 (neurotrofina-3), NT-4/5 (neurotrofina-4/5), NT-6 (neurotrofina-6) e NT-7 (neurotrofina-7) irão auxiliar na maturação axonal, fornecendo

suporte trófico para esta proliferação celular. Após a regeneração, essas células de Schwann ainda atuam na produção de mielina em torno dos axônios regenerados, os alvos musculares voltam a ser inervados de maneira adequada e o corpo celular volta a ter sua aparência normal (LEE & WOLFE, 2000; SOFRONIEW, HOWE & MOBLEY, 2001; WOOD et al., 2013).

Figura 1 - Processo de degeneração e de regeneração de um nervo periférico

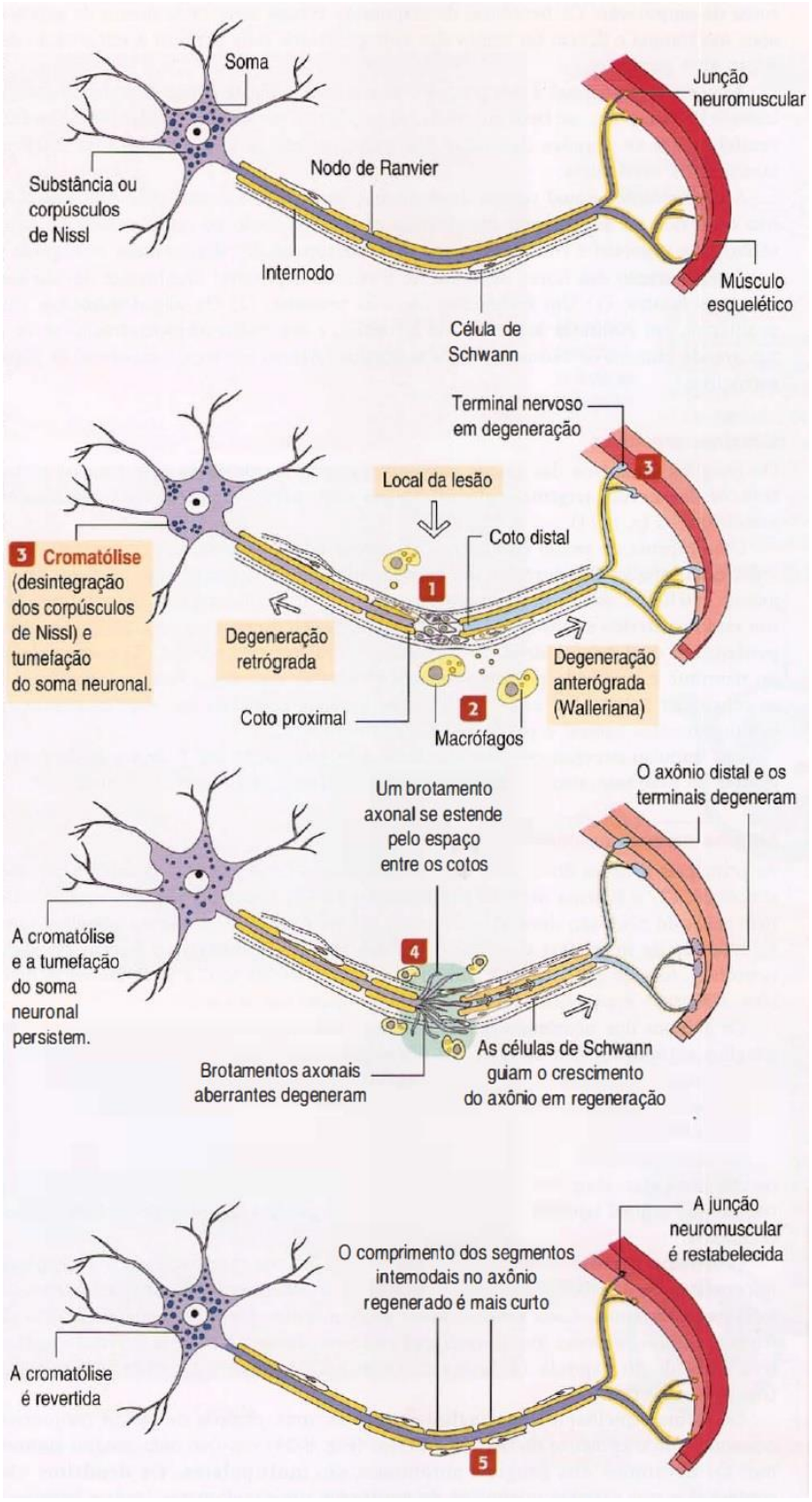

Figura 1. Figura esquemática mostrando resumidamente o processo de degeneração e de regeneração no SNP. Após uma lesão axonal por esmagamento, as células de Schwann sofrem divisão mitótica e preenchem o espaço entre os cotos proximais e distais do axônio (1). Estas células fagocitam a mielina. Gotículas de mielina são excretadas por estas células de Schwann e, em seguida, fagocitadas pelos 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) (modifidado de KIERSZENBAUM, 2008).

As axonotmeses ocasionadas após uma lesão por esmagamento afetam as pequenas e grandes fibras aferentes e eferentes (ROBINSON, 2000; HÖHNE et al., 2011), provocando dor e trazendo prejuízo à função sensorial e motora da célula muscular (DIAO et al., 2004; TAYLOR et al., 2008; RONCHI et al., 2009; RONCHI et al., 2010; NEAL & FIELDS, 2010; NOVAK et al., 2011). Essas alterações resultarão em altos níveis de comprometimento funcional e consequente impacto negativo na qualidade de vida das pessoas lesionadas (NOVAK et al., 2011; HADJ-SAÏD et al., 2012). Atrasos na recuperação após uma LNP trarão ainda mais prejuízos musculares e, como consequência, maiores prejuízos das atividades funcionais (CARSON & SWINNEN, 2002; HADJ-SAÏD et al., 2012). Como o nervo isquiático inerva grande parte dos músculos dos membros inferiores que são responsáveis pela flexão e extensão das articulações do joelho e tornozelo (IJKEMA-PAASSEN et al., 2004), uma lesão neste nervo ocasionará disfunções sensitivas e motoras e consequentes limitações de equilíbrio e de locomoção (UZUN et al., 2006; UDINA et al., 2011; HADJ-SAÏD et al., 2012). Com relação à locomoção em humanos após uma lesão do nervo isquiático, a principal alteração da marcha ocorre na fase do contato inicial, com a mudança da posição do tornozelo afetando seriamente a absorção de impacto e estabilidade do membro (PERRY, 1990).

Após uma lesão do nervo periférico, a recuperação completa irá depender do grau de lesão (VALERO-CABRÉ & NAVARRO, 2002; TAYLOR et al., 2008) e embora as axonotmeses tenham uma grande capacidade de regeneração após uma lesão por esmagamento, esta regeneração ocorre de maneira lenta (WOOD et al., 2013). Como consequência, a recuperação funcional também ocorrerá de maneira lenta e incompleta na maioria das vezes (ALBORNOZ et al., 2011; ENGLISH, WILHELM & SABATIER, 2011; KHUONG & MIDHA, 2013; WOOD et al., 2013). No entanto, a maioria destas lesões são tratáveis (UZUM et al., 2006) e o sucesso deste tratamento dependerá do método eleito para o

tratamento desta patologia (WANG et al., 2013). Muitos estudos sobre o tema estão sendo realizados, com resultados promissores e progressos significativos no entendimento do processo de degeneração e regeneração nervosa e muscular, e recuperação funcional após uma LNP. Embora nem todas as abordagens testadas no campo da pesquisa tenham se transformado em realidade clínica, as expectativas pelas novas descobertas são animadoras (KHUONG & MIDHA, 2013).

1.3. A atividade física no tratamento das LNPs

Em humanos, a atividade física tem sido cada vez mais utilizada como abordagem terapêutica após uma LNP (YING et al., 2003; UDINA et al., 2011). Consequentemente, diferentes estratégias e protocolos de treinamento físico estão sendo utilizadas para promover a recuperação nervosa, muscular e funcional após uma lesão nervosa periférica (BOBINSKI et al., 2011; HADJ-SAÏD et al., 2012). Em modelos animais, diversos protocolos de treinamento físico estão sendo testados, destacando-se o treinamento aeróbico moderado em esteira ergométrica adaptada (MARQUESTE et al., 2004; ILHA et al., 2008, SABATIER et al., 2008; ENGLISH et al., 2009; BOBINSKI et al., 2011; CUNHA et al., 2011; ENGLISH, WILHELM & SABATIER et al., 2011; UDINA, PUIGDEMASA & NAVARRO, 2011; HADJ-SAÏD et al., 2012; BOELTZ et al., 2013; PARK & HÖKE, 2014), o treinamento aeróbico de alta intensidade e curta duração também realizado em esteira ergométrica adaptada (SABATIER et al., 2008; COBIANCHI et al., 2013), o treinamento passivo na bicicleta adaptada (UDINA, PUIGDEMASA & NAVARRO, 2011), o treinamento através do exercício voluntário (MOLTENI et al., 2004), o treinamento de resistência (ILHA et al.,

2008), o treinamento de força (ANSENSIO-PINILLA et al., 2009), o treinamento de natação (VAN MEETEREN et al., 1997; TEODORI et al., 2011) e o treinamento de equilíbrio e coordenação (BONETTI et al., 2011).

No entanto, estes estudos demonstram evidências conflitantes sobre os efeitos benéficos e deletérios dos treinamentos físicos na regeneração do nervo periférico (UDINA, PUIGDEMASA & NAVARRO, 2011). Não há consenso sobre qual o tipo de treinamento é o mais adequado, sobre o tempo ideal de início do treinamento após a lesão, sobre a intensidade desses protocolos de treinamento, etc. (TEODORI et al., 2011; UDINA, PUIGDEMASA & NAVARRO, 2011). Estudos prévios realizados em nosso laboratório revelaram que o treino aeróbico moderado (ILHA et al., 2008) e o treinamento de equilíbrio e coordenação (BONETTI et al., 2011), apresentaram ótimos resultados na recuperação funcional, na regeneração nervosa e na muscular após uma lesão por esmagamento do nervo isquiático, mesmo esses estudos tendo períodos de início, tempo e intensidade dos treinamentos distintos.

Estes protocolos de treinamento têm o objetivo principal de otimizar o processo de recuperação nervosa, muscular e funcional (MARQUESTE et al., 2004; UDINA et al., 2011; UDINA, PUIGDEMASA & NAVARRO, 2011). Entretanto, o entendimento da regeneração nervosa e muscular é apenas uma etapa da recuperação total após uma LNP (ALVAREZ et al., 2010). Embora seja mais comum o estudo da recuperação local após uma LNP, a reorganização do sistema nervoso não ocorre apenas neste nível (WANG et al., 2013). Essa reorganização ou plasticidade do sistema nervoso também ocorre em diferentes níveis do sistema nervoso central (SNC): no encéfalo e na medula espinal (CHEN, COHEN & HALLETT, 2002; HANSSON & BRISMAR, 2003; BURNETT & ZAGER, 2004; SADOWSKY & MCDONALD, 2009). Então, uma falha no reestabelecimento de novas sinapses, por exemplo, pode interferir significativamente na função motora normal do indivíduo lesionado (ALVAREZ et al., 2010).

O entendimento da plasticidade sináptica central após uma LNP é muito importante para a recuperação total da funcionalidade, pois essa também depende das novas conexões sinápticas que ocorrem em outros níveis do sistema nervoso (VALERÓ & NAVARRO, 2001; LUNDBORG, 2003; STEIN et al., 2003). Se estas conexões sinápticas não ocorrerem de maneira adequada, os movimentos do segmento lesionado continuarão prejudicados, mesmo que este nervo seja responsável pela atividade de apenas um ou dois músculos (ALVAREZ et al., 2010). Além disto, após uma LNP, a plasticidade no SNC poderá compensar funcionalmente alguma falha no processo de regeneração do nervo e do músculo (NAVARRO, VIVÓ & VALERO-CABRÉ, 2007). Então, a escolha do protocolo de treinamento após uma LNP deve levar em consideração não apenas os aspectos de regeneração nervosa e muscular no foco da lesão, mas também toda a plasticidade do SNP e SNC (UDINA et al., 2011).

1.4. A sinaptofisina, os fatores de crescimento neurotróficos e a neurotrofina-3 após uma LNP

A sinaptofisina é a uma proteína integral da membrana das vesículas sinápticas (OKAJIMA et al., 1993; SUDHOF, 1995; SUN et al., 2006), a maior (THIELE et al., 2000), e acredita-se que tenha um papel múltiplo e importante na regulação da exocitose vesicular durante uma transmissão sináptica (OKAJIMA et al., 1993; SHIBAGUCHI et al., 2000; VANGUILDER et al., 2010). Essa proteína está amplamente distribuída pelo SNC, desempenha um papel importante na formação e estabilização das sinapses (MATUTE et al., 2007), sendo utilizada como um marcador específico de vesículas sinápticas, de terminais sinápticos (THIELE et al., 2000; VANGUILDER et al., 2010) e da liberação de

neurotransmissores (KWON & CHAPMAN, 2011). Devido a isto, em diversos estudos tem sido consistentemente utilizada como um marcador para a plasticidade sináptica (GUNTINAS-LICHIUS et al., 1994; MATUTE et al., 2007; KWON & CHAPMAN, 2011). A sinaptofisina é facilmente detectável por técnicas de imunoistoquímica em áreas específicas do SNP (ZIMMERMANN & VOGT, 1989; OKAJIMA et al., 1993) e do SNC (WALAAS, JAHN & GREENGARD, 1988; CHOU et al., 2002; SEO et al., 2010).

Com relação aos fatores de crescimento, uma importante classe destes são os fatores de crescimento neurotróficos, o NGF (*nerve growth factor*), BDNF (*brain-derived neurotrophic factor*), NT-3 (neurotrofina-3), NT-4/5 (neurotrofina-4/5), NT-6 (neurotrofina-6) e NT-7 (neurotrofina-7) (GU et al., 2011; WOOD et al., 2013). Estes fatores neurotróficos usualmente se ligam a duas classes de receptores: p75 e receptores da família tirosina-quinase (GU et al., 2011). Entretanto, as diferentes neurotrofinas demonstram grande especificidade para os receptores tirosina-quinase (NGF com a tirosina-quinase A, BDNF e NT-4/5 com a tirosina-quinase B e a NT-3 com a tirosina-quinase C) (CHAO, 2003) e baixa especificidade para os receptores p75 (CHAO, 1994; CASACCIA-BONNEFIL et al., 1999). Nos últimos anos, muita atenção tem sido dada aos fatores de crescimento neurotróficos e seus receptores após uma LNP, pois estes fatores de crescimento desempenham um importante papel na sobrevivência e no crescimento neuronal (LEE, ZHUOA & HELKEA, 2001; NAVARRO, VIVÓ & VALERO-CABRÉ, 2007). As LNPs causam prejuízos não apenas na inervação eferente direcionada ao funcionamento do músculo, mas também na inervação aferente, que também é responsável para que os movimentos ocorram de maneira adequada (VALERO-CABRÉ & NAVARRO, 2007). Imediatamente após uma transecção do nervo isquiático, aproximadamente 10 a 50% dos neurônios do gânglio da raiz dorsal (GRD) são perdidos, mas imagina-se que em uma lesão por esmagamento do nervo estas taxas sejam menores (TANDRUP, WOOLF & COGGESHALL, 2000). Como consequência, o efeito combinado

da redução significativa do número de sinapses centrais com as aferências sensitivas dos neurônios do tipo Ia que se ligam no fuso muscular também influenciará nas alterações de funcionalidade após uma LNP (ALVAREZ et al., 2010).

Especificamente sobre a NT-3, tem sido proposto que este fator de crescimento neurotrófico é importante na regulação do desenvolvimento sensorial muscular e na manutenção proprioceptiva muscular (GOROKHOVA, GAILLARD & GASCON, 2009). A NT-3 é indispensável para as células sensoriais do tipo Ia, pois transmite sinais tróficos das células alvo para os neurônios do SNC após uma transecção nervosa periférica (NITTA et al., 1999) e após uma LNP por esmagamento, também regula as interações axônio-alvo (TAYLOR et al., 2005). Devido a isto, a NT-3 é de extrema importância para que a aferência ao SNC ocorra de maneira adequada, pois modula a conexão entre o fuso muscular e o motoneurônio após uma LNP (XIE et al., 1997; MENDELL et al., 2001). A NT-3 é considerada essencial para a manutenção dos neurônios proprioceptivos e indispensável para a execução correta de uma tarefa motora (FAN, JAENISCH & KUCERA, 1999). Ratos mutantes com deficiência de NT-3 não se desenvolvem normalmente e apresentam disfunção neurológica grave, expressando perda de neurônios sensoriais e anormalidades em estágios iniciais de desenvolvimento destes neurônios (TESSAROLLO et al., 1994), alteração na postura, deficiência na percepção da colocação dos membros no espaço (ERNFORS et al., 1994) e consequentemente movimentos descoordenados (ERNFORS et al., 1995). Apesar de já ter sido demonstrado que a atividade física aumenta os níveis de NT-3 em regiões seletivas da medula espinal e que a repetição dos exercícios tem um efeito na magnitude e na estabilidade destas respostas, pouco se sabe sobre a efetividade de outros protocolos de treinamento sobre os níveis de NT-3 na medula espinal (GOMEZ-PINILLA et al., 2001),

Estas questões envolvendo a NT-3 confirmam a importância do sistema sensorial para o adequado movimento humano e também durante o processo de reabilitação após uma LNP

(COBIANCHI et al., 2013). A compreensão da plasticidade central, com seus princípios de conectividade que coordenam o movimento ao nível medular, tornará mais viável o entendimento do papel desta organização central na produção de um movimento correto e coordenado (FAN, JAENISCH & KUCERA, 1999; MENDELL et al., 2001; LEVINE, LEWALLEN & PFAFF, 2012). Além disto, o melhor entendimento sobre o sistema sensorial irá auxiliar no desenvolvimento de protocolos de treinamento físico que considerem a importância do estímulo sensorial, e assim, o melhor resultado funcional possível poderá ser alcançado (FAN, JAENISCH & KUCERA, 1999; UDINA et al., 2011b; COBIANCHI et al., 2013).

Após a apresentação dos Objetivos deste trabalho, os Materiais e Métodos, assim como os Resultados, serão apresentados na forma três artigos científicos.

2 OBJETIVOS DA TESE

2.1. Objetivo Geral

Este estudo teve como objetivo geral estudar os efeitos do treinamento de equilíbrio e coordenação e do treinamento aeróbico sobre a recuperação funcional e plasticidade neuromuscular após uma lesão por esmagamento do nervo isquiático.

2.2. Objetivos Específicos

- Examinar os efeitos do treinamento de equilíbrio e coordenação e do treinamento aeróbico sobre a recuperação da função sensoriomotora, utilizando dois testes específicos, Teste da Escada Horizontal e o Teste da Barra Estreita, após uma lesão por esmagamento do nervo isquiático.

- Avaliar os efeitos do treinamento de equilíbrio e coordenação e do treinamento aeróbico sobre a recuperação da função locomotora dos membros posteriores, por meio do índice de funcionalidade do nervo isquiático, após uma lesão por esmagamento do nervo isquiático.

- Analisar os efeitos do treinamento de equilíbrio e coordenação e do treinamento aeróbico na regeneração do nervo isquiático e no trofismo das fibras do músculo sóleo após uma lesão por esmagamento desse nervo.

- Verificar os efeitos do treinamento de equilíbrio e coordenação e do treinamento aeróbico sobre o padrão de imunorreação da sinaptofisina no corno dorsal da medula espinal (L4-L6) após uma lesão por esmagamento do nervo isquiático.

- Verificar os efeitos do treinamento de equilíbrio e coordenação e do treinamento aeróbico sobre o padrão de imunorreação da neurotrofina-3 no corno dorsal da medula espinal (L4-L6) após uma lesão por esmagamento do nervo isquiático.

3 COLETÂNEA DE ARTIGOS

3.1 ARTIGO 1

Balance and coordination training and endurance training after nerve injury – publicado na revista *Muscle & Nerve*

BALANCE AND COORDINATION TRAINING AND ENDURANCE TRAINING AFTER NERVE INJURY

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ABSTRACT: *Introduction*: Different rehabilitation treatments have proven useful in accelerating regeneration. *Methods*: After sciatic nerve crush in rats, we tested balance and coordination training (BCT) and endurance training (ET) through sensorimotor tests and analyzed nerve and muscle morphology. *Results*: After BCT and ET, rats performed better in sensorimotor tests than did non-trained animals. However, only BCT maintained sensorimotor function during training. Furthermore, BCT and ET produced significantly larger muscle area than in non-trained animals. *Conclusions*: These findings indicate that BCT and ET, when initiated in the early phase after sciatic nerve injury, improve morphological properties of the soleus muscle and sciatic nerve, but only the task-oriented BCT maintained sensorimotor function. The success of rehabilitative strategies appears to be highly task-specific, and strategies that stimulate sensory pathways are the most effective in improving balance and/or coordination parameters.

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Peripheral nerve injuries (PNIs) cause motor and sensory impairment.[1] Axonotmesis is a consequence of nerve injury and occurs after nerve crush, stretch, and percussion injuries.[2] Skeletal muscle inactivity occurs after these injuries due to disruption of nerve–muscle communication, and reductions in muscle use impact muscle performance negatively.[3]

Recently, different rehabilitation approaches using movement therapies have been shown to be useful for increasing axonal regeneration, maintaining muscle activity, and increasing functional recovery after nerve lesions.[4] Treadmill training[5–7] passive cycling,[5] resistance training,[6] forced exercises,[8] swimming exercises,[9] and balance and coordination training[10] are some of these movement therapies.

Moreover, previous studies from our laboratory revealed that both endurance training[6] and balance and coordination training[10] improve sensorimotor performance and morphological properties of the sciatic nerve after nerve crush; however, initiation of these training programs and the period of treatment were different. Thus, this study was designed to analyze the effects of 4-week endurance and balance and coordination training programs initiated early (48 h after the lesion) on functional recovery and morphology of sciatic nerve and soleus muscle fibers after crush injury of the sciatic nerve.

Abbreviations: BCT, balance and coordination training; ET, endurance training; HLRWT, horizontal ladder rung walking test; ICBS, Instituto de Ciências Básicas da Saúde; MET, maximal exercise test; NBT, narrow beam test; NT, non-trained; PB, phosphate buffer; PNI, peripheral nerve injury; SEM, standard error of the mean

Key words: balance; coordination; endurance; muscle; nerve

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METHODS

Experimental Design and Surgical Procedures. The experiments were performed on 23 3-month-old male Wistar rats weighing 280–330 g (initial age and weight) obtained from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul, Brazil). The rats were housed in standard plexiglass boxes (5 or 6 rats per box) under a 12-h light/12-h dark cycle in a temperature-controlled environment ($20 \pm 1^{\circ}C$) with food and water available *ad libitum*. All of the procedures were approved by the animal ethics committee of the Federal University of Rio Grande do Sul (Protocol No. 21794), and all animals were handled in accordance with Brazilian laws. The animals were randomly divided into the following 4 groups: (1) sham-operated rats that did not receive sciatic crush and were unexercised (sham, $n = 5$); (2) rats that received sciatic crush and were unexercised (non-trained, NT, $n = 6$); (3) rats that received sciatic crush and endurance training (ET, $n = 6$); and (4) rats that received sciatic crush and balance and coordination training (BCT, $n = 6$). Before the surgical procedures, the animals were placed in contact with the training program apparatus for 5 min/day over a period of 5 days in an attempt to avoid stress due to newness. For the surgical procedures, the animals were anesthetized using ketamine and xylazine (90 and 15 mg/kg intraperitoneally, respectively; Vetbrands, Brazil), and the sciatic nerve was exposed via an incision through the skin that extended from the greater

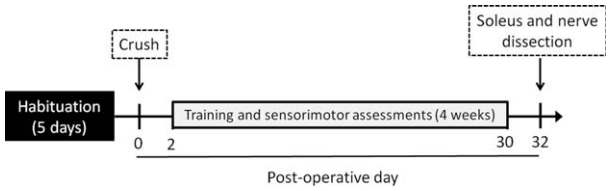

FIGURE 1. Timeline of the experimental procedures.

trochanter to the mid-thigh followed by splitting of the overlying gluteal muscle. The nerve crush injury was performed using 1-mm hemostat forceps for 30 s (as previously described by Bridge *et al.*[11]) 10 mm above the bifurcation into the tibial and common fibular nerves. The muscle and skin were then closed with 4-0 nylon sutures (Somerville, Brazil), and the animals were placed in their cages to rest. Forty-eight hours after surgery, animals from the ET and BCT groups began specific 4-week training programs. The sham and NT animals were placed in the same locations as the ET and BCT animals for a few minutes to equalize the handling of all groups as much as possible; however, the ET and BCT animals did not perform any form of motor activity training (Fig. 1).

Training Protocols. The balance and coordination training program was identical to that utilized in our previous study.[10] The animals were required to traverse 5 different elevated obstacles per day; these obstacles included suspension bridges, rope bridges, and parallel bars (each 100 cm in length) and ended in a dark box. These obstacles required motor learning, balance, and coordination, and each rat from this group crossed the obstacles 25 times, which amounted to walking 2500 cm on each day of training. The difficulty of the tracks was increased as the training progressed such that the tracks eventually included obstacles that were more unstable and more challenging than those that the rats encountered in the first training week.

The endurance training program was identical to that utilized in a previous study in our laboratory.[6] This exercise program was performed on a treadmill that was designed for human use (Runner, Brazil) and modified for use by rats. Two days before the surgical procedures, the rats were subjected to a maximal exercise test (MET). The test consisted of graded exercise on the treadmill during which the speed was incremented from 5 m/min by 5 m/min every 3 min to the maximum intensity attained by each rat. This training program consisted of running on the treadmill for 20 min on the first day, and this period was progressively increased every day up to 50 min on the fifth day and 60 min in the next 4 weeks. Each training session included a warm-up period of 5 min at 30% of the maximum speed reached in the MET (5.5 m/min), 10–50 min at 45–55% (∽9 m/min), and 5 min of recovery at 30% (5.5 m/min). There were 5 sessions per week, and these occurred once daily over a period of 5 weeks. This training program was considered a moderate-intensity endurance regimen, because the animals ran for extended periods at 45–55% of the maximum speed reached in the MET; that is, approximately 9 m/min.

Sensorimotor Studies. At the end of the second, third, and fourth training weeks, the horizontal ladder rung walking test (HLRWT)[12] and the narrow beam test (NBT)[13] were used to examine hindlimb sensorimotor function. Before the first test day, the animals were adapted to the test apparatus and, for each test, the animals were filmed from the side during 3 trials. The HLRWT apparatus was 100 cm long and 5 cm wide with horizontal parallel metal rungs (3 mm in diameter) that could be inserted to create a horizontal ladder with a minimum distance of 1 cm between rungs that was elevated 30 cm above the floor. A small dark box was located at the end of the apparatus. In this test, the animals were required to walk along the horizontal ladder, which had an irregular pattern (the distance between the rungs varied from 1 to 5 cm) that was changed between the adaptation phase and the testing phase to prevent the animals from learning the pattern. In the NBT, the rats were required to walk along a 100-cm-long, 3-cm-wide flat surface beam (in the adaptation phase) and a 100-cm-long, 2.6-cm-wide surface beam (in the test phase) that was elevated 30 cm above the floor to reach a small dark box at the end. In both tests, the number of hindlimb step slips in each trial was counted by 2 blinded observers, and these counts were used for analysis.

Histological and Morphometric Studies. Forty-eight hours after the end of the training programs, the animals were anesthetized with sodium thiopental (50 mg/kg intraperitoneally; Cristália, Brazil), injected with 1000 IU of heparin (Cristália, Brazil), and transcardially perfused with 300 ml of saline solution followed by 0.5% glutaraldehyde (Sigma Chemical Co., St. Louis, Missouri) and 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (PB, pH 7.4), at room temperature. A short segment (∽2 mm) of the right sciatic nerve 5 mm distal to the crush injury site was excised rapidly.[6] The right soleus muscle was dissected carefully from the surrounding tissue, and small samples (2 × 1 mm) of the central part of each soleus muscle were selected.[14] The specimens were post-fixed by immersion in the same fixative solution at 4°C until processed. The samples were then washed in

0.1 M PB and post-fixed in 1% OsO_4 (Sigma) in 0.1 M PB for 30 min. The samples were washed again in 0.1 M PB, dehydrated in a graded series of acetone, embedded in resin (Durcupan; ACM-Fluka, Switzerland), and polymerized at 60°C. Semi-thin cross-sections (1 μm) were obtained using an ultramicrotome (MT 6000-XL; RMC, Tucson, Arizona) and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil).[6,10,14]

Images of the right hindlimb soleus muscle and the distal portion of the right sciatic nerve were then captured and digitized (initially the muscles and nerves were examined at 200× and 1000× magnification, respectively, and both were further amplified 200% for analysis) using an optical microscope (Eclipse E-600; Nikon, Japan) coupled to a CMOS camera (Accu-Scope, Inc.) and processed using Image Pro Plus 6.0 software (Media Cybernetics).[6,10,14]

For morphometric evaluation of the soleus muscle, a set of 5 images from 1 slice was chosen through random sampling and later digitized. Morphometric measurements of the soleus included: (1) percentage of muscle tissue area, connective tissue area, and blood vessel area (%); (2) muscle fiber density (fibers/mm^2); and (3) average muscle fiber area (μm^2) (20 different muscle fibers from each image were selected, and 100 fibers from each animal were analyzed).

For morphometric evaluation of nerves, the distal portion of the right sciatic nerve was analyzed separately, and a set of 8 images from 1 slice was chosen through random sampling. Morphometric measurements of the sciatic nerve included the following: (1) percentage of nerve tissue area, connective tissue area, and blood vessel area (%); (2) myelinated fiber density (fibers/mm^2); (3) average myelinated fiber area (μm^2); (4) average myelinated fiber diameter (μm); (5) average axon diameter (μm) of myelinated fibers; (6) average myelin sheath thickness (μm); and (7) the g ratio (the ratio of the axon diameter to the fiber diameter, which is a measure of the degree of myelination).

The average myelin sheath thickness was estimated using the measurement tools provided by the Image Pro Plus software. The area measurements of the percentage of muscle tissue area, muscle connective tissue area, muscle blood vessel area, nerve tissue area, nerve connective tissue area, nerve blood vessel area, and muscle fiber and myelinated nerve fiber areas were estimated through a point-counting technique[6,10,14] using grids with point densities of 1 point per 106.28 μm^2 or 4 μm^2 (for muscles and nerves, respectively) and the following equation: $\hat{A} = \Sigma p \times a/p$, where $\hat{A}$ is the area, Σp is the sum of the points, and a/p is the area/point value. To estimate the axon and myelinated nerve fiber diameters, the area of each individual fiber was converted to the diameter of a circle with an equivalent area.

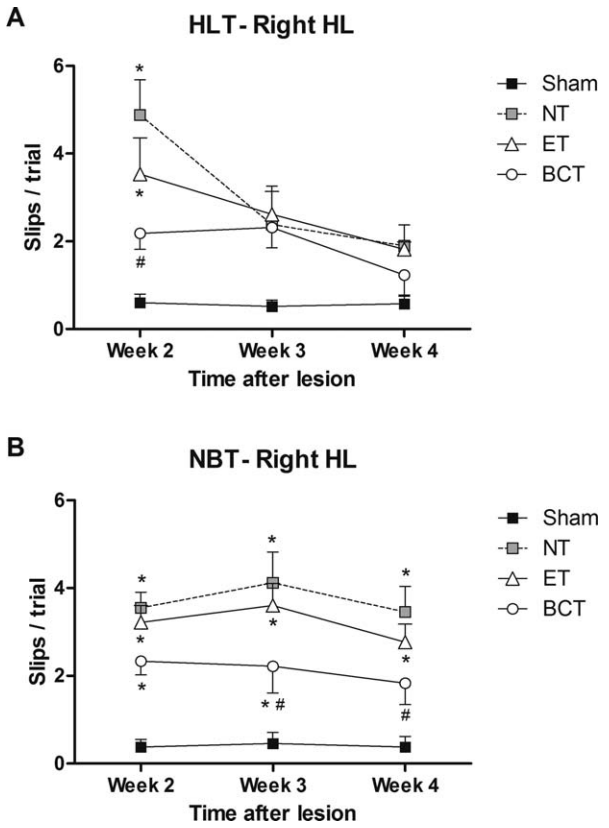

FIGURE 2. Comparison of sensorimotor tests at the end of the second, third, and fourth weeks of training. Data are expressed as mean ± SEM. **(A)** HLRWT: $^*P < 0.05$ compared with the sham group; $^{\#}P < 0.05$ compared with the NT group. **(B)** NBT: $^*P < 0.05$ compared with the sham group; $^{\#}P < 0.05$ compared with the NT group. HLWRT = horizontal ladder rung walking test; NBT = narrow beam test; sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group.

Statistical Analysis. The sensorimotor tests were analyzed through 2-way repeated-measures analyses of variance (ANOVAs) followed by Bonferroni tests for multiple comparisons. The morphometric measurements of the soleus muscle and sciatic nerve were analyzed using 1-way ANOVA followed by *post-hoc* Tukey tests. Data were run on GraphPad Prism 5.0 software (GraphPad, Inc.), with significance set at $P < 0.05$. All data are presented as mean ± standard error of the mean (SEM).

RESULTS

Sensorimotor Tests. In the horizontal ladder rung walking test (HLRWT; Fig. 2A), the NT and ET groups exhibited significantly greater numbers of hindlimb slips (4.9 ± 0.8 and 3.5 ± 0.8, respectively;

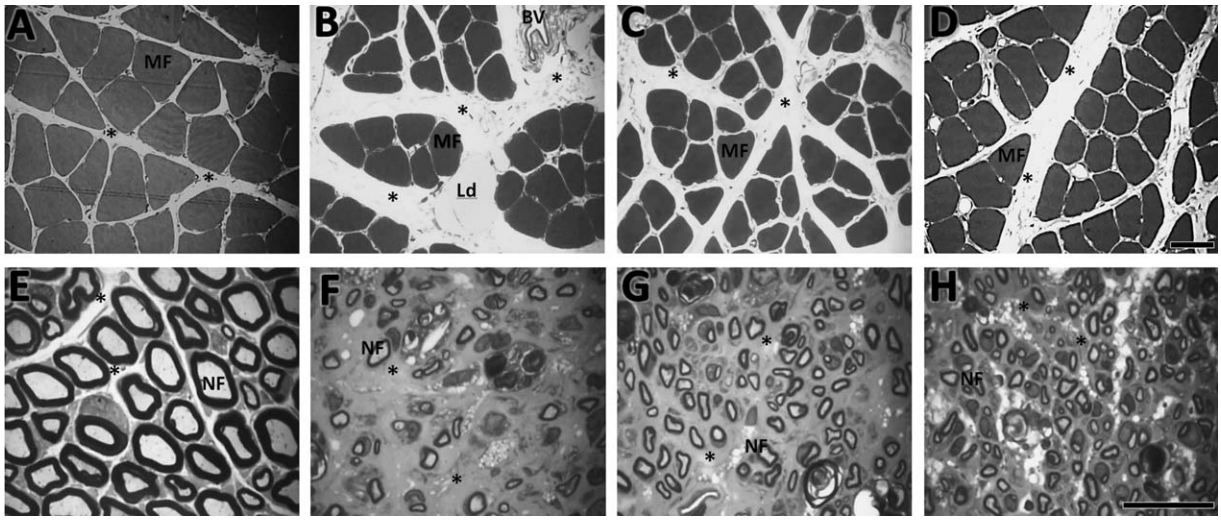

FIGURE 3. Digitized images of transverse semi-thin sections (1 μm) obtained from the central part of the right soleus muscle and of transverse semi-thin sections (1 μm) obtained from the right distal portion of the regenerating sciatic nerve 32 days after the nerve crush lesion. **(A)** Central part of a soleus muscle from the sham group. **(B)** Central part of a soleus muscle from the NT group. **(C)** Central part of a soleus muscle from the ET group. **(D)** Central part of a soleus muscle from the BCT group. Scale bar = 50 μm. **(E)** Distal portions from normal nerves of the sham group. **(F)** Distal portions of regenerating nerves from the NT group. **(G)** Distal portions of regenerating nerves from the ET group. **(H)** Distal portions of regenerating nerves from the BCT group. Scale bar = 20 μm. MF = muscle fiber; NF = myelinated nerve fiber; Sc = Schwann cell; asterisk (*) = endoneurial connective tissue; BV = blood vessel; Ld = lipid droplet; sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group. The sections were stained with toluidine blue.

$P < 0.05$) than the sham group (0.6 ± 0.2), and the NT group showed significantly more hindlimb slips than the BCT group (2.2 ± 0.4; $P < 0.05$) in the second week. In the third and fourth weeks, there were no significant differences in number of hindlimb slips in the HLRWT between groups.

In the narrow beam test (NBT; Fig. 2B), the NT (3.5 ± 0.3 and 4.1 ± 0.7), ET (3.2 ± 0.4 and 3.6 ± 0.5), and BCT (2.3 ± 0.3 and 2.2 ± 0.6) groups showed significantly greater numbers of hindlimb slips than the sham group (0.4 ± 0.4 and 0.5 ± 0.2; $P < 0.05$; respectively) in the second and third weeks. In the fourth week, only the NT (3.4 ± 0.6) and ET (2.8 ± 0.4) groups showed significantly more hindlimb slips than the sham group (0.4 ± 0.2; $P < 0.05$). Furthermore, the BCT group (2.2 ± 0.6 and 1.8 ± 0.5) showed significantly fewer hindlimb slips than the NT group ($P < 0.05$) in the third and fourth weeks.

Histological and Morphometric Studies. Histological analysis of the soleus muscle (Fig. 3A–D) revealed differences between the groups. In the NT group, the percentages of muscle tissue ($68.1 \pm 2.5\%$) and muscle connective tissue ($29.9 \pm 2.5\%$) areas differed significantly (Fig. 4A and B; $P < 0.05$) from those of the sham ($83.1 \pm 2.1\%$ and $15.8 \pm 1.9\%$, respectively), ET ($76.6 \pm 2.2\%$ and $20.8 \pm 2.3\%$, respectively), and BCT ($76.4 \pm 0.8\%$ and $20.3 \pm 1.0\%$, respectively) groups. However, there were no significant differences in the percentages of muscle tissue and muscle connective tissue areas between the sham, ET, and BCT groups. Furthermore, the percentage of muscle blood vessel area in the BCT group ($3.3 \pm 0.4\%$; $P < 0.05$) was significantly greater than that of the sham ($1.3 \pm 0.3\%$) and NT ($1.9 \pm 0.3\%$) groups, but there were no significant differences between the BCT and ET groups (Fig. 4C).

Analysis of muscle fiber density (Fig. 5A) shows that the NT group (578.8 ± 42.7 fibers/mm^2; $P < 0.05$) and the BCT group (531 ± 31.8 fibers/mm^2; $P < 0.05$) had a greater average density than that of the sham group (390.8 ± 22.5 fibers/mm^2). However, there was no significant difference in the average muscle fiber density between the ET (488.8 ± 26.7 fibers/mm^2) and sham groups. The average fiber area (Fig. 5B) was significantly larger in the sham group (2046 ± 137.7 μm^2) than in the NT group (1349 ± 80 μm^2; $P < 0.05$). However, there were no significant differences in mean area of the soleus muscle fibers between the sham, ET (1761 ± 107 μm^2), and BCT (1700 ± 130.4 μm^2) groups or between the ET, BCT, and NT groups.

Histological analysis of the distal portions of the regenerating sciatic nerves (Fig. 3E–H) revealed differences between the sham and experimental groups (i.e., the NT, ET, and BCT groups). These differences were confirmed by the analysis of percentages of nerve tissue area, nerve connective tissue area, and nerve blood vessel area (Fig. 6). In the sham group, the average percentages of

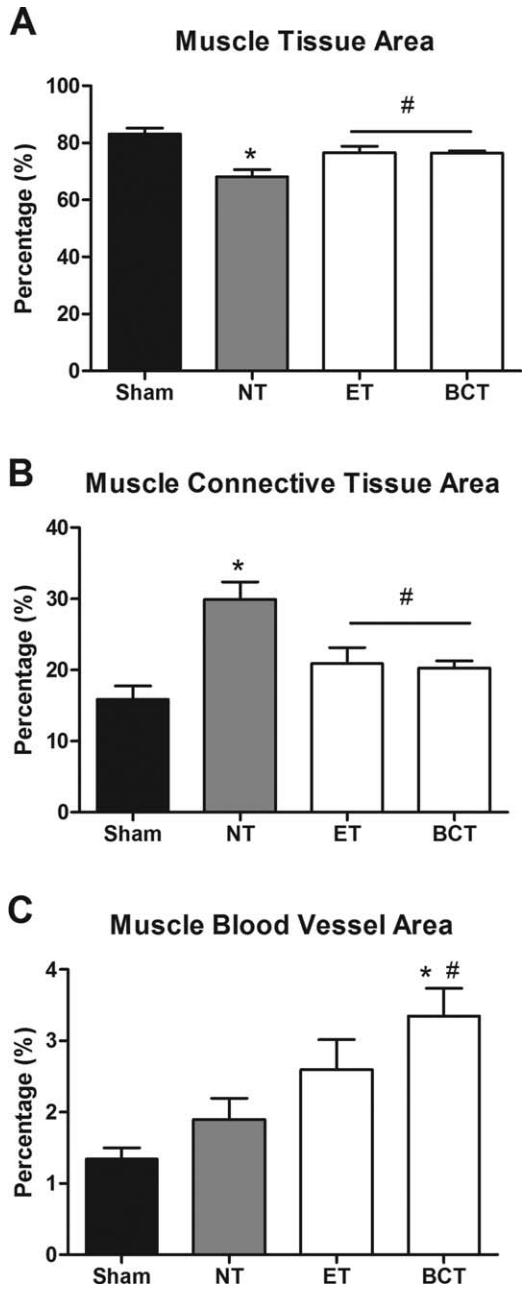

FIGURE 4. Percentage of muscle tissue area **(A)**, muscle connective tissue area **(B)**, and muscle blood vessel area **(C)** of the central part of the right soleus muscle 32 days after sciatic nerve crush lesion. Data are expressed as mean ± SEM. $^{*}P < 0.05$ compared with the sham group; $^{\#}P < 0.05$ compared with the NT group. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group.

nerve tissue (61.8 ± 1.2%) and nerve connective tissue (36.9 ± 1.2%) areas were significantly different from those of the NT (23.6 ± 1.1% and 74.5 ± 1.9%, respectively), ET (24.4 ± 1.8% and 74.3 ± 1.8%, respectively), and BCT (27.3 ± 2.2.8% and 71.6 ± 2.3%, respectively) groups ($P < 0.05$; Fig. 6A and B). However, there were no significant differences in the percentages of nerve tissue and nerve connective tissue areas between the NT, ET, and BCT groups. The percentage of nerve blood vessel area (Fig. 6C) in the NT group (0.9 ± 0.1%) was significantly lower than that in the sham group (1.3 ± 0.1%; $P < 0.05$). Furthermore, the percentage of nerve blood vessel area in the ET group (1.4 ± 0.1%; $P < 0.05$) was significantly larger than that in the NT group (0.9 ± 0.1%). There were no significant differences in percentage of nerve blood vessel area in the BCT group (3.3 ± 0.4%) when compared with the other groups.

The average myelinated fiber density (Fig. 7A) in the NT group (21,612 ± 1387 fibers/mm^2; $P < 0.05$) was significantly greater than that of the sham (16,472 ± 823.7 fibers/mm^2) and ET (16,207 ± 1112 fibers/mm^2; $P < 0.05$) groups. Furthermore, the ET group showed a significantly lower myelinated fiber density than the NT group ($P < 0.05$). However, there were no significant differences between the BCT group (19,619 ± 1341 fibers/mm^2) when compared with the other groups.

The average area of myelinated nerve fibers (Fig. 7B) of the sham group (37.83 ± 1.77 μm^2) was significantly greater than that of the NT (11.68 ± 0.65 μm^2; $P < 0.05$), ET (14.96 ± 0.40

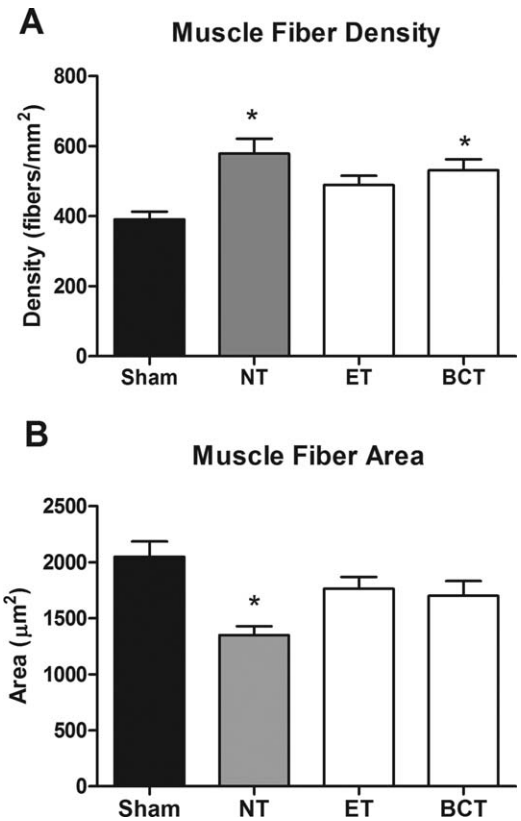

FIGURE 5. Morphometric parameters of the central part of the right soleus muscle 32 days after sciatic nerve crush lesion. **(A)** Muscle fiber density of the different groups. **(B)** Average cross-sectional areas of the soleus muscle fibers. Data are expressed as mean ± SEM. $^{*}P < 0.05$ compared with the sham group. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group.

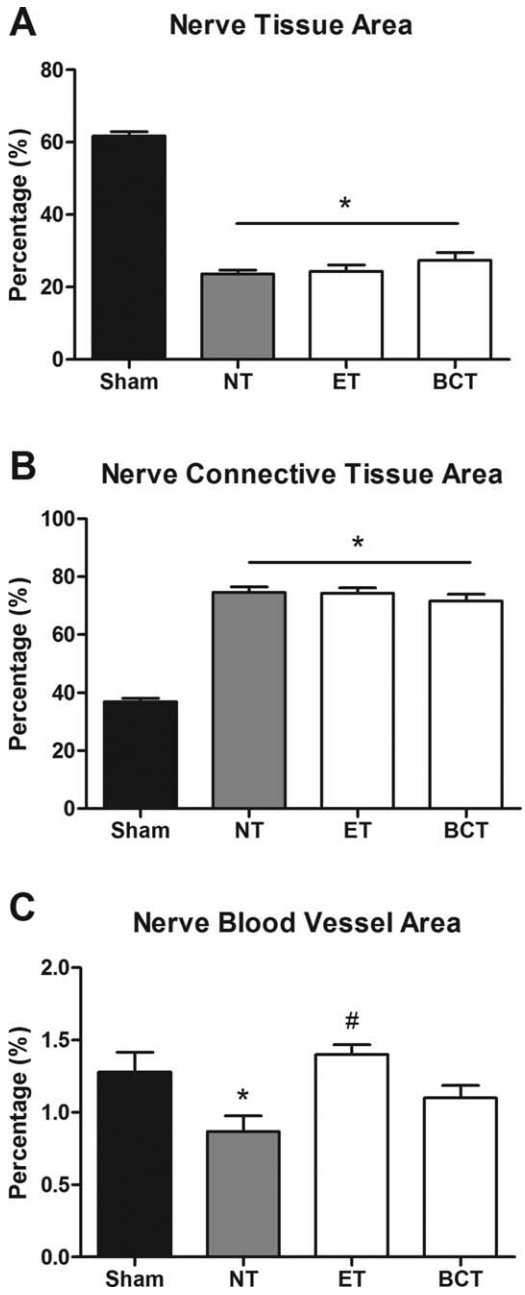

FIGURE 6. Percentage of nerve tissue area **(A)**, nerve connective tissue area **(B)**, and nerve blood vessel area **(C)** of the distal portion of the sciatic nerves 32 days after nerve crush lesion. The data are expressed as mean ± SEM. $^{*}P < 0.05$ compared with the sham group; $^{\#}P < 0.05$ compared with the NT group. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group.

μm^2; $P < 0.05$), and BCT (13.90 ± 0.37 μm^2; $P < 0.05$) groups. There were no significant differences between the NT, ET, and BCT groups. The analysis of average myelinated fiber diameter (Fig. 7C) revealed that the sham group (6.57 ± 0.14 μm) exhibited a greater mean diameter ($P < 0.05$) than the NT (3.70 ± 0.10 μm), ET (4.27 ± 0.05 μm), and BCT (4.14 ± 0.05 μm) groups. Furthermore, the ET and BCT groups had greater myelinated fiber diameters than the NT group.

Analysis of average axon diameters (Fig. 7D) revealed that the mean value of the sham group (4.52 ± 0.15 μm) was significantly greater ($P < 0.05$) when compared with the NT (2.91 ± 0.11 μm), ET (3.28 ± 0.07 μm), and BCT (3.01 ± 0.13 μm) groups. Comparison of mean thickness of myelin sheaths (Fig. 7E) reveals that the sham group (1.02 ± 0.01 μm) had significantly greater values ($P < 0.05$) than the NT (0.45 ± 0.02 μm), ET (0.49 ± 0.01 μm), and BCT (0.56 ± 0.05 μm) groups, but there were no significant differences between the NT, ET, and BCT groups. Analysis of *g* ratios (Fig. 7F) revealed no significant differences between groups.

DISCUSSION

Traumatic PNI is a common injury that occurs predominantly in young men of productive age and contributes to major social and economic problems.[15] A 16-year retrospective study in Brazil showed that axonotmesis (45%) is the most common result of nerve injury, followed by neurotmesis (41%) and neuropraxia (14%).[16] These nerve injuries affect small- and large-sensory afferent fibers[17] and result in loss of motor, sensory, and autonomic functions.[5]

Different rehabilitation approaches using movement-based therapy have been used to enhance axonal and muscle recovery. However, variations in the type of nerve injury and the type, time, and intensity of the exercise programs likely explain the discrepant results found in the literature. Thus, we conducted this study to determine whether balance and coordination training or endurance training, both of which were initiated 2 days after crush injury and were performed over the course of 4 weeks, alter the outcomes of sensorimotor tests (HLRWT and NBT) and morphometric analysis of sciatic nerve and soleus muscle of the affected hindlimb. Morphological analysis of nerve and muscle are important and can provide quantitative measurement of regeneration in animal models.

As expected for this experimental injury model, all groups showed improvement in the behavioral tests from the second to the fourth weeks after sciatic nerve crush. However, balance and coordination training promoted the maintenance of sensorimotor performance when compared with no training and endurance training. This was evident, because the animals in the BCT group showed no significant differences from the sham group (control) in mean hindlimb slips during the HLWRT after the second week, whereas the ET and NT groups were not significantly different

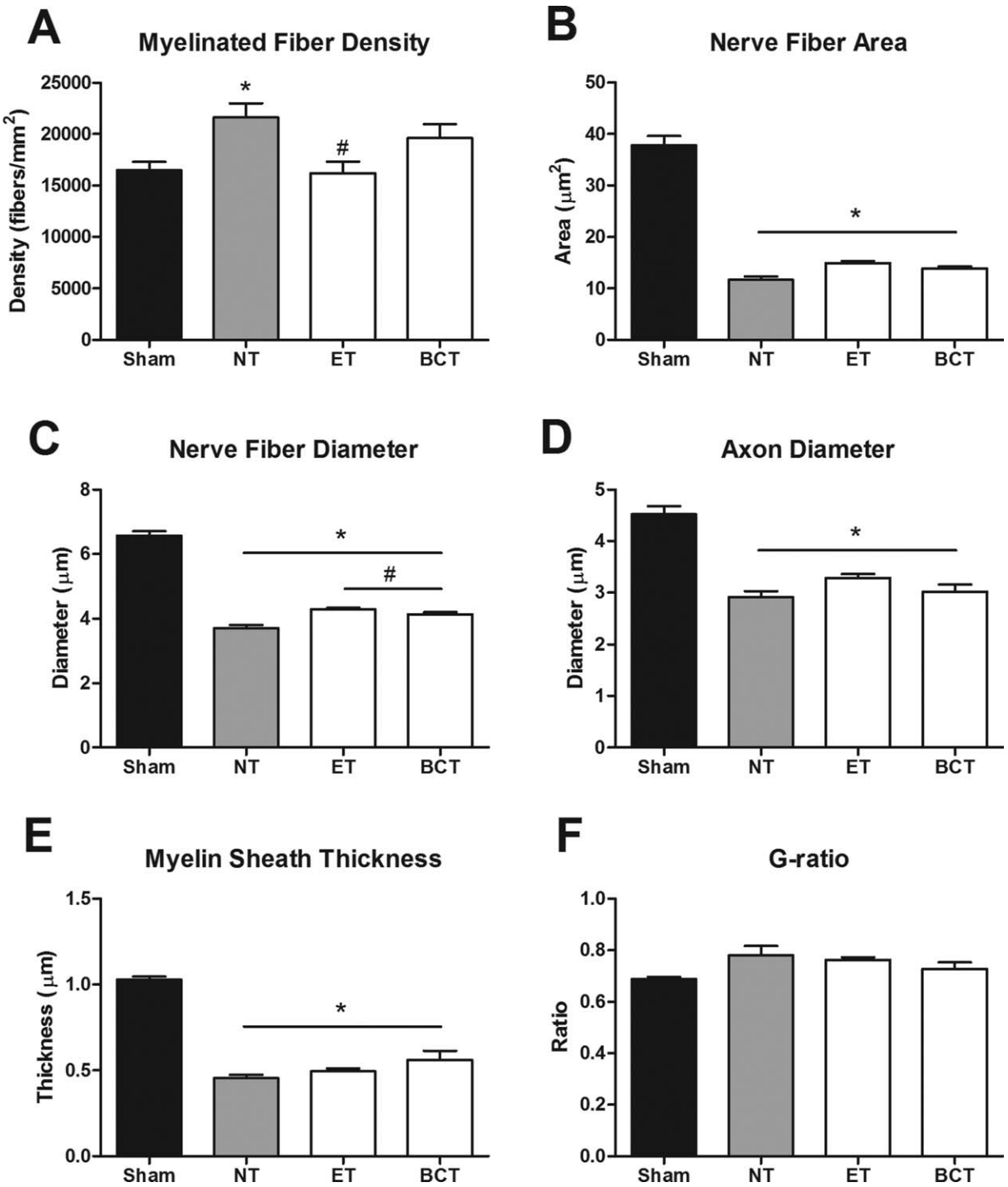

FIGURE 7. Morphometric parameters of the distal portion of the sciatic nerves 32 days after nerve crush lesion. **(A)** Average myelinated fiber density of the different groups. **(B)** Average areas of the nerve fibers. **(C)** Mean diameters of the nerve fibers. **(D)** Mean axon diameters of the nerve fibers. **(E)** Average myelin sheath thicknesses of the nerve fibers. **(F)** The *g* ratios of the nerve fibers. Data are expressed as mean ± SEM. $^{*}P < 0.05$ compared with the sham group; $^{\#}P < 0.05$ compared with the NT group. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group.

from the sham group only after the third and fourth weeks of training. Furthermore, only the animals in the BCT group exhibited reduced numbers of hindlimb slips in the second week after sciatic crush compared with the injured NT animals. In the NBT, the animals in the NT, ET, and BCT groups showed significant differences in mean hindlimb slips compared with the sham group in the second and third weeks, but in the fourth week only the NT and ET groups were different from the sham group. When we compared the NT group with the trained groups, only the BCT group

demonstrated a significant difference in mean hindlimb slips, and this difference occurred in the third and fourth weeks; the NT group was similar to the ET group in the second, third, and fourth weeks.

Our findings indicate that BCT was more effective than ET in sensorimotor evaluations. The exact mechanisms through which BCT exerts its superior positive effects are not understood completely. The HLRWT and NBT tasks require body balance, fine sensorimotor control, and precise foot placement.[12,18] It is possible that some adaptations in the central and peripheral nervous systems may occur after this training and restore normal coordination of muscle activity.[19,20] Moreover, the rats that received specific motor training exhibited improved performance in the motor control tasks for which they were previously trained (balance and coordination training), but it is not known whether other non-specific training protocols may also improve coordination.[21] The behavioral results show that, in contrast to endurance training, specific skilled balance and coordination training sustained fine sensorimotor coordination during the training period. The specificity of the training task is an essential concept in rehabilitation[22] and supports the use of task-oriented therapy in the treatment of PNI.

The ET and BCT groups had larger areas of muscle tissue and smaller areas of muscle connective tissue compared with the NT group. Moreover, in the BCT group, the muscle blood vessel area was significantly larger than in the sham and NT groups and was similar to that of the ET group. The sham and ET groups showed similar percentages of muscle fiber density, which were lower in comparison to the NT and BCT groups. In addition, the mean muscle area was larger in the trained animals than in the non-trained animals, and there were no differences in the mean muscle area between the trained groups. The muscle values found in the trained groups were better than in the NT group, indicating that the 2 training protocols prevented soleus muscle atrophy when the programs were initiated rapidly after sciatic nerve crush and continued for 4 weeks. Reduced muscle activity negatively affects intrinsic muscle performance.[3] After a PNI, disuse has many effects on skeletal muscles and can promote muscle atrophy.[23] During the reinnervation period, many strategies have been used to maintain muscle activity. Active treadmill walking or passive cycling, initiated 5 days after nerve injury and continued for 4 weeks, improves muscle reinnervation.[5] Furthermore, treadmill running, when initiated 1 week after nerve injury, promotes increased functional muscle afferent recovery.[24] Locomotor training on a treadmill, when initiated 2 days after nerve crush injury and continued for 4 weeks, enhances fatigue resistance of reinnervated muscles; however, 2 weeks of training does not enhance fatigue resistance, and the training duration reduces neither muscle force deficits nor muscle atrophy.[3]

Morphological analysis of the sciatic nerve revealed that the sham group had significantly less connective tissue between nerve fibers and significantly more nerve tissue area than the experimental groups. The nerve blood vessel area was similar in all trained groups, but in the ET group it was significantly greater than in the NT group and similar to that of the sham group. In addition, in the NT group, the myelinated fiber density was greater than in the sham and ET groups. Other studies have also demonstrated increases in myelinated fiber density after a nerve injury in non-trained animals.[6,25,26] The greater myelinated fiber density in non-trained animals may be explained by the fact that these animals were in an early stage of regeneration, with smaller axons than the ET group. However, treadmill training can accelerate regeneration and maturation, showing larger axons and, consequently, lower myelinated fiber density.[6,25]

Furthermore, all the experimental groups (NT, ET, and BCT) exhibited reduced myelin sheath thickness and axon diameter compared with control animals. However, analysis of nerve fiber diameter revealed that the trained groups (ET and BCT) exhibited significantly greater average values than the NT group.

Many validated outcome measures have been established to assess muscle and nerve regeneration, including morphological parameters.[4,6,8,10,25] Morphometric analysis can be quantified during all phases of nerve recovery and can distinguish between normal and abnormal.[27–29]

Treadmill training enhances axon regeneration when initiated on the third day after nerve repair surgery,[7] and our previous study showed that balance and coordination training improves the morphological properties of the sciatic nerve when initiated 2 days after the lesion.[10] Active treadmill walking or passive cycling improves muscle reinnervation and increases the number of regenerated axons in the distal nerve when initiated 5 days after nerve injury,[5] and treadmill training improves nerve regeneration when initiated 2 weeks after injury.[6] There is no consensus about the time to initiate an exercise program after injury. For example, nerve fiber diameters have been shown to be increased significantly by both acutely initiated swimming exercise (24 h after the injury) and delayed swimming exercise (initiated after the injury and our good results were observed).

In conclusion, these data show that both endurance training and balance and coordination

training can improve the morphological properties of soleus muscle and sciatic nerve when initiated soon after experimental traumatic injury of the sciatic nerve. An analysis of morphological properties is important because it may suggest nerve regeneration after a nerve injury. However, only balance and coordination training maintained sensorimotor function during the training period. It appears that the efficacy levels of the rehabilitative strategies used in this study are highly task-specific, and strategies that specifically stimulate sensory pathways are the most effective for improving balance and/or coordination parameters. Indeed, several lines of evidence support the conclusion that the greatest training effects occur when the same type of exercise is used both in testing and in training.

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3.2 ARTIGO 2

The effects of two different training exercises on ultrastructural characteristics of sciatic nerve and soleus muscle after sciatic crush – submetido à revista *Histology and Histopathology*

The effects of two different training exercises on ultrastructural characteristics of sciatic nerve and soleus muscle after sciatic crush

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Running title: Nerve & Muscle Ultrastructure

Keywords: Training; Injury; Nerve; Muscle; Ultrastructure.

The effects of two different training exercises on ultrastructural characteristics of sciatic nerve and soleus muscle after sciatic crush

Abstract

INTRODUCTION: Peripheral nerve injuries constitute a significant medical problem and although peripheral nerves have the capacity to regenerate, recovery is critically dependent on post-injury treatment. MATERIALS AND METHODS: Following sciatic nerve crush, we investigated the effect of 4 weeks of endurance training (ET) and balance and coordination training (BCT) on the ultrastructural characteristics of the sciatic nerve and soleus muscle. Transverse sections of sciatic nerve and soleus muscle were used for ultrastructural analyses. RESULTS: Electron micrographs of the sciatic nerve and soleus muscle of the ET and BCT groups showed similar characteristics to the non-trained group. These characteristics are similar to those previously described in nerve and muscle regeneration. CONCLUSIONS: These data showed no effects of either endurance training or balance and coordination training on the ultrastructural characteristics of the sciatic nerve and the soleus muscle after a crush injury.

Keywords: Training; Injury; Nerve; Muscle; Ultrastructure.

Introduction

Peripheral nerve injuries (PNIs) constitute a significant medical problem (Uzun et al., 2006). A 16-year retrospective study in Brazil showed that vehicle accidents are the most common causes of PNI, most patients were men (74%) and the mean age was 32.4 years (Kouyoumdjian, 2006). Lower limb PNIs are less common than upper limb PNIs, however, the sciatic nerve is the most frequently injured in the lower limb, followed by peroneal and, rarely, tibial or femoral nerves (Robinson, 2000). Thus, the sciatic nerve originates in the sacral plexus and bifurcates in the thigh region into the tibial and common peroneal nerve and innervates many hind leg muscles (IJkema-Paassen et al., 2004). Crush injuries appear to have the highest incidence of associated nerve injury (Taylor et al., 2008). The experimental model of crush injuries of rat's sciatic nerve consistently produces axonotmesis (Ronchi et al., 2009; Ronchi et al., 2010) and this is a good model for experimental studies of nerve and muscle regeneration (Mazzer et al., 2008; Wang et al., 2008). Although peripheral nerves have the capacity to regenerate (Wood et al., 2011), recovery from nerve injury is critically dependent on post-injury treatment (Wang et al., 2013). Endurance training (Udina et al., 2011; Ilha et al., 2008; English et al., 2009), passive cycling (Udina et al., 2011), resistance training (Ilha et al., 2008), forced exercises (Asensio-Pinilla et al., 2009), swimming exercises (Teodori et al., 2011), and balance and coordination training (Bonetti et al., 2011; Bonetti et al., 2015) are different rehabilitation movement therapies used after sciatic crush injury studies. Our previous studies demonstrate that endurance training and balance and coordination training improve some morphological properties of the soleus muscle and sciatic nerve (Bonetti et al., 2015), however, the ultrastructural analyses were not realized. Ultrastructural changes occurring in muscle and nerve, after a nerve crush injury, are not fully characterized (Diao et al., 2004). Thus, the aim of this study was to analyze the effects of 4-week endurance and balance and coordination training programs, initiated early after crush injury of the sciatic

nerve (48 h after the lesion), on ultrastructural alterations of the soleus muscle and sciatic nerve.

Material and methods

Experimental design and surgical procedures

The experiments were performed on 23 three-month-old male Wistar rats weighing 280–330 g (initial age and weight) obtained from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul, Brazil). The rats were housed in standard Plexiglass boxes (five or six rats per box) under a 12-h light/12-h dark cycle in a temperature-controlled environment (20 ± 1 °C) with food and water available *ad libitum*. All of the procedures were approved by the Animal Ethics Committee at the Federal University of Rio Grande do Sul (protocol number 21794), and all of the animals were handled in accordance with Brazilian laws. The animals were randomly divided into the following four groups: (1) Sham-operated rats that did not undergo sciatic crush and were unexercised (Sham, $n = 5$); (2) rats that underwent sciatic crush and were unexercised (non-trained, NT, $n = 6$); (3) rats that underwent sciatic crush and endurance training (ET, $n = 6$); and (4) rats that underwent sciatic crush and balance and coordination training (BCT, $n = 6$). Before the surgical procedures, the animals were placed in contact with the training program apparatus for 5 minutes for 5 days in order to avoid newness stress. For the surgical procedures, the animals were anesthetized using ketamine and xylazine (90 and 15 mg/kg, i.p., respectively; Vetbrands, Brazil), and the sciatic nerve was exposed via an incision through the skin that extended from the greater trochanter to the mid-thigh followed by splitting of the overlying gluteal muscle. The nerve crush injury was performed using 1 mm hemostatic forceps for 30 seconds (as previously described by Bridge et al., 1994) 10 mm above the bifurcation into the tibial and common fibular nerves. The muscle and skin were then closed with 4-0 nylon sutures (Somerville,

Brazil), and the animals were placed in their cages to rest. Forty-eight hours after the surgery, the animals from the ET and BCT groups began specific training programs that lasted 4 weeks, whereas the Sham and NT animals were placed in the same locations as the ET and BCT animals for a few minutes to equalize the handling of all of the groups as much as possible; however, the sham and NT animals did not perform any form of motor activity training (Figure 1).

Training protocols

The balance and coordination training program was identical to that utilized in our previous study (Bonetti et al., 2011; Bonetti et al., 2015) and this training program was adapted from acrobatic training (Black et al., 1990; Anderson et al., 1996; Kleim et al., 1996). Animals were required to traverse five different elevated obstacles per day; these obstacles included suspension bridges, rope bridges, parallel bars, and so on (each 100 cm long), and ended in a dark box. These obstacles required motor learning and balance and coordination from the animals, and each rat from this group crossed these obstacles 25 times, which amounted to walking 2500 cm on each day of training. The difficulty of the tracks was increased as the training progressed such that the tracks eventually included obstacles that were more unstable and more challenging than those encountered by the rats in the first training week.

The endurance training program was identical to that utilized in a previous study in our laboratory (Ilha et al., 2008; Bonetti et al., 2015). This exercise program was performed on a treadmill that was designed for human use (Runner, Brazil) and modified for use by rats. Two days before the surgical procedures, rats were submitted to a maximum exercise test (MET). The test consisted of graded exercise on the treadmill during which speed was incremented by 5 m/min every 3 min, starting at 5 m/min and increasing to the maximal

intensity attained by each rat. This training program consisted of running on the treadmill for 20 min on the first day, and this period was progressively increased every day up to 50 min on the fifth day and 60 min for the next 4 weeks. Each training session included a warm-up period of 5 min during which the rats ran at 30% of the maximum speed reached in the MET (5.5 m/min), 10 to 50 min running at 45 to 55% (~9 m/min) and 5 min recovery at 30% (5.5 m/min). There were five sessions per week occurring once a day over 5 weeks. This training program was considered a moderate-intensity endurance regimen because the animals ran for extended periods at 45 to 55% of the maximum speed that they reached in the MET; that is, approximately 9 m/min.

Morphological nerve and muscle studies

Forty-eight hours after the end of the training programs, the animals were anesthetized with sodium thiopental (50 mg/kg, i.p.; Cristália, Brazil), injected with 1000 IU of heparin (Cristália, Brazil), and transcardially perfused with 300 ml of saline solution followed by 0.5% glutaraldehyde (Sigma Chemicals Co., St Louis, MO, USA) and 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (pH 7.4, PB) at room temperature. A short segment (~ 2 mm) of the right sciatic nerve 5 mm distal to the crush injury site was excised rapidly (Ilha et al., 2008; Bonetti et al., 2011; Bonetti et al., 2015). The right soleus muscle was dissected carefully from the surrounding tissue, and small samples (2 x 1 mm) of the central part of each soleus muscle were selected (Marcuzzo et al., 2008; Bonetti et al., 2011; Bonetti et al., 2015). The specimens were post-fixed by immersion in the same fixative solution at 4°C until processed. The samples were then washed in 0.1 M PB and post-fixed in 1% OsO4 (Sigma Chemicals Co., St Louis, MO, USA) in 0.1 M PB for 30 min. The samples were washed again in 0.1 M PB, dehydrated in a graded series of acetone, embedded in resin (Durcupan, ACM-Fluka, Switzerland), and polymerized at 60°C. For ultrastructural

evaluation, ultra-thin transverse sections (70–85 nm) of the right sciatic nerve distal to the crush injury and the right soleus muscle were obtained with an ultramicrotome (Ultracut UCT 2.0) and mounted on copper grids (200-mesh). These sections were stained with 1% uranyl acetate (Merck, Germany) followed by 1% lead citrate (Merck, Germany) (Reynolds, 1963); and examined using an electron microscope (JEM 1200 EXII; JEOL, Japan). Digitized images of right sciatic nerve distal and soleus muscle from rats in the Sham, NT, ET, and BCT groups (*n* = three per group) were analyzed comparatively (and compared between groups) (Malysz et al., 2011).

Results

Cross-sections of the right sciatic nerve of the Sham group (Figure 2A–C) showed normal myelinated nerve fiber structure, with normal myelin sheath and compact myelin lamellae, and inside the axon there were neurofilaments and few mitochondria. These nerve fibers presented normal Schwann cells cytoplasm with rough endoplasmic reticulum and mitochondria and eucromatic nucleus (Figure 2B). Normally, these sections contain mixture of myelinated and bundle of unmyelinated nerve fibers, both with variable diameters (Figure 2A–C). Cross-sections of the right sciatic nerve distal to the crush injury of the NT, ET, and BCT groups are presented in Figure 2D–L. This material showed similar characteristics between the crushed groups, with normal myelinated axons, however, some ultrastructural alterations were noted including apparent decrease of axonal diameter, myelin sheaths relatively thinner, increase of area occupied by connective tissue and alterations in the round aspect of the nerve fibers. The axoplasm demonstrated loss of neurotubules, loss of neurofilaments, swelling of the mitochondria, and changes in the granularity of the axoplasm (Figure 2E, 2H, and 2K). Collagen was abundant and the myelin sheath was disintegrated configuring the myelin debris (Figure 2F, 2J, and 2K) which almost immediately plugged the

space taken by the denervated axon. Axonal debris engulfed within macrophages or within Schwann cells was found and the formation of lipid droplets was present (Figure 2E, 2I, and 2L). The Schwann cells of myelinated nerves presented lipid droplets, cytoplasm with abundant granular endoplasmic reticulum, prominent nucleoli, small round mitochondria, neurotubules, and neurofilaments. Unmyelinated fibers (Figure 2D, 2G, and 2J) exhibited no significant changes, with normal mitochondria, neurotubules, neurofilaments, and the axoplasm also contained rare small vesicles.

Cross-sections of the right soleus muscle of the Sham group revealed normal aspect of blood vessels and normal myofibers similar to those observed in soleus muscle of the rats. In the cytoplasm, nucleus shape with a normal appearance and the presence of polyribosomes, glycogen, sarcoplasmic reticulum and mitochondria (Figure 3A–C). In the crush injury of the NT (Figure 3D–F), ET (Figure G–I), and BCT (Figure J–L) groups, some morphological alterations were noted. Myofibrils cross-sectional areas were apparently reduced in association with enlarged intermyofibrillar spaces (Figure 3D–E). These electron micrographs also showed increasingly dense fields of collagen fibers around muscle fibers (Figures 3D and 3L). Mitochondria were smaller and rounded, presence of glycogen deposits, polyribosomes and sarcoplasmic reticulum occurred between myofibrils and next to the nucleus. The nucleus contained highly heterochromatic nuclei and the blood vessels presented very thick walls (Figures 3D and 3L).

Discussion

A recent bibliographic review found that the sciatic nerve injury model still represents a widely used method for the study of nerve repair and regeneration (Geuna, 2015), since different rehabilitation methods using exercise therapies have been used for increasing axonal

and muscle regeneration after a peripheral nerve injury (PNI) (Udina et al., 2011; Ilha et al., 2008; Asensio-Pinilla et al., 2009; English et al., 2009; Teodori et al., 2011; Bonetti et al., 2011; Bonetti et al., 2015). However, there are few studies that relate the effects of exercise therapies to nerve and muscle ultrastructure after nerve crush injury. Thus, we conducted this study to determine whether endurance training and/or balance and coordination training, both of which were initiated 2 days after crush injury and were performed over the course of 4 weeks, alter ultrastructural properties of the sciatic nerve and soleus muscle.

In this study, the Sham group presented normal structure of unmyelinated nerve fibers and myelinated nerve fiber, with normal myelin sheath, mitochondria and axofilaments, normal Schwann cell cytoplasm and nucleus with rough endoplasmic reticulum and mitochondria, as exhaustively discussed in previous ultrastructural normal nerve studies (Ohmi, 1961; Bardosi, 1989; Hildebrand et al., 1994). Nevertheless, all injured groups (NT, ET, and BCT) demonstrated the same appearances and showed Wallerian degeneration features in the distal portion of the sciatic nerve. The ultrastructure of Wallerian degeneration has been discussed for a long time by classical studies and these features included myelin sheaths with thin caliber and evaginations, axoplasm with loss of neurotubules and neurofilaments, swelling mitochondria, abundant collagen fibers, much axonal debris, lipid droplet and altered blood vessels, and Schwann cells (Ohmi, 1961; Satinsky et al., 1964; Jacobson, 1965; Morris et al. 1972; Calabretta et al., 1973; Sea and Peterson, 1975; Sunderland, 1978). With regard to unmyelinated axons, in Wallerian degeneration there was no apparent degeneration of these fibers (Calabretta et al., 1973; Fried et al., 1989). In crush injuries, the nerve regeneration is influenced by the injury characteristics and time of the crush. In ultrastructural analyses 30 days after the injured crush for 3 seconds, most distal portion nerve fibers appear to be normal, but such myelin debris fragments are still found (Gershenbaum and Roisen, 1978). However, after the injured crush for 30 seconds, the

ultrastructural analyses 2 weeks after the injury demonstrated many small nerve fibers with thin myelin sheath at the beginning of the myelination process (Varejão et al., 2004; Bobinski et al., 2011) while 8 weeks after the injury the distal portion of the injury demonstrated nerve fibers at an advanced stage of regeneration with only a few degeneration features (Varejão et al., 2004).

In the soleus muscle electron micrograph analyses, the Sham group showed normal structure similar to that described previously, normal organization of myofibers and myofilaments, reduced intermyofibrillar spaces, polyribosomes occurring between the plasma membrane, around the nuclei and, in lesser amounts, between the myofibers, mitochondria occurring between myofibers and around the nuclei, and blood vessels with regular walls (Pellegrino and Franzini, 1963; Price, 1963; Mair and Tomé, 1972). The injured animals (NT, ET, and BCT groups) presented the same appearances, showing characteristics of muscle regeneration (Heck and Davis, 1988). These ultrastructural alterations of muscle regeneration included myofiber disorders with muscle-reduced areas and many collagen fiber areas, an increase in glycogen granules and polyribosomes, the presence of sarcoplasmic reticulum, the nucleus and nuclei presenting highly heterochromatic, and blood vessels with thick walls (Pellegrino and Franzini, 1963; Miledi and Slater, 1968; Schmalbruch, 1976; Cullen and Pluskal, 1977; Irintchev et al., 1990; Peña et al., 1995; Lu et al., 1997; Borisov and Carlson, 2000; Borisov et al., 2001; Malysz et al., 2011). Our findings indicate, through the analysis of these sciatic nerve and soleus muscle electron micrographs, no ultrastructural differences between the NT, ET, and BCT groups. The morphometric analyses of our previous study demonstrated that trained groups had better results than the NT group only in fiber diameter analyses (Bonetti et al., 2015). However, this same study[18] showed that myelinated fiber density of the NT group was significantly greater than just the ET group. Many other previous studies that have utilized physical training after sciatic nerve injury have demonstrated good

results in morphometric parameters of sciatic nerve regeneration and soleus muscle regeneration (Udina et al., 2011; Ilha et al., 2008; Asensio-Pinilla et al., 2009; English et al., 2009; Teodori et al., 2011; Bonetti et al., 2011), nevertheless the ultrastructural changes were not verified. Ultrastructural studies are important to study morphological characteristics of the nerve and muscle and provide evidence concerning the differentiated characteristics and fine details of the structure of these cells (Borisov and Carlson, 2000). In conclusion, these data show no effects of either endurance training or balance and coordination training on the ultrastructural characteristics of the sciatic nerve and the soleus muscle after a crush injury. The consequences of nerve injury are a complex and often poorly understood mechanism and although morphological analyses are not definitive in determining whether different physical exercise protocols are responsible for alterations after a peripheral nerve injury, these methodological procedures are important in regeneration studies. However, ultrastructural analyses after PNI and exercise rehabilitation are still rare and our findings may influence future studies aimed at characterizing the different specific physical activities on sciatic nerve and soleus muscle regeneration.

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Figures Legends

Figure 1. Timeline of the experimental procedures.

Figure 2. Electron micrographs of the right sciatic nerve from Wistar rats. The images show nerve fibers (myelinated and unmyelinated) and the connective tissue (#) between them of the Sham (A–C), SE (D–F), ET (G–I) and BCT (J–L) groups. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group. uf, unmyelinated nerve fiber; ax, axoplasm; (#) connective tissue; sc, Schwann cell; n, Schwann cell nucleus; bv, blood vessel; ld, lipid droplet; md, myelin debris, ad, axonal debris; mit, mitochondria. Scale bars in (A), (D), (F), (G) and (J) = 5 µm; (B), (C), (I) and (K) = 2 µm; (E), (H) and (L) = 1 µm.

Figure 3. Electron micrographs of the right soleus muscle from Wistar rats. The images show myofibers and the extracellular space (*) between them of the Sham (A–C), SE (D–F), ET (G–I) and BCT (J–L) groups. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group. n, nucleus; nu, nucleolus; myo, myofilaments; mit, mitochondria, bm, basement membrane; bv, blood vessel; en, vascular endothelium. Scale bars in (L) = 5 µm; (A), (B), (C), (D), (E), (F), (G), (I) and (J) = 1 µm; (H) and (K) = 0.5 µm.

Figures

Figure 1.

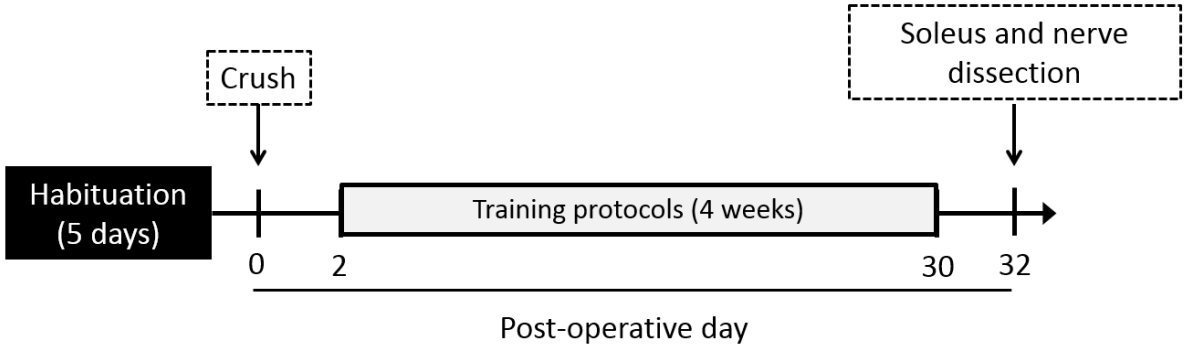


Figure 2.

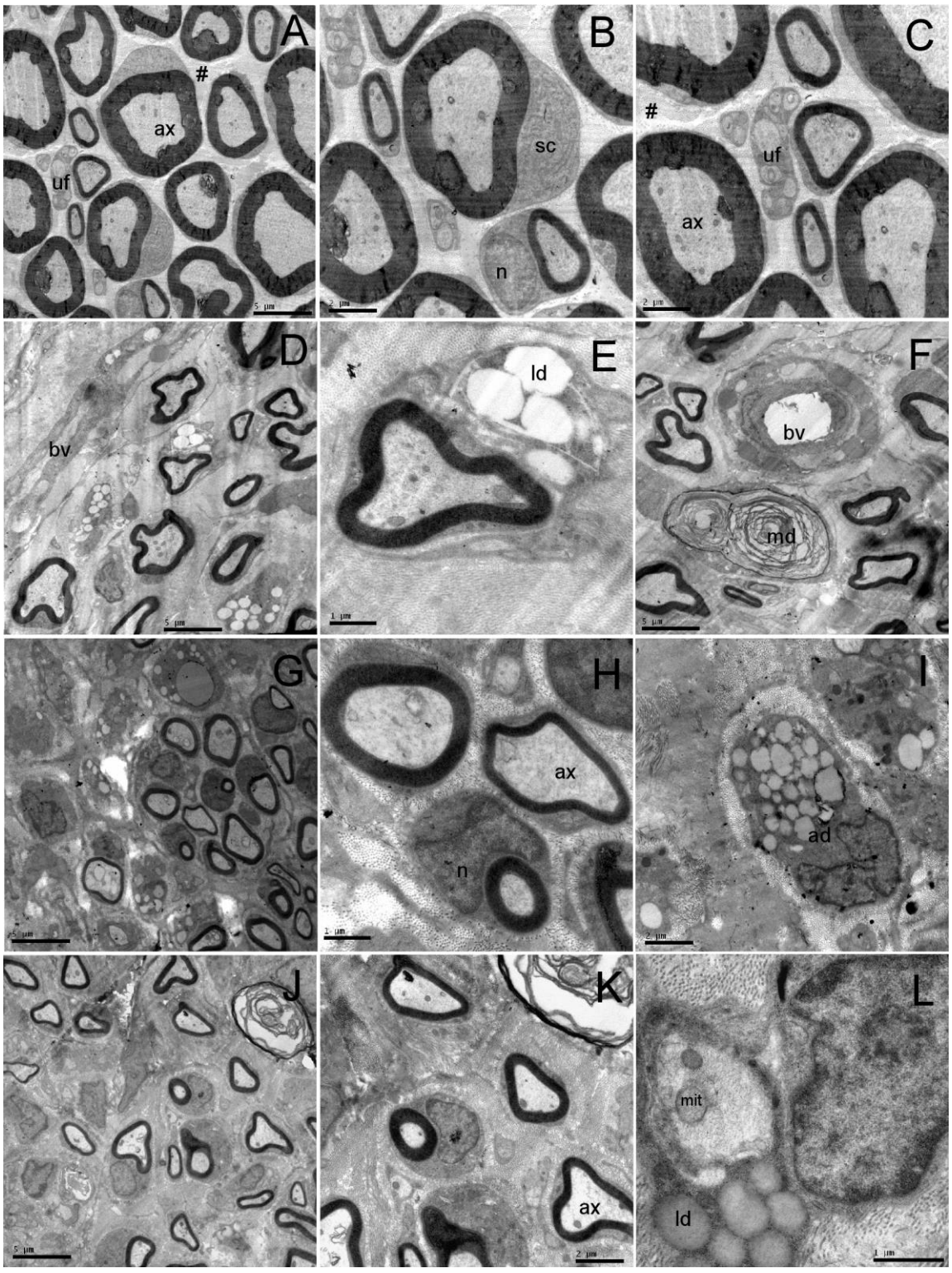
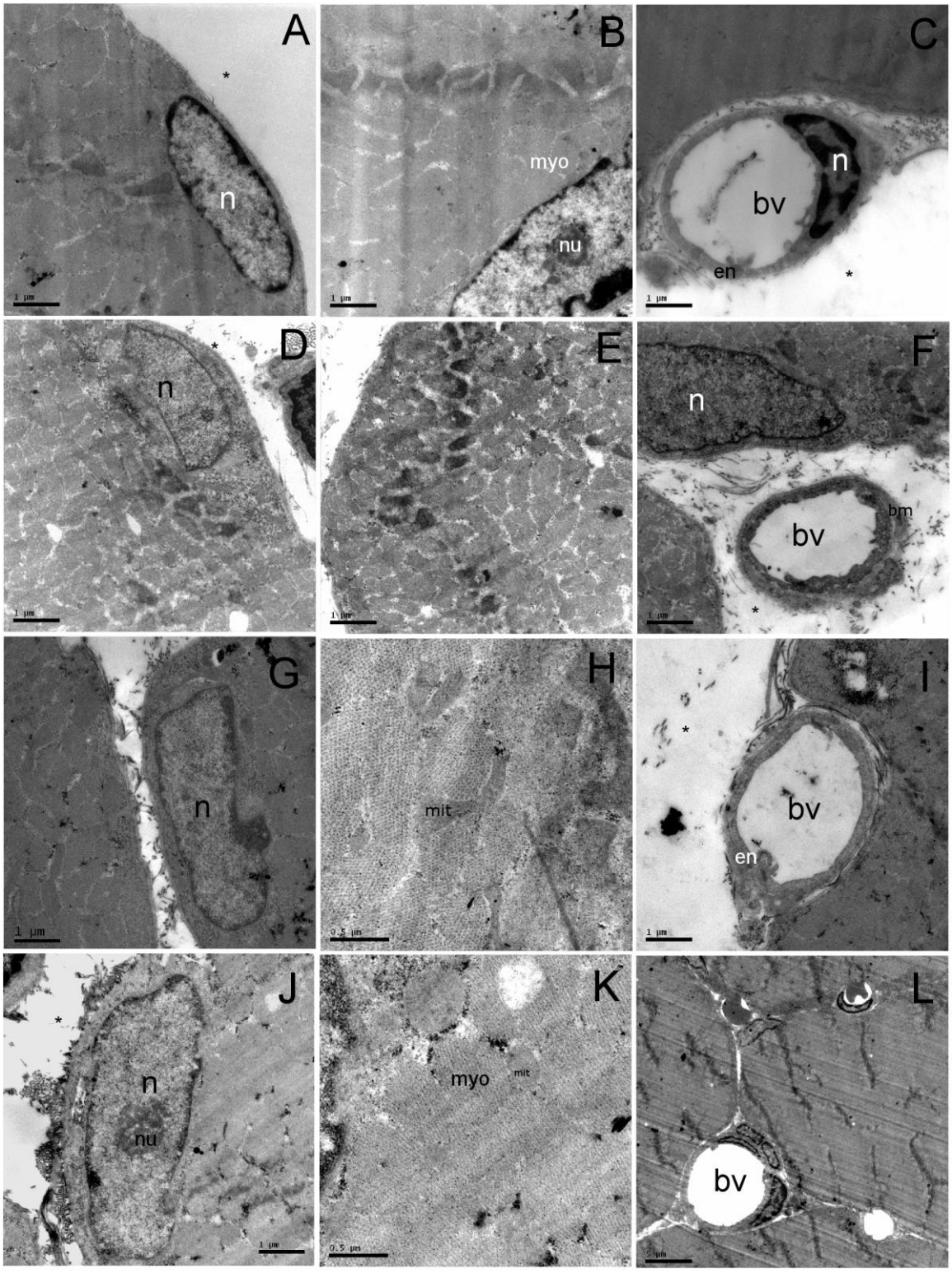


Figure 3.



3.3 ARTIGO 3

Balance and coordination training, but not endurance training, enhances synaptophysin and neurotrophin-3 immunoreactivity in the lumbar spinal cord after sciatic nerve crush – submetido à revista *Muscle & Nerve*

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Balance and coordination training, but not endurance training, enhances synaptophysin and neurotrophin-3 immunoreactivity in the lumbar spinal cord after sciatic nerve crush

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Keywords:	Balance, Coordination, Endurance, Synaptophysin, Neurotrophin

Balance and coordination training, but not endurance training, enhances synaptophysin and neurotrophin-3 immunoreactivity in the lumbar spinal cord after sciatic nerve crush

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Conflict of interest

The authors declare that there are no conflicts of interest.

Balance and coordination training, but not endurance training, enhances synaptophysin and neurotrophin-3 immunoreactivity in the lumbar spinal cord after sciatic nerve crush

Abstract

INTRODUCTION: Numerous rehabilitation treatments have been shown to be useful for peripheral and central restoration after a peripheral nerve injury (PNI). METHODS: Following sciatic nerve crush, we investigated four weeks of endurance training (ET) and balance and coordination training (BCT) in sciatic function index and hind paw stride length, as well as the spinal cord dorsal horn synaptophysin and neurotrophin-3 immunoreactivity. RESULTS: Our results demonstrated no significant differences between the non-trained (NT), ET and BCT groups in sciatic functional index and in the stride length analysis, the ET demonstrated greater values compared to the NT group. The synaptophysin immunoreactivity was higher in the BCT compared to the NT group, and the neurotrophin-3 immunoreactivity in the BCT was greater compared to the others groups. CONCLUSION: The BCT can positively affect spinal cord plasticity after a PNI and these modifications are important in the rehabilitation process.

Keywords: Balance; Coordination; Endurance; Synaptophysin; Neurotrophin.

Introduction

Peripheral nerve injury (PNI) is a common nerve injury and is mostly caused by laceration or penetration of the nerve[1]. Laceration by a sharp object and road traffic trash are the most common causes of PNI[2], and young males are more affected than females[2,3]. Disability, delay of function recovery and activity return[4] and specific functional deficits are common consequences of this neuromuscular pathology[5]. Although peripheral nerve injuries can devastate movement function, most of these injuries are often treatable[3]. Locomotor training is a good treatment option in the context of peripheral nerve injury[6] and physical activity is beneficial for overall neural system function[7].

Many previous studies have demonstrated the effectiveness of physical treatment on muscle recovery, nerve regeneration or functional recovery after PNI[8-14]. Although these studies examined physical activity on nerve regeneration and muscle regeneration, reorganization after an injury does not occur only at the site of injury. This reorganization can occur at different levels: cortical, subcortical, spinal cord, and in the peripheral nervous system[15]. Nevertheless, the role of functional reorganization following PNI is unclear[16].

The plasticity of central connections may functionally compensate for the lack of specificity in reinnervation[17]. Synaptophysin, an integral membrane component of synaptic vesicles, is a presynaptic vesicle protein that facilitates the exocytosis of vesicles during synaptic release[18,19]. In nerve terminals, synaptophysin is easily detectable by immunoreactivity and is useful in the identification of axonal nerve terminals[20] and synaptogenesis in the central nervous system[20-22]. Other important elements of central neuroplasticity after injury include neurotrophic factors[23-24]. These neurotrophic factors stimulate and control neurogenesis and are an important class of growth factors. They consist of four structurally related factors: nerve growth factor

(NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5)[23]. Neurotrophins, particularly neurotrophin-3, can stimulate spinal axon elongation; modulate the muscle spindle-motoneuron connection after peripheral nerve injury and during development[24], and play a role in mediating synaptic transmission[25].

Moreover, previous studies in our laboratory have revealed that endurance training, and balance and coordination training improve sensorimotor performance, muscle regeneration and sciatic nerve regeneration after sciatic nerve crush[8,13,14]. However, we hypothesized that plasticity in the spinal cord dorsal horn after peripheral nerve injury is dependent on specific physical training. Thus, this study was designed to investigate the effects of a four-week endurance training program as well as a balance and coordination training program after sciatic nerve crush injury, which were initiated early (48 hours after lesion) on hindlimb locomotor function recovery and on synaptophysin and neurotrophin-3 immunoreactivity distribution in L4-L6 lumbar levels of the spinal cord.

Materials and Methods

Experimental design and surgical procedures

The experiments were performed on 23 three-month-old male Wistar rats weighing 280-330 g (initial age and weight) obtained from a local breeding colony (ICBS, Universidade Federal do Rio Grande do Sul, Brazil). The rats were housed in standard Plexiglass boxes (five or six rats per box) under a 12-h light/12-h dark cycle in a temperature-controlled environment (20 ± 1 °C) with food and water available *ad libitum*. All of the procedures were approved by the Animal Ethics Committee at the Federal University of Rio Grande do Sul (protocol number 21794), and all of the animals were handled in accordance with Brazilian laws. The animals were randomly

divided into the following four groups: (1) sham-operated rats that did not undergo sciatic crush and were unexercised (Sham, n = 5); (2) rats that underwent sciatic crush and were unexercised (non-trained, NT, n = 6); (3) rats that underwent sciatic crush and endurance training (ET, n = 6); and (4) rats that underwent sciatic crush and balance and coordination training (BCT, n = 6). Before the surgical procedures, the animals were placed in contact with the training program apparatus during five minutes for five days in order to avoid the newness stress. For the surgical procedures, the animals were anesthetized using ketamine and xylazine (90 and 15 mg/kg, i.p., respectively; Vetbrands, Brazil), and the right sciatic nerve was exposed via an incision through the skin that extended from the greater trochanter to the mid-thigh followed by splitting of the overlying gluteal muscle. The nerve crush injury was performed using 1 mm hemostatic forceps for 30 seconds (as previously described by Bridge[26]) 10 mm above the bifurcation into the tibial and common fibular nerves. The muscle and skin were then closed with 4-0 nylon sutures (Somerville, Brazil), and the animals were placed in their cages to rest. Forty-eight hours after the surgery, the animals from the ET and BCT groups began specific training programs that lasted four weeks, whereas the Sham and NT animals were placed in the same locations as the ET and BCT animals for a few minutes to equalize the handling of all of the groups as much as possible; however, the Sham and NT animals did not perform any form of motor activity training (Figure 1).

Training Protocols

The balance and coordination training program was identical to that utilized in our previous study[13,14], and this training program was adapted from acrobatic training[27-29]. Animals were required to traverse five different elevated obstacles per day; these obstacles included suspension bridges, rope bridges, parallel bars, etc. (each 100 cm long) and ended in a dark box. These obstacles required motor learning, balance and

coordination from the animals, and each rat from this group crossed these obstacles 25 times, which amounted to walking 2,500 cm on each day of training. The difficulty of the tracks was increased as the training progressed such that the tracks eventually included obstacles that were more unstable and more challenging than those that the rats encountered in the first training week.

The endurance training program was identical to that utilized in a previous study in our laboratory[8]. This exercise program was performed on a treadmill that was designed for human use (Runner, Brazil) and modified for use by rats. Two days before the surgical procedures, rats were submitted to a maximum exercise test (MET). The test consisted of graded exercise on the treadmill during which speed was incremented by 5 m/min every 3 min, starting at 5 m/min and increasing to the maximal intensity attained by each rat. This training program consisted of running on the treadmill for 20 min on the first day, and this period was progressively increased every day up to 50 min on the fifth day and 60 min for the next 4 weeks. Each training session included a warm-up period of 5 min during which the rats ran at 30% of the maximum speed reached in the MET (5.5 m/min), 10 to 50 min running at 45% to 55% (~9 m/min) and 5 min recovery at 30% (5.5 m/min). There were five sessions per week that occurred once per day over 5 weeks. This training program was considered a moderate-intensity endurance regimen because the animals ran for an extended periods at 45% to 55% of the maximum speed reached they reached in the MET; i.e., approximately 9 m/min.

Hindlimb locomotor function analysis

All animals were subjected to a series of motor activity assessments 1, 2, 3 and 4 weeks after completion of physical training protocols. Recovery of right hindlimb locomotor function confirmed adequate muscle reinnervation and functional recovery, which was monitored by analysis of the free-walking pattern. The sciatic function index

(SFI) and hind paw stride length were determined. The SFI is the most common method used to analyze hindlimb function after peripheral nerve injury and was originally described by De Medinaceli, Freed and Wyatt[30] and modified by Bain, Mackinnon and Hunter[31]. This index is based on measurements of the footprints of walking rats, which provides a reliable and easily quantifiable method of evaluating the functional condition of the sciatic nerve. Briefly, rats were trained to walk over a white sheet of paper, which covered the bottom of a 100 cm in length, 8.5 cm in width track that ended in a dark box. Next, the ventral hind feet of the animals were painted with dark dye, and the animals were subsequently placed on the track to walk. The rats' footprints were used to determine the following measurements: (1) distance from the heel to the third toe, the print length (PL); (2) distance from the first to the fifth toe, the toe spread (TS); and (3) distance from the second to the fourth toe, the intermediary toe spread (ITS). These three measurements were obtained from the experimental (E) and normal (N) sides[32,33] (Figure 2A). Several prints of each foot were obtained on each track, but only three prints of each foot were used to determine the mean measurements on the experimental and normal sides. Next, these means were included in the SFI formula.

SFI = –38.3 (EPL – NPL) / NPL + 109.5 (ETS – NTS) / NTS + 13.3 (EITS – NITS) / NITS –8.8.

The results obtained were used as an index of the functional conditions of the sciatic nerve, where zero (±11) represented normal function and -100 represented functional loss.

Analyses on other walking patterns included the right hindlimb paw stride length. The stride length was determined as the distance from the metatarsal head of the right foot to the metatarsal head of right foot. The right hindlimb paw stride length of each rat was obtained from the mean values of three consecutive footprints obtained

from each side, which was adapted from Kunkel-Bagden et al.[34] and Marcuzzo et al.[35] (Figure 2B).

Immunohistochemical procedure

Forty-eight hours after the end of the training programs, the animals were anesthetized with sodium thiopental (50 mg/kg, i.p.; Cristália, Brazil), which was injected with 1000 IU heparin (Cristália, Brazil), and transcardially perfused with 300 ml of saline solution followed by 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (pH 7.4, PB) at room temperature. The L4-L6 lumbar levels of the spinal cord were quickly dissected and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer solution for 4 h at room temperature. Next, these lumbar samples were cryoprotected in 15% and 30% sucrose (Synth, Brazil) solutions in phosphate buffer at 4ºC. The spinal cord segments were quickly frozen in isopentane (Merck, Germany) cooled in liquid nitrogen and stored in a freezer (-70ºC) until further analyses. Serial coronal sections (40 µm) were obtained using a cryostat (CM1850, Leica, Germany) at -20ºC and collected in PB saline (PBS).

Free-floating sections were washed in PBS, pre-treated with 3% hydrogen peroxide for 30 min, washed in PBS and incubated in PBS containing 0.4% Triton X-100 (PBS-T) for 30 min, followed by incubation with the primary monoclonal anti-synaptophysin antibody (Sigma Chemical Co., USA) diluted 1:200 in PBS containing 0.4% Triton X-100 (PBS-T) and 3% normal goat serum for 48 h at 4ºC or incubated with primary polyclonal rabbit anti-neurotrophin-3 antibody (AB1532SP Chemicon, Temecula, CA, USA) diluted 1:100 in PBS containing 0.4% PBS-T and 3% normal goat serum for 48 h at 4ºC (as previously described by Gómez-Pinilla et al.[36]). The primary antibody was then removed, and the sections were washed in PBS-T for 30 min. Next, the sections were immersed in secondary anti-mouse IgG-peroxidase antibody (Sigma

Chemical Co, USA) diluted 1:500 in PBS-T, for 2 hours at room temperature with gentle stirring. After washing with PBS-T for 30 min, the samples were then washed in PBS and incubated in a solution of 3,3 diaminobenzidine tetrahydrochloride (60 mg/100 ml, Sigma Chemical Co., USA) and 0.005% v/v hydrogen peroxide in PBS.

Finally, the sections were washed, mounted onto gelatinized slides, dehydrated, cleared and covered with Entellan (Merck, Germany) and coverslips. Specific immunostaining was abolished when the primary antibody was omitted from the staining sequence.

Semi-quantitative analysis by optical densitometry

Semi-quantitative densitometry analysis was performed to measure the intensity of the synaptophysin and neurotrophin-3 immunoreaction. Digitized images of the spinal cord dorsal horn were captured at 10x for optical densitometry analysis using a Nikon Optiphot-2 microscope (Tokyo, Japan) equipped with a Micrometrics camera (Accu Scope, Commack, NY, USA). The digitized images obtained from the selected areas were converted to an 8-bit gray scale (0-255 gray levels) with the Image Pro Plus 6.0 (Media Cybernetics, USA) software for further analysis. All lighting conditions and magnifications were kept constant during the process of capturing the images, and the investigator was blind to the experimental group conditions. The pixels used to measure the optical density (OD) were obtained from squares measuring 2057.92 μm^2 (area of interest, AOI), where each was overlaid onto the gray scale image. The AOI delimitated a region of interest in the Rexed laminae II, III and IV of the right dorsal horn of the spinal cord (Figure 3A - adapted from Paxinos and Watson[37] - and Figure 3B). One digitized image was obtained from each spinal cord section, and six sections from each animal were analyzed. Background correction and background staining subtraction were performed[38]. To perform the background correction, a background image was captured

and opened. The background image was generated with the slide removed from the microscope stage. Background staining was subtracted from OD measurements using averaged values of the tissue sections in which the primary antibody was omitted.

The optical density (OD) was calculated using the following formula:

$$OD(x,y) = -\log [(INT(x,y) - BL)] / (INC-BL)$$

where "OD(x,y)" is the optical density of each pixel(x,y), "INT(x,y)" or intensity is the intensity of the pixel(x,y), "BL" or black is the intensity generated when no light is transmitted through the material, and "INC" is the intensity of incidental light.

Statistical analysis

Hindlimb locomotor function tests were analyzed using two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni tests for multiple comparisons. Immunohistochemistry measurements were analyzed using one-way ANOVA followed by post hoc Tukey's tests. Data were run on GraphPad Prism 5.0 software (USA), and G*Power 3 software (Institut Für Experimentelle Psychologie, Heinrich Heine Universitat, Dusseldorf, Germany) was used to calculate the statistical power analysis[39]. To analyze the correlation between the hindlimb locomotor function analysis (SFI and right hindlimb paw stride length) and immunoreactivity data (synaptophysin and neurotrophin-3 immunoreactivity), the Pearson's correlation coefficient was calculated. These values are presented as the mean ± standard errors of the mean (SEM) and the significance was established at $p<0.05$.

Results

To calculate the Sciatic Functional Index (SFI), a total of 552 footprints were analyzed after 1, 2, 3 and 4 weeks of training. The SFI data are presented as the mean ±

SEM in Figure 4. The crush lesion was established, as evidenced by an SFI value in the range of -80 to -100 (complete denervation) on the 1st post-training week record in the sciatic nerve crushed groups (NT, ET and BCT). However, the injured groups demonstrated improvements in SFI mean values from the 1st to the 4th week after sciatic nerve crush, but these values were significantly lower in the 1st, 2nd and 3rd weeks compared to the sham group and there was no significant difference between the NT, ET and BCT groups in all evaluations. In the sham group, the SFI remained stable at around - 5 to 0.5, which was considered normal, throughout the experiment.

The right hindlimb paw stride length data are presented as the mean ± SEM in Figure 5. The NT group had a significantly shorter stride length in the 1st and 2nd post-training weeks compared to sham animals ($P<0.001$). However, the ET group showed a similar stride length to the sham group since the 1st post-training week and was significantly greater compared to the NT group in all evaluations ($P<0.05$). The BCT group still had a significantly shorter stride length in the 1st post-training week compared to the sham group ($P<0.01$); however, from 2 to 4 weeks after training, the right stride length was similar to sham animals. Furthermore, the BCT group demonstrated a significantly greater stride length compared to the NT group in the 2nd post-training week ($P<0.001$).

The synaptophysin and neurotrophin-3 immunoreactivity in the lumbar spinal cord are presented in Figure 6. This spinal cord area showed synaptophysin immunoreactivity, which was normally distributed throughout the spinal cord gray matter, and neurotrophin-3 immunoreactivity was highly concentrated in the dorsal horn of the spinal cord. The immunohistochemical analysis of the dorsal horn of the spinal cord revealed that the synaptophysin immunoreaction intensity (Figure 7) was higher in the BCT group (0.385 ± 0.035) compared to the NT group (0.227 ± 0.019; $P<0.05$). However, there were no significant differences between the sham (0.295 ± 0.052), NT

(0.227 ± 0.019) and ET (0.341 ± 0.050) groups and between the sham (0.295 ± 0.052), ET (0.341 ± 0.050) and BCT (0.385 ± 0.035) groups. For neurotrophin-3, immunohistochemical analysis of the dorsal horn of the spinal cord demonstrated that immunoreactivity (Figure 8) in the BCT group (0.310 ± 0.019) was significantly greater compared to the other groups ($P<0.05$). However, there were no differences between the sham (0.115 ± 0.022), NT (0.144 ± 0.010) and ET (0.153 ± 0.028) groups.

The findings of the statistical power analysis for the SFI, right hindlimb paw stride length, synaptophysin immunoreactivity and neurotrophin-3 immunoreactivity were 34.4%, 74.5%, 40.1% and 55.6%, respectively. The Pearson's correlation coefficients were presented in Table 1, and were also calculated to determine the relation between the results of the fourth week of assessment of the hindlimb locomotor function analysis (SFI and right hindlimb paw stride length) and immunoreactivity data (synaptophysin and neurotrophin-3 immunoreactivity).

Discussion

Different traumas to the extremities can result in damage to specific nerves and can cause severe disabilities[4,40]. Due to the superficial position and long course, specific peripheral nerves demonstrate an increased risk of injury[41], and one of these is the sciatic nerve. Injuries in the sciatic nerve results in physical and social disabilities[2] and has an effect on the public health system, thus requiring further research[2,3].

In recent years, many exercise protocols have been used to enhance locomotor function and regeneration of the nerve and muscle after PNI in rats. However, differences in the nature of the nerve injury and the type, time, and intensity of the exercise programs likely explain the discrepant results found in the literature. Another question is that these studies only consider the plasticity of the site of injury; this does not consider the plasticity of other parts of the nervous system. Thus, the present study

was designed to investigate whether two different exercise programs, the endurance training and the balance and coordination training, both of which were initiated two days after the crush injury and performed over the course of four weeks, altered the outcomes of hindlimb locomotor function recovery and synaptophysin and neurotrophin-3 immunoreactivity patterns in the L4-L6 lumbar spinal cord levels of rats after sciatic nerve crush injury.

In the evaluation of hindlimb locomotor function, the walking track analysis was utilized and the sciatic function index (SFI) and right hindlimb paw stride length were analyzed. Walking track analysis is a quantitative, useful[33] and precise method used to assess functional locomotor nerve recovery[42]. Since its introduction by De Medinaceli et al.[30] in 1982, analysis of a rat's walking pattern by documenting their footprints has been a well-established and widely used method. The SFI method is simple, noninvasive[43], easy to apply, and less expensive compared to other methods and should be a component of functional evaluation in neural regeneration studies[33,44]. Our results demonstrated that all injured groups (NT, ET and BCT) showed SFI mean values significantly lower to the sham group in the first three weeks and reached to normal levels just in fourth week. Therefore, 4 weeks of ET and BCT training, initiated 48 hours after the lesion were not sufficient to differ to the non-trained animals. However, other previous studies using exercise protocols after sciatic nerve crush obtained controversial results in SFI analyses. Swimming training initiated 1 day after sciatic crush showed normalization of the SFI after 4 weeks[45], and another study revealed that swimming training initiated 1 day after injury produced better SFI results and functional recovery was faster compared to the group initiated 14 days after injury[12]. Treadmill training initiated 2 days after injury and performed over 12 consecutive days was effective in increasing SFI values[46]. Endurance training initiated 2 weeks after the lesion was more effective compared to resistance and endurance-resistance training at 3 weeks

post-lesion, but in the 6th week, no additional differences between the groups were observed[8]. A preoperative program of low-intensity aerobic exercise for 2 weeks and exercise postoperative for 2 weeks and preoperative-postoperative training protocols for 4 weeks showed that preoperative-postoperative training produced a significant effect in SFI analyses when compared with the other two groups[47]. The other hindlimb locomotor function tests that included the right stride length analyses revealed that the ET group showed better results compared to the NT group in all evaluations. Nevertheless, the BCT group showed betters results compared to the NT group just in 2nd post-training week. This poor result in the 2nd post-training week in the NT group occurred during the most intense period of pain after sciatic crush injury[48] and may be associated with the lack of physical activity in this group, which was promoted by a protective response to potential hyperalgesia. Stride length is an important measure, and the gradual recovery of this measurement exhibited a strong correlation with the post-nerve repair time course[49].

The results of functional locomotor nerve recovery indicated significant hindlimb impairment and loss of motor function in the right hindlimb for injured groups at the end of the training period. Using this same methodology, our previous study showed that treadmill training and balance and coordination training were not sufficient to recover the morphological parameters of the nerves and soleus muscle[14]. These morphological parameters indicated an inefficient nerve and muscle regeneration and consequently impaired locomotor function of the injured hindlimb, which were consistent with the results obtained in this study. However, in sensorimotor tests, another type of analysis, our previous studies demonstrated that balance and coordination training improved sensorimotor function since the first week[13,14], demonstrating the specific beneficial effects of this training. In addition, the promotion of regeneration and acceleration of nerve regeneration and muscle reinnervation is

crucial, but it is also important to modulate plastic changes at the spinal cord level to improve functional recovery[10]. Plasticity of central connections is established to accompany axotomy of peripheral axons[50] and may functionally compensate for the lack of specificity in target reinnervation[17]. One limitation of the present study was not performing hindlimb locomotor function analysis immediately after the injury and/or before the beginning of the training programs. However, sciatic nerve injuries often cause joint contractures, such that the rats use the dorsum of the affected foot making measurement compromised for few days do not provide adequate results[33,51], and this seems to be a limitation of the study design. Furthermore, the few studies that performed hindlimb locomotor function analysis prior to the injury using the sciatic nerve crush injury model showed similar results between the groups[12,43,52].

Immunohistochemical analysis of the spinal cord dorsal horn revealed that the synaptophysin immunoreactivity of the BCT group was significant greater to the NT group. Synaptophysin represents increased plasticity in the spinal cord, and some studies have confirmed this finding after sciatic nerve injury[21,53-56]. A model for neuropathic pain using peripheral nerve injury increases the excitability of sensory circuits[53], and it has been associated with a synaptic increase in the spinal cord[54]. Chronic constriction injury or resection injury of the sciatic nerve may cause long-lasting synaptic plasticity changes in the spinal cord[57]. After transection, synaptophysin immunoreactivity was increased in the substantia gelatinosa ipsilateral to the injury[58] and after crush injury of rat sciatic nerve a solid synaptophysin immunoreactivity was demonstrated in the regenerating sprouts that emerged from the proximal nodes of Ranvier[18]. However, in the central nervous system, a slight reduction in synaptophysin immunoreactivity, in L3-L6 levels of the spinal cord, was observed after sciatic nerve transection[59].On the other hand, this study revealed that physical activity, especially

balance and coordination training, promoted more synaptic plasticity than the unexercised animals with sciatic nerve crush.

With regard to neurotrophin-3, immunohistochemical analysis of the spinal cord dorsal horn revealed that neurotrophin-3 immunoreactivity in the BCT group was significantly greater compared to the other groups. In past years, much attention has been focused on the role of neurotrophic factors in the maintenance and survival of neurons and in promoting axonal regeneration after nerve injury[17,60]. Neurotrophin-3 is indispensable for trophic signal transmission from target cells to neurons[61] and regulates the target axon after peripheral nerve injury[40]. Numerous studies have suggested that neurotrophin-3 is required for the maintenance of proprioceptive neurons and is indispensable for the development of limb proprioceptive neurons[62]. Mutant mice with neurotrophin-3 deficiency do not develop normally and exhibit severe neurological dysfunction, sensory neuron loss and abnormalities in early stages of the development of these neurons[63]. Neurotrophin-3-deficient mice exhibit abnormal body postures, limb movements[64] and movement defects due to severe deficits in the peripheral sensory system[65]. Thus, physical treatments may provide therapeutic benefit after neuropathy by enhancing neurotrophic factor expression[66], increasing the expression of BDNF[36] and neurotrophin-3[7] in the intact spinal cord, resulting in subsequent effects on synaptic plasticity.

Although synaptophysin immunoreactivity was found in the spinal cord gray matter, neurotrophin-3 immunoreactivity was concentrated in the spinal cord dorsal horn. The projection of proprioceptors to their target-derived neurotrophin-3 in the spinal cord dorsal horn is important for signal transmission to motoneurons, thereby shaping the monosynaptic proprioceptor/motoneuron circuit[67]. Synaptic connectivity between muscle sensory and motor neurons is regulated by neurotrophin-3[68]. Consequently, neurons located in the dorsal horn region of the lumbar spinal cord are

important and necessary for the production of good locomotion[69]. However, the significantly greater neurotrophin-3 immunoreaction in these areas was only demonstrated in the balance and coordination training, and our previous studies demonstrated that this training program also improved findings obtained from sensorimotor tests[13,14].

In the present study, the statistical power analysis indicates that the results for the SFI, right hindlimb paw stride length, synaptophysin immunoreactivity and neurotrophin-3 immunoreactivity were 34.4%, 74.5%, 40.1% and 55.6%, respectively. The statistical power analysis can be used to assess the real significance of the results obtained, and, thus, our results demonstrated a weak statistical power. Moreover, the results of Pearson's correlation coefficients between hindlimb locomotor function and immunoreactivity data demonstrated weak correlations between these parameters, also an unexpected inverse correlation statistically significant between SFI and neurotrophin-3 for the group BCT. These statistical analyses demonstrated that an increase of the number of animals per group in future research is extremely important to have these results confirmed. However, in conclusion, our data showed that both endurance training and balance and coordination training, initiated soon after experimental traumatic injury of the right sciatic nerve, did not differ to non-trained group in the sciatic function index evaluation, while the right hind paw stride length values of the endurance training group was better compared to the non-trained group in all evaluations. Although balance and coordination training did not demonstrate good results in hindlimb locomotor function analysis, this training promoted synaptic plasticity and increased neurotrophin-3 immunoreactivity in the spinal cord dorsal horn. Neurotrophin-3 is more involved with sensorimotor neurons, and this may account for our previous good results obtained from the balance and coordination training on sensorimotor function during the trained period, while the muscle and nerve

regeneration did not reach significant indices. Thus, the balance and coordination training would be a good choice after a peripheral nerve injury, since this training can positively affect spinal cord plasticity and these modifications are important in the rehabilitation process. However, our results should inspire new studies with an increase in the number of animals to confirm our results as well as studies about neural regeneration, reorganization research and physical rehabilitation practice in human treatment.

Abbreviations

AOI .....area of interest

BCT .....balance and coordination training

BDNF.....brain-derived neurotrophic factor

BL.....black

ºC .....Celsius degrees

cm .....centimeters

E.....experimental

ET .....endurance training

g.....grams

h.....hours

i.p. .....intra-peritoneal

ICBS .....Instituto de Ciências Básicas da Saúde

INC .....incidental light

INT .....intensity

ITS .....intermediary toe spread

M .....molar

MET.....maximal exercise test

mg/kg .....milligrams/kilograms

ml .....milliliters

mm .....millimeter

m/min .....meters/minutes

n .....number

N .....normal

NGF .....nerve growth factor

NT .....non-trained

NT-3 .....neurotrophin-3

NT-4/5 .....neurotrophin-4/5

OD .....optical density

PB .....phosphate buffer

PBS .....phosphate buffer saline

PBS-T .....phosphate buffer saline Triton-X

PL .....print length

PNI .....peripheral nerve injury

SEM .....standard error of the mean

SFI .....sciatic function index

TS .....toe spread

µm .....micrometer

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Figure Legends

Figure 1. Timeline of the experimental procedures.

Figure 2. Hindlimb locomotor function analysis. (A) Sciatic function index (SFI) measurement. (B) Right hindlimb paw stride length measurement. EPL = experimental print length (distance from the heel to the third toe of the injured hindlimb); ETS = experimental toe spread (distance from the first to the fifth toe of the injured hindlimb); EITS = experimental intermediary toe spread (distance from the second to the fourth toe of the injured hindlimb); NPL = normal print length (distance from the heel to the third toe of the normal hindlimb); NTS = normal toe spread (distance from the first to the fifth toe of the normal hindlimb); NITS = normal intermediary toe spread (distance from the second to the fourth toe of the normal hindlimb).

Figure 3. Semi-quantitative analysis by optical densitometry. (A) A diagram of the right dorsal horn of the spinal cord section (L5) demonstrating Rexed laminae adapted from Paxinos and Watson[37]. Scale bar = 500 μm. (B) The AOI (area of interest) delimitated a region of interest for synaptophysin and neurotrophin-3 optical densitometry analysis in the digitized image of the right spinal (captured at 10x). Scale bar = 100 μm. D = dorsal; V = ventral; L = lateral; M = medial.

Figure 4. Comparison of the hindlimb locomotor function recovery determined by the Sciatic Functional Index (SFI) after 1, 2, 3, and 4 weeks of training. Data are expressed as the mean ± SEM. *$P < 0.05$ compared with the Sham group. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group.

Figure 5. Comparison of the hindlimb locomotor function recovery determined by the right hindlimb paw stride length after 1, 2, 3, and 4 weeks of training. Data are expressed as the mean ± SEM. "a" $P < 0.05$ when NT was compared with the Sham group; "b" $P < 0.05$ when BCT was compared with the Sham group; "c" $P < 0.05$ when ET was compared with the NT; "d" $P < 0.05$ when BCT was compared with the NT. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group.

Figure 6. Digitized image of synaptophysin and neurotrophin-3 immunoreactivity in the right dorsal horn of spinal cord (captured at 4x). Note the strong synaptophysin immunoreactivity that is normally distributed throughout the spinal cord gray matter and that the neurotrophin immunoreactivity is highly concentrated in the dorsal horn of the spinal cord. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group; D = dorsal; V = ventral; L = lateral; M = medial. Scale bar = 500 μm.

Figure 7. Synaptophysin immunoreactivity optical densitometry measures. Data are expressed as the mean ± SEM. *$P < 0.05$ compared with the NT group. Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group; O.D. = optical densitometry.

Figure 8. Neurotrophin-3 immunoreactivity optical densitometry measures. Data are expressed as the mean ± SEM. #$P < 0.05$ compared with the Sham group; *$P < 0.05$ compared with the NT group; π $P < 0.05$ compared with the ET group. Sham = sham

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Table 1. Pearson's correlation coefficients between hindlimb locomotor function and immunoreactivity data

	All (*n = 23*)		Sham (*n = 5*)		NT (*n = 6*)		ET (*n = 6*)		BCT (*n = 6*)	
	SNPT	NT-3	SNPT	NT-3	SNPT	NT-3	SNPT	NT-3	SNPT	NT-3
SFI	-0.096	-0.197	-0.037	0.007	0.208	-0.090	-0.444	0.870	0.322	-0.880*
RHPSL	0.184	0.066	-0.722	-0.264	0.539	-0.065	0.093	0.427	-0.276	0.285

*SFI = Sciatic Functional Index; RHPSL = right hindlimb paw stride length; SNPT = synaptophysin immunoreactivity; NT-3 = neurotrophin-3 immunoreactivity; Sham = sham group; NT = non-trained group; ET = endurance training group; BCT = balance and coordination training group. * Significant (P < 0.05).*

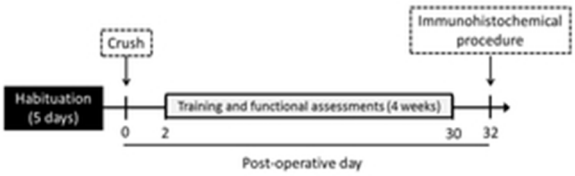

Figure 1. Timeline of the experimental procedures.
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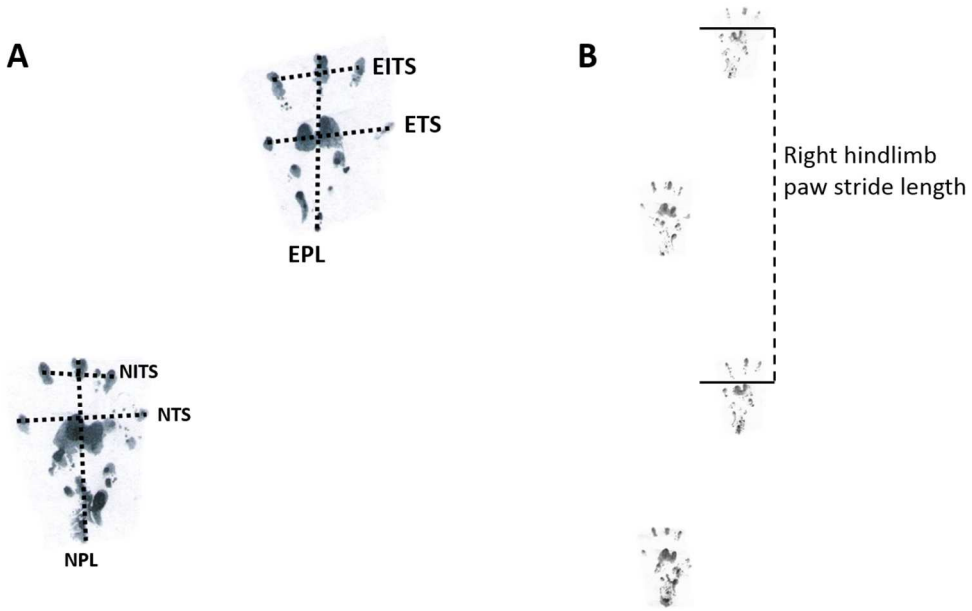

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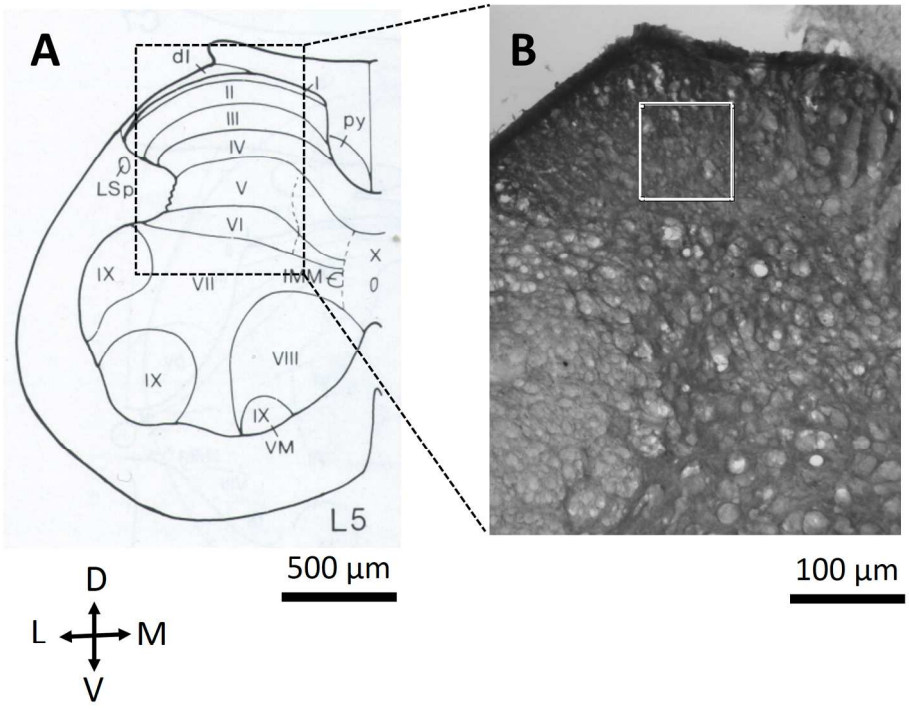

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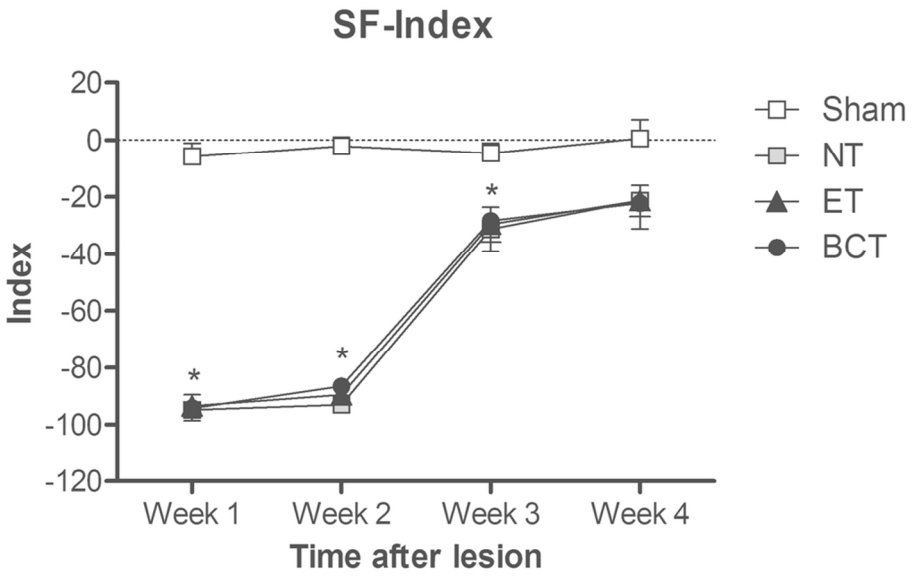

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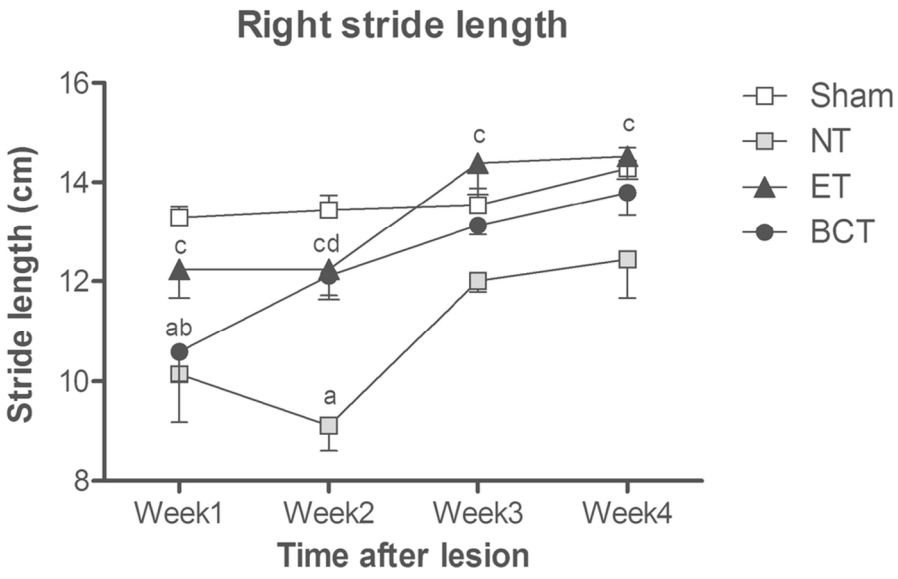

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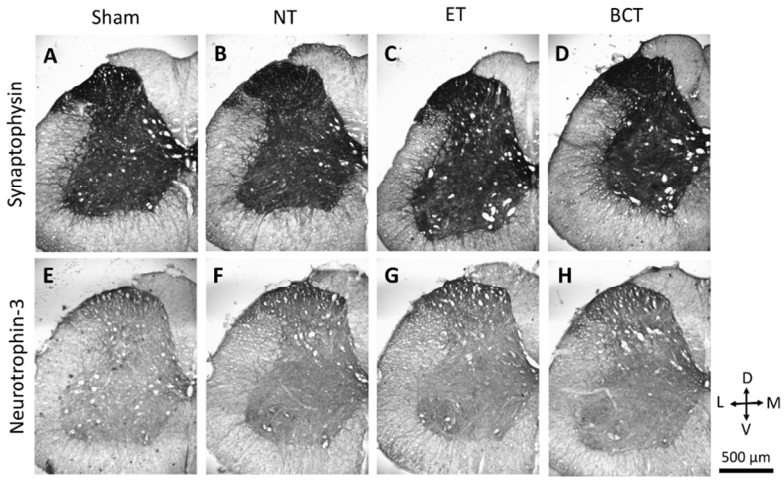

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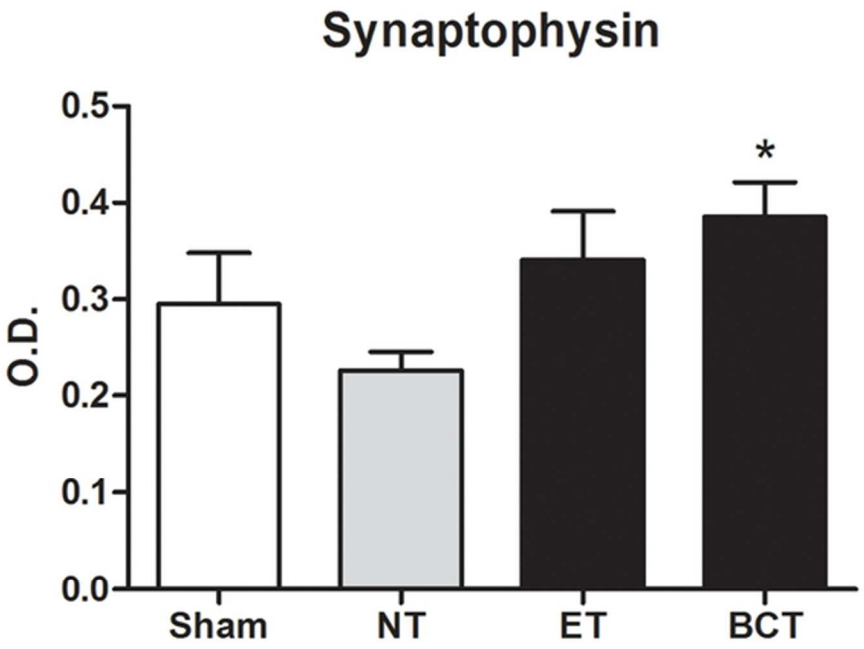

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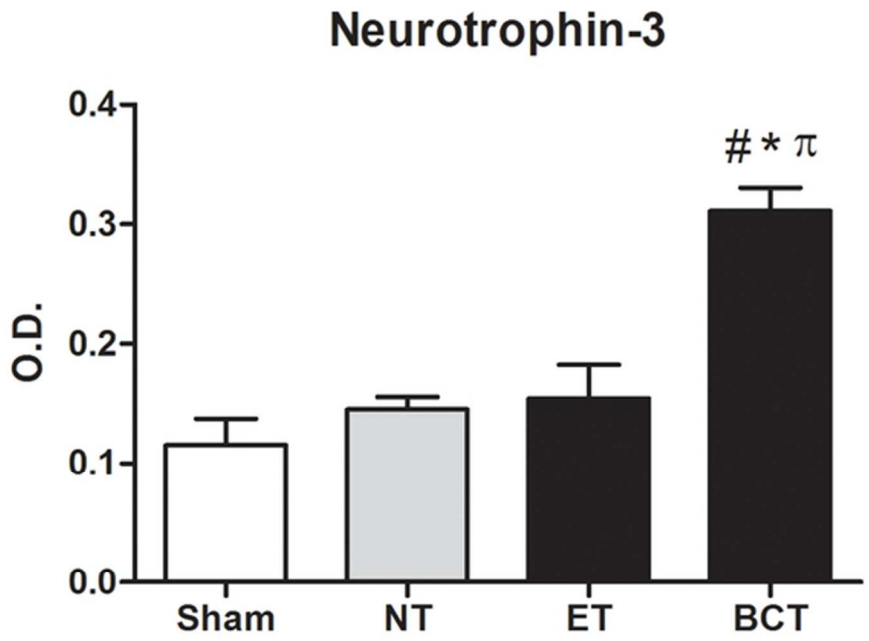

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55x39mm (300 x 300 DPI)

4 DISCUSSÃO GERAL

Estes estudos tiveram o objetivo geral estudar os efeitos do treinamento aeróbico e do treinamento de equilíbrio e coordenação sobre as variáveis funcionais, morfológicas do nervo isquiático e do músculo sóleo, e de imunorreação na medula espinal L4-L6 após uma lesão por esmagamento do nervo isquiático de ratos adultos machos. O experimento foi conduzido da seguinte maneira: primeiramente, os animais foram randomicamente divididos nos quatro grupos experimentais: (1) animais Sham-operados (SH), (2) animais com lesão por esmagamento do nervo isquiático e Não-treinados (NT), (3) animais com lesão por esmagamento do nervo isquiático e que realizaram o Treinamento Aeróbico (*Endurance Training* – ET) e (4) animais com lesão por esmagamento do nervo isquiático e que realizaram o Treinamento de Equilíbrio e Coordenação (*Balance and Coordination Training* – BCT). Após, estes animais foram adaptados nos seus respectivos locais de treinamento e passaram pelos procedimentos cirúrgicos da lesão por esmagamento.

Quarenta e oito horas após o procedimento cirúrgico de esmagamento do nervo isquiático, os animais dos grupos BCT e ET iniciaram seus respectivos protocolos de treinamento, 5 vezes por semana, durante 4 semanas. O protocolo de treinamento aeróbico, também já foi utilizado em estudos prévios em nosso laboratório, consistiu num programa de treinamento diário realizado em uma esteira para humanos (ILHA et al., 2008). Este programa de treinamento é considerado um treino aeróbico de intensidade moderada. O protocolo de treinamento de equilíbrio e coordenação foi adaptado do protocolo do treinamento acrobático (BLACK et al., 1990; ANDERSON, ALCANTARA & GREENOUGH, 1996; KLEIM et al., 1996). Esse protocolo, que já foi utilizado anteriormente em nosso laboratório, foi ajustado para que os animais com lesão por esmagamento do nervo isquiático pudessem realizar atividades de equilíbrio e coordenação sem prejuízos (BONETTI et al., 2011). As pesquisas prévias de Ilha e cols. (2008) e de Bonetti e cols. (2011), que utilizaram os protocolos de treinamento aeróbico e de equilíbrio e coordenação após uma lesão nervosa periférica por

esmagamento, demonstraram ótimos resultados na recuperação locomotora, sensoriomotora e nos parâmetros morfológicos do nervo lesionado e do músculo sóleo. No entanto, o período de início dos protocolos de treinamento e o próprio período de treinamento foram variados, o que dificulta uma melhor análise e comparação destes dois tipos de treinamento. Outra questão importante é que esses estudos, assim como a maioria dos estudos relacionados à recuperação após uma lesão nervosa periférica (LNP), consideram em suas análises estruturais apenas as alterações periféricas, não avaliando alterações em outras áreas do sistema nervoso.

No presente estudo, as variáveis funcionais foram analisadas por meio de testes sensoriomotores e testes locomotores. Os testes sensoriomotores foram realizados ao final da 2ª, 3ª e 4ª semanas de treinamento, enquanto os testes locomotores foram realizados ao final de cada uma das 4 semanas de treinamento. Os testes sensoriomotores utilizados neste trabalho foram o Teste da Escada Horizontal (*Horizontal Ladder Rung Walking Test* – HLRWT) (METZ & WHISHAW, 2002) e o Teste da Barra Estreita (*Narrow Beam Test* – NBT) (ALLBUTT & HENDERSON, 2007), que são tarefas que exigem controle sensoriomotor fino, equilíbrio corporal e colocação precisa do pé (KLEIM et al., 1996; METZ & WHISHAW, 2002). Os resultados destas análises estão apresentados no 1º Artigo, publicado na revista *Muscle & Nerve*. Na análise do Teste da Escada Horizontal, os resultados mais expressivos foram dos animais do grupo BCT, que desde a 2ª semana de treinamento não apresentaram diferenças significativas no número médio de erros do membro posterior lesionado quando comparado ao grupo Sham. Além disso, o grupo BCT exibiu valores médios estatisticamente inferiores ao grupo NT na 2ª semana enquanto o outo grupo treinado, o grupo ET, não apresentaram diferença significativa do grupo NT em nenhuma das três avaliações realizadas.

Com relação aos resultados do outro teste sensoriomotor, o Teste da Barra Estreita, os animais dos grupos lesionados tiveram desempenho significativamente inferior ao grupo Sham na 2ª e 3ª semanas, mas na 4ª semana, apenas os grupos NT e ET apresentaram-se diferentes do grupo Sham. Interessantemente, o BCT apresenta valores médios de erros significativamente menores que o grupo NT na 3ª e 4ª semanas, enquanto o outro grupo treinado, o ET, apresentou valores muito similares aos do NT, ficando distante dos valores médios do grupo BCT. Então, os resultados dos testes sensoriomotores demonstram que o os animais que realizaram o treinamento de equilíbrio e coordenação (BCT) tiveram um desempenho sensoriomotor muito superior ao dos animais do grupo aeróbico (ET) e aos animais não treinados (NT). Entretanto, apesar de entendermos que o treino de equilíbrio e coordenação alcançou os melhores resultados por ser uma tarefa específica (SADOWSKY & MCDONALD, 2009), acreditamos que estes não sejam os únicos agentes envolvidos nestes resultados. É possível que algumas adaptações no sistema nervoso periférico (SNP) e no sistema nervoso central (SNC) tenham ocorrido durante este treinamento para que tenha sido possível restaurar a coordenação da atividade motora destes animais (COWAN et al., 2003; RASOOL & GEORGE, 2007).

A funcionalidade dos animais também foi avaliada por meio de testes locomotores para avaliação do membro posterior lesionado. Os testes utilizados foram o Índice de Funcionalidade do Nervo Isquiático (IFNI) (DE MEDINACELI, FREED & WYATT, 1982; BAIN, MACKINNON & HUNTER, 1989) e por meio da medida do Comprimento da Passada do Membro Lesionado (KUNKEL-BAGDEN, DAI & BREGMAN, 1993; MARCUZZO et al., 2008). Os resultados das análises destes testes locomotores estão apresentados no 3º Artigo, que foi submetido à revista *Muscle & Nerve*. Este tipo de análise de padrões locomotores é considerado uma análise quantitativa (VAREJÃO et al., 2001) e precisa de avaliação da regeneração funcional do nervo lesionado (IJKEMA-PAASSEN,

JANSEN & MEEK, 2004; DIJKSTRA et al., 2000; LUÍS et al., 2007). Nossos resultados demonstraram uma evolução da função locomotora do membro posterior dos animais lesionados (grupos NT, ET e BCT), no entanto, ao final das 4 semanas de treinamento os valores médios do IFNI destes grupos continuaram inferiores aos do grupo Sham, apesar destas diferenças não terem sido estatisticamente significativas. Outra questão importante, é que os grupos lesionados apresentaram valores similares em todas as avaliações, demonstrando que os protocolos de treinamento dos grupos ET e BCT, iniciados 48 horas após uma lesão por esmagamento do nervo isquiático e realizados durante 4 semanas, não foram eficientes para a recuperação locomotora desses animais. Diferentemente destes resultados, outros estudos demonstraram a eficiência de protocolos de treinamento na recuperação locomotora, como o treinamento de natação iniciado um dia após a lesão e com duração de 4 semanas (VANN MEETEREN et al., 1997) ou com duração de 2 semanas (TEODORI et al., 2011). O próprio treinamento aeróbico demonstrou resultados satisfatórios na recuperação do IFNI quando iniciado dois dias após a lesão e realizado durante 12 dias (BYUN et al., 2005). Os resultados da avaliação do Comprimento da Passada do Membro Lesionado demonstraram que o grupo NT teve um desempenho significativamente inferior aos animais do grupo Sham nas duas primeiras avaliações, enquanto o grupo BCT apresentou desempenho inferior ao grupo Sham apenas na 1ª semana de avaliação. Este mesmo grupo BCT apresentou valores estatisticamente superiores ao grupo NT na 2º semana e voltou a apresentar valores similares a este grupo na 3º e 4º semanas de treinamento. Entretanto, os melhores resultados foram alcançados pelo grupo ET, que apresentou resultados semelhantes aos do grupo Sham durante todo o período de treinamento, e neste mesmo período também apresentou valores médios significativamente superiores aos do grupo NT. Outro dado importante a ser destacado, é que na 2ª semana o grupo NT apresentou valores médios inferiores aos valores médios dos outros grupos. Segundo Martins e cols. (2011), este fato se

deve a este período ser o período de dor mais intenso após uma lesão traumática por esmagamento e a falta de atividade física deste grupo pode ter acentuado a resposta protetora e prejudicando o desempenho dos animais deste grupo nesta avaliação. Os resultados da função motora demonstram que na análise dos testes sensoriomotores (HLWRT e NBT) os animais do grupo BCT apresentaram melhores resultados que os animais dos outros grupos; enquanto na análise do teste locomotor do Comprimento da Passada do Membro Lesionado os animais do ET apresentaram resultados mais satisfatórios.

Análises morfológicas da porção distal do nervo isquiático lesionado e do músculo sóleo também foram realizadas neste estudo. Essas análises morfológicas são importantes e podem fornecer informações quantitativas e qualitativas da regeneração, fornecendo ainda uma relação com a recuperação funcional em modelos animais (MICHAILOV et al., 2004; GROVES et al., 2005; AYDIN et al., 2006; DA SILVA, JORDÃO & FAZAN, 2007; MAGILL et al., 2007; BAPTISTA et al., 2008; MAZZER et al., 2008; SABATIER et al., 2008; TEODORI et al., 2011; BARGHASH et al., 2013; RADUCAN et al., 2013; STA et al., 2014). As análises morfológicas quantitativas foram avaliadas por meio de parâmetros morfométricos do nervo isquiático e do músculo sóleo e os resultados estão expostos no 1º Artigo, publicado na revista *Muscle & Nerve*. Com relação à análise do músculo, os valores médios encontrados para as áreas de tecido muscular, de tecido conjuntivo, de vasos sanguíneos, de densidade de fibras musculares e da área das fibras musculares foram similares e estatisticamente semelhantes entre os grupos treinados (ET e BCT). Este resultado é importante, pois após uma lesão nervosa periférica o desuso muscular pode provocar atrofia nos músculos envolvidos (MATSUURA et al., 2001) reduzindo ativamente o desempenho muscular (HADJ-SAÏD et al., 2012). Muitas estratégias são utilizadas com o intuito de manter a atividade muscular e evitar atrofias durante o período de reinervação em modelos animais. Os resultados deste estudo mostraram que tanto o treinamento aeróbico como o treinamento

de equilíbrio e coordenação apresentaram resultados semelhantes entre si, entretanto, os dois protocolos de treinamento preveniram a atrofia muscular quando iniciados na fase aguda da lesão traumática periférica e realizados durante 4 semanas.

Com relação às análises morfométricas do nervo isquiático, o grupo Sham apresentou uma quantidade de tecido conjuntivo significativamente menor entre as fibras nervosas e também uma maior área média de tecido nervoso quando comparado aos demais grupos experimentais. A área de vasos sanguíneos neste tecido nervoso foi similar entre os grupos treinados (ET e BCT), entretanto, o grupo ET apresentou a área dos vasos sanguíneos significativamente maior que o grupo NT. O grupo NT apresentou uma densidade de fibras mielínicas maior que os grupos Sham e ET. Quanto maior a densidade de fibras mielínicas, menor a densidade de fibras axonais nesta região e isto demonstra que o grupo NT estava num estágio de regeneração mais atrasado quando comparado ao grupo ET (ILHA et al., 2008; CUNHA et al., 2011). Outros estudos também demonstraram que maiores densidades de fibras mielínicas são encontradas em animais não treinados (CUNHA et al., 2011; RADUCAN et al., 2013), corroborando com os nossos achados. Com relação à análise dos diâmetros das fibras nervosas, os grupos ET e BCT apresentaram valores médios significativamente superiores aos do grupo NT, demonstrando que estes treinamentos iniciados imediatamente após a lesão podem aumentar a velocidade de regeneração nervosa, como demonstrado anteriormente (ENGLISH et al., 2009; BONETTI et al., 2011). Também já foi demonstrado um aumento no número de axônios em regeneração em animais submetidos ao treinamento aeróbico e do treinamento passivo na bicicleta quando iniciados 5 dias após o episódio de esmagamento do nervo isquiático (UDINA et al., 2011) e o treinamento aeróbico na esteira também apresentou um número maior de axônios em regeneração quando iniciado 2 semanas após a lesão (ILHA et al., 2008). Estes dados

demonstram que não há consenso quanto ao início dos protocolos de treinamento após uma lesão traumática do nervo isquiático.

Análises morfológicas qualitativas foram realizadas pela da observação da ultraestrutura da porção distal do nervo isquiático e do músculo sóleo. A análise ultraestrutural foi feita pelos das análises de cortes transversais ultrafinos dessas estruturas. Essas análises estão apresentadas no 2º Artigo, submetido à revista *Histology and Histopathology*. Na observação do músculo sóleo, os animais do grupo Sham demonstraram características ultraestruturais de um músculo normal, como descrito previamente em estudos clássicos de microscopia eletrônica, com miofibrilas, núcleo, polirribossomos, retículo sarcoplasmático, vasos sanguíneos e mitocôndrias com organização, forma, tamanhos e aparência normais (PELLEGRINO & FRANZINI, 1963; PRICE, 1963; MAIR & TOMÉ, 1972). Já os animais dos grupos lesionados (NT, ET e BCT) mostraram características de um músculo em regeneração, com reduzidas áreas musculares e com muitas áreas de colágeno, presença de gotículas de lipídeos, aumento de colágenos e polirribossomos, retículo sarcoplasmático, vasos sanguíneos com paredes delgadas e núcleo e nucléolos com heterocromatina (PELLEGRINO & FRANZINI, 1963; MILEDI & SLATER, 1968; SCHMALBRUCH, 1976; CULLEN & PLUSKAL, 1977; IRINTCHEV, DRAGUHN & WERNIG, 1990; PEÑA et al., 1995; LU, HUANG & CARLSON, 1997; BORISOV & CARLSON, 2000; BORISOV, DEDKOV & CARLSON, 2001; MALYSZ et al., 2011). Na observação da porção distal do nervo isquiático, os animais do grupo Sham demonstraram características ultraestruturais de fibras amielínicas e mielínicas com características normais. Essas características normais incluem bainhas de mielina, mitocôndrias, axofilamentos, células de Schwann apresentando retículo endoplasmático rugoso tanto no núcleo como no citoplasma e mitocôndrias normais, como demonstrado em estudos prévios (OHMI, 1961; BARDOSI, 1989; HILDEBRAND, BOWE & REMAHL, 1994). Já os animais dos grupos

lesionados (NT, ET e BCT) demonstraram características de um nervo em degeneração Walleriana, com bainhas de mielina com um diâmetro menor e com evaginações, axoplasma com perda de neurotúbulos e neurofilaments, muitas fibras colágenas, debris axonais, gotículas de lipídeos mitocôndrias e vasos sanguíneos com formato alterado (MORRIS, HUDSON & WEDDELL, 1972; SATINSKY, PEPE & LIU, 1964; JACOBSON, 1965; SEA & PETERSON, 1975; CALABRETTA, MUNGER & GRAHAM, 1973; SUNDERLAND, 1978; DUBOVY, 2011). Estudos prévios demonstraram que na análise da porção distal de um nervo após uma lesão, as alterações ultraestruturais estão fortemente relacionadas às características dessa lesão e ao tempo de esmagamento do nervo (PELLEGRINO & FRANZINI, 1963; GERSHENBAUM & ROISEN, 1978; VAREJÃO et al., 2004; BOBINSKI et al., 2011). Entretanto, embora haja modificações benéficas em nível óptico, não foram encontradas evidências de que os protocolos de treinamento aeróbico e de treinamento de equilíbrio e coordenação, iniciados na fase aguda da lesão traumática periférica e realizados durante 4 semanas, sejam suficientes para que os animais desses grupos apresentassem alterações na ultraestrutura do músculo sóleo e do nervo isquiático lesionado quando comparado com o grupo não treinado.

Os resultados das análises morfológicas quantitativas e qualitativas da porção distal do nervo isquiático lesionado e do músculo sóleo não demonstraram diferenças entre o protocolo de treinamento aeróbico e o protocolo de treinamento de equilíbrio e coordenação. Entretanto, os resultados dos testes sensoriomotores mostraram que o grupo que realizou o treinamento de equilíbrio e coordenação apresentou resultados melhores e mais consistentes quando comparado ao grupo que realizou a atividade aeróbica. Neste contexto, este estudo também buscou abranger outras áreas do sistema nervoso para melhorar o entendimento sobre como os exercícios específicos podem ser mais eficazes na melhora de determinadas tarefas, como as tarefas sensoriomotoras. Para tanto, a técnica de imunoistoquímica foi utilizada para verificar

a expressão da sinaptofisina e da neurotrofina-3 na medula espinal, nos níveis de origem do nervo isquiático lesionado (L4-L6), após uma lesão por esmagamento desse nervo. Os resultados dessas análises estão apresentados no 3º Artigo, submetido à revista *Muscle & Nerve*. A sinaptofisina foi escolhida para o estudo imunoistoquímico por ser um indicador preciso de sinaptogênese no SNC (WALAAS, JAHN & GREENGARD, 1988; CHOU et al., 2002; SEO et al., 2010). A densitometria óptica do corno dorsal da medula espinal nos níveis L4-L6 revelaram que a imunorreação dos grupos treinados (ET e BCT) foi maior quando comparada ao grupo não treinado (NT), no entanto apenas o BCT foi significativamente superior ao grupo NT. O grupo NT também mostrou níveis inferiores ao grupo Sham, mas essa diferença não foi estatisticamente significativa. O aumento dos níveis de sinaptofisina nos grupos treinados foi um importante achado, demonstrando uma relação entre o treinamento e a plasticidade sináptica. Com relação à redução dos níveis de sinaptofisina no grupo NT, apesar dessa redução não ser estatisticamente significativa, outros estudos já demonstraram que ocorre esta diminuição e ela representou uma perda de contato sináptico após a ruptura do nervo facial (GEHLERT et al., 1997) e do nervo isquiático (ARBAT-PLANA et al., 2015). Entretanto, o que parece controverso é que alguns estudos demonstraram aumento nos níveis de sinaptofisina após uma lesão por constrição ou secção do nervo isquiático (CHOU et al., 2002; ENGLISH, WILHELM & SABATIER, 2011; CHEN et al., 2009; LIN et al., 2011; TIRAIHI & REZAIE, 2004; MATSUDA et al., 2010), assim como após uma lesão por esmagamento, demonstrando a sinaptofisina auxilia na regulação do crescimento axonal após uma lesão (OKAJIMA et al., 1993). É importante lembrar que a marcação imunorreativa da sinaptofisina ocorreu em toda a substância cinzenta de L4-L6 demonstrando atividade sináptica em toda esta região, não apenas na área de interesse selecionada para essas análises.

Na análise imunoistoquímica da neurotrofina-3, a densitometria óptica demonstrou que a imunorreação nessa região foi significativamente maior no grupo que realizou o treinamento de equilíbrio e coordenação quando comparado aos demais grupos, mesmo quando comparado ao grupo que realizou o treinamento aeróbico. A neurotrofina-3 tem grande importância na manutenção dos neurônios proprioceptivos, que são responsáveis pela coordenação dos membros durante os movimentos e são indispensáveis pelo desenvolvimento desses neurônios (FAN, JAENISCH & KUCERA, 1999). Alguns estudos com uso de modelo animal já demonstraram que animais com deficiência de neurotrofina-3 não se desenvolvem normalmente, apresentando disfunções neurológicas graves (TESSAROLLO et al., 1994), posturas corporais e movimentos dos membros anormais (ERNFORS et al., 1994; ERNFORS et al., 1995). Estudos prévios demonstraram a importância da neurotrofina-3 local após uma lesão nervosa periférica (NITTA et al., 1999; LEE, ZHUO & HELKE, 2001; BOYD & GORDON 2003), entretanto, esse fator de crescimento neurotrófico também é importante no SNC, fazendo parte da plasticidade sináptica após uma lesão (CHAO, 2003). A neurotrofina-3 é responsável por regular a conectividade sináptica entre os neurônios sensoriais e motores (CHEN, TOURTELLOTTE & FRANK, 2002), então, os neurônios localizados no corno dorsal da medula espinal da região lombar são importantes para a locomoção (KIEHN & KJAERULFF, 1998). No entanto, o aumento significativo na imunorreação da neurotrofina-3 nessa região foi demonstrado apenas nos animais que realizaram o treinamento específico de equilíbrio e coordenação, demonstrando que esse tipo de treinamento induz maior plasticidade na medula espinal que o exercício aeróbico.

Hipotetizamos que essa imunorreação específica do grupo BCT está diretamente relacionada com os melhores resultados deste grupo nos testes sensoriomotores. Vários estudos mostraram que o treinamento de habilidades motoras complexas, como as realizadas durante o treino de equilíbrio e coordenação, melhora a funcionalidade e estimula a

plasticidade do córtex motor (KLEIM et al., 1996; BURY & JONES, 2002; ADKINS et al., 2006; MALDONADO et al., 2007; GARCIA et al., 2012), do córtex cerebelar (BLACK et al., 1990; ANDERSON, ALCANTARA & GREENOUGH, 1996; KLEIM et al., 1997; KLEIM et al., 1997; KLEIM et al., 1998; KLEIM et al., 2007) e da medula espinal (ADKINS et al., 2006) de ratos saudáveis. Em animais com lesões centrais, este protocolo de treinamento também estimulou a plasticidade das áreas lesionadas (JONES et al., 1999; CHU & JONES, 2000; SAKATA & JONES, 2003).

A forte correlação entre o treinamento de equilíbrio e coordenação e os melhores resultados nos testes sensoriomotores pode ser explicado por conceito básico muito utilizado na área da reabilitação física, o conceito do treinamento específico, no qual o treinamento em uma tarefa específica auxilia na melhor execução dessa tarefa (MALDONADO et al., 2007; SADOWSKY & MCDONALD, 2009). Esse conceito justifica a grande utilização dos exercícios de equilíbrio e coordenação na prática clínica diária durante a reabilitação de patologias neuromusculares. Entretanto, como discutido previamente, as alterações morfológicas em diferentes níveis do SNP e do SNC ajudam a esclarecer a eficiência da especificidade de cada tipo de treinamento. Diante disto, os resultados desse estudo ampliam o conhecimento científico acerca de uma lesão nervosa periférica por esmagamento e as consequências funcionais e de alterações morfológicas no SNP e SNC relacionadas aos diferentes protocolos de reabilitação propostos.

5 CONCLUSÕES E PERSPECTIVAS

Os resultados apresentados por meio dos estudos desenvolvidos nesta Tese permitem concluir que:

- O treinamento de equilíbrio e coordenação foi mais eficiente, quando comparado ao treinamento aeróbico, na recuperação sensoriomotora de ratos adultos machos após uma lesão nervosa por esmagamento do nervo isquiático;

- Tanto o treinamento aeróbico como treinamento de equilíbrio e coordenação, iniciados 48 horas após a lesão por esmagamento do nervo isquiático:

  - Melhoraram as propriedades morfométricas do músculo sóleo quando comparado aos animais não treinados;

  - Melhoraram as propriedades morfométricas da porção distal à lesão do nervo isquiático quando comparado aos animais não treinados;

  - Não foram suficientes para que as propriedades ultraestruturais do músculo sóleo e da porção distal à lesão do nervo isquiático se diferenciassem dos animais do grupo não treinado.

- O treinamento de equilíbrio e coordenação aumentou a imunorreação da sinaptofisina, quando comparado ao grupo não treinado, e da neurotrofina-3 quando comparado ao grupo sham-operado, não treinado e ao grupo que realizou o treinamento aeróbico, na região do corno dorsal da medula espinal lombar;

- Dessa forma, acreditamos que essa imunorreação específica dos animais que realizaram o treinamento de equilíbrio e coordenação está diretamente relacionada com os melhores resultados deste grupo nos testes sensoriomotores. Vários estudos demostraram que o treinamento de habilidades motoras complexas, como as realizadas durante o treino de equilíbrio e coordenação, melhorou a funcionalidade e a plasticidade em diferentes regiões do sistema nervoso central.

Colocamos como perspectivas futuras:

- Estudar a relação de diferentes padrões temporais para o início das atividades físicas e suas correlações funcionais, morfológicas de nervo e músculo e de imunorreação na medula espinal após uma lesão por esmagamento do nervo isquiático;

- Realizar uma análise ultraestrutural longitudinal do músculo sóleo e da porção distal à lesão do nervo isquiático;

- Analisar imunoistoquimicamente a expressão da sinaptofisina e da neurotrofina-3 em outras regiões do SNC, como o corno ventral da medula espinal e os córtex sensorial e motor, e as relações com os diferentes protocolos de exercício físico utilizados nesta pesquisa;

- Estudar as possíveis alterações ultraestruturais do corno dorsal e do corno ventral da medula espinal e do gânglio da raiz dorsal da região lombar e as relações com os diferentes protocolos de exercício físico utilizados nesta pesquisa;

- Em futuras pesquisas sobre a influência de diferentes protocolos de atividade física e suas correlações funcionais, morfológicas de nervo e músculo e de imunorreação, o aumento do número de animais por grupo se faz necessário para que se tenha um maior poder estatístico sobre os resultados.

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