UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

# REABILITAÇÃO E PLASTICIDADE NEUROMUSCULAR APÓS LESÃO MEDULAR: EFEITOS DO TREINO DE MARCHA EM ESTEIRA E TRANSPLANTE DE GLIA EMBAINHANTE OLFATÓRIA

**TESE DE DOUTORADO**

**Jocemar Ilha**

**Porto Alegre, RS, Brasil**
**2011**

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**por**

**Jocemar Ilha**

Tese apresentada 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.**

**Orientadora: Profa Dra Matilde Achaval**
**Coorientadora: Profa Dra Carmem Gottfried**

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# APRESENTAÇÃO

Esta tese é apresentada de acordo com normas para estrutura e apresentação de monografias, dissertações e teses (MDT; UFSM, 2006) e as recomendações da NBR 14724 (ABNT, 2005). A estrutura física deste trabalho científico, em sua caracterização geral, compreende os elementos pré-textuais, textuais e pós-textuais.

Os elementos pré-textuais antecedem o texto e apresentam as informações que ajudam na visualização geral deste trabalho.

Os elementos textuais estão dispostos em três partes fundamentais: introdução, desenvolvimento e conclusão.

A introdução apresenta a delimitação e problemática do tema, a justificativa e os objetivos do estudo. Esta primeira parte da tese está estruturada em um capítulo intitulado "Introdução geral".

O desenvolvimento está estruturado em 6 capítulos contemplando o referencial teórico, os artigos científicos e a discussão do trabalho em ordem a responder os objetivos propostos. Os dois primeiros são capítulos temáticos e contêm as informações provenientes da literatura científica atual. Os três capítulos seguintes apresentam os artigos científicos e são intitulados Artigo 1, 2 e 3. A discussão contempla a retomada e análise crítica dos resultados apresentados nos artigos, fundamentando-se em fatos amparados por conhecimentos científicos em razão da problemática estabelecida. Além disso, uma discussão sobre o modelo de estudo e sobre e protocolo de treinamento empregado é apresentada neste capítulo que é intitulado "Discussão geral".

Na parte final do texto são apresentadas as conclusões do trabalho com vistas a mostrar o alcance dos objetivos propostos. Este capítulo é intitulado "Conclusões e perspectivas" e também contém as perspectivas para novas pesquisas.

Por fim, encontra-se o capítulo "Referências". Este faz parte dos elementos pós-textuais e lista os textos científicos consultados para a produção da introdução e discussão desta tese. As bibliografias consultadas para os artigos encontram-se listadas ao final de cada trabalho.

*Há uma grandeza nesta visão da vida, com seus diversos poderes tendo sido originalmente insuflados em algumas poucas formas ou em uma só; e, enquanto este planeta esteve revolucionando de acordo com a fixa lei da gravidade, a partir de um início tão simples, infinitas formas, as mais belas e mais maravilhosas, evoluíram e continuam evoluindo.*

CHARLES DARWIN, *A ORIGEM DAS ESPÉCIES.*

Nota do autor: este trecho citado de Darwin foi retirado de Landim e Moreira (2009).

# RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Neurociências
Universidade Federal do Rio Grande do Sul

## REABILITAÇÃO E PLASTICIDADE NEUROMUSCULAR APÓS LESÃO MEDULAR: EFEITOS DO TREINO DE MARCHA EM ESTEIRA E TRANSPLANTE DE GLIA EMBAINHANTE OLFATÓRIA

AUTOR: JOCEMAR ILHA
ORIENTADORA: MATILDE ACHAVAL
COORIENTADORA: CARMEM GOTTFRIED

Porto Alegre, 04 de abril de 2011.

O objetivo desta Tese foi analisar os efeitos do treino de marcha isolado e em combinação com transplante de glia embainhante olfatória (GEO) na recuperação funcional e na plasticidade neuromuscular dependente da atividade em um modelo experimental de paraplegia.

Para tanto, foram realizados 2 experimentos. No 1º experimento foi realizada completa transecção da medula espinal (TME) em ratos Wistar adultos e após 5 dias iniciou-se um protocolo de 9 semanas de treino de marcha em esteira com suporte de peso corporal. No 2º experimento, os animais receberam, imediatamente após a TME, transplante de células gliais embainhantes olfatórias (GEO) e, como no primeiro experimento, iniciaram o treino de marcha 5 dias após a lesão/transplante. Durante o período dos experimentos, estudos comportamentais para acompanhamento da recuperação da função sensório-motora dos animais foram periodicamente realizados. Além disso, ao término da fase de treinamento (10 semanas após a lesão/transplante), análises histológicas e bioquímicas foram realizadas em amostras de tecido retiradas da medula espinal e músculo sóleo.

Os resultados mostram que o treino de marcha em esteira promove melhora da função sensório-motora nos membros posteriores (MPs) de ratos com completa transecção da medula espinal (TME). Os animais treinados apresentaram escores mais altos na escala BBB e normalização do reflexo flexor de retirada. Além disso, os animais com TME apresentaram atrofia do soma celular nos motoneurônios alfa, redução na expressão de sinaptofisina e na atividade da $Na^+,K^+$-ATPase na região lombar. Os animais treinados mostraram soma motoneuronal, expressão de sinaptofisina e atividade da bomba de $Na^+,K^+$-ATPase similares aos controles.

No músculo sóleo, a TME causou severa atrofia muscular, que foi acompanhada pela redução na expressão do fator neurotrófico derivado do encéfalo (BDNF) neste músculo. Por outro lado, o treino de marcha foi capaz de parcialmente impedir/reverter a atrofia provocada pela paralisia muscular e promover um significante aumento na expressão do BDNF, o qual teve positiva correlação com o trofismo muscular dependente da atividade motora no músculo sóleo.

O transplante de glia embainhante olfatória (GEO) promoveu significativo aumento nos escores da escala BBB nos animais com completa TME. Entretanto, o treino de marcha foi capaz de acelerar este ganho funcional. Apesar de não ser observada significativa regeneração axonal através do local da lesão, sugerindo que as melhoras funcionais ocorreram independentemente da existência de regeneração axonal.

Estes resultados sugerem que o treino de marcha após a TME promove plasticidade morfológica e bioquímica dependente da atividade nos tecidos neuromusculares. A melhora funcional ocorreu concomitantemente a estas alterações plásticas. Além disso, a terapia de transplante de GEO mostrou resultados positivos na recuperação da função motora dos MPs que foi acelerada pelo treino de marcha, mesmo na ausência de regeneração axonal através da lesão. Estes dados mostram importantes informações neurobiológicas que fornecem base neurocientífica para o uso seguro e eficaz destas terapias na reabilitação após LME.

**Palavras-chaves:** Lesão da medula espinal; Paraplegia; Treino de marcha; Glia embainhante olfatória; Plasticidade neuromuscular

# ABSTRACT

Doctoral Thesis
Programa de Pós-Graduação em Neurociências
Universidade Federal do Rio Grande do Sul

## REHABILITATION AND NEUROMUSCULAR PLASTICITY AFTER SPINAL CORD INJURY: EFFECTS OF TREADMILL STEP TRAINING AND OLFACTORY ENSHEATHING GLIA TRANSPLANTATION

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MATILDE ACHAVAL
CARMEM GOTTFRIED

Porto Alegre, April 4th, 2011.

The aim of this thesis was to study the effects of treadmill step training alone and in combination with olfactory ensheathing cells (OEC) on functional recovery and activity-dependent neuromuscular plasticity in a traumatic paraplegia model.

For this, we made two experiments. In the 1st experiment, complete spinal cord transection (SCT) was made in adult Wistar rats and after 5 days the spinal animals were underwent a 9 week body-weight-supported treadmill training (BWSTT) program. In the 2nd experiment, the spinal animals received acute olfactory ensheathing cell (OEC) transplantation and, similar to the 1st experiment, started a BWSTT 5 days after the injury/transplantation. Behavioral tests were periodically performed in order to study the hindlimb sensorimotor functions in both experiments. Furthermore, after 9 weeks of the training (10 weeks after SCI/transplantation), histological and biochemical analysis were performed in spinal cord and soleus muscle tissues.

The results show that treadmill step training improves hindlimb sensorimotor function in rats with complete spinal cord transection (SCT). The trained animals showed higher BBB scores and normalization of the withdrawal reflex. Furthermore, spinal animals showed alpha motoneuron soma size atrophy, decrease in synaptophysin expression and $Na^+,K^+$-ATPase activity in lumbar spinal cord. Trained SCT animals showed motoneuron soma size, synaptophysin expression and $Na^+,K^+$-ATPase activity values similar to controls.

In soleus muscle, SCT led to severe muscular atrophy, which was accompanied by a decrease in brain-derived neurotrophic factor (BDNF) expression in this muscle. On the other hand, treadmill step training was able to revert/prevent this paralysis-induced muscular atrophy and promote significant improvement in soleus BDNF expression, which was positively correlated to activity-dependent muscular trophism.

Olfactory ensheathing cell (OEC) transplantation promotes significant improvements in the BBB scores of animals with SCT. However, treadmill step training was able to accelerate this functional gain. There was no significant axonal regeneration that traversed the injury site, which suggests that functional gains occurred in a manner independent of axonal regeneration.

Taken as a whole, these results suggest that treadmill step training after SCT promotes activity-dependent morphological and biochemical plasticity in neuromuscular tissues. The functional improvements occurred concomitantly to these plastic changes. Moreover, OEC therapy showed positive results on hindlimb motor function recovery which was accelerated with treadmill step training even in the absence of axonal regeneration across the lesion site. These results represent important neurobiological information for the neuroscientific basis that supports these therapies as an efficient and safe approach in spinal cord injury rehabilitation.

**Keywords:** Spinal cord injury; Paraplegia; Treadmill step training; Olfactory ensheathing cell; Neuromuscular plasticity

# LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| ASIA | *American Spinal Cord Injury Association Impairment* |
| ATPase | Adenosina trifosfatase |
| AVD | Atividades de vida diária |
| BBB | Escala locomotora de Basso, Beattie e Bresnahan |
| BDNF | Fator neurotrófico derivado de encéfalo (*brain-derived neurotrophic factor*) |
| NT | Neurotrofina |
| BO | Bulbo olfatório |
| BWSTT | *Body-weight-supported treadmill training* |
| DW | Degeneração walleriana |
| GEO | Glia embainhante olfatória |
| GFAP | Proteína glial fibrilar ácida (*Glial fibrillary acid protein*) |
| GPC | Gerador de padrão central |
| h | Hora |
| L | Lombar |
| LME | Lesão da medula espinal |
| m | Metro |
| ME | Medula espinal |
| min | Minuto |
| MN | Motoneurônio |
| MNI | Motoneurônio inferior |
| MNS | Motoneurônio superior |
| MP | Membro posterior |
| NO | Nervo olfatório |
| SN | Sistema nervoso |
| SNC | Sistema nervoso central |
| SNP | Sistema nervoso periférico |

| | |
|---|---|
| T | Torácica |
| TME | Transecção da medula espinal |
| Trk | Receptor tirosina cinase |
| 5-HT | Serotonina |
| GAP-43 | Proteína associada ao crescimento |

# SUMÁRIO

# 1 INTRODUÇÃO GERAL

A execução das funções motoras e sensoriais necessita da integridade estrutural e funcional da medula espinal (ME), desta forma condições patológicas que afetem esta estrutura podem levar a importantes impactos na sua funcionalidade. Estas condições vão desde a lenta evolução de doenças crônicas até insultos agudos ocasionados por traumas físicos ou acidentes vasculares que podem causar severos comprometimentos funcionais, resultando em desabilidades permanentes e a necessidade de cuidados por toda a vida do paciente (FREDERICKS, 1996).

As lesões da medula espinal (LME) são consideradas as principais causas de complicações clínicas, sociais e econômicas dentre as afecções neurológicas. Embora sua incidência mundial, estimada em 130.000 novos casos por ano, seja relativamente baixa comparada com outras doenças ou traumas, elas apresentam grande repercussão social uma vez que geralmente ocorrem em indivíduos jovens em torno dos 30 anos, sendo a maior ocorrência aos 19 anos (RICK HANSEN FOUNDATION, 2006). Com os crescentes avanços da medicina, cresce também o número de pacientes que sobrevivem ao traumatismo da ME e iniciam uma "nova e diferente forma de vida" fadada ao uso de cadeira de rodas para seu deslocamento por 40 anos ou mais. Embora a expectativa de vida seja muito boa, pacientes com LME sofrem de importantes complicações associadas ao nível e a severidade da lesão. Estas complicações diminuem sua qualidade de vida e se apresentam sob a forma de espasticidade e paralisia muscular, perda sensorial, obesidade, perda de massa óssea, dor, úlceras de pressão e infecções do trato urinário.

O impacto socioeconômico relativo aos custos com cuidados clínicos e previdência social por longo tempo soma mais de dez milhões de dólares por ano, gerando um importante problema financeiro mundial (RICK HANSEN FOUNDATION, 2006). Até recentemente, o mau prognóstico em tratamentos efetivos para a recuperação da função, fez com que a comunidade e governos focassem seus esforços na atenção básica, provendo serviços voltados aos cuidados médicos por longo tempo. Dado o entendimento científico anterior aos anos 1990 de que a regeneração no sistema nervoso central (SNC) era impossível, essa estratégia clínica voltada à manutenção através de cuidados básicos e preventivos das complicações

associadas às LME era adequada. Entretanto, hoje estamos frente a um novo horizonte e nossa estratégia deve mudar para que possamos desenvolver tratamentos efetivos na busca da cura da paralisia e consequente recuperação da perda funcional causada pelo traumatismo da ME.

Neste contexto, pesquisadores têm aprendido importantes lições da neurobiologia experimental a respeito das pistas de que o SNC pode ser alterado estrutural e bioquimicamente, propiciando alterações plásticas decorrentes do aprendizado de novas habilidades e regeneração axonal após lesões. Como resultado, o potencial para redução das complicações neurológicas e desabilidades funcionais das pessoas com lesões traumáticas da ME tem crescido nos últimos anos.

## 1.1 Justificativa

O treinamento específico da tarefa de marcha em esteira ergométrica vem sendo empregado no retreino da marcha em pacientes com lesões medulares (WERNIG et al., 1998; HICKS et al., 2005; FIELD-FOTE; ROACH, 2011). Embora existam estudos animais mostrando os efeitos do treinamento desta tarefa de reabilitação físico-motora sobre o sistema neuromuscular, a neurobiologia desta intervenção ainda não é completamente conhecida.

Por outro lado, a busca por estratégias que possibilitem o reparo neural através da regeneração axonal e promovam a reabilitação funcional após lesões medulares tem sido um intrigante campo de estudo das neurociências. Modelos experimentais têm sido usados para proporcionar maior conhecimento sobre a regeneração nervosa e desenvolvimento de terapias que promovam uma adequada recuperação funcional. Recentes estudos em animais têm relatado resultados promissores da utilização do transplante de células gliais embainhantes olfatórias (GEO) após lesão medular completa (RAMÓN-CUETO et al., 1998; 2000; LU et al., 2001). Embora esta terapia celular mostre importantes ganhos funcionais, estes ainda são considerados modestos pela comunidade científica.

Dessa forma, a presente tese justifica-se por buscar uma maior compreensão neurobiológica da plasticidade neuromuscular dependente da atividade após lesões medulares. Além disso, a hipótese de que o treino de marcha pode otimizar os possíveis resultados benéficos obtidos com o transplante celular de GEO é testada.

## 1.2 Objetivos

### 1.2.1 Objetivo geral

Este trabalho teve como objetivo geral estudar os efeitos do treino de marcha isolado e em combinação com o transplante de células gliais embainhante olfatória (GEO) na recuperação neurológica e plasticidade neuromuscular em um modelo experimental de paraplegia.

### 1.2.2 Objetivos específicos

(i) Avaliar a recuperação sensório-motora dos membros posteriores (MP), através da análise dos movimentos em campo aberto e da testagem do reflexo flexor de retirada, durante o período do treinamento de marcha em esteira ergométrica em ratos machos adultos após transecção da medula espinal (TME) ao nível de T8-9 (Artigo 1);

(ii) Caracterizar os efeitos da TME e analisar os efeitos do treino específico da tarefa de marcha em esteira no tamanho dos motoneurônios alfa (lâmina IX), na plasticidade sináptica e atividade da bomba de $Na^+$,$K^+$-ATPase na região lombar de L5 (Artigo 1);

(iii) Caracterizar os efeitos da TME e analisar os efeitos do treino específico da tarefa de marcha em esteira no trofismo muscular e na expressão do fator neurotrófico derivado do encéfalo (BDNF) (Artigo 2);

(iv) Avaliar a recuperação funcional dos membros posteriores (MP), através da análise dos movimentos em campo aberto, durante o período do treinamento de marcha em esteira ergométrica em ratos machos adultos após TME ao nível de T8-9 e submetidos ao transplante agudo de GEO (Artigo 3);

(v) Avaliar a regeneração axonal na medula espinal, através da marcação imunoistoquímica das fibras serotoninérgicas e da proteína associada ao crescimento axonal (GAP-43), nas regiões cranial e caudal à lesão em ratos machos adultos, após TME, submetidos ao transplante agudo de GEO e ao treino de marcha em esteira (Artigo 3).

# 2 LESÃO TRAUMÁTICA DA MEDULA ESPINAL

A integridade da ME é necessária para muitas das funções motoras e sensoriais normais em mamíferos. Importunamente, uma variedade de condições patológicas pode afetar tanto a estrutura como a função da ME. Estas condições variam desde distúrbios crônicos a insultos agudos ocasionados por trauma físico. As LME interrompem a informação descendente do encéfalo para os níveis medulares abaixo da lesão, bem como a informação ascendente das estruturas sensoriais causando importante déficit motor e sensorial caudal ao nível da lesão.

## 2.1 Incidência

Os traumatismos da ME causam desabilidades em mais de oito mil pessoas por ano tanto no Brasil como nos Estados Unidos da América (RICK HANSEN FOUNDATION, 2006). As causas mais frequentes incluem lesões penetrantes por armas de fogo e outras formas de violência (26%), acidentes de carro (38%), acidentes esportivos (7%), bem como quedas (22%), especialmente em pessoas idosas (DOBKIN; HAVTON, 2004). O comprometimento funcional pode ser severo, resultando em permanentes desabilidades funcionais e a necessidade de cuidados por toda uma vida. Aproximadamente 50% das vítimas apresentam completa perda sensório-motora distalmente ao nível da LM e menos de 5% destes pacientes poderão ter alguma habilidade de caminhar (DOBKIN; HAVTON, 2004).

## 2.2 Classificação neurológica

O grau de severidade das complicações imediatas, decorrente da lesão inicial, é depende do nível espinal (Figura 1) lesado e do grau de extensão da lesão. Lesões ao nível cervical determinam uma distribuição topográfica dos déficits neurológicos, motores e/ou sensoriais, que envolvem os membros superiores, o tronco e os membros inferiores, sendo topograficamente classificadas como tetraplegia ou quadriplegia. O termo paraplegia refere-se ao comprometimento da função motora e/ou sensorial ocasionada por LME aos níveis

torácico, lombar ou sacral. Na paraplegia a função sensório-motora dos membros superiores é preservada e, dependendo do nível da lesão, o tronco e os membros inferiores são funcionalmente comprometidos (MAYNARD et al., 1997) (Figura 2).

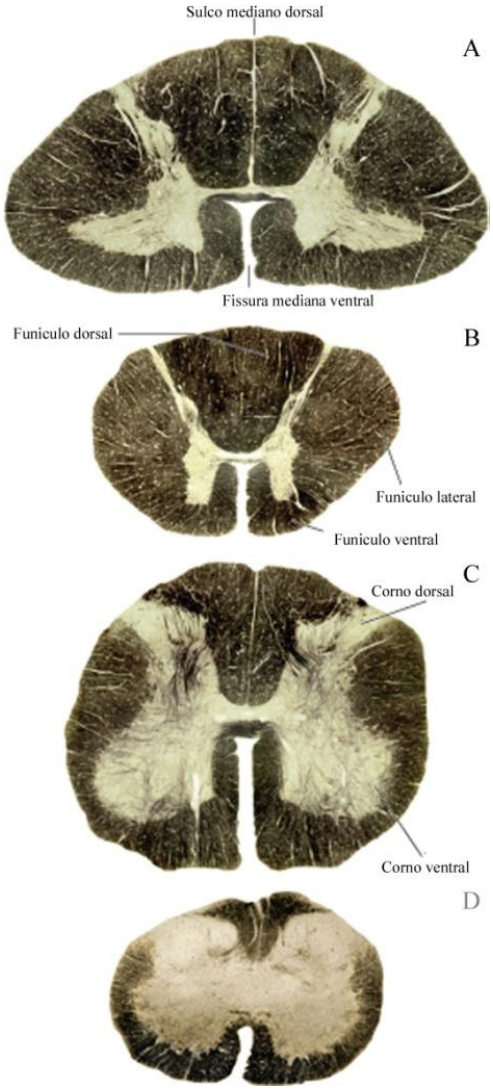


**Figura 1.** Secções transversas da medula espinal representativas dos níveis espinais: (A) cervical, (B) torácico, (C) lombar e (D) sacral. Coloração de hematoxilina de Weigert. Aumento aproximado de 5x (modificada de STANDRING et al., 2008).

Segundo o grau de extensão, a Associação Americana de Lesões da Medula Espinal (*American Spinal Cord Injury Association Impairment*, ASIA) define lesões completas e incompletas funcionalmente. O termo lesão completa descreve a ausência de função motora e sensorial caudal ao nível da lesão. A lesão incompleta é o termo que descreve preservação

parcial das funções motoras e/ou sensoriais caudal ao nível da lesão (MAYNARD et al., 1997).

Utilizando estes critérios, a escala funcional da ASIA define o grau de perda neurológica como: A – completa perda sensório-motora caudal ao nível da LME, incluindo ausência da sensação sacral; B – não há função motora, mas a função sensorial está preservada caudal ao nível da LME; C – alguma função motora preservada, mas a maioria dos músculos caudais à LME apresentam menos de 3 pontos em uma escala de 5 pontos para avaliação da força muscular; e, D – a maioria dos músculos caudais à LME apresentam 3 ou mais pontos na escala de força.

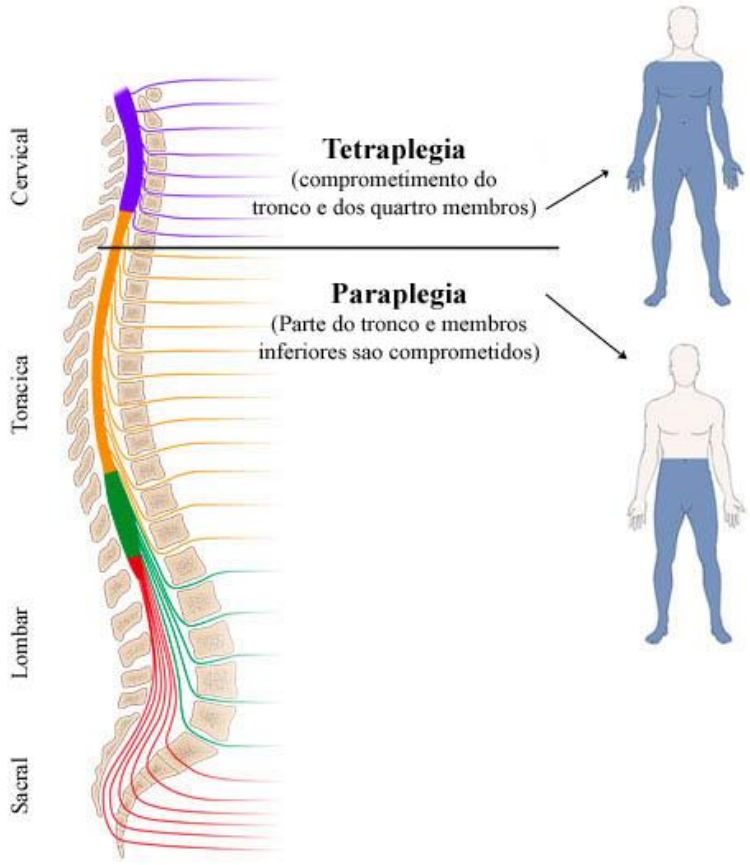


**Figura 2.** Desenho esquemático mostrando a classificação topográfica funcional (tetraplegia e paraplegia) nas lesões medulares em humanos (modificada de UAMS, 2011).

Dessa forma, pacientes que apresentam tetraplegia possuem lesões em um dos oito segmentos cervicais da medula espinal, por outro lado, aqueles com paraplegia possuem lesões nas regiões torácicas, lombares ou sacrais. Desde o ano 2000, a mais frequente

classificação neurológica reportada nas bases de dados epidemiológicos é a tetraplegia incompleta (34,1%), seguida por paraplegia completa (23%), tetraplegia completa (18,3%) e paraplegia incompleta (18,5%) (RICK HANSEN FOUNDATION, 2006).

Clinicamente, as lesões podem ser consideradas incompletas ou completas dependendo da presença ou ausência de funções neurológicas caudais ao nível da lesão. No nível segmental, a paralisia é caracterizada pela lesão do motoneurônio inferior (MNI), resultando da lesão à substância cinzenta da ME. Entretanto, é a interrupção dos longos tratos da substância branca (Figura 3) que são mais importantes na determinação do quadro clínico em LME. Caudal à lesão, a paralisia é caracterizada pela lesão do motoneurônio superior (MNS), com aumento do tônus muscular (espasticidade), reflexos exagerados (hiperreflexia) e sinal de Babinski positivo (KAKULAS, 2004).

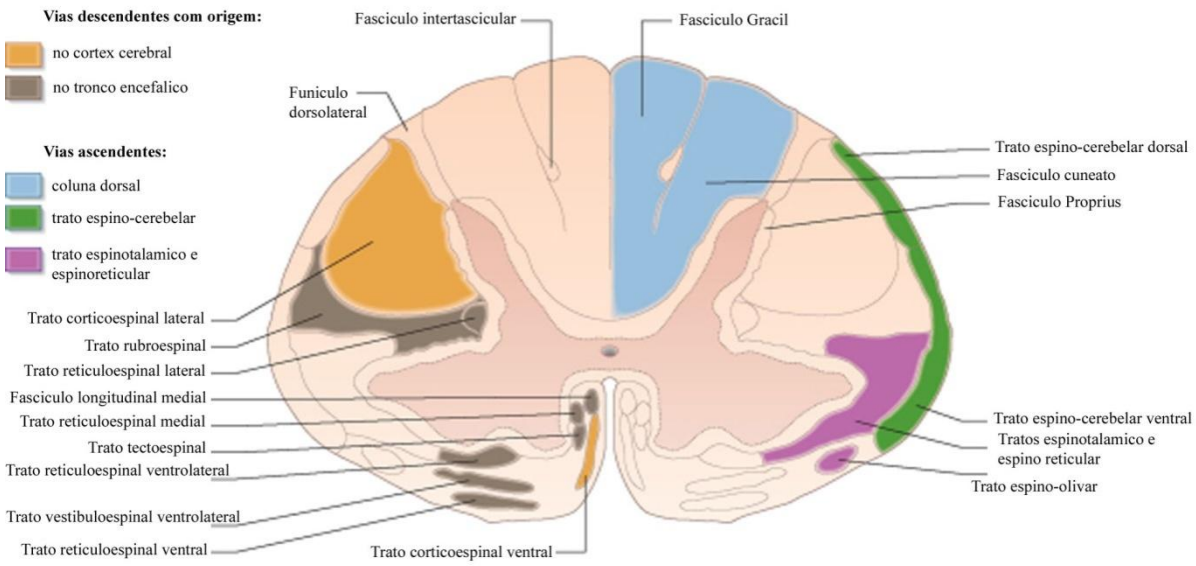


**Figura 3.** Desenho esquemático mostrando a posição aproximada das vias espinais de fibras nervosas ao nível cervical médio de humanos (modificada de STANDRING et al., 2008).

## 2.3 Alterações neuromusculares

As principais causas de incapacidade funcional em indivíduos com LME são a ausência de movimento voluntário associada às contrações musculares involuntárias e sustentadas. Estes comprometimentos são características do aumento da excitabilidade dos circuitos reflexos espinais e compõem o sinal clínico de disfunção neurológica chamado de

espasticidade (ADAMS; HICKS, 2005; ELBASIOUNY et al., 2010). Em geral, a espasticidade é classificada como um sinal da síndrome do MNS, caracterizado por um exagero do reflexo de estiramento secundário à hiperexcitabilidade dos circuitos reflexos espinais (ADAMS; HICKS, 2005).

A hiperatividade reflexa apresentada pelos pacientes com LME também se manifesta clinicamente como uma atividade anormal aumentada dos reflexos flexores de retirada (*withdrawal reflexes*). Estes reflexos são mediados por vias oligossinápticas que agem sobre MNI oriundas de fibras nervosas não mielinizadas nociceptivas C e de fibras mielinizadas de pequeno calibre A delta. Estas fibras nervosas são ativadas por estímulos nocivos. Fisiologicamente, os reflexos flexores de retirada são limitados aos músculos responsáveis pela retirada da região corporal em contato com o estímulo. Entretanto, após a lesão medular tornam-se difusos e hiperativos e assim contribuem para os espasmos musculares e anormalidades posturais visíveis nos pacientes (WOLPAW; TENNISSEN, 2001).

Os MNS originam-se no encéfalo e em centros motores do tronco encefálico e projetam-se principalmente para os MNI do tronco encefálico e ME. Os MNI localizam-se predominantemente no corno ventral da ME, Lâmina IX de Rexed (Figura 4), e são classificados em: motoneurônios alfa que se projetam para as fibras musculares extra-fusais e motoneurônios gama que se projetam para as fibras musculares intra-fusais (STANDRING, 2008).

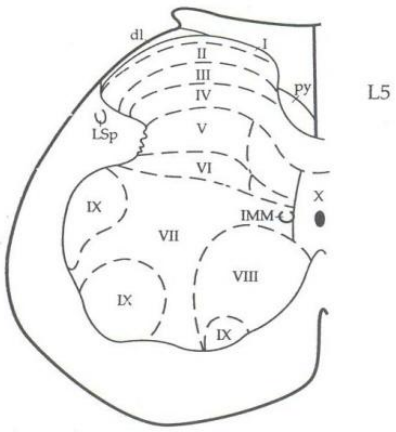


**Figura 4.** Desenho esquemático de uma secção transversa da ME ao nível lombar (L5) mostrando a divisão das lâminas I à X de Rexed (imagem retirada de GRANT; KOERBER, 2004).

A LME provoca uma interrupção na transmissão de sinais enviados dos MNS para os MNI e interneurônios locais relacionados. Imediatamente após a lesão medular, existe um período em que o indivíduo apresenta uma paralisia muscular flácida e perda dos reflexos

tendíneos abaixo do nível da lesão. Este período, conhecido como “choque medular” dura de dias (1 a 3) a poucas semanas após a lesão e, é seguido pelo desenvolvimento gradual de hiperreflexia e espasmos involuntários (ELBASIOUNY et al., 2010).

Neste contexto, pensa-se que o desenvolvimento da espasticidade é causado pela perda da inibição dos impulsos supraespinais sobre os interneurônios e MNIs, que, por sua vez, causaria a hiperatividade dos circuitos reflexos espinais (GOLDSTEIN, 2001). Por outro lado, alterações nas propriedades eletrofisiológicas intrínsecas dos MNI, tais como diminuição da amplitude do potencial de repouso dessas células (despolarização), têm sido relatadas e podem ser um importante componente da hiperreflexia espinal (BEAUMONT et al., 2004). Embora o exato mecanismo neurofisiológico responsável por esta hiperreflexia não seja completamente conhecido, é possível que ambas, a perda da inibição dos MNSs sobre os MNIs e as alterações das propriedades eletrofisiológicas, estejam envolvidas.

As LME causam uma das mais incapacitantes condições neurológicas, caracterizando-se por importante paresia e/ou plegia muscular caudal ao nível da lesão. O desuso muscular provocado pela paralisia causa uma severa atrofia muscular (GIANGREGORIO; McCARTNEY, 2006; BIERING-SORENSEN et al., 2009; QIN; BAUMAN; CARDOZO, 2010). Esta rápida perda de massa muscular após LME possui um significante impacto nos cuidados e no estilo de vida desses pacientes. Além disso, o risco de desenvolver complicações associadas como perda de massa óssea, aparecimento de doenças cardiovasculares e diabetes, é grande nesses pacientes (QIN; BAUMAN; CARDOZO, 2010).

A atrofia muscular presente nos pacientes com LME é caracterizada pela perda de massa muscular e redução na área de secção transversa tanto de músculos como das fibras musculares (ADAMS et al., 2006; JAYARAMAN et al., 2008). Embora essas variáveis estejam reduzidas em vários músculos abaixo do nível da lesão, os maiores graus de atrofia são encontrados nos músculos posturais, compostos predominantemente por fibras musculares do tipo I (BIERING-SORENSEN et al., 2009).

Associada a atrofia da musculatura, as fibras musculares lentas oxidativas tipo I sofrem alterações metabólicas e passam a apresentar características histoquímicas de fibras glicolíticas rápidas, ou seja, do tipo II. Este processo se inicia meses após as alterações morfológicas que caracterizam a atrofia do tecido muscular e evolui por vários anos. Além disso, o tecido muscular demonstra alterações fundamentais nas propriedades fisiológicas que incluem marcado aumento na fatigabilidade e alteradas taxas de contração e relaxamento muscular após LME (QIN; BAUMAN; CARDOZO, 2010).

## 2.4 Aspectos fisiopatológicos

Traumas agudos da ME em humanos frequentemente produzem hematoma central que, nos casos mais severos, é facilmente visível nas imagens por ressonância magnética. A compressão ou a avulsão da ME leva à destruição da substância cinzenta dos cornos ventral e dorsal por um ou mais níveis espinais e a interrupção parcial ou completa dos tratos ascendente e descendente na substância branca. Além disso, raízes dorsais e ventrais podem sofrer avulsão no nível da lesão. Dessa forma, o dano é maior no local da lesão, mas pode estender-se por vários segmentos craniais ou caudais à lesão primária (FREDERICKS, 1996; DOBKIN; HAVTON, 2004).

O primeiro sinal da lesão medular é o edema do tecido nervoso, manifestado como um inchaço no local causado pelo extravasamento de fluídos dos capilares. Isto ocorre dentro de minutos após a lesão e normalmente é acompanhado por hemorragia parenquimal da substância branca (KAKULAS, 2004). Nesta fase aguda, a necrose e, em menor grau, a apoptose celular, são responsáveis pela morte de neurônios e células gliais do tecido lesionado. Além disto, há um efeito do choque excitotóxico com a devastação ocasionada pelos radicais livres ao nível molecular. Alterações reativas vasculares, iniciadas pelas citocinas liberadas pelo tecido lesionado, podem ser evidentes nas primeiras 12 h e são representadas pela migração de leucócitos polimorfonucleares e dilatação vascular. Linfócitos aparecem logo em seguida e macrófagos também estão presentes entre 24 e 48 h pós-lesão. Em 72 h, macrófagos carregados de gordura são abundantes, englobando fragmentos de mielina e enzimaticamente convertendo-os à gordura neutra para remoção local (KAKULAS, 2004).

Dentro de horas após o trauma inicial, um processo progressivo de destruição tecidual inicia-se na ME e pode estender a região de dano neural por vários segmentos no sentido longitudinal (crânio – caudal). Estas reações secundárias levam à isquemia, ao edema, à desmielinização e à necrose tecidual. Na busca por terapias mais eficazes, estudos têm sido realizados tanto em animais de laboratório, como *post mortem* em humanos, com objetivo de definir os mecanismos fisiopatológicos desses efeitos secundários. Entre os fatores estudados estão a hemorragia, a isquemia, o extravasamento de eletrólitos locais, a reação inflamatória e o acúmulo de varias substâncias biorreativas no local. Após este período agudo, a região de necrose da ME é reabsorvida e substituída por tecido cicatricial e formação de cistos ou cavidades em estágios mais crônicos (FREDERICKS, 1996; WEBB; NGAN; FOWLER, 2010).

## 2.5 Modelos animais

Modelos animais de LME permitem aos pesquisadores estudar ou manipular alterações biológicas específicas em resposta a intervenções terapêuticas. A maioria dos estudos de LME utiliza um de três modelos gerais de lesão: contusão, compressão ou transecção da ME (TME) (ROSENZWEIG; McDONALD, 2004).

A realização da contusão medular é um dos modelos de lesão mais utilizados, o qual é realizado através de um impacto aplicado sobre a região dorsal da ME após laminectomia dos processos vertebrais (BASSO; BEATTIE; BRESNAHAN, 1996). No modelo de compressão, um clip de aneurisma é colocado subduralmente após laminectomia (cervical ou torácica) por um minuto e então é retirado (POON et al., 2007; MARQUES et al., 2009). Ambos os modelos de compressão e contusão são principalmente realizados em roedores (ratos ou camundongos) e são considerados modelos reprodutíveis e clinicamente relevantes por provocarem características morfológicas e funcionais similares aos encontrados em humanos com LME.

Entretanto, esses são modelos de lesões incompletas, tanto sob o aspecto morfológico quanto neurológico, que mantêm a integridade de fibras dos tratos espinais de controle motor, que por sua vez, permite uma recuperação funcional rápida e quase completa em roedores (BASSO; BEATTIE; BRESNAHAN, 1996; POON et al., 2007; MARQUES et al., 2009). Dessa forma, quando esses modelos são utilizados para estudar a plasticidade espinal induzida pelo treino de uma tarefa específica, a recuperação funcional pode ocorrer tanto pelo crescimento e/ou brotamento colateral das fibras remanescentes que passam pelo local da lesão e inervam os MNIs caudais, quanto por processos plásticos das interconexões neurais locais e alterações das propriedades eletroquímicas dos MNI.

Nos modelos de transecção, após a realização de uma laminectomia, a ME é completamente transeccionada por um bisturi ou uma tesoura cirúrgica (BASSO; BEATTIE; BRESNAHAN, 1996; BOUYER, 2005). Em roedores, a transecção pode ser realizada em qualquer nível medular. Quando esta transecção é realizada ao nível torácico em animais, um modelo de lesão completa da ME é produzido já que desconecta permanentemente das influencias supraespinais os circuitos neuronais e os grupos de motoneurônios caudais ao nível da lesão, causando uma severa paralisia dos membros posteriores (MPs) (BASSO; BEATTIE; BRESNAHAN, 1996). Esta distribuição corporal dos comprometimentos sensório-motores, associada ao desenvolvimento de hiperreflexia dos circuitos espinais de

forma dependente do tempo, similar ao apresentado por humanos após lesões completas da ME torácica, o caracterizam como um modelo experimental de paraplegia (YATES et al., 2008a).

Este modelo é amplamente empregado no estudo do controle e adaptação da locomoção. A compreensão dos efeitos do treinamento de tarefas específicas, tais como a marcha ou a postura, na completa ausência do controle supraespinal pode aumentar o conhecimento de como as estruturas espinais se adaptam em decorrência da atividade para reexpressar um padrão locomotor similar ao existente anterior à lesão (BOUYER, 2005). Estudos utilizando o modelo de completa TME em ratos mostraram que a ME desconectada das estruturas supraespinais mantém grande capacidade de aprendizado motor ao nível espinal quando uma tarefa motora é treinada (BEAUMONT et al., 2004; BIGBEE et al., 2007). Estes resultados mostram que o padrão motor aprendido é dependente da atividade treinada, ou seja, animais que realizam treino de marcha melhoram a realização de passos, enquanto que os que treinam a acomodação de peso sobre os membros posteriores melhoram a capacidade de sustentar o peso corporal (BIGBEE et al., 2007). Além disso, alterações nas propriedades eletrofisiológicas dos MNI foram mostradas após exercícios cíclicos passivos dos membros posteriores em ratos com completa TME (BEAUMONT et al., 2004). Estes estudos enfatizam a remarcada neuroplasticidade da circuitaria neural da ME e fornecem embasamento para as técnicas de reabilitação físico-motora baseadas no retreinamento da marcha.

Além disso, pesquisas de neuroplasticidade após lesões do SNC encontram na completa TME um modelo experimental adequado para estudar a regeneração axonal. Para que as terapias celulares mostrem eficiência e comprovem uma adequada regeneração, as fibras necessitam atravessar o local da lesão e reinervar funcionalmente seus alvos. Neste contexto, o modelo de lesão completa tem sido utilizado em estudos sobre os efeitos do transplante de células gliais embainhantes olfatórias (GEO) na regeneração das vias espinais. Estas pesquisas têm mostrado promissores resultados experimentais desta terapia de reparo tecidual após LME (RAMÓN-CUETO et al., 2000; LU et al., 2001; LÓPEZ-VALES et al., 2006a; AOKI et al., 2010).

# 3 RECUPERAÇÃO APÓS LESÃO DA MEDULA ESPINAL

A maior parte da reabilitação de pacientes com grande perda funcional compreende a compensação do déficit motor. Estes pacientes aprendem a usar, independentemente da função sensório-motora residual, equipamentos assistivos que os tornam mais independentes em sua mobilidade diária, cuidados pessoais e participação social. A prática orientada à tarefa, desenvolvimento da habilidade de resolver problemas, cuidado da pele, manejo do intestino e bexiga, bem como o uso de cadeira de rodas são prioridades na fase inicial do tratamento. O relativo aumento na expectativa de vida dos pacientes e a redução da severidade da lesão, ocasionadas pelas melhoras nos serviços clínicos prestados, contribuem para um maior foco na restauração da mobilidade e otimização das capacidades funcionais remanescentes após LME. A restauração da mobilidade, que inclui recuperação da capacidade de caminhar, é um componente importante na melhora do desempenho das atividades de vida diária (AVDs) e na qualidade de vida de pacientes com disfunções neurológicas (ANNEKEN et al., 2010).

A intrínseca habilidade da ME de se reorganizar e possibilitar a recuperação funcional tornou-se mais conhecida recentemente. Com este avanço no conhecimento da neurobiologia da reabilitação, novas estratégias terapêuticas têm surgido nos últimos anos. Uma estratégia de reabilitação física que vem sendo amplamente estudada e tem mostrado ser eficientemente capaz de suportar e/ou direcionar essa recuperação funcional é o treinamento específico da tarefa de marcha com auxílio de esteira ergométrica após LME (WESSELS et al., 2010).

Além disso, são crescentes as evidências que suportam as terapias celulares como promissoras estratégias terapêuticas na recuperação morfofuncional após LME. Recentes estudos realizados em modelos animais e em humanos têm mostrado o potencial benéfico do transplante de células gliais embainhantes olfatórias (GEO) no local da lesão neurológica como uma eficiente terapia para reparo anatômico e ganho funcional na paraplegia (RAMÓN-CUETO et al., 2000; LIMA et al., 2010).

## 3.1 Treino de marcha em esteira

De maneira geral, a locomoção é uma função motora que por meio da produção de movimentos coordenados e rítmicos assegura o deslocamento ativo de um organismo no ambiente. Em humanos, a capacidade de caminhar compõe o nível mais elevado do controle motor e representa um componente fundamental da independência funcional, dando suporte e favorecendo uma eficiente interação com o ambiente.

O modo como o ato locomotor ocorre é prioritariamente determinado pela arquitetura esquelética sobre a qual os músculos vão exercer suas forças através das inserções osteotendíneas (BIOULAC et al., 2004). Durante a locomoção, o SNC é capaz de coordenar quais articulações irão mover-se, a que distância e durante quanto tempo o movimento ocorrerá (DUYSENS; Van de CROMMERT, 1998). Este comportamento motor é controlado por mecanismos espinais e supraespinais que recebem entradas sensoriais e geram comandos motores (Figura 5) (ARMSTRONG, 1988; DUYSENS; Van de CROMMERT, 1998; BIOULAC et al., 2004).

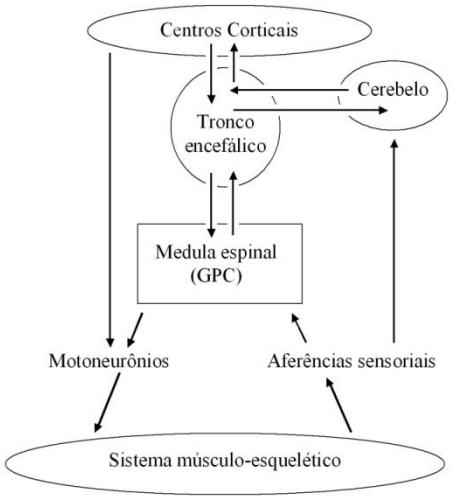


**Figura 5**. Representação esquemática das relações dentro de diversas regiões que integram o sistema sensório-motor envolvido na locomoção (GPC, gerador de padrão central) (modificado de GASC, 2001).

O programa de comando locomotor é feito por circuitos neuronais locais na medula espinal, conhecidos como geradores de padrão central (GPCs) para locomoção (GRILLNER, 1985; DUYSENS; Van de CROMMERT, 1998; BIOULAC et al., 2004). Dentro da medula

espinal, estes circuitos de interneurônios, que recebem comandos motores oriundos do córtex motor e de núcleos do tronco encefálico, são capazes de ativar motoneurônios em uma sequência apropriada. Esta ativação controla a sincronização temporal e a coordenação de movimentos complexos, bem como ativa outros tipos de interneurônios envolvidos na transmissão de informações das vias descendentes e dos aferentes sensoriais, possibilitando ajustes durante as várias fases do ciclo locomotor. Este modelo é complexo e envolve a ativação bilateral sequencial de músculos que agem em diferentes articulações, cada uma tendo suas próprias características de amplitude e velocidade de movimento (GRILLNER, 1985; GRILLNER; WALLÉN, 2002; TRESCH et al., 2002; DIETZ, 2003).

Entretanto, a locomoção só pode ser apropriadamente realizada se um conjunto de atos biomecânicos ocorrerem de forma adequada. Para que isso ocorra, conjuntos específicos de músculos devem ser recrutados por um padrão de sinais eletroquímicos produzidos nos centros geradores e moduladores da atividade motora em diversos níveis do SNC que são enviados através dos motoneurônios (MN) ao tecido muscular (DUYSENS; Van de CROMMERT, 1998; BIOULAC et al., 2004). Desta forma, as atividades motoras, como a locomoção necessitam, além da integridade dos centros geradores e moduladores da atividade motora em diversas regiões do SNC, uma adequada comunicação entre as regiões mais craniais, como o córtex e o tronco encefálico, com as regiões mais caudais, MNs e circuitos espinais. As LME causam uma interrupção anatomofisiológica entre estas regiões, determinando assim, uma importante perda da função motora caudal ao nível espinal da lesão.

Após anos de pesquisas nas ciências básicas, a comprovação da existência de neuroplasticidade no SNC decorrente de lesões e/ou estímulos ambientais fez com que os velhos e enraizados princípios da reabilitação através da compensação e da adaptação funcional começassem a mudar nas últimas décadas. O campo da neurorreabilitação vem sofrendo importantes mudanças.Terapias físicas que estimulem a plasticidade do sistema nervoso (SN) vêm sendo estudadas e empregadas na tentativa de otimizar a recuperação funcional e o desenvolvimento de habilidades antes tidas como perdidas após as lesões neurológicas (EDGERTON et al., 2008; KNIKOU, 2010).

Desde a década de 1980, o grupo do pesquisador canadense Hugues Barbeau vem propondo que a remoção parcial do peso corporal pode facilitar a expressão do padrão de marcha, podendo ser considerado como uma ferramenta terapêutica no retreino da marcha em pacientes com disfunções neuromotoras (VISINTIN; BARBEAU, 1989; FINCH et al., 1991). Em virtude disto, importante atenção vem sendo dedicada aos efeitos do treino de marcha com suporte parcial do peso corporal em esteira (Figura 6) na reabilitação neurofuncional.

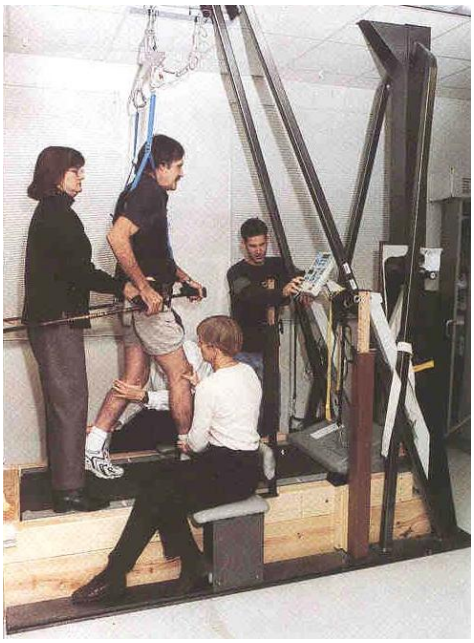

**Figura 6.** Treino de marcha com suporte de peso corporal em esteira ergométrica (body-weight-supported treadmill training, BWSTT) (imagem retirada de BEHRMAN; HARKEMA, 2000).

Esta estratégia de reabilitação físico-motora tem mostrado importantes ganhos funcionais em pacientes com disfunções neuromotoras após acidente vascular encefálico (MALOUIN et al., 1992; BARBEAU; VISINTIN et al., 2003; DRUŻBICKI et al., 2010), doença de Parkinson (LO et al., 2010), paralisia cerebral (MATTERN-BAXTER et al., 2010) e LME (WERNIG et al., 1998; FIELD-FOTE; ROACH, 2011).

Na reabilitação de pacientes paraplégicos, o treino de marcha com suporte do peso corporal em esteira ergométrica mostrou ser capaz de melhorar a habilidade de realizar passos e consequentemente a qualidade de vida desses sujeitos (WERNIG et al., 1998; BEHRMAN; HARKEMA, 2000; HICKS et al., 2005; NOOIJEN et al., 2009, FIELD-FOTE; ROACH, 2011). Após o retreinamento da marcha nestes pacientes, vários deles caminharam com uma maior cadência, apresentando passos mais longos e mais velozes. A progressiva redução da porcentagem de peso corporal suportada, aumento na velocidade da esteira e da distância percorrida, além de melhora do desempenho de marcha em solo, são alguns dos benefícios relatados desta terapia de reabilitação físico-motora (HICKS et al., 2005). Estudos recentes têm mostrado que indivíduos com LME apresentam um aumento na satisfação pessoal de seu desempenho físico e com sua qualidade de vida com a prática de atividades físicas. Variáveis

estas que estão significativamente correlacionadas com um melhor desempenho na habilidade de caminhada em esteira (ANNEKEN et al., 2010).

## 3.2 Regeneração do sistema nervoso central e glia embainhante olfatória (GEO)

Contrariamente a conhecida capacidade de regeneração presente nos nervos periféricos, as lesões do SNC adulto caracterizam-se como devastadoras pela inabilidade dos neurônios maduros em realizar uma adequada regeneração axonal e correta reconexão dendrítica. Entretanto, graças a uma série de estudos prévios que mostraram a capacidade de regeneração axonal dos neurônios do SNC quando um ambiente favorável é fornecido, este estigma vem mudando e desafiando os neurocientistas. Na década de 1980, Albert Aguayo e seus colaboradores (DAVID; AGUAYO, 1981) forneceram evidências de que o SNC pode regenerar-se por longas distâncias após o transplante de pontes feitas com nervos periféricos em ratos adultos.

Após LME, os axônios passam por uma série de alterações: o coto axonal proximal se retrai e quase não apresenta regeneração espontânea, enquanto que o axônio distal passa por um processo conhecido como degeneração walleriana (DW) (KERSCHENSTEINER et al., 2005, VARGAS; BARRES, 2007). A DW é um conjunto de eventos celulares e moleculares pelos quais os axônios e a mielina se degeneram e são removidos. Ela ocorre tanto no SNP como no SNC. Entretanto, no central a remoção dos restos de degeneração axonal e da mielina é mais demorada e menos eficaz, o que cria um ambiente altamente inibitório à regeneração axonal (VARGAS; BARRES, 2007). Desta forma, a falha dos neurônios do SNC em regenerar seus axônios não decorre de uma deficiência intrínseca dos neurônios, mas de um fator característico do ambiente, que além de não dar suporte à regeneração, ainda a impede. Segundo Schwab e Thoenen (1985), o contraste entre a capacidade regenerativa do sistema nervoso periférico (SNP) e o déficit regenerativo apresentado pelo SNC ocorre devido à presença de constituintes inibitórios do crescimento neurítico no central. Os oligodendrócitos e a mielina foram subsequentemente identificados como fontes desses fatores inibitórios (SCHWAB; CARONI, 1988).

Estratégias regenerativas vêm sendo desenvolvidas a partir desse conhecimento sobre a degeneração e regeneração axonal no SNC. Entre estas, uma surge da observação de que a falha no crescimento axonal no SNC não ocorre no bulbo olfatório (BO) de mamíferos adultos. Esta é uma estrutura do SNC onde axônios normais e seccionados são capazes de crescerem por longas distâncias e reestabelecer seus contatos sinápticos com seus neurônios

alvo durante toda a vida (DOUCETTE et al., 1983). A marcante diferença entre o BO e outras regiões do SNC reside na presença de um tipo diferenciado de células gliais embainhantes, a GEO (DOUCETTE et al. 1991). A via olfatória primária consiste dos neurônios na cavidade nasal cujos axônios projetam-se pela placa cribriforme entrando no SNC e realizando sinapses com as células mitrais no BO. Neste percurso das fibras olfatórias amielínicas são acompanhadas pelas células GEOs (Figura 7), que estendem processos citoplasmáticos para compartimentar os axônios olfatórios em fascículos (DOUCETTE et al., 1983).

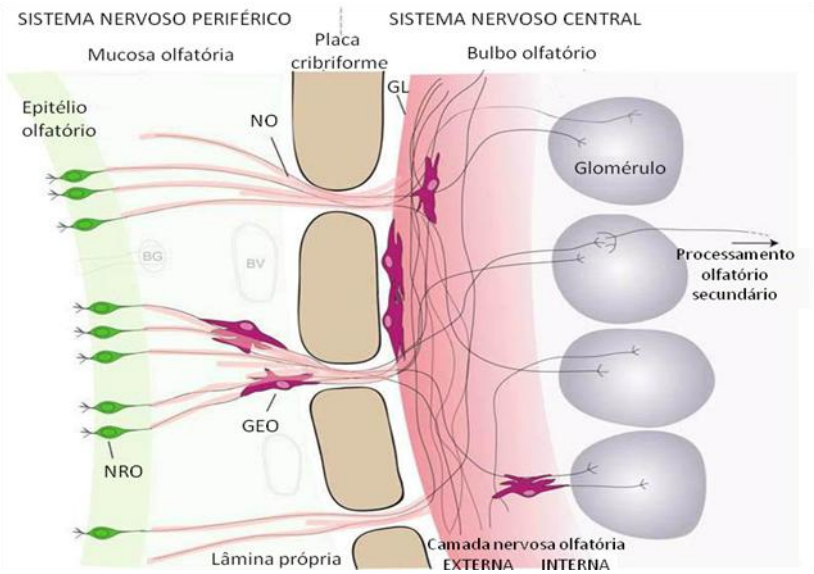


**Figura 7.** Desenho esquemático representando o sistema olfatório primário adulto dos mamíferos. Glia embainhante olfatória (GEO, áreas em rosa) embainhando feixes de axônios dos neurônios receptores olfatórios (NRO) formando os nervos olfatórios (NO) em seu trajeto pela lâmina própria no SNP. Os NOs e suas GEOs cruzam pela placa cribriforme do crânio em direção ao SNC e contornam o BO para formar a camada nervosa olfatória. Esta é composta por uma região externa e uma interna e apresentam células GEO guiando os axônios olfatórios em direção aos glomérulos (imagem modificada de VINCENT et al., 2005).

Esta célula glial é um tipo único de macroglia exclusivamente localizada no nervo olfatório (NO) e no BO e possui propriedades que promovem o crescimento axonal por toda a vida. As células GEOs produzem fatores neurotróficos e fatores promotores do crescimento neurítico que podem mediar à sobrevivência e o crescimento axonal (WOODHALL et al., 2001). Este crescimento axonal também pode ser auxiliado pela secreção de componentes da matriz extracelular, tais como laminina, fibronectina e colágeno tipo IV (FAIRLESS; BARNETT, 2005). Além disso, as GEOs são capazes de envolver e migrar ao longo dos

axônios em crescimento, impedindo-os de entrar em contato com as moléculas inibitórias do crescimento axonal presentes no ambiente extracelular do SNC adulto (RAMÓN-CUETO; VALVERDE, 1995; RAMÓN-CUETO; AVILA, 1998).

Estas propriedades pró-regenerativas colocam as células GEO como um dos mais promissores recursos experimentais para reparo celular mediado por transplante. Ramón-Cueto e Nieto-Sampedro (1994) foram os pioneiros em relatar que esta estratégia poderia estimular o crescimento axonal após lesões do SNC quando mostraram a ocorrência de regeneração de axônios no SNC 8 semanas após a transecção de raízes torácicas dorsais (T10) e transplante de GEO em ratos. Desde então, vários estudos utilizando o transplante de GEO como terapia de reparo celular em modelos animais de completa TME vêm mostrando evidências de regeneração axonal (RAMÓN-CUETO et al., 1998; 2000; LU et al., 2001; LÓPEZ-VALES et al., 2006a; 2007). Os resultados morfológicos deste tipo de terapia são acompanhados por promissoras melhoras funcionais dos MPs destes animais (RAMÓN-CUETO et al., 2000; TOFT et al., 2007; AOKI et al., 2010).

Os promissores resultados alcançados nos experimentos realizados em modelos animais, levou alguns centros a realizarem recentes estudos com autotransplante de GEO em humanos. Na última década, alguns destes estudos discutiram a segurança deste tipo de transplante (HUANG et al., 2006) e replicaram, na clínica, alguns dos resultados experimentais previamente reportados (LIMA et al., 2006; HUANG et al., 2009; BOHBOT, 2010). Mesmo com promissores efeitos sobre a regeneração após o transplante de GEO na LME, a recuperação funcional ocorre apenas parcialmente e ainda é considerada como modesta. Entretanto, novos estudos têm buscado a associação deste tipo de transplante com outras terapias na expectativa de potencializar os efeitos da GEO. Entre estas combinações, estão o uso de pontes feitas com células de Schwann no local da lesão para realizar a união entre os cotos (FOUAD et al., 2005), o uso combinado de transplante de células tronco (AO et al., 2007), as intervenções neuroprotetoras com uso de fármacos (LÓPEZ-VALES et al., 2006b) ou ainda treinamento de habilidades físico-motoras (KUBASAK et al., 2008). Os crescentes avanços neurocientíficos da terapia celular de reparo neural com transplante de GEO vêm fazendo com que as terapias de reabilitação físico-motoras ganhem um importante papel na fase pré e pós-transplante (LIMA et al., 2010).

# 4 ARTIGO 1

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ORIGINAL PAPER

# The Beneficial Effects of Treadmill Step Training on Activity-Dependent Synaptic and Cellular Plasticity Markers After Complete Spinal Cord Injury

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**Abstract** Several studies have shown that treadmill training improves neurological outcomes and promotes plasticity in lumbar spinal cord of spinal animals. The morphological and biochemical mechanisms underlying these phenomena remain unclear. The purpose of this study was to provide evidence of activity-dependent plasticity in spinal cord segment (L5) below a complete spinal cord transection (SCT) at T8–9 in rats in which the lower spinal cord segments have been fully separated from supraspinal control and that subsequently underwent treadmill step training. Five days after SCT, spinal animals started a step-training program on a treadmill with partial body weight support and manual step help. Hindlimb movements were evaluated over time and scored on the basis of the open-field BBB scale and were significantly improved at post-injury weeks 8 and 10 in trained spinal animals. Treadmill training also showed normalization of withdrawal reflex in trained spinal animals, which was significantly different from the untrained animals at post-injury weeks 8 and 10. Additionally, compared to controls, spinal rats had alpha motoneuronal soma size atrophy and reduced synaptophysin protein expression and $Na^+$, $K^+$-ATPase activity in lumbar spinal cord. Step-trained rats had motoneuronal soma size, synaptophysin expression and $Na^+$, $K^+$-ATPase activity similar to control animals. These findings suggest that treadmill step training can promote activity-dependent neural plasticity in lumbar spinal cord, which may lead to neurological improvements without supraspinal descending control after complete spinal cord injury.

**Keywords** Spinal cord injury · Treadmill training · Neurological disorder · Motoneuronal soma size · Synaptic plasticity · $Na^+$, $K^+$-ATPase

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## Introduction

Complete spinal cord injury (SCI) disrupts the neural inputs between the upper and lower regions of the spinal cord, leading to permanent sensorimotor impairments below the injury site. Locomotor impairments are particularly prejudicial to quality of life and are the result of disrupted cortical descending control after a SCI. Beneficial effects on step ability and quality of life have been reported after long-term body-weight-supported treadmill training (BWSTT) rehabilitation in paraplegic patients [1–4]. After training, several patients with SCI walked at a higher cadence and had longer step and stride lengths. Reduction in external body weight support, increase in treadmill speed, distance walked/session and improved capacity to walk over ground

are also reported in patients with SCI after BWSTT [3]. Furthermore, there were accompanying increases in satisfaction with life and satisfaction with physical performance [5], both of which were significantly correlated with improvements in treadmill walking ability.

Thoracic spinal cord transection (SCT) in animals is a model of complete SCI that produces severe hindlimb paralysis and time-dependent development of hyperreflexia similar to those reported in human paraplegia. Animal models of SCT have therefore been widely employed in the development of experimental therapies [6, 7]. Repetitive motor training, such as wheel running, stationary bicycle or treadmill training, have shown encouraging results in functional and morphological outcomes after partial SCI models [8–12]. Locomotor treadmill training has also shown improvements in stability and weight bearing in chronic spinalized adult cats [13, 14]. Moreover, improvements in hindlimb movements and step abilities after step training have also been recently documented in rats with complete SCI [15–20].

After complete SCT, supraspinal control descending connections to motoneurons are absent, so motor learning at spinal level may depend on the nature of the peripheral input provided to the spinal cord and on the dynamic cellular plasticity that remains within the neural circuitry caudal to the lesion site. Changes in electrophysiological properties [21] and preserved motor learning capabilities [22] have been documented in spinal motoneurons in the absence of supraspinal inputs when appropriate sensory stimuli associated with hindlimb activity are repeatedly provided. However, the impact of activity-dependent motoneuron morphological and biochemical plasticity markers provided by repeated task-specific training is still not fully understood. This might be relevant to the comprehension of the physiological bases that underlie spinal motor learning, sensorimotor improvement and treadmill training rehabilitation after SCI.

The present study was designed to assess the possible effects of a treadmill step training program on neurological function and activity-dependent neural plasticity markers of spinal cord in rats that received a complete SCT. For neurological assessment, we studied the hindlimb movements and withdrawal reflex. In addition, alpha motoneuron size, synaptic protein changes and $Na^+$, $K^+$-ATPase activity were analyzed in order to study lumbar spinal cord plasticity in adult rats fully spinalized at the mid-thoracic level.

## Experimental procedures

### Animals

Experiments were performed on adult male Wistar rats (2.5-month-old) from a local breeding colony (ICBS, *Universidade Federal do Rio Grande do Sul*, Brazil). The rats were housed in standard plexiglass boxes (2 per cage), under a 12:12-h light/dark cycle, in a temperature-controlled environment (22 ± 1°C), and given free access to food and water. All procedures were in accordance with Brazilian laws and the recommendations of the Brazilian Society of Neurosciences and the International Brain Research Organization. This study was approved by the Ethics Committee of our institution (Nr. 2007738).

Animals were randomly divided into the following groups: (a) rats without spinal cord transection, sham-operated (control, $n = 10$); (b) untrained rats with spinal cord transection (untrained SCT, $n = 10$); and (c) step-trained rats with spinal cord transection (trained SCT, $n = 10$).

### Surgical procedures

Animals were anesthetized using pentobarbital (40 mg/kg, i.p., Cristália, Brazil) and subjected to a vertebral laminectomy at thoracic levels (T8–T9). Spinal cord transection was performed using microscissors, and the completeness of the transection was ensured by passing a sickle probe (n° 3, White, Brazil) through the lesion site. The same surgical procedure, though without SCT was performed in the uninjured group (control). The surgical procedure was concluded by suturing the muscle plane and skin (6–0 and 4–0 nylon sutures, respectively; Somerville, Brazil). The skin surface was then disinfected with 2% iodine solution.

### Post-operative care

Following the surgery, rats were kept in a warm environment and monitored until they recovered from anesthesia. Animals were then returned to standard conditions. All animals were treated for 14 days with Baytril (Enrofloxacin 2.5 mg/kg, subcutaneously; Bayer S.A., Brazil) to prevent urinary tract infections. Furthermore, bladders were manually expressed twice a day until the bladder was no longer distended and palpable, indicating that the animal had developed an automatic bladder voidance reflex (10–14 days). Inspection for general health, skin irritation, decubitus ulcers or evidence of autophagia, was carried out daily throughout the post-injury survival period.

### Step training rehabilitation

The training program was performed on a treadmill designed for human use (Runner, Brazil) and modified for use by rats. Before the SCT, animals were familiarized with the treadmill apparatus at 5 m/min for 5 min a day on three consecutive days and at post-operative day 6 the trained SCT animals started a 9-week step-training program. The training program consisted of step-training on a

treadmill (band speed 6–7 m/min) with partial body weight support (BWS), once a day, 5 sessions per week. The first training day began with 5 min of step training. The training time was progressively increased every day up to 20 min on the second week and 30 min over the following 7 weeks. The design of this treadmill training regime took into account a previously published study using a complete spinal cord lesion model in rats [17].

The step training was carried out using a manually adjustable weight-supporting counterbalance system to provide weight support assistance. Each rat was fitted with a Lycra vest that was closed with Velcro, and placed into a BWS harness, thereby supporting the thorax, while the head, forelimbs and hindlimbs had full range of movement. For step training, rats were placed in a quadripedal position, bearing ~15% of their body weight on their hindlimbs (i.e., ~85% BWS). Each spinal animal was individually trained and the hindlimbs were manually moved in a step pattern by the researcher holding the ankle region (as previously performed by Laird et al. [20]). During the step training, special care was taken to place the rats' feet in a plantar stepping position and to keep the toes extended to ensure the footpad made contact with the treadmill band during the stance phase.

### Hindlimb motor function

Hindlimb movements were evaluated at post-operative day 5 and post-operative weeks 2, 4, 6, 8 and 10 during spontaneous activity. For this, one animal at a time was allowed to move freely inside an open-field (60 × 30 × 40 cm) for 5 min. One blinded examiner observed the hindlimb movements of the rat, and scored according to the Basso, Beattie and Bresnahan scale (BBB scale [23]), which ranges from 0 (paralysis) to 21 points (normal gait). The average value from the right and left hindlimbs was calculated and taken as the animal score.

### Reflex testing

To assess neurological functionality, the reflex responses of the hindlimbs were evaluated using withdrawal reflex at post-operative day 5 and post-operative weeks 2, 4, 6, 8 and 10. For the test, the rats were held and the hind foot briefly pinched with thin forceps between the 1st and 2nd toes of both the right and left limbs. The mechanical stimulus applied was not of sufficient strength or duration to cause injury. For consistency, a single experienced examiner observed the reflex responses, and scored ranges from 0 (no withdrawal) to 3 (hyperactive withdrawal reflex) according to Gale et al. [24]. The average value from the right and left hindlimbs was calculated and taken as the animal score.

### Morphological study

The day after the last training session, five animals from each group were deeply anesthetized with pentobarbital (100 mg/kg, i.p.;Cristália, Brazil) and transcardially perfused using a peristaltic pump (Milan, Brazil) with 300 ml of saline solution, followed by 400 ml of 0.5% glutaraldehyde (Merck, Germany) and 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (PB, pH 7.4) at room temperature. The spinal cord segments at L4–5 level were removed after careful laminectomy, post-fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in PB solution at room temperature for 1 h and at 4°C until processed. The samples were washed in PB and post-fixed in 1% OsO4 (Sigma, USA) in PB for 2 h. They were then washed with PB and dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Sciences, USA), embedded in resin (Durcupan, ACM-Fluka, Switzerland), maintained in vacuum for 24 h, and, afterwards, polymerized for 72 h at 60°C. Serial transverse-semithin sections (1 µm) were obtained using an ultramicrotome (MT 6000-XL, RMC, USA). Every 10 µm, one section was collected and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil).

Images of the spinal cord were captured (initially 20× and further amplified 200% for analysis) using a Nikon Eclipse E-600 microscope (Japan) coupled to a digital camera and Image Pro Plus Software 6.0 (Media Cybernetics, USA). Digital images from lamina IX in the right and left ventral horns were taken and the cross-sectional areas of the alpha motoneurons in which the nucleolus was visible were estimated (~60 motoneurons per animal were analyzed). In accordance with Roy et al. [25], motoneurons in which the soma size was greater than 490 µm$^2$ (i.e., ~25 µm in diameter) were assumed to be alpha motoneurons and included in the study.

The area of each individual motoneuron was estimated by the point-counting technique [26, 27] using grids with a point density of one point per 26.29 µm$^2$ and the equation: $\hat{A} = \Sigma p \cdot a/p$, where $\hat{A}$ is area, $\Sigma p$ is the total of counted areas/point and $a/p$ is the area/point value (26.29 µm$^2$). This is an unbiased estimate of the area. The average of the cross-sectional areas of each individual rat was based on the mean of the motoneuron areas measured per animal.

### Biochemical studies

The day after the last training session, five animals from each group were deeply anesthetized with pentobarbital (100 mg/kg, i.p.; Cristália, Brazil) and the lumbar enlargements were quickly removed after laminectomy. Under ice, the lumbar enlargements were transversally cut (~1 mm) and 2 non-consecutive lumbar slices were taken

for western blot or $Na^+$, $K^+$-ATPase activity analysis. The samples were stored at −70°C until processed.

### Western blot analysis

The lumbar spinal cord samples were homogenized and equal amounts (30 µg) of proteins from each whole nuclei sample were boiled in sample buffer (0.0625 M Tris–HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (w/v) $\beta$-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue) and electrophoresed in 10% (w/v) SDS–polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma, USA). In order to study the synaptic plasticity an anti-synaptophysin antibody (Santa Cruz Biotechnology, USA) was used at a dilution of 1:1,000. After incubating with the primary antibody for 1 h at room temperature, filters were washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG, Sigma, USA) at a dilution of 1:2000. Furthermore, to confirm equal loading of protein in each lane, the blots were stripped using 1 N NaOH solution for 10 min, washed in distilled $H_2O$, and reprobed with mouse monoclonal anti-$\beta$-tubulin antibody (1:200; Sigma, USA). The chemiluminescence signal was detected using an ECL kit from Amersham (USA). The films were digitally scanned and the optical density measured using Image Pro Plus Software 6.0. Synaptophysin values for each sample were normalized to $\beta$-tubulin and combined for each group. Final data are presented as a percentage of the control group, which was assigned a value of 100%.

### $Na^+$, $K^+$-ATPase activity assay

The lumbar spinal cord samples were homogenized in 10 vol. 0.32 mM sucrose solution containing 5.0 mM HEPES (Sigma, USA) and 1.0 mM EDTA (Sigma, USA), pH 7.4. The reaction mixture for the $Na^+$, $K^+$-ATPase activity assay contained 5.0 mM MgCl2, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 µl. The reaction was initiated by the addition of ATP (Sigma, USA) to a final concentration of 3.0 mM, and incubated for 20 min. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain (Sigma, USA). $Na^+$, $K^+$-ATPase activity was calculated by the difference between the two assays, as previously described by Wyse et al. [28]. Released inorganic phosphate (Pi) was measured using the method from Chan et al. [29] Incubation times and protein concentrations were chosen in order to ensure the linearity of the reaction. Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein and shown as a percentage of the control values.

### Protein determination

For the Western blot analysis, protein content was measured using Lowry's method [30] and for the $Na^+$, $K^+$-ATPase activity assay using the Comassie Blue method [31]. In both, bovine serum albumin (BSA) was used as a standard.

### Statistical analysis

The BBB and withdrawal reflex scores were analyzed using repeated measures analysis of variance (ANOVA) with *time* as the repeated measure. Morphometric measurements and biochemical analysis were analyzed using one-way ANOVA. Analyses were followed by Tukey test. For all comparisons statistical significance was defined as $P < 0.05$. Data were expressed as means ± SEM. Pearson Correlation was used to determine the relationship between withdrawal reflex scores with both lumbar $Na^+$, $K^+$-ATPase activity and motoneuronal soma size.

## Results

### Hindlimb motor function

Experimental rats underwent complete SCT and, after 5 days of recovery from surgery, hindlimb movements were evaluated over time and scored on the basis of the open-field BBB scale. Animals in the control group exhibited normal hindlimb locomotor activity, scored as 21 in the BBB scale, during the entire observation period (data not showed). By contrast, at post-operative day 5, there was complete absence of hindlimb movement in untrained and trained spinalized animals and the BBB scores were equal to zero (see methods) as with flaccid paralysis (Fig. 1a). However, the BBB scores of the trained animals increased overtime and were significantly higher than those of the untrained SCT group (1.6 ± 0.5 and 2.5 ± 0.5) at post-operative weeks 8 and 10 (trained group scores: 3.6 ± 0.6 and 4.4 ± 0.8, respectively; $P < 0.05$; Fig. 1a). At these evaluation points (8 and 10 weeks after lesion), the trained spinalized rats showed slight (less than half of joint motion) to extensive (more than half of joint motion) movements in all three hindlimb joints.

### Withdrawal reflex

Withdrawal reflex was elicited by a mechanical stimulus and used to assess neurological functionality 5 days after a SCT and over time. Several animals in the untrained SCT group exhibited hyperreflexive withdrawal response (scorings around 3) to stimuli, usually manifested after

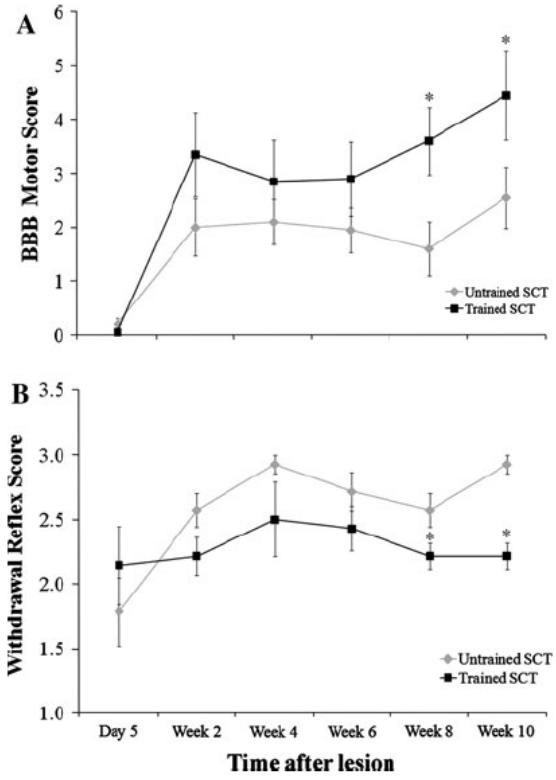


**Fig. 1** Effects of treadmill step training on time course of neurological outcomes. **a** BBB scale for hindlimb movements shown that trained SCT group has higher scores than untrained SCT group at 8 and 10 weeks after the spinal cord injury. **b** Withdrawal reflex response shown that training reduces the hyperreflexia at 8 and 10 weeks after the spinal cord injury. Values are expressed as mean ± SEM *Asterisks* (*) mark significant differences ($P < 0.05$) between trained and untrained SCT groups

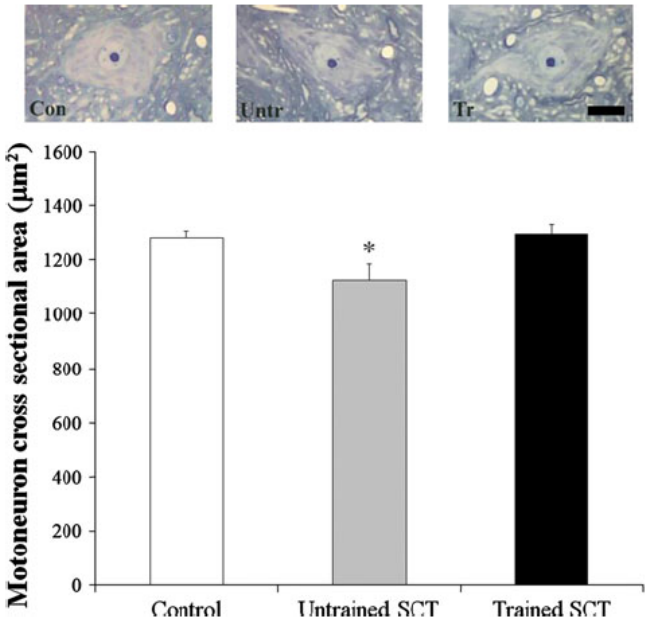


**Fig. 2** Effects of treadmill step training on alpha motoneuronal soma size. Digitalized images showing the alpha motoneurons in lamina IX of the ventral horn at L5 spinal cord segments of the control (*Con*), untrained SCT (*Untr*) and trained SCT (*Tr*) groups are shown at the *top* of figure. Motoneuron size was significantly reduced in the untrained group when compared to control and step-trained rats. Motoneuron soma size in trained animals was not significantly different from controls. Toluidine blue-stained sections. *Scale bar* = 20 µm. Graph confirming this result by morphometry of alpha motoneuron cross-sectional areas (see Experimental Procedures). Values are expressed as mean ± SEM. *Asterisks* (*) mark significant differences ($P < 0.05$) when compared with control and trained SCT groups

post-operative week 2 onward. This reflex response increased at 4, 6 and 10 weeks ($P < 0.05$), showing the time-dependent development of hyperreflexia in untrained SCT animals. Furthermore, at post-operative weeks 8 and 10, the untrained SCT group exhibited a hyperreflexive withdrawal response, when compared with trained SCT and control groups ($P < 0.05$; Fig. 1b). However, the trained SCT group showed normal average withdrawal responses (scores around 2) throughout the 10-week test period, similar to those of the control animals.

## Alpha motoneuronal soma size

In order to assess changes in motoneuron size, representative transverse sections of alpha motoneurons from the ventral horn at L5 spinal cord segment for each experimental group are shown at the top of Fig. 2. Analysis of morphometric data revealed that SCT significantly reduced the alpha motoneuronal cross-sectional area in untrained animals compared to controls (1126. ± 60.13 and 1284.99 ± 24.41 µm$^2$, respectively; $P < 0.05$; Fig. 2). In step-trained group, alpha motoneuronal cross-sectional area (1297.5 ± 37.07 µm$^2$) was greater ($P < 0.05$) than in the untrained spinalized animals and similar to that of the control group (Fig. 2).

## Synaptophysin expression

Representative Western blot analyses with synaptophysin-stained bands to examine changes in lumbar spinal cord expression of this synaptic protein for each experimental group are shown at the top of Fig. 3. Optical densitometry (OD) analysis revealed a significant reduction in synaptophysin protein expression in untrained animals compared to controls and trained animals ($P < 0.05$, Fig. 3). Although synaptophysin expression was reduced by approximately 9% in trained animals, this value was not significantly different from controls (Fig. 3).

## $Na^+$, $K^+$-ATPase activity

$Na^+$, $K^+$-ATPase activity contributes towards the maintenance of the electrochemical gradient across the plasma membrane and plays an important role in synaptic

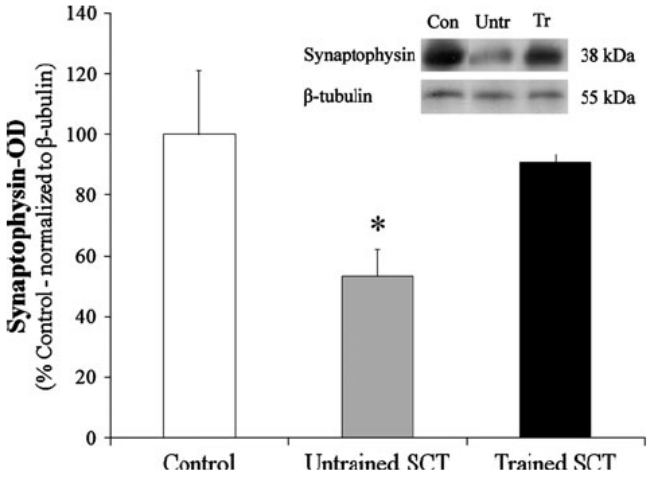


**Fig. 3** Effects of treadmill step training on synaptophysin expression. Western blot analysis with synaptophysin-stained bands to illustrate changes in lumbar synaptic plasticity of the untrained SCT (*Untr*) compared to control (*Con*) and trained SCT (*Tr*) groups are shown at the top of figure. Graph showing the quantification of the synaptophysin in control and experimental groups. Synaptophysin expression was significantly reduced in the untrained group when compared to control and step-trained rats. Synaptophysin expression in trained animals was not significantly different from controls. Values are shown as a percentage of the control values and expressed as mean ± SEM. *Asterisks* (*) mark significant differences ($P < 0.05$) when compared with control and trained SCT groups

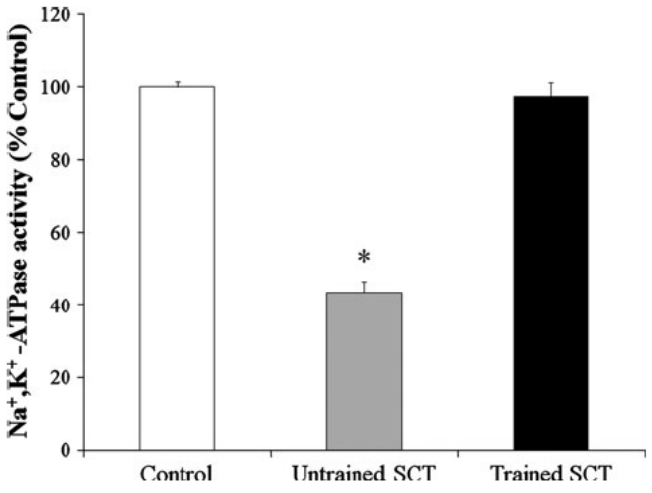


**Fig. 4** Graph showing the $Na^+$, $K^+$-ATPase activity in control and experimental groups. $Na^+$, $K^+$-ATPase activity was significantly reduced in the untrained group when compared to control and step-trained rats. $Na^+$, $K^+$-ATPase activity in trained animals was not significantly different from controls. *Asterisks* (*) mark significant differences ($P < 0.05$) when compared with control and trained SCT groups

transmission and plasticity. Biochemical analysis of the $Na^+$, $K^+$-ATPase activity showed that SCT reduced this enzyme activity by 56% in untrained animals compared to controls ($P < 0.05$, Fig. 4). Furthermore, the trained spinalized animals showed 54% more $Na^+$, $K^+$-ATPase activity than the untrained ($P < 0.05$) and similar values to those of the controls, which demonstrates that the treadmill step training have a positive effect on this enzyme activity (Fig. 4).

### Correlations

In order to study the relation of $Na^+$, $K^+$-ATPase activity and alpha motoneuronal size in lumbar spinal cord with withdrawal reflex responses in paralyzed hindlimbs, we examine the Pearson correlation between these variables. Correlation analysis showed that there is a strong positive relationship between withdrawal reflex scores with the $Na^+$, $K^+$-ATPase activity ($r = 0.837$; $P = 0.003$). Although there is a tendency, no correlation is showed between withdrawal reflex scores with the motoneuronal soma size ($r = 0.472$; $P = 0.06$).

## Discussion

Neural structures are known to be able to change with experience. In the context of motor learning and neurological rehabilitation, activity-dependent plasticity can be assessed by the neuronal mechanisms changed with neuromuscular practice. The present study was designed to assess some of the morphological and molecular mechanisms that contributed to activity-dependent plastic changes paralleling partial recovery of motor function following a specific motor training after complete SCT.

In our study, step-trained paraplegic rats achieved better BBB scores than untrained injured animals at 8 and 10 weeks after the spinal lesion. Similarly, recent studies using manual treadmill step-training have shown improvements in hindlimb motor performance in the same open-field test evaluation after thoracic SCT in rats [17, 20]. In addition, de Leon et al. [16] showed that robotic-assisted treadmill training increased the quantity of step movements 10 weeks after SCT. Also, robotic step training showed improved stepping ability after 4 and 8 weeks of training in adult rats spinalized as neonates (P5) [19].

Furthermore, paraplegic rats showed hyperreflexiveness in withdrawal responses (scores around 3; according to Gale et al. [24]) and in spinal-trained animals, motor training promoted the maintenance of reflex responses close to control values. Previous studies showed that the H-reflex exhibits lower negative modulation at low frequency stimulations (from 0.2 to 10 Hz) from post-injury week 2 onward in spinal adult rats compared with intact animals and that motorized bicycle exercise training, started prior this period, normalizes this hyperreflexia [32–34].

A component involved in the attenuating effects of exercise on the sensorimotor system of spinally transected

rats may reside in the spinal cord and may include the afferent connections to motoneurons, or the properties of motoneurons themselves. It is thought that treadmill training may activate a neural network located within the lumbar spinal cord, the central pattern generator (CPG), which is capable of generating rhythmic locomotor activity without descending control. The sensorial stimulation provided by Ia and Ib fiber groups during step training could play an important role in the normalization of motoneuron electrophysiological properties after spinal cord injuries and reinforce the efficacy of specific sensorimotor pathways. This plasticity could result in a more selective and stable network of neurons capable of controlling locomotion in spinal rats [35–37].

Given that the motoneuronal size has an important effect on its electrophysiological properties [38], it is interesting that motoneuron size is affected by training after SCT. Our findings showed that lumbar alpha motoneurons were larger in trained spinal-transected rats than in untrained animals and that their values were similar to those of the control subjects. The reduction in motoneuron size found after SCI would making this cell more excitable after some afferent stimuli. Motoneuronal soma enlargement has been reported after chronic treadmill training (10 weeks) in intact adult rats. This increase in soma size was accompanied by an increase in succinate dehydrogenase (SDH) activity in motoneurons, indicating an enhanced protein synthesis capacity in these cells after training [39].

In addition, the number and diameter of dendritic stems were positively correlated to the cell body size of the hindlimb motoneurons [40]. In agreement with our results, Kitzman [41] showed that sacrocaudal motoneuronal soma size and overall dendritic branching decreased in chronically injured rats (between 4 and 12 weeks post-injury). This study also showed that maintenance of the motoneuron soma size in the early stages after SCI could be dependent on the dendritic branches preserved during this period. Gazula et al. [42] demonstrated that motoneurons deprived of excitatory stimulation from descending cortical and brainstem inputs display marked dendritic-tree atrophy and that acute limb exercise training imposed by a motorized device for 5 days can prevent this pathological change in rats. On the other hand, no changes in motoneuron soma size or SDH activity were observed 5 or 30 days after complete SCI [25, 42]. Alterations to the motoneuronal soma size seem to occur in a more chronic stage, as showed by Moshonkina et al. [15] and Kitzman [41].

Furthermore, treadmill training has been shown to produce significant changes in the electrophysiological properties of motoneurons, their synaptic input from spinal white matter, muscle spindle afferents in spinal transected rats [18, 32] and in the modulation of glycinergic and GABAergic inhibition in spinalized animals [19, 43]. The amplitude of both action potential after hyperpolarization and synaptic inputs to motoneurons were correlated with the effectiveness of step training in improving stepping [18].

In order to evaluate the activity-dependent synaptic plasticity in lumbar spinal cord after complete transection at the thoracic level, the expression of synaptophysin was measured in step-trained animals. Our data showed that the treadmill step training may prevent or recovery the synaptic loss produced by complete SCI in this model. Synaptic plasticity of the lumbar spinal cord has been related with the spontaneous recovery of locomotion after incomplete SCI [44]. Studies have shown that motor training can restore the synaptic protein levels after incomplete [9, 45] or complete [46] thoracic SCI, similar to our results.

Synaptophysin is the most abundant integral pre-synaptic vesicle protein and is therefore often measured in attempts to quantify synapses plasticity during neuroanatomic remodeling and neural development [47–49]. This pre-synaptic protein binds synaptobrevin, which cannot simultaneously bind synaptophysin and the SNARE complex, suggesting that synaptophysin plays a role in regulating SNARE assembly and vesicle fusion [50, 51]. There is evidence that also supports a mechanism by which synaptophysin is involved in endocytosis and vesicle recycling [52]. These studies support the important role of this protein in synaptic function and plasticity. In this context, qualitative and quantitative analyses of the immunoreactivity of the synaptic protein synaptophysin have been performed in the spinal cord motor nuclei of rats in order to assess the changes in synaptic activity during the course of nervous system pathology [53] and activity dependent plasticity [46]. The up-regulation of the synaptic proteins may be a result of the lumbar circuitry synaptic plasticity and the regeneration of supraspinal descending axons upon lower neural structures in incomplete SCI [54]. However, rats receiving a complete SCT at a neonatal stage spontaneously recover significant stepping ability without nerve fiber regeneration across the lesion site. This recovery is attributable to changes in the lumbosacral neural circuitry and not to regeneration of axons across the lesion [55]. Furthermore, Macias et al. [46] showed that treadmill training can increase the synaptic inputs to motoneurons and may explain the cellular trophism showed by these cells after training in our study.

One of the most interesting and new findings in the present study was that $Na^+$, $K^+$-ATPase activity in lumbar spinal cord was reduced after long term complete SCT and that after 9 weeks of training this ion pump activity was similar to control values in spinal trained animals. Furthermore, we showed for the first time a strong positive correlation between a hindlimb reflex response and the

lumbar $Na^+$, $K^+$-ATPase activity. To our knowledge there is no previous reported data on these subjects.

The ion pump $Na^+$, $K^+$-ATPase activity significantly contributes with the maintenance of the electrochemical gradient across the plasma membrane underlying resting and action potentials as well as the modulation of neurotransmitter release and uptake. As a consequence, an inhibition of this electrogenic pump would lead to depolarization and enhanced excitability in neurons similar to the hyperexcitability shown by down-regulation of the potassium-chloride cotransporter KCC2 after SCI [56–58]. A previous electrophysiological study showed that complete spinalization in rats significantly depolarizes the resting membrane potential (RMP) compared to control animals. Nevertheless, passive cycling exercise attenuated the transection-induced depolarization of the RMP in injured rats [21]. Altogether, these reposts and our results suggest that the maintenance/recovery of $Na^+$, $K^+$-ATPase activity promoted by treadmill training after complete SCI in our study may be, in part, responsible for the attenuation of the transection-induced motoneuronal hyperexcitability [21] and normalization of specific spinal reflexes [32–34] in injured rats. In agreement with this hypothesis, Boulenguez et al. [58] showed that disruption of the normal electrochemical gradient in motoneurons reduces the rate-dependent depression of the H-reflex, as is observed in spasticity after SCI.

Among other factors, the reduction in $Na^+$, $K^+$-ATPase activity could be promoted by decreased synthesis or increased degradation of this enzyme. In this context, thoracic SCT showed acute reduction in the expression of mRNAs of the $\alpha 3$ and $\beta 1$ subunits of the $Na^+$, $K^+$-ATPase in ventral horn motoneurons [59, 60]. Though the exact mechanism of reversal of $Na^+$, $K^+$-ATPase inhibition by exercise training remains unknown, acute and chronic treadmill training is known to promote increases in the mRNA of these isoforms in equine muscle, representing an adaptive response to exercise [61] and giving clues of how training can modulate the $Na^+$, $K^+$-ATPase activity.

In summary, our results provide evidence that step training started at an early stage after SCT in rats enhances hindlimb movements and promotes normalization of withdrawal reflex responses. Additionally, the training protocol employed in our study prevents/recovery lumbar motoneuronal size atrophy, synaptic marker loss and $Na^+$, $K^+$-ATPase inhibition. Several biochemical and morphometric parameters have been shown to change in a activity-dependent manner in the spinal cord of transected animals. Many of those have been cited in the present report. The most important data here is the exercise-dependent modulation of the $Na^+$, $K^+$-ATPase activity. These findings have important implications for the comprehension of the neurobiological substrate that promotes lumbar activity-dependent plasticity during motor learning without supraspinal control and supports motor training as a therapeutic approach in SCI. Furthermore, with the development of neural repair therapies, this rehabilitative strategy may have a key role in counteracting chronic morphophysiological deterioration below the injury site, maintaining functional properties of the neural tissue and ensuring functional interaction between caudal spinal structures and regenerative fibers.

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# 5 ARTIGO 2

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# Treadmill step training-induced adaptive muscular plasticity in a chronic paraplegia model

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ABSTRACT

The purpose of this study was to provide evidence that treadmill step training is capable of attenuating muscle atrophy and may regulate brain derived neurotrophic factor (BDNF) in soleus muscle after complete spinal cord transection (SCT) at T8–T9 in rats. Five days after SCT, spinal animals started a 9-week step-training program on a treadmill with partial body weight support and manual step help. The muscular trophism was studied by analyzing muscle weight and myofiber cross-sectional area of the soleus, while Western blot analysis was used to detect BDNF expression in the same muscle. Step training, initiated immediately after SCT in rats, may partially impede/revert muscular atrophy in chronic paralyzed soleus muscle. Moreover, treadmill step training promoted upregulation of the BDNF in soleus muscle, which was positively correlated with muscle weight and myofiber cross-sectional size. These findings have important implications for the comprehension of the neurobiological substrate that promotes exercise-induced effects on paralyzed skeletal muscle and suggests treadmill training is a viable therapeutic approach in spinal cord injuries.



Skeletal muscle is a dynamic tissue that can adapt to mechanical stimulus such as reduced neural activity after nervous system injuries and/or muscular training [24]. Spinal cord injury (SCI) is a devastating neurological condition that produces muscular pareses/paralyses caudal to the lesion level, leading to a pronounced loss of muscle mass and severe muscle atrophy [9,31]. This paraplegia-induced muscle atrophy increases the risk of developing secondary health problems such as cardiovascular disease and diabetes in paraplegic patients [7].

Though the muscle to body weight ratio, muscle and myofiber cross-sectional area (CSA) and muscle function are reduced in several muscles after SCI in animal models [9,22,23,25,31], the greatest damage is mainly seen in postural muscles composed predominantly of type 1 fibers [5,22].

Activity-based restorative strategies have been used in attempts to restore muscle mass and preserve some muscle functions after SCI. Functional electrical stimulation employed after SCI has been shown to capable of improving muscular trophism [12]. Body weight-supported treadmill training (BWSTT) has been shown to be effective in restoring muscle mass and function. Using this procedure, muscle atrophy was partially reversed and greater muscular activation promoted [1,10,11]. Though these studies have shown the promising beneficial effects of BWSTT in muscular trophism after SCI, further research is needed in order to establish the biological mechanisms involved in this rehabilitation strategy [12].

Among other factors, muscular disuse severely reduces the expression of brain-derived neurotrophic factor (BDNF) protein and mRNA levels in both lumbar spinal cord and soleus muscle in acute and chronic stages after SCI [15,33]. This trophic factor can activate the rapamycin (mTOR), the protein that participates in mammalian cell size control and plays an important role in muscular trophism [6]. Furthermore, paraplegia-induced muscle atrophy in rats has been associated with downregulation of the mTOR signaling pathway [8].

Studies have shown that repetitive motor activity, such as cycling exercise training accelerates muscle size restoration after complete SCI in rats [9,25,26]. Furthermore, treadmill training has been shown to diminish the extent of muscle atrophy [23,31] and restores BDNF levels in both the lumbar spinal cord and soleus muscles [15] in moderate SCI models. In contrast to the well reported effects of treadmill training on several growth factors and muscular trophism in intact and after moderate SCI models, few studies have attempted to examine the effectiveness of this activity-based therapy on the neuromuscular system after complete SCI. Since, mTOR

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is the downstream effector of the action of BDNF on cell size, the correlation of the regulation of this growth factor and trophism in paralyzed muscle may shed some light on the mechanism by which treadmill step training affects muscular tissue and promotes maintenance/restoration of the muscle mass in spinal subjects. In this context, the main aim of this study was to test the hypothesis that treadmill step training is capable of attenuating the loss of muscle mass and myofiber atrophy and modifying the BDNF content in soleus muscle after SCT in rats.

Experiments were performed on 30 adult male Wistar rats (2.5 months old) from a local breeding colony (ICBS, UFRGS, Brazil). The rats were housed in standard plexiglass boxes (2 per cage), under a 12:12-h light/dark cycle, in a temperature-controlled environment ($22 \pm 1\,^{\circ}C$), and given free access to food and water.

Animals were randomly divided into the following groups: (a) rats without spinal cord transection, sham-operated (control, $n = 10$); (b) untrained rats with spinal cord transection (untrained SCT, $n = 10$); and (c) step-trained rats with spinal cord transection (trained SCT, $n = 10$).

Procedures were in accordance with Brazilian laws and the recommendations of the Brazilian Society of Neurosciences and the International Brain Research Organization. This study was approved by the Ethics Committee of our institution (Nr. 2007738).

Animals were anesthetized using pentobarbital (40 mg/kg, i.p., Cristália, Brazil) and subjected to a vertebral laminectomy at thoracic levels T8–T9. Spinal cord transection was performed using microscissors, and the completeness of the transection was ensured by passing a sickle probe (no. 3, White, Brazil) through the lesion site. The same surgical procedure, though without SCT was performed in the uninjured group (control). The surgical procedure was concluded by suturing the muscle plane and skin (6–0 and 4–0 nylon sutures, respectively; Somerville, Brazil). The skin surface was then disinfected with 2% iodine solution.

Following the surgery, rats were kept in a warm environment and monitored until they recovered from anesthesia. Animals were then returned to standard conditions. All animals were treated for 14 days with Baytril (Enrofloxacin 2.5 mg/kg, subcutaneously; Bayer S.A., Brazil) to prevent urinary tract infections. Furthermore, bladders were manually expressed twice a day until the bladder was no longer distended and palpable, indicating that the animal had developed an automatic bladder voidance reflex (10–14 days). Inspection for general health, skin irritation, decubitus ulcers or evidence of autophagia, was carried out daily throughout the post-injury survival period.

The training program was performed on a treadmill designed for human use (Runner, Brazil) and modified for use by rats. Before the SCT, the animals were familiarized with the treadmill apparatus at 5 m/min for 5 min a day on three consecutive days and at post-operative day 6 the trained SCT animals started a 9-week step-training program. The training program consisted of step training on a treadmill (band speed 6–7 m/min) with partial body weight support (BWS), once a day and 5 sessions per week. The first training day began with 5 min of step training. The training time was progressively increased every day up to 20 min on the second week and 30 min over the following 7 weeks. The design of this treadmill training regime took into account a previously published study using complete SCT in rats [34].

The step training was carried out using a manually adjustable weight-supporting counterbalance system to provide weight support assistance. Each rat was fitted with a Lycra vest that was closed with Velcro, and placed into a BWS harness, thereby supporting the thorax, while the head, forelimbs and hindlimbs had full range of movement. For step training, rats were placed in a quadripedal position, bearing ~15% of their body weight on their hindlimbs (i.e., ~85% BWS). Each spinal animal was individually trained and the hindlimbs were manually moved in a step pattern by the researcher holding the ankle region (as previously performed [20]). During the step training, special care was taken to place the rats' feet in a plantar stepping position and to keep the toes extended to ensure the footpad made contact with the treadmill band during the stance phase.

The day after the last training session, five animals from each group were deeply anesthetized with pentobarbital (100 mg/kg, i.p.; Cristália, Brazil) and the right soleus muscles were carefully dissected from the surrounding tissue and rapidly weighed. Results are presented as a percentage of total body mass and were calculated by dividing the weight of the soleus muscle by the weight of the animal. Additionally, samples of the central part of each muscle were excised under ice and stored at $-70\,^{\circ}C$ until processed for biochemical analysis.

The soleus muscle was chosen for the purposes of this study of muscular adaptations because it is predominantly composed of slow-twitch muscle fibers (type 1), which makes it more vulnerable to disuse atrophy after SCI (see review [5]). Moreover, the rat soleus muscle is used during standing and locomotion and intensively recruited during treadmill training [2].

Other five animals from each group were deeply anesthetized with pentobarbital and transcardially perfused with 300 mL of saline solution, followed by 400 mL of 0.5% glutaraldehyde (Merck, Germany) and 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (PB, pH 7.4) at room temperature. The right soleus was carefully dissected from the surrounding tissue. Small samples (~2 mm × 1 mm) from the central part of each muscle were selected and post-fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in PB solution at room temperature for 1 h and at $4\,^{\circ}C$ until processed. The samples were washed in PB and post-fixed in 1% $OsO_4$ (Sigma, USA) in PB for 2 h. They were then washed with PB and dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Sciences, USA), embedded in resin (Durcupan, ACM-Fluka, Switzerland), maintained in vacuum for 24 h, and, afterwards, polymerized for 72 h at $60\,^{\circ}C$. Serial transverse-semithin sections (1 μm) were obtained using an ultramicrotome (MT6000-XL, RMC, USA).

Images of the muscles were captured (20×) using a Nikon Eclipse E-600 microscope (Japan) coupled to a digital camera and Image Pro Plus Software 6.0 (Media Cybernetics, USA). For soleus myofiber morphometric evaluation, a set of 6 images was obtained for each muscle using random sampling of one slice and the transverse sectional areas of 120 muscle fibers, randomly chosen from the 6 digitalized images, were estimated. The area of each individual myofiber was estimated using a point-counting technique [24] using grids with a point density of one point per 58.56 $\mu m^2$ and the equation: $\hat{A} = \sum p \cdot a/p$. Where $\hat{A}$ is area, $\Sigma p$ is the total of counted areas/point and $a/p$ is the area/point value (58.56 $\mu m^2$). This is an unbiased estimate of the area. The average of the cross-sectional areas of each individual rat was based on the mean obtained for the soleus myofiber areas measured per animal.

The muscle samples stored at $-70\,^{\circ}C$ were homogenized and equal amounts (30 μg) of proteins from each sample were boiled in sample buffer (0.0625 M Tris–HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (w/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromphenol blue) and electrophoresed in 10% (w/v) SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma, USA). Anti-BDNF antibody (Santa Cruz Biotechnology, USA) was used at a dilution of 1:200. After incubating with the primary antibody for 2 h at room temperature, membranes were washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG, Sigma, USA) at a dilution of 1:2000 for 1 h. The chemiluminescence signal was detected using an ECL kit (Amersham, USA). The films were digitally scanned and the optical density measured using Image Pro

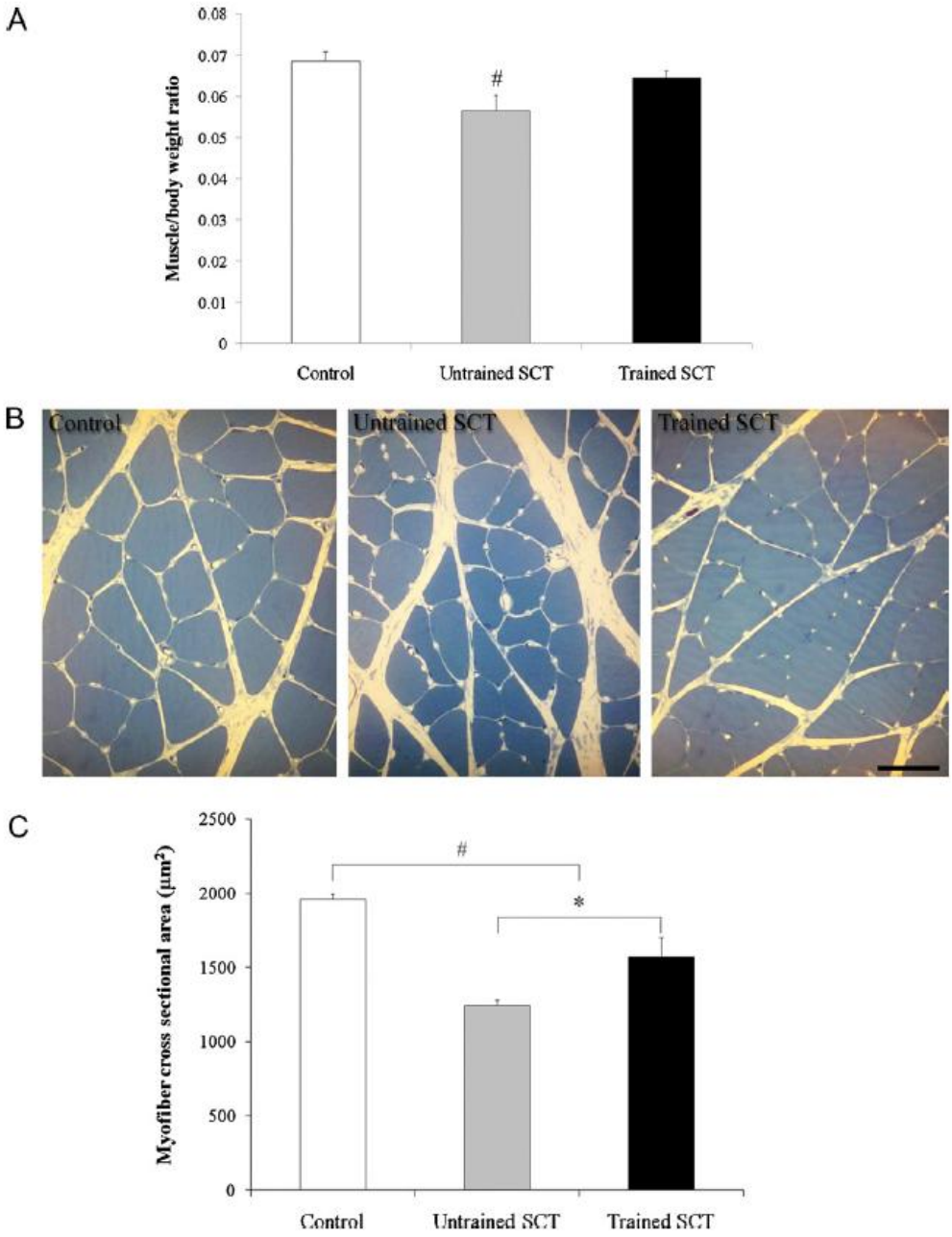


**Fig. 1.** Effects of treadmill step training on soleus muscle trophism. (A) Soleus muscle to body weight ratio. Soleus to body weight ratio was significantly reduced in the untrained group when compared to controls. There was no significant difference between the step-trained spinal animals and controls. (B) Digitalized images showing soleus muscle myofibers of the control and experimental groups. Untrained spinal animals showed myofiber atrophy compared with controls. Note that in the step-trained animals, the soleus myofibers were larger when compared with the untrained rats. Toluidine blue-stained semithin sections. Scale bar = 50 µm. (C) Soleus myofiber cross-sectional areas. Myofiber size was significantly reduced in the untrained and trained group when compared to control rats. In the step-trained animals the myofiber sizes were larger when compared with untrained rats. Values in graphs (A) and (C) are expressed as means ± S.E.M. Symbol "#" marks significant differences ($P < 0.05$) when compared with the control group and asterisk (*) marks significant differences ($P < 0.05$) when compared with the step-trained group.

Plus Software 6.0 and shown as a percentage of the control values. The protein content was measured using Lowry's method using bovine serum albumin (BSA) as a standard.

To test for statistically significant differences, morphological and biochemical data were analyzed using one-way ANOVA and, in the case of significant differences, the Tukey *post hoc* test was applied. Statistical significance was assumed at $P < 0.05$. Data were expressed as means ± S.E.M. Pearson's Correlation was used to determine the relationship between BDNF expression with both the soleus to body weight ratio and soleus myofiber size.

Analysis of the soleus muscle to body weight ratio revealed that SCT significantly reduced the soleus muscle mass in untrained animals compared with controls (0.056 ± 0.003 and 0.068 ± 0.002; respectively; $P < 0.05$; Fig. 1A). There was no difference in terms of the soleus muscle to body weight ratio between the step-trained group (0.064 ± 0.001) and the controls (Fig. 1A).

In order to assess changes in the cross-sectional myofiber size, transverse-semithin sections of the soleus muscle for each experimental group are shown in Fig. 1B. Analysis of the morphometric data revealed that soleus cross-sectional myofiber area was signif-

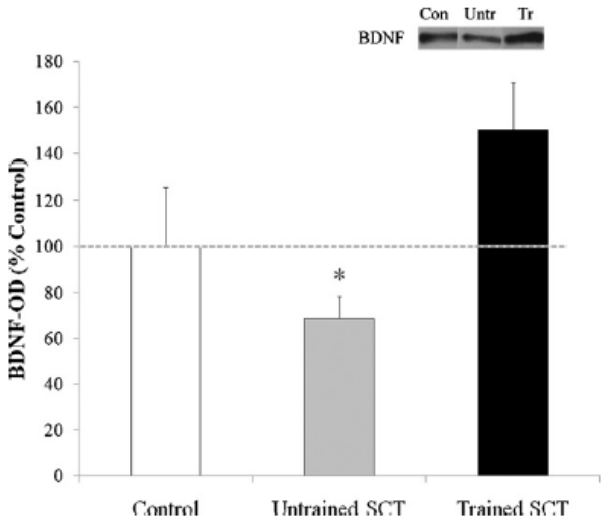


**Fig. 2.** Effects of treadmill step training on soleus BDNF expression. Western blot analysis with BDNF-stained bands to illustrate changes in muscular expression of this protein in the trained SCT (Tr) compared to the untrained SCT (Untr) and control (Con) groups are shown at the top of figure. Graph showing the quantification of the BDNF in the control and experimental groups. BDNF expression was significantly enhanced in the step-trained when compared with untrained spinal animals. Values are shown as a percentage of the control values and expressed as mean ± S.E.M. Asterisks (*) mark significant differences ($P < 0.05$) when compared with the trained SCT group.

icantly reduced with SCT in both the untrained and step-trained animals ($1245.47 \pm 34.08\ \mu m^2$ and $1573.06 \pm 131.69\ \mu m^2$, respectively) compared with controls ($1960.23 \pm 33.69\ \mu m^2$; $P < 0.05$; Fig. 1C). The step-trained group showed an enhanced myofiber cross-sectional area in the soleus muscle when compared with the untrained SCT group ($P < 0.05$).

Representative Western blot analyses with BDNF-stained bands that facilitate the examination of the changes in the expression of this protein in the soleus for each experimental group are shown at the top in Fig. 2. Although optical densitometry (OD) analysis revealed that SCT reduced BDNF expression by about 31% in soleus muscle, there was no statistically significant difference between the untrained SCT and control groups (Fig. 2). In the step-trained group, soleus BDNF expression was enhanced by between/about 50% and 80% when compared respectively with control and untrained spinal animals, and this value was significantly different from the untrained SCT group (Fig. 2).

In order to study the relationship between muscular expression of BDNF and soleus trophism, we examined the correlation between the expression of this protein and the soleus to body weight ratio and myofiber cross-sectional area in spinal animals. Pearson's Correlation showed that there is a strong positive relation between muscular BDNF expression with the muscle to body weight ratio ($r = 1$; $P < 0.001$) and with the myofiber size ($r = 0.959$; $P < 0.001$) in soleus muscle.

Muscle atrophy as detected by a reduction in muscle mass caudal to the site of the spinal lesion is an important hallmark of spinal cord injury. In this study, the SCT caused a severe decrease in soleus muscle weight and myofiber size. The untrained spinal animals showed a greater loss of soleus weight compared with controls. However, the treadmill step training prevented and/or reverted this muscle loss. Moreover, SCT lead to a reduction of ~37% in soleus myofiber size in the untrained spinal rats when compared with controls. Additionally, the step training was effective in partially maintaining and/or restoring muscle myofiber size, given that the average cross-sectional area of the soleus myofibers in the trained spinal animals was ~17% greater than that in the untrained group.

Severe muscle atrophy caused by the complete or incomplete SCI has been well documented in experimental studies [21,23,25,26]. Five days of locomotor training, starting one week after midthoracic contusion SCI, resulted in significant enlargement of the soleus cross-sectional myofiber area, with the trained animals having muscle fiber sizes 23% larger than the untrained [31]. Additionally, magnetic resonance imaging has shown that long-term locomotor training enhances the cross-sectional area and accelerates soleus muscle recovery in spinal cord contusion injured rats [22,23]. While few studies have evaluated the effects of treadmill training in fully spinalized rats, other exercise paradigms, such as cycling exercises, when started 5 days after midthoracic SCT restored skeletal muscle to body mass ratio and cross-sectional myofiber area in soleus muscle to control values [25,26].

Skeletal muscle is known to be an important secretor of growth factors. In our study, step training led to an increase of 80% in soleus BDNF expression in the trained spinal animals when compared with the untrained spinal animals. Other studies have shown that motor training has an intrinsic potential to enhance the production of neurotrophins. The expression of BDNF, neurotrophin-3 (NT-3), and their tyrosine kinase receptors (TrkB and TrkC, respectively) in both the spinal cord and soleus muscle of rats increases with locomotor exercise training in the intact spinal cord [14,30,32]. Moreover, locomotor training has been shown to restore BDNF levels in both the lumbar spinal cord and soleus muscle, which were severely reduced in the acute and chronic stages after SCI [15,33].

Muscular trophism in the soleus is correlated with muscle BDNF expression in our study. We showed that both soleus muscle weight and soleus myofiber size had a positive correlation with muscular expression of the BDNF protein in step-trained and untrained spinal animals 10 weeks after SCT. BDNF activates mTOR, the protein that participates in mammalian cell size control, and the down-regulation of this protein is associated with the muscle atrophy after SCI [8,17,27,29]. Skeletal muscle trophism is controlled by the regulation of cellular signaling pathways that involve muscle protein synthesis, breakdown and cellular proliferation [13]. The mTOR signaling pathway is capable of regulating translation initiation and cellular protein synthesis [18,19]. In this context, the level of mRNA translation is the primary muscle protein synthesis regulator [18,19]. Therefore, activation of mTOR by BDNF signaling may be involved in beneficial exercise-induced effects that may underlie the partial maintenance and/or recovery of the muscular trophism seen in the soleus from trained spinal animals in our study.

Stepping-based rehabilitation programs, such as wheel running, stationary bicycle or treadmill training, may activate a neural network located within the lumbar spinal cord, the central pattern generator (CPG), which is capable of generating rhythmic locomotor activity without descending control. The sensorial stimulation provided by Ia and Ib fiber groups during step training could play an important role in the normalization of motoneuron electrophysiological properties after SCI and reinforce the efficacy of specific sensorimotor pathways in promoting neuromuscular activity [3,4,28]. This plasticity could result in a more selective and stable network of neurons capable of controlling the limb muscles activated during locomotion in spinal rats [16].

In summary, our results provide evidence that step training in rats, when started immediately after SCT, may partially impede and/or revert the muscular atrophy in chronic paralyzed soleus muscle. Moreover, the treadmill step training promoted upregulation of BDNF in the soleus muscle, which was positively correlated with muscle weight and myofiber cross-sectional area in our study. We believe that the beneficial effects of treadmill training on soleus muscle trophism could be promoted by BDNF-induced mTOR activation.

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# 6 ARTIGO 3

*Early treadmill step training accelerates the partial recovery of hindlimb motor functions after spinal cord transection provided by olfactory ensheathing cell transplantation* – será submetido à Neuroscience

# Early treadmill step training accelerates the partial recovery of hindlimb motor functions after spinal cord transection provided by olfactory ensheathing cell transplantation

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**Abstract**

Several studies have reported that olfactory ensheathing cell (OEC) transplantation after spinal cord injury (SCI) represents a promising therapeutic approach. However, the functional gains obtained remain modest and combined strategies have been tested in order to enhance the results achieved with OEC therapy. The present study was performed to test the hypothesis that early treadmill step training could be used as a combined rehabilitation therapy to increase the beneficial effects of OEC transplantation after a complete spinal cord transection (SCT) at T8-9 in rats. Five days after SCT, OEC-transplanted spinal animals started a step-training program on a treadmill with partial body weight support and manual step help. Hindlimb movements, were evaluated over time and scored on the basis of the open-field BBB scale and showed significant improvement as from post-transplant weeks 10 and 4 in OEC-untrained and trained spinal animals, respectively. Histological results revealed that OEC transplantation facilitated serotinergic brain stem axon sprouting, which is more evident in OEC-trained animals. However, there was scant regrowth of serotonin immunoreactive fibers that traversed the injury site and into caudal spinal cord segments. In conclusion, acute OEC transplantation after SCT improves the hindlimb movements. Early treadmill step training accelerates the OEC effects when associated with this cellular therapy. Additionally, the functional gains are not accompanied for axonal regeneration studied by serotonin and GAP-43 immunoreactivity. These results further support the use of this combined therapeutic strategy for neurorehabilitation of SCIs.

**Key words**: Spinal cord injury; Olfactory ensheathing cell; Treadmill training; Paraplegia; Hindlimb motor function; Rehabilitation

**Introduction**

Thoracic spinal cord transection (SCT) results in a significant neurologic dysfunction and disability and determines one of the most severe neurological disorders, paraplegia. This disorder is a result of the permanent loss in neural connectivity between the upper and lower regions of the spinal cord and a consequent loss of central control of motor and somatosensory functions caudal to the injury site. The failure of the spinal cord to show spontaneous anatomical and functional repair after spinal cord injury (SCI) can be attributed to the extremely limited capacity of the mature neurons to regenerate their axons in the hostile extracellular environment of injured central nervous system (CNS) (Schwab, 2002). Astroglial scar formation and tissue ischemia, upregulation of proteoglycans and myelin associated inhibitors together with a decrease in growth-promoting molecules are the main factors associated with the inhibited repair capability of spinal cord (Schwab, 2002; McDonald and Sadowsky, 2002; for review see Vargas and Barres, 2007).

Several animal studies have suggested that the transplantation of olfactory ensheathing cells (OEC) to the injury site after SCT constitutes a promising therapeutic approach. Spinal rats have shown OEC-supported supraspinal descending axonal regeneration after transplantation (Ramón-Cueto et al., 1998; 2000; Lu et al., 2001; López-Vales et al., 2006a; 2007). However, the most encouraging results have been found with functional improvement, as partial hindlimb motor function restoration was demonstrated after OEC transplantation in paraplegic animals (Ramón-Cueto et al., 2000; Toft et al., 2007; Aoki et al., 2010). These numerous successes in terms of reported functional gains make OECs an inviting target for human studies. Over the last decade, several human trials have been performed in order to replicate the successes seen in animal studies. Most of these trials have shown that OEC transplantation is safe (Huang et al., 2006) and have shown some potential effects of transplantation in terms of neurologic outcomes (Lima et al., 2006; Huang et al., 2009;

Bohbot, 2010). Despite these promising beneficial functional and morphological effects produced by OEC transplantation after SCI in animal and human design studies, the reported recovery of lost neurological function is still modest. For this reason, in order to enhance its therapeutic potential, OEC transplantation has been combined with other therapeutic strategies such as the use of Schwann cell bridges to join disrupted stumps (Fouad et al., 2005), combined stem cell transplantation (Ao et al., 2007), pharmacological neuroprotection (López-Vales et al., 2006b) and physical training (Kubasak et al., 2008).

The use of activity-based therapy to improve functional and morphological outcomes after SCI is well documented in patients (Wernig et al, 1998; Behrman and Harkema et al., 2000; Hicks et al., 2005; Nooijen et al., 2009) and animal models of paraplegia (Barbeau and Rossignol, 1987; Leon et al., 1998; Moshonkina et al., 2004; de Leon and Acosta, 2006; Zhang et al., 2007; Macias et al., 2009; Ilha et al., 2011a;b). Furthermore, cycling exercise combined with neurotrophin-secreting transplants results in improved hindlimb motor function (Nothias et al., 2005). Although, both OEC and exercise training therapies have shown to have beneficial effects on neurological outcomes after SCI, little data is available on the effect of combining OEC transplantation and physical training. In this context, there is only one study, conducted by Kubasak et al. (2008), which showed that OEC transplantation combined with step training enhanced hindlimb-stepping abilities in spinal rats. However, the training program was initiated one month after SCT and transplantation. As we have reported that early exercise training (initiated 5 days after complete SCI) has beneficial effects in paraplegic rats (Ilha et al., 2011a;b), we hypothesized that acutely initiated treadmill step training could also produce synergic beneficial effects when combined with OEC therapy after SCI. Thus the present study was performed in order to determine whether the restorative properties of OEC transplantation might be enhanced when combined with early treadmill step training after complete SCT in adult rats.

**Experimental Procedures**

All procedures were in accordance with Brazilian laws and the recommendations of the Brazilian Society of Neurosciences and the International Brain Research Organization. This study was approved by the Ethics Committee of our institution (Nr. 2007738).

*OEC culture*

Primary OEC cultures were prepared from the olfactory bulb (OB) of adult male Wistar rats (2.5 months old, UFRGS, Brazil) based on a previously described method (Nash et al., 2001). Isolation of the different types of glia was accomplished by the different rates of cell attachment. Briefly, animals were decapitated and the OBs were removed and transferred into Hank's balanced salt solution (HBSS, Sigma Chemical Co., USA). After removal of the meninges and vessels, the medioventral superficial aspects of the bulbs (mainly olfactory nerve and glomerular layer) were collected, minced, and incubated with 0.1% trypsin (Gibco, USA) at 37°C for 10-15 min. The trypsinization was stopped by the addition of culture medium containing serum: Dulbecco's modified Eagle's medium (DMEM)/Ham's/F-12 (1:1 mixture, pH 7.4; Sigma Chemical Co., USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 0.1% amphotericin B (Fungizone; Sigma Chemical Co., USA) and 0.032% Garamycin (from a local commercial supplier). Following two washes in the medium, tissue fragments were mechanically dissociated by sequential passage through a fire-polished glass Pasteur pipette. The resulting cell suspension was centrifuged at 1000 rpm for 5 min, the supernatant discarded and the pellet resuspended in the same culture medium. The cells were then plated onto uncoated Petri dishes. Some fibroblasts, present in the cell culture became attached to the Petri dishes during this first 18-h incubation period (37°C, 5% CO2). The supernatants, enriched by astrocytes and OEC, were transferred to an uncoated culture flask

and incubated for another 4-6 h to allow for the attachment of astrocytes. As OECs do not attach to uncoated surfaces, the remaining cells in the supernatant were then transferred onto poly-D-lysine-coated (PDL, 0.1 mg/mL, Sigma Chemical Co., USA) culture flasks. OECs were allowed to grow to confluency in 10–14 days on PDL-coated flasks. On the day of transplantation, the cells were trypsinized with 0.25% trypsin for 5 min at 37°C. The detached cells were transferred to DMEM containing FBS and centrifuged at 3000 r.p.m. for 8 min. After washing, the cell pellet was resuspended in 10 mL DMEM and the cells were counted. Finally the cells were centrifuged at 3000 r.p.m. for 8 min before the volume was adjusted to the appropriate cell concentration for transplantation (100,000 cells per µL).

To determine the purity of the OECs in the primary cultures, additional cell cultures were plated onto well glass chamber slides (PDL-coated) and processed for anti-nerve growth factor receptor (NGFR p75) immunohistochemistry. Following a week in culture, cells were fixed for 20 min with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), washed several times in PBS, and preincubated in blocking solution containing 5% bovine serum albumin (BSA; Sigma Chemical Co., USA) and 0.2% Triton X-100 in PBS for 1 h at room temperature. Staining was performed using primary antibodies raised against the low affinity neurotrophin receptor p75 ($p75^{NTR}$, rabbit, 1:200; Sigma Chemical Co., USA), diluted in blocking solution. Following overnight incubation at room temperature, cells were washed three times in PBS and incubated with goat anti-rabbit IgG fluorescein conjugate antibody diluted in PBS (1:1000; Calbiochem, USA) for 2 h. Afterwards, slides were washed three times in PBS and incubated with a solution containing 4,6-diamidino-2-phenylindole (DAPI, 0.2 µg/mL; Calbiochem, USA) for 1 h, washed several times in PBS and coverslipped with mounting medium (Fluoromount; Sigma Chemical Co., USA). The purity of the cell cultures was determined by counting the $p75^{NTR}$ immunoreactive cells attached to the slides. Cells

with p75 $^{NTR}$ immunoreactivity were designated OECs as has been previously reported (Ramón-Cueto and Nieto-Sampedro, 1992; Nash et al., 2001).

Six sample areas per well and 3 wells in total at 20× magnification were used to calculate the average percentage of p75 $^{NTR}$ immunoreactive cells. Using this culture protocol, we obtained ~85% of p75 $^{NTR}$ immunoreactive cells in OEC culture (Fig. 1).

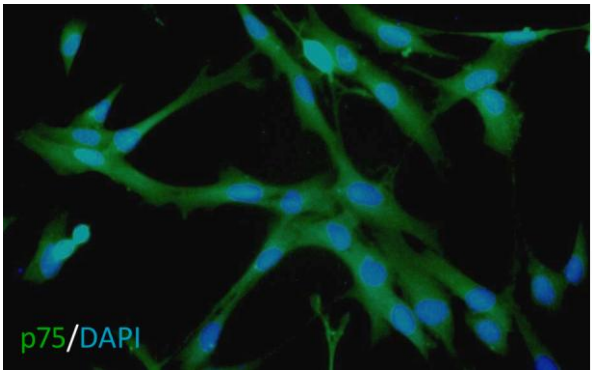


**Figure 1.** Image of primary cultured OECs stained with anti-p75$^{NTR}$ antibody (green) and DAPI (blue).

*Surgical procedure and OEC transplantation*

Adult male Wistar rats (2.5-month-old; UFRGS) were anesthetized using pentobarbital (40 mg/kg, i.p., Cristália, Brazil) and subjected to a vertebral laminectomy at thoracic levels (T8–T9). Spinal cord transection was performed using microscissors, and the completeness of the transection was ensured by passing a sickle probe (n° 3, White, Brazil) through the lesion site (Ilha et al., 2011a,b). For transplantation, a suspension of OECs was stereotaxically injected with a sterile glass needle (80 μm i.d.; 100 μm o.d.) connected to a Hamilton syringe. Twelve transected rats received injections of suspended OECs into four sites of the midline of both spinal cord stumps (coordinates 0.15, 0.12, 0.09 and 0.06 mm below the dural surface), at 1 mm (cranial and caudal) from the transection site (similar to the procedure performed by

Ramón-Cueto et al., 2000). Each site received 0.5 μL of a suspension containing ~50,000 cells injected over 1 minute and each transplanted rat received a total of ~400,000 cells. Additionally, six transected animals received 4 μL of DMEM distributed at the same coordinates and under the same conditions. The surgical procedure was concluded by suturing the muscle plane and the skin (6-0 and 4-0 nylon sutures, respectively; Somerville, Brazil). The skin surface was then disinfected with 2% iodine solution.

After the surgery, animals were randomly divided into the following groups: (a) untrained rats with spinal cord transection that only received DMEM (medium, n = 6); (b) with spinal cord transection and OEC transplantation (untrained OEC, n = 6); and (c) rats with spinal cord transection and OEC transplantation that underwent to step-training (trained OEC, n = 6).

*Post-operative care*

Following the surgery, rats were kept in a warm environment and monitored until they recovered from anesthesia. The animals were then returned to standard conditions. All animals were treated for 14 days with Baytril (Enrofloxacin 2.5 mg/kg, subcutaneously; Bayer S.A., Brazil) to prevent urinary tract infections. Furthermore, bladders were manually expressed twice a day until the bladder was no longer distended and palpable, indicating that the animal had developed an automatic bladder voidance reflex (10-14 days). Inspections for general health, skin irritation, decubitus ulcers or evidence of autophagia, were carried out daily throughout the post-injury survival period.

*Step training rehabilitation*

The training program was performed on a treadmill designed for human use (Runner, Brazil) and modified for use by rats. Before the SCT, the animals were familiarized with the treadmill apparatus at 5 m/min for 5 min a day on three consecutive days and on post-operative day 6 the trained OEC animals started a 9-week step-training program. The training program consisted of step training on a treadmill (band speed 6-7 m/min) with partial body weight support (BWS), once a day and 5 sessions per week. The first training day began with 5 minutes of step training. The training time was progressively increased every day up to 20 minutes on the second week and 30 minutes over the following 7 weeks. The design of this treadmill training regime took into account previously published studies using complete SCT in rats (Zhang et al., 2007; Ilha et al., 2011a,b).

The step training was carried out using a manually adjustable weight-supporting counterbalance system to provide weight support assistance. Each rat was fitted with a Lycra vest that was closed with Velcro, and placed into a BWS harness, thereby supporting the thorax, while the head, forelimbs and hindlimbs had full range of movement. For step training, rats were placed in a quadripedal position, bearing ~ 15% of their body weight on their hindlimbs (i.e., ~ 85% BWS). Each spinal animal was individually trained and the hindlimbs were manually moved in a step pattern by the researcher holding the ankle region (according to Laird et al., 2009 and Ilha et al., 2011a,b). During the step training, special care was taken to place the rats' feet in a plantar stepping position and to keep the toes extended to ensure the footpad made contact with the treadmill band during the stance phase.

*Hindlimb motor function*

Hindlimb movements were evaluated over time and scored on the basis of the open-field BBB scale during spontaneous activity. For this, one animal at a time was allowed to

move freely inside an open-field (60 x 30 x 40 cm) for 5 minutes. One blinded examiner observed the hindlimb movements of the rat, and scored according to the Basso, Beattie and Bresnahan scale (BBB scale; Basso et al.,1995), which ranges from 0 (paralysis) to 21 points (normal gait). The average value from the right and left hindlimbs was calculated and taken as the animal's score.

*Histological evaluation*

The day after the last training session, animals were deeply anesthetized with pentobarbital (100 mg/kg, i.p.;Cristália, Brazil). Heparin (1000 IU; Cristalia, Brazil) was injected into the left cardiac ventricle, then the animals were transcardially perfused through the left ventricle using a peristaltic pump (Milan, Brazil, 20 mL/min) with 300 mL of 0.9% saline solution, followed by 400 mL of 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (PB, pH 7.4) at room temperature. The spinal cord segments were removed after careful laminectomy, post-fixed in the same fixative solution at room temperature for 4 h and cryoprotected in 15 and 30% sucrose (Synth, Brazil) solution in PB at 4 °C until they sank. After that, a ~1.2 mm block containing the injury/transplant site and extending 6 mm cranially and 6 mm caudally to the epicenter was photographed with a digital camera (Sony Cyber-shot S730 7.2 Megapixels, China) on a dark background. Then, the samples were embedded in TissueTek, quickly frozen in isopentane (Merck, Germany) cooled in liquid nitrogen and stored at −70°C. Using a cryostat (CM1850, Leica, Germany), 25-µm-thick sagittal sections of the block, including the transection site, were cut and all consecutive spinal cord sections collected onto different gelatin-coated glass slides so that each slide contained a series of sections that were 100 µm apart.

Each slice was immunolabeled with different primary antibodies for histological analysis. Briefly, after several washes with PB saline (PBS; pH 7.4) sections were pre-treated

with 3% hydrogen peroxide for 30 min, carefully washed and treated with 2% BSA in PBS containing 0.4% Triton X-100 (PBS-Tx) for 30 min and then incubated for 24-48 h at 4°C with the following primary antibody: rabbit polyclonal antibody against serotonin (5-HT; 1:5000; Sigma Chemical Co., USA) and mouse monoclonal antibody against growth associated-protein (GAP-43; 1:500; Santa Cruz Biotechnology, USA). Sections were again washed in PBS-Tx and incubated with anti-rabbit IgG (1:100; Sigma Chemical Co., USA) or anti-mouse antibody conjugated with peroxidase (1:500; Sigma Chemical Co., USA) diluted in PBS-Tx for 2 h at room temperature. After several washes with PBS-Tx, the sections previously incubated with rabbit polyclonal antibody against 5-HT were again incubated with rabbit PAP (1:500; Sigma Chemical Co., USA) diluted in PBS for 1.5 h at room temperature. The reaction was revealed in a medium containing 0.06% 3,3′-diaminobenzidine (DAB, Sigma Chemical Co., USA) dissolved in PBS for 10 min and then 1μL of 3% $H_2O_2$/mL was added to the DAB medium for an additional 10 min. Finally, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xylene and covered with Entellan (Merck, Germany) and coverslips.

Control sections were prepared omitting the primary antibody by replacing it with PBS. Furthermore, the samples were post-fixed for the same time in identical solutions, rigorously processed at the same time and incubated in the same medium for the same period of time. This precaution was taken to avoid overreaction, differences in chromogen reaction, saturation of optical density or changes in background staining levels.

For histological measurements, images from stained sagittal spinal cord sections were captured from the lesion epicenter as well 1 mm cranially and 1 mm caudally to the lesion edge using a Nikon Optiphot-2 microscope (20×, Japan) coupled to a Micrometrics camera (Accu Scope, USA) and Image Pro Plus Software 6.0 (Media Cybernetics, USA). Semi-quantitative densitometric analysis was used to measure the intensity of the serotonin and

GAP-43 immunoreactions. The digitized images obtained from the selected areas were converted to an 8-bit gray scale (0–255 gray levels). All lighting conditions and magnifications were held constant. Picture elements (pixels) employed to measure optical density were obtained from 3 squares measuring 179,200 μm2 (area of interest, AOI) overlaid on dorsal, central and ventral regions in the sagittal spinal cord section gray scale images immediately before (cranial) or after (caudal) the lesion edge. Background staining subtraction and correction were done in accordance with our previous published protocol (Xavier et al., 2005).

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 at pixel(x,y), "INT(x,y)" or intensity is the intensity at pixel(x,y), "BL" or black is the intensity generated when no light goes through the material and "INC" is the intensity of the incidental light. Around 8-13 images were analyzed from each rat.

*Data analysis*

All measurements were performed in a blinded manner. A code was used for the rats and histological slices. Then, a mean value was calculated for each group based on the average value for each animal in the group. The functional and histological measurements were analyzed using repeated measures analysis of variance (ANOVA) with *time* or *site*, respectively, as the repeated measure. The Bonferroni test was used to adjust the results of the multiple comparisons at $P < 0.05$. Descriptive data were expressed as means ± S.E.M. (standard error of the mean). Data were run on SPSS 11.5 (Statistical Package for the Social Sciences, Inc., USA).

## Results

*Hindlimb motor function*

Over time, the OEC-transplanted groups showed improved hindlimb movement, so that both the OEC-trained and untrained animals showed higher BBB scores at 4 and 10 weeks compared to 5 days after lesion/transplantation (Fig. 2A). Significant functional recovery in hindlimb usage was observed in the OEC-transplanted group compared to the medium animals at post-operative week 10 (Fig. 2B). Additionally, treadmill step training combined with OEC transplantation accelerated hindlimb motor function improvements in the BBB test. The OEC-trained group showed higher BBB scores at post-operative weeks 4 and 10 compared to the medium group (Fig. 2B).

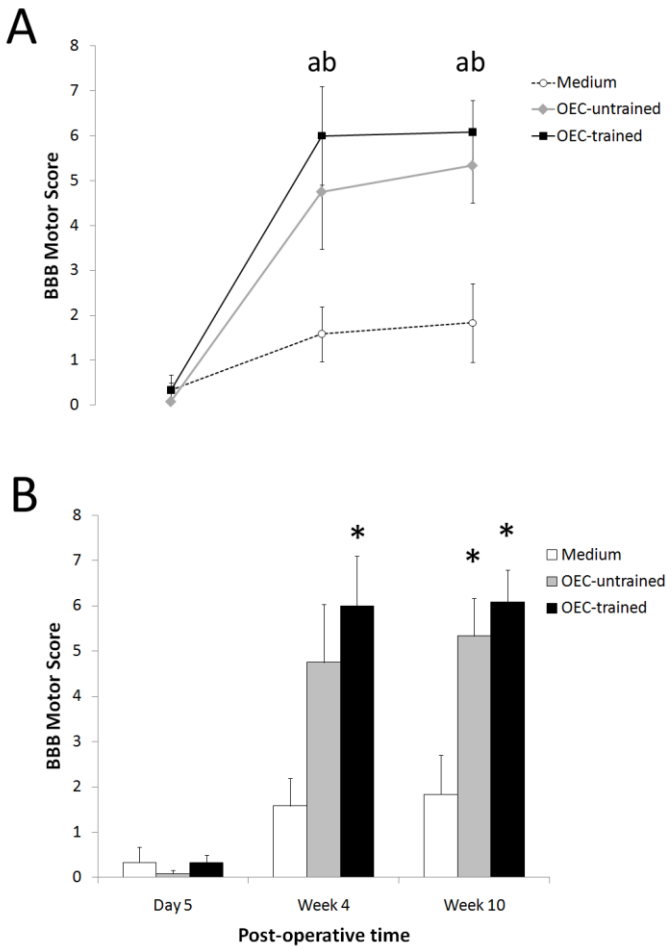


**Figure 2.** Effects of treadmill step training combined with OEC transplantation after complete SCT on hindlimb motor function. Graphs showing (A) time course and (B) group comparisons of functional recovery in hindlimb movements at post-operative day 5 and weeks 4 and 10. Repeated measure ANOVA showed *time* [$F_{(2,30)}$=22,989; $P$<0.001] and *group* [$F_{(2,15)}$=18,309; $P$<0.001] effects. Letters "a" and "b" mark significant differences ($P$<0.05) when compared 4 and 10 weeks with 5 days after SCT in the OEC-untrained and trained groups, respectively. Asterisks (*) mark significant differences ($P$<0.05) when compared with the medium group.

*Morphology of the lesion sites*

Figure 3 shows images of spinal cord lesion sites from animals that received DMEM (medium group) or OEC transplantation after SCT in both the untrained and trained groups. Substantial tissue continuity between cranial and caudal segments was visible in several animals. Interestingly, this tissue continuity seems to be more evident in both untrained and trained OEC-transplanted animals compared to the medium group (Fig. 3).

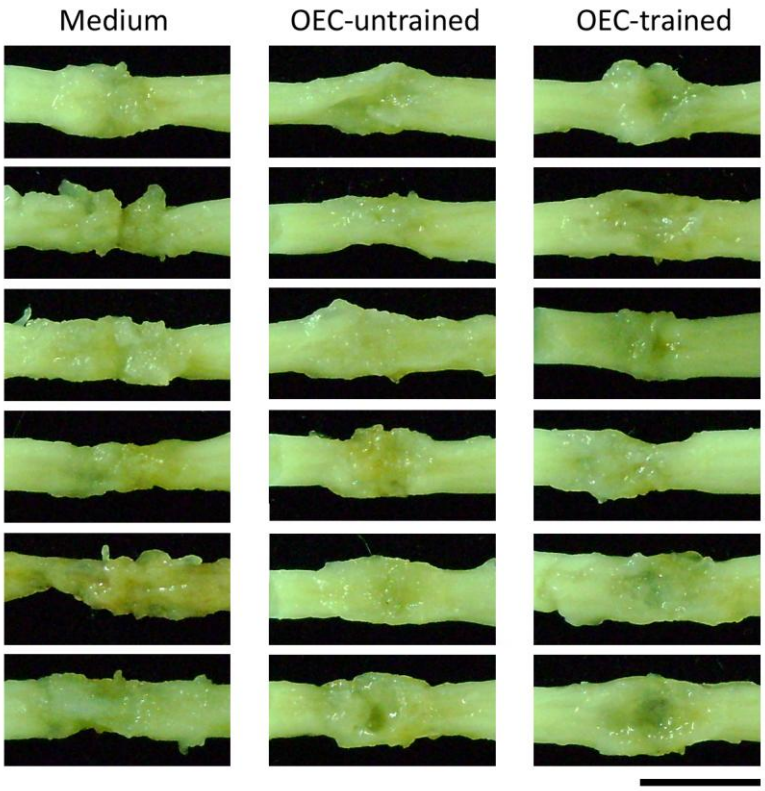


**Figure 3.** Macroscopic morphology of dorsal spinal cord from animals in the medium, OEC-untrained and OEC-trained groups. Note that transected spinal cords show tissue continuity, the lesion site is filled with tissue that forms a gap between the cranial (on the left) and caudal (on the right) segments. Scale bar = 5 mm.

*Axonal regeneration*

In order to study axonal regeneration after combined OEC transplantation and treadmill step training, 5-HT and GAP-43 immunostaining was performed in injured spinal cords. In the histology analysis, numerous 5-HT immunoreactive fibers were observed in the grey and white matter of the spinal cord cranial to the lesion in both medium and OEC-transplanted animals (Fig. 4). However, no serotonergic fibers were observed in the caudal stump of the medium and OEC-transplanted rats. The immunoreactivity, analyzed by means of optical densitometry (OD), showed an increase in 5-HT staining cranial to the lesion in the OEC-transplanted animals (Fig. 4). Furthermore, the rats that underwent treadmill step training showed significantly higher values for 5-HT immunoreactivity cranial to the lesion, when compared to the medium group ($P$=0.006; Fig. 4). However, there were no statistical differences between the OEC untrained (0.0432 ± 0.003) and trained groups (0.0465 ± 0.005; $P$=1.0), or between the OEC untrained and medium groups (0.0284 ± 0.003; $P$=0.07).

Additionally, OD analysis revealed an absence of 5-HT staining caudal to the lesion in medium and OEC-transplanted animals. OEC-transplanted rats that were subjected to early treadmill training showed very little 5-HT immunoreactivity caudal to the lesion (Fig. 4). The 5-HT OD values caudal to the lesion were significantly different to the cranial values in all experimental groups ($P$<0.001; Fig. 4).

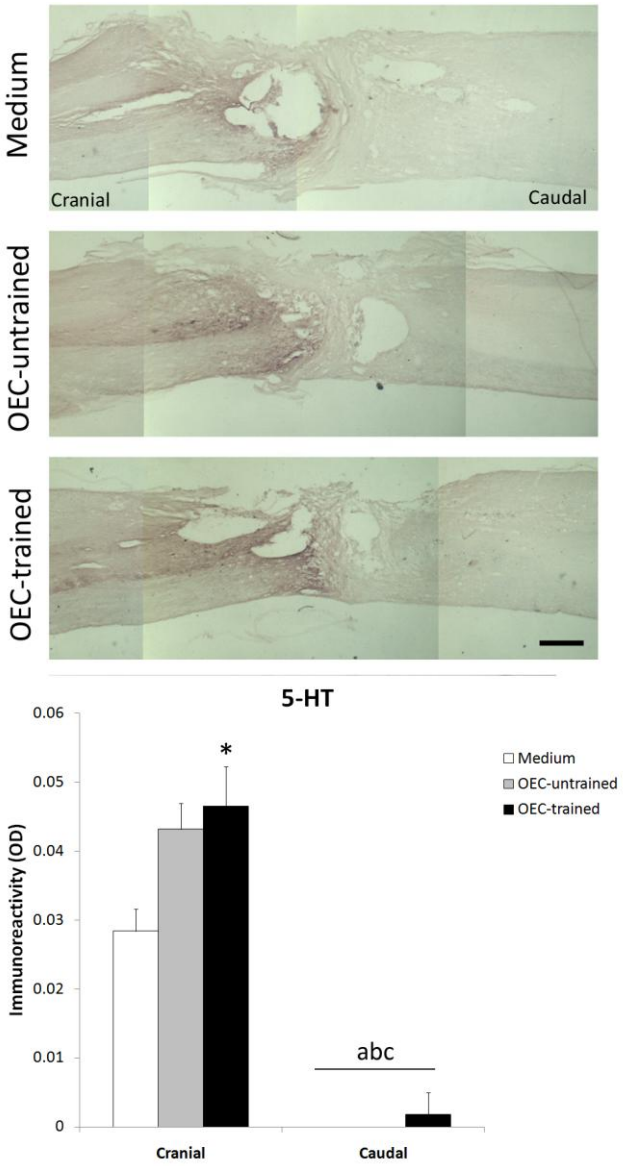


**Figure 4.** Effects of treadmill step training combined with OEC transplantation after complete SCT on serotonin (5-HT) immunoreactivity cranial and caudal to lesion epicenter. Digitalized images showing 5-HT stained sagittal spinal cord sections of medium, OEC-untrained and OEC-trained groups are shown at the top of figure. Scale bar = 500 μm. Repeated measure ANOVA showed *site* [$F_{(1,11)}$=177.188; $P$<0.001] and *group* [$F_{(2,11)}$=5.167; $P$=0.026] effects. In the graph, asterisks (*) mark significant differences ($P$<0.05) when compared with the medium group. Letters “a”, “b” and “c” mark significant differences ($P$<0.05) when the caudal and cranial regions are compared in the medium, OEC-untrained and trained groups, respectively.

Immunostaining against GAP-43 showed very little immunoreactivity cranially or caudally to the lesion in the non-transplanted, the trained and untrained OEC-transplanted animals. Although OEC-trained group showed higher values both cranially and caudally to the lesion sites when compared to the medium and OEC-untrained groups, there were no statistical differences between the experimental groups (Fig. 5).

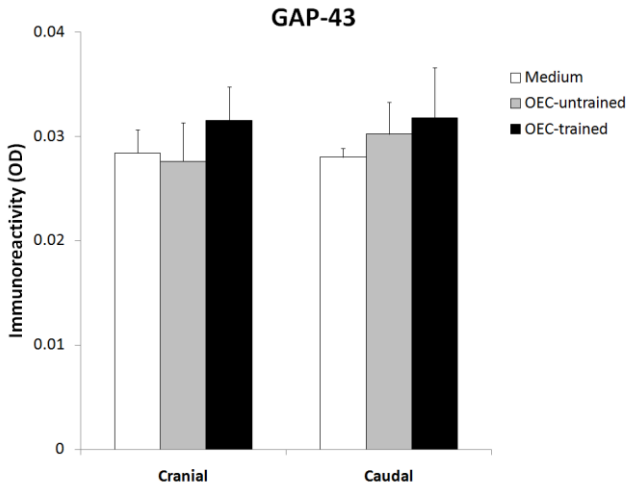


**Figure 5.** Effects of treadmill step training combined with OEC transplantation after complete SCT on GAP-43 immunoreactivity cranially and caudally to the lesion epicenter. Repeated measure ANOVA showed no *site* [$F_{(1,11)}$=0.320; *P*=0.583] or *group* [$F_{(2,11)}$=0.386; *P*=0.688] effects.

## Discussion

The present study was performed in order to assess whether acute transplantation of OECs combined with early treadmill step training after complete transection of spinal cord was able to produce functional recovery and axonal regeneration. The results reported herein show that OEC transplantation produced a modest but significant recovery of hindlimb movement, 10 weeks after SCI. Additionally, the early treadmill step training was able to

accelerate this partial recovery of hindlimb motor function to 4 weeks after lesion. To our knowledge, only one previous study has tested the hypothesis that step training combined with OEC transplantation may enhance the restorative properties of OECs after SCI. In that study, Kubasak et al. (2008) reported that training enhanced hindlimb-stepping abilities in spinal OEC-transplanted rats only 4 months post-injury and transplantation. The most substantial difference between that study and the present one is the time post injury/transplantation that physical training was initialized. We started the training 5 days after injury/transplantation, in the acute phase of the injury while Kubasak et al. (2008) only started the training 1 month post lesion, in a more chronic stage (Houle and Tessler, 2003). The potential of acute and delayed OEC transplantation to promote functional recovery after complete SCI, are well reported in spinal rats (Ramón-Cueto et al., 2000; Lu et al., 2001; 2002; Fouad et al., 2005; López-Vales et al., 2006a; 2007; Toft et al., 2007; Aoki et al., 2010). In this context, studies using OEC transplantation in acute (López-Vales et al., 2006a) or sub-acute (Aoki et al., 2010) stages after complete SCT have shown progressive partial recovery of hindlimb motor skills using an open field BBB score, as employed in our study. Similar to our results, Aoki et al. (2010) reported OEC-induced hindlimb motor recovery over the first 8 weeks post lesion in spinal rats.

The most novel finding of the present study is that combining acute OEC transplantation with early treadmill step training after complete SCI resulted in enhanced functional outcomes. The beneficial effects of locomotor training on functional recovery and morphological plasticity in paraplegic models has been widely reported in previous studies from our and other research groups (Barbeau and Rossignol, 1987; Leon et al., 1998; Moshonkina et al., 2004; de Leon and Acosta, 2006; Zhang et al., 2007; Macias et al., 2009; Ilha et al., 2011a;b). Several neural repair strategies have been studied in experimental models of SCI. Although some of these have shown promising results, the functional recovery

achieved is modest. Combined strategies to enhance function and promote the repair of injured spinal cord have provided new insights into paralysis after SCI (Fouad et al., 2005; Bunge, 2008). Treatment strategies based on the combination of rehabilitation with other biological therapies, such as peripheral nerve grafting (Ma et al., 2010) or antibody-mediated suppression of the growth inhibitory protein Nogo-A (Gonzenbach et al., 2010) suggest that physical therapy combined with other approaches could have beneficial effects on neurological outcomes.

In the present study, we used immunostaining for serotonin (5-HT) and GAP-43 to analyze axonal regeneration after SCI. Serotonergic fibers in spinal cord emerge from brain stem nuclei (Holstege and Kuypers, 1987) and may modulate locomotor spinal cord circuits (Barbeau and Rossiggnol, 1990). In addition, GAP-43 is associated with filopodial extension and neurite branching, which clearly allows a regenerating neuron to grow (Denny, 2006). In SCI models, both serotonergic fiber and GAP-43 immunoreactivity have been studied as axonal regeneration markers (Lu et al., 2001; López-Vales et al., 2006a).

While previous studies have reported that acute OEC transplantation promotes greater brain stem serotonergic tract regrowth (Ramón-Cueto et al., 2000; Lu et al., 2001; López-Vales et al., 2006a), our results showed only a little 5-HT immunoreactivity in the caudal stump after OEC transplantation and treadmill step training in spinal rats. The OD analysis revealed that the immunoreactivity in the caudal segment is also lower when compared to the cranial segment in all studied groups. On the other hand, 5-HT immunoreactivity tends to be higher in the cranial stump of OEC-transplanted animals. In the OEC-trained group, this increase is statistically higher when compared with the medium group and similar to OD values in OEC-untrained animals. This greater increase in 5-HT fibers near the face of the lesion in cranial stump probably showed the enhanced serotonergic fiber sprouting and regrowth capabilities after OEC transplantation, which is more pronounced in animals

subjected to treadmill step training. On the other hand, although there is an increase in GAP-43 immunoreactivity (measured using OD analysis) in both the cranial and caudal stumps of the spinal lesion in the OEC-trained group, there was no significance statistical difference between the groups. Similar to our results, Kubasak et al. (2008) found numerous 5-HT-positive axons immediately cranial to the transection site in all the examined spinal rats. Additionally, in the OEC-injected animals, they observed few 5-HT-positive axons traversing the injury site. Since medium-injected rats did not show any serotonergic fibers spanning the injury site, they inferred that the presence of 5-HT-positive axons traversing the injury site in the OEC-treated rats most likely represented regeneration.

In addition, Guest et al (2008) in an interesting study showed that xenografts of cultured primate OEC (POEC) led to important functional recovery in the BBB test from 8 weeks after lesion/transplant in rats. However, the most surprising result from this study is the lack of correlation between functional gains and serotonergic or corticospinal tract (CST) regeneration. As with our results, they observed sprouting and limited regeneration of 5-HT fibers in transplanted animals. Furthermore, although the CST in the POEC transplanted spinal cord showed collateralization and the appearance of the terminals, regeneration did not occur.

Apart from promoting axonal regeneration, OEC transplants may contribute to spinal cord repair in several other ways. They are reported to secret several neurotrophic factors (Woodhall et al., 2001; Pastrana et a., 2007), reduce cavitation (Ramer et al., 2004a, b; López-Vales et al., 2006b), improve vascularization (Lopez-Vales et al., 2004; Ramer et al., 2004a, b), myelinate experimentally demyelinated and regenerating axons (Franklin et al., 1996; Sasaki et al., 2004) and promote sprouting of intact axons near the transplant (Chuah et al., 2004). All of these mechanisms could potentially contribute to improved functional recovery

(Franssen et al., 2007), as seen in our study by the increase in hindlimb movement after OEC transplantation.

Additionally, our findings support the hypothesis that the effects of OEC transplantation can be enhanced when combined with task-specific training. While the exact mechanisms by which training improves performance in spinal OEC-injected rats remain unknown, physical exercise could facilitate axon regeneration, and/or provide protection for the neurons and glia near the injury site by stimulating the secretion of growth factors produced by the neuromuscular system after locomotor training (Hutchinson et al., 2004; Ying et al., 2005; Ilha et al., 2011). This activity-dependent increase in neurotrophic release could promote spinal cord reorganization and improve hindlimb motor functions after SCI. Thus we believe that our OEC-transplanted animals trained or untrained may have benefited from neurotrophic factors. Furthermore, the final outcome of combining early treadmill step training with OEC transplantation may be influenced by activity of the neuromuscular structures that generate the practiced motor tasks and lead a motor learning in spinal level.

**Conclusion**

In summary, our study reproduces some of the beneficial morphofunctional effects of OEC transplantation previously reported after complete SCI in animal models. Additionally, it shows that early treadmill step training combined with OEC transplantation is able to enhance the partial recovery of hindlimb movements, although this is not accompanied by greater nerve regeneration in our study. While further studies are required in order to elucidate the biological mechanisms by which task-specific training improves motor performance when combined with OEC transplantation, our preliminary results support its beneficial effects on rehabilitation after this cellular intervention in SCI.

**Acknowledgments**

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# 7 DISCUSSÃO GERAL

Este trabalho teve como objetivo geral estudar os efeitos do treino de marcha na recuperação neurológica e na plasticidade neuromuscular em animais submetidos a lesão medular e ao transplante de GEO. Para obtenção dos resultados, os experimentos foram conduzidos em 2 fases distintas. A primeira englobou a análise dos efeitos do treino de marcha em esteira sobre a recuperação sensório-motora dos membros posteriores (MP) e a plasticidade dependente da atividade no sistema neuromuscular de ratos paraplégicos. Estes resultados foram apresentados e individualmente discutidos nos Capítulos 4 e 5 deste trabalho (Artigos 1 e 2). Na segunda fase, o protocolo de treino de marcha foi combinado ao transplante agudo de GEO com o objetivo de potencializar os efeitos desta terapia celular que vem sendo reportada como uma técnica promissora para no reparo do SNC lesionado. Os resultados da segunda fase foram apresentados e individualmente discutidos no Capítulo 6 (Artigo 3). Na presente seção, são abordadas as discussões gerais pertinentes aos leitores que se interessem pelo campo da neurociências da reabilitação.

O modelo experimental adotado para estudo foi o de completa transecção da medula espinal (TME) ao nível torácico em ratos Wistar machos adultos. Esta lesão determina uma importante disfunção físico-motora caudal ao nível da lesão, que inclui a paralisia e hiperreflexia neuromuscular em MPs (BOUYER, 2005; ZHANG et al., 2007; YATES et al., 2008a). Este modelo foi escolhido por apresentar uma completa interrupção entre os centros de controle motor craniais ao local da lesão e os circuitos neuronais espinais, bem como as unidades motoras caudalmente localizadas. Desta forma, as alterações plásticas, morfológicas e bioquímicas encontradas na região espinal lombar após o treino de marcha são dependentes da prática específica desta tarefa de reabilitação. Além disso, este modelo propiciou o estudo da regeneração axonal das vias espinais após a TME e o transplante da GEO, tanto em animais treinados e não treinados.

Apesar de ser um modelo com um procedimento cirúrgico relativamente simples, ele exige uma série de cuidados no período pós-operatório imediato e até mesmo no crônico. Entre estes, destacamos a profilaxia das infecções urinárias. Em nosso estudo, os animais com TME recebiam uma suave massagem na região abdominal baixa para estimular o

esvaziamento da bexiga urinária 2 vezes ao dia, durante 10 a 14 dias, ou até que o reflexo de micção fosse reestabelecido. Neste período, antibiótico terapia foi realizada com Baytril (Enrofloxacin 2.5 mg/kg, subcutaneamente; Bayer S.A., Brasil). Estes procedimentos são considerados padrão nos estudos experimentais de lesão medular em ratos (STEWARD et al., 2006; SHARP et al., 2010). Entretanto, mesmo com estes cuidados, aproximadamente 15% dos animais morreram por infecções urinárias, apresentando sangue e pus na urina, durante os experimentos.

Interessantemente, nossos animais não apresentaram episódios de autofagia dos MPs após a lesão, como relatado por alguns estudos. Porém, podemos perceber que os animais com TME perdem grande quantidade de massa corporal nas primeiras semanas e depois voltam a ganhar peso. Ao final dos experimentos, os animais lesionados pesavam, aproximadamente, apenas 13% a menos do que os intactos (dados não mostrados nos artigos).

O treino de marcha foi realizado em uma esteira ergométrica de humanos (RUNNER; Joinvile, SC, Brasil) adaptada com um inversor digital (DANFOSS; SP, Brasil) de velocidade para o uso com animais. Para tanto, os animais tiveram parte de seu peso corporal (85%) suspenso por um sistema confeccionado em nosso próprio laboratório. Este consistia em uma cinta elástica presa com um velcro ao redor do tórax e abdômen superior dos animais interligado a uma haste mecânica que fazia a suspensão parcial do peso corporal. Para calibração da quantidade de massa corporal suportada, os animais eram pesados sem o suporte e posteriormente o sistema era individualmente colocado e ajustado sobre uma balança digital (TOLEDO, Brasil). Dessa forma, pôde-se calcular a porcentagem de massa corporal sobre os MPs que os animais teriam que suportar durante o treino.

Os animais foram individualmente treinados e auxílio manual era dado pelo treinador para que o animal conseguisse realizar uma boa acomodação de peso dos MPs sobre a lona (solo), com os artelhos em extensão e abdução. Dessa forma, os animais recebiam de forma adequada os estímulos proprioceptivos providos pelo movimento da esteira que são capazes de desencadear o movimento rítmico de marcha (Figura 8) através da ativação do GPC, mesmo após a TME em mamíferos (BARBEAU; ROSSIGNOL, 1987). Este treino foi pensado tomando como base os protocolos empregados em estudos anteriores com treino de marcha em esteira, suporte de peso corporal e auxílio manual dos pesquisadores (ZHANG et al. 2007; LAIRD et al., 2009).

Em nosso estudo, o treino teve início na fase aguda, 5 dias após a lesão da ME. Este período foi esperado apenas para que os tecidos cutâneos e musculares, seccionados durante a cirurgia, pudessem apresentar uma melhor cicatrização no momento da intervenção física.

Durante o treinamento, os animais realizavam passos na esteira ergométrica com velocidade entre 6 e 7 m/min, uma vez ao dia (período da tarde), 5 dias por semana. O tempo de treino foi progressivamente aumentado, começando com 5 min no primeiro dia até atingir 20 min na segunda semana e 30 min nas outras 7 semanas de duração do estudo, totalizando 9 semanas de treino.

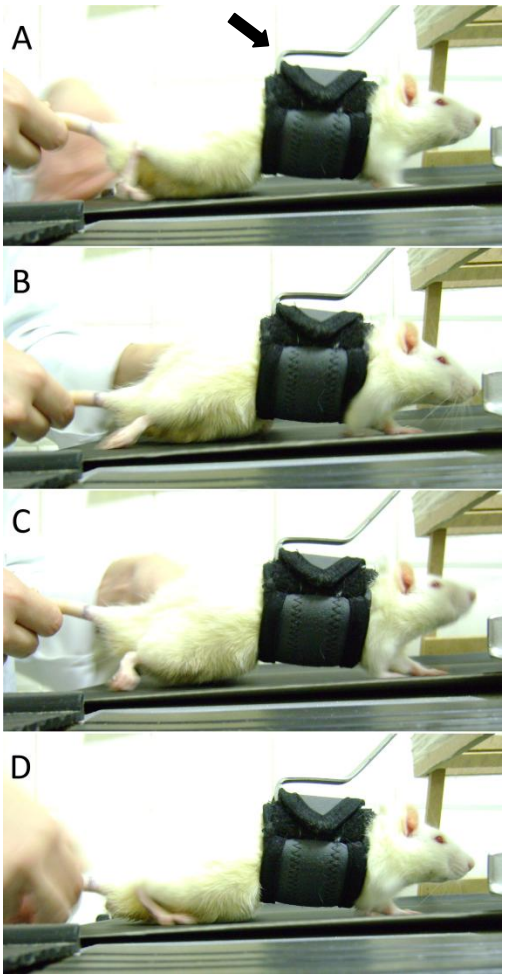


**Figura 8.** Fotografias mostrando o treino de marcha após 10 semanas da completa transecção da medula espinal. Note que de A-B o movimento da lona da esteira faz com que ocorra o alongamento dos músculos da região anterior do membro posterior (MP) direito. Após este alongamento, o MP direito inicia o movimento de balanço da marcha (C) através da flexão do quadril, joelho e tornozelo que culmina com avanço do membro e novo contato (D) com o solo (lona). Flecha indicando o equipamento utilizado para suporte do peso corporal dos animais (Laboratório de Histofisiologia Comparada, ICBS, UFRGS).

A recuperação funcional foi estudada periodicamente através do comportamento de livre caminhada em campo aberto. Neste, os movimentos dos MPs dos animais foram observados e escores foram atribuídos conforme o índice descrito por Basso, Beattie e Bresnahan (1995; escala BBB). Esta escala avalia a locomoção, apresentando escores que

variam de 0 a 21 pontos, representando, respectivamente, a total ausência de movimentos (paralisia) à marcha normal. Para análise da hiperreflexia, utilizamos o teste do reflexo de retirada MPs através de um suave estímulo mecânico realizado entre o primeiro e o segundo artelhos dos MPs. Obtém-se como resposta normal, a flexão vigorosa das articulações do MP e extensão/abdução dos artelhos. A resposta foi observada e escores de 0 a 3 foram atribuídos em conformidade com o estudo de Gale, Kerasidis e Wrathall (1985), onde 0 representa a ausência de reposta e 3 a hiperreflexia. As respostas foram consideradas como hiperreflexas, e consequentemente recebiam escore 3, quando após a aplicação do estímulo uma resposta exagerada era observada. A resposta exagerada foi, muitas vezes, acompanhada de flexão seguida de um espasmo em padrão extensor total dos MPs, ou ainda, caracterizada pela presença de movimentos rápidos e alternados entre flexores e extensores, considerados como sinal de clônus neuromuscular.

Os resultados do presente trabalho mostram que a TME causa severa paralisia muscular e que o treino de marcha em esteira, iniciado na fase aguda após a lesão, promove melhora da função neuromotora dos MPs. Este ganho foi evidenciado pelos escores mais elevados alcançados entre 8 e 10 semanas após a lesão na escala BBB pelos animais que realizaram treino de marcha. Além disso, os animais com TME desenvolveram hiperreflexia observada como hiperatividade do reflexo flexor de retirada que se desenvolveu de forma dependente do tempo, em torno de 2 semanas após a lesão. Cabe ressaltar que o treino de marcha manteve os valores do teste dentro dos padrões reportados como normais (em torno de 2 na escala) durante todo o período de estudo nos animais treinados.

Estes resultados encontram-se em acordo com os estudos de Zhang et al. (2007) e Laird et al. (2009), onde ratos submetidos a completa LME ao nível torácico que realizaram similar protocolo de treino de marcha em esteira mostraram melhora nos valores da escala BBB (ZHANG et al. 2007; LAIRD et al., 2009). Além disso, os efeitos benéficos do treino de marcha em esteira já foram bem relatados em modelos de lesões parciais da ME (BEAUMONT et al., 2008; HENG; de LEON, 2009).

Da mesma forma, a normalização da hiperreflexia nos circuitos espinais após LME em ratos tem sido relatada em estudos eletrofisiológicos com o uso de exercícios cíclicos em bicicleta estacionária como terapia físico-motora (SKINNER et al., 1996; REESE et al., 2006; YATES et al., 2008b). Nestes trabalhos, os animais são submetidos ao exercício passivo dos MPs uma semana após a completa TME, ou seja, ainda na fase aguda, antes do desenvolvimento da hiperreflexia. De forma similar, Côté et al. (2011) relataram normalização da hiperreflexia após completa LME em ratos submetidos tanto ao treino em

bicicleta quanto ao treino de marcha em esteira. Nossos resultados estão em conformidade com estes estudos e mostraram que o treino de marcha, com início 5 dias após a LME, ou seja, antes do aparecimento da hiperreflexia, é capaz de promover a normalização da resposta reflexa.

Nossos resultados (Capítulo 1) mostraram o treino de marcha por 9 semanas preveniu e/ou reverteu a atrofia somática dos MN alfa, a perda sináptica e a inibição da atividade da bomba de Na+,K+-ATPase na região lombar (L5) após completa TME. Além disso, as respostas obtidas no reflexo flexor de retirada foram positivamente correlacionadas com a atividade da bomba iônica de Na+,K+-ATPase.

A manutenção do gradiente eletroquímico através da membrana plasmática, incluindo a dos MNIs, é regulada pela atividade da bomba de Na+,K+-ATPase. A atividade normal desta enzima contribui para a manutenção da transmissão sináptica e do potencial de membrana celular. A inibição da atividade desta bomba iônica leva a uma despolarização e significativo aumento na excitabilidade neuronal (PHILLIS, 1992; VAILLEND et al, 2002). A despolarização e hiperexcitabilidade motoneuronal é correlacionada com aumento da hiperreflexia neuromuscular (BOULENGUEZ et al., 2010). Desta forma, a atenuação da redução na atividade da Na+,K+-ATPase após o treino de marcha em esteira pode em parte estar envolvido na normalização da atividade reflexa neuromuscular, como mostrado pelos resultados deste trabalho. Este pensamento é reforçado pela correlação positiva entre estas variáveis. Em concordância com nossa hipótese, Boulenguez et al. (2010) mostrou que a alteração do gradiente eletroquímico em MN produz alterações no reflexo H que são compatíveis com as alterações eletroneuromiográficas observadas na espasticidade após lesões medulares.

Além disso, mostramos (Artigo 2) que este treino é capaz de controlar parcialmente a hipotrofia do músculo sóleo de forma positivamente correlacionada ao aumento na expressão de BDNF nesta estrutura. Um grande número de estudos, incluindo o nosso, vem mostrando que treinamento motor intensivo possui intrínseca capacidade de aumentar a produção de neurotrofinas no tecido neuromuscular. As neurotrofinas desempenham um papel chave na regulação da sobrevivência e metabolismo celular, na síntese proteica e plasticidade sináptica (SCHMELZLE; HALL, 2000; OLDHAM; HAFEN, 2003; JOHNSON-FARLEY et al., 2007, YING et al., 2005; 2008). Esta produção aumentada de neurotrofinas vem sendo correlacionadas com as alterações plásticas neuromusculares e recuperação funcional.

A expressão do BDNF, Neurotrofina 3 (NT-3) e seus receptores tirosina cinase (TrkB e TrkC, respectivamente) na ME e músculo sóleo de ratos intactos aumenta com treinamento

locomotor intenso (GÓMEZ-PINILLA et al. 2001; SKUP et al. 2002; YING et al., 2003). Além disso, após lesões moderadas da ME, o treinamento locomotor promove recuperação dos níveis lombares e muscular de BDNF (HUTCHINSON et al., 2004; YING et al., 2005). Em resumo, o aumento na produção destes fatores neurotróficos associados ao crescimento/plasticidade fortalecem o conceito de que o treinamento reabilitativo promove plasticidade neuromuscular no nível molecular, e que este provavelmente ocorre de uma maneira dependente da atividade motora.

No estudo apresentado no Artigo 3, relatamos os efeitos do transplante de GEO e sua combinação com o treino de marcha iniciado na fase aguda após a LME. Nossos resultados mostram que o transplante melhora o desempenho funcional no teste locomotor em campo aberto. Além disso, o treino de marcha mostrou ser capaz de potencializar este efeito, pois os animais treinados mostram um maior aumento na atividade motora dos MPs. Os escores da escala BBB nos animais transplantados e treinados foram significativamente maiores do que os dos animais controle lesionados não treinados na 4ª e 10ª semanas após a LME. Enquanto que nos animais que somente receberam o transplante de GEO, os escores do BBB foram significativamente maiores do que os dos animais lesionados apenas na 10ª semana após a lesão/transplante.

Neste contexto, estudos com transplante agudo de GEO após LME vêm mostrando importantes e promissores resultados, especialmente os relacionados aos ganhos funcionais (RAMÓN-CUETO et al., 2000; LU et al., 2001; LÓPEZ-VALES et al., 2006a; TOFT et al., 2007; AOKI et al., 2010). Da mesma forma que em nosso estudo, utilizando-se da escala BBB, López-Vales et al. (2006a) e Aoki et al. (2010) mostraram uma progressiva melhora nos movimentos dos MP após transplante de GEO em ratos.

Embora o potencial terapêutico da GEO tenha se solidificado na última década, a recuperação funcional ainda é limitada, em especial nas lesões completas da ME. Desta forma, a combinação de terapias reconhecidamente benéficas, tais como o treino de marcha e o transplante de GEO, realizado em nosso estudo, vem despertando a curiosidade dos neurocientistas. Muito pouco se sabe sobre esta combinação terapêutica, Kubasak et al. (2008) reportaram que o treino de marcha iniciado um mês após a completa LME e transplante de GEO resulta em uma maior recuperação funcional. Nosso experimento foi realizado para estudar se o inicio do treino em uma fase mais aguda também poderia potencializar os benéficos efeitos da GEO. Tendo em vista nossos estudos anteriores (Artigos 1 e 2) que mostraram que o início agudo do treino específico da tarefa de marcha melhora a função sensório-motora dos MPs em animais paraplégicos, acreditávamos que intervenções na fase

inicial seriam cruciais para o sucesso da reabilitação. Esta hipótese se consolidou com os resultados positivos da combinação do treino de marcha com o transplante de GEO na função sensório-motora dos MPs dos animais lesionados.

Um dos mecanismos mais comumente sugeridos como uma explicação para a recuperação funcional após LME e transplante de GEO é a regeneração axonal. Entretanto, há grande discordância na literatura sobre a extensão desta regeneração. Enquanto alguns estudos relatam regeneração axonal cruzando a lesão e estendendo-se por longas distâncias (RAMÓN-CUETO et al., 2000; LU et al., 2001), outros mostram regeneração restrita ao local do transplante (RAMER et al. 2004a;b). Nós estudamos a regeneração axonal após LME e transplante de GEO através da marcação imunoistoquímica para serotonina (5-HT) e proteína associada ao crescimento neurítico (GAP-43). As fibras serotonérgicas na ME tem origem em núcleos do tronco encefálico (HOLSTEGE; KUYPERS, 1987) e podem modular os circuitos locomotores na medula espinal (BARBEAU; ROSSIGGNOL, 1990), tendo assim, grande importância para a tarefa de reabilitação estudada (marcha).

Nossos resultados mostraram apenas uma baixa 5-HT imunorreatividade na região caudal à lesão após o transplante de GEO nos animais treinados ou não. A análise da densitometria óptica revelou um aumento na imunoreatividade a 5-HT na região cranial à lesão em ambos os grupos. Entretanto, nos animais transplantados que realizaram o treino este aumento foi significativamente maior do que nos animais com LME sem tratamento. Isto sugere um aumento na tentativa de regeneração das fibras serotonérgicas nestes animais transplantados, potencializada pelo treinamento físico-motor.

Concomitantemente, nossos resultados falham em mostrar aumento na imunoreatividade à proteína GAP-43. Embora tenha ocorrido um leve aumento, tanto no coto cranial quanto no caudal à lesão nos animais transplantados/treinados, nenhuma significância foi evidenciada na análise estatística. Estes resultados nos fornecem embasamento para sugerir que os ganhos funcionais alcançados (relatados no Artigo 3) pela terapia combinada de transplante da GEO com o treino de marcha não decorram da regeneração axonal através da lesão em nosso estudo. De forma similar, Guest et al. (2008) após transplantar GEO de primatas em ratos, mostraram importantes ganhos funcionais (escores mais elevados na escala BBB) nos animais transplantados. Este estudo também falhou em mostrar significativa regeneração axonal, estudada pela marcação das fibras serotonérgica e da via corticoespinal, através da lesão. Este resultado sugere não haver correlação entre a melhora da função e a regeneração axonal. Além disso, os autores realizaram uma nova TME ao final do

experimento que não levou a redução nos escores da escala BBB. Assim, a melhora funcional após o transplante, não ocorreu pela regeneração das vias motoras interrompidas pela lesão.

Neste contexto, recentes estudos têm relatado que a GEO pode participar da recuperação aos LME através de outros fatores, entre os quais destacamos a melhora na vascularização e a produção de fatores neurotróficos (WOODHALL et al., 2001; PASTRANA et a., 2007; LOPEZ-VALES et al., 2004; RAMER et al., 2004a, b) que também podem ter influência do treinamento físico. Assim, acreditamos que haja potencialização dos efeitos GEO com o treino reabilitativo iniciado agudamente, e que este resultado possa acontecer pela somação desses fenômenos biológicos.

Além destes fatores, o treino específico da tarefa de marcha na esteira consiste de movimentos alternados que ritmicamente "estiram e encurtam" os músculos dos MPs, reforçando um padrão de movimentos locomotores gerados pelas redes neuronais lombares (o GPC da locomoção) que, ativadas pela retroalimentação (*feedback*) oriunda das aferências sensoriais proprioceptivas e cutâneas, são capazes de desencadear o padrão de marcha. Esta ativação pode levar a um reforço ou uma remodelação dos circuitos neurais envolvidos com o programa locomotor mesmo na ausência dos comandos motores descendentes. Esta idéia ganha força com resultados do Artigo 1 onde um significativo aumento na proteína sináptica sinaptofisina foi mostrado após o treino de marcha em animais com completa LME. Havendo, assim, a indicação de um aprendizado motor dependente da atividade que pode ocorrer mesmo nos circuitos espinais isolados (para uma maior revisão veja FOUAD; TETZLAFF, 2010).

# 8 CONCLUSÕES E PERSPECTIVAS

Os resultados apresentados nesta Tese nos permitem concluir que:

- Após completa LME em ratos adultos, o treino específico da tarefa de marcha em esteira, iniciado precocemente, promove significativo aumento nos movimentos dos membros paralisados destes animais. Além disso, esta terapia de reabilitação físico-motora mostrou ser capaz de propiciar a normalização do reflexo flexor de retirada nos animais treinado;
- Paralelamente aos ganhos sensório-motores, o treino de marcha foi capaz de impedir e/ou reverter a atrofia dos MN alfa, a perda sináptica e a redução na atividade da enzima Na+,K+-ATPase na região lombar de L5 induzidas pela LME. Além disso, a paralisia muscular leva a uma severa atrofia do músculo sóleo, que foi parcialmente revertida e/ou impedida pelo treinamento motor de uma maneira dependente da atividade e positivamente correlacionada com a expressão do BDNF pelo mesmo músculo;
- O início precoce do treino de marcha potencializa os efeitos benéficos do transplante de GEO na recuperação sensório-motora dos MP. Embora esta combinação de terapias tenha aumentado a tentativa de regeneração axonal serotonérgica (maior imunoreatividade na região cranial próxima a borda da lesão) a regeneração no local da lesão e no coto caudal não foi significativa. Esses resultados sugerem que o treinamento possa ter potencializado os efeitos da GEO por mecanismos plásticos dependentes da atividade nos próprios circuitos espinais isolados;
- Desta forma, o uso do treino de marcha em esteira com suporte parcial do peso corporal mostrou-se potencialmente seguro e benéfico como uma terapia de reabilitação físico-motora após LME e transplante de GEO em animais e incentiva a aplicação destas terapias em humanos.

Colocamos como perspectivas futuras:

- Estudar o padrão temporal da redução da atividade da enzima Na+,K+-ATPase e sua participação no desenvolvimento da hiperreflexia após LME;
- Analisar a expressão da proteína mTOR no músculo sóleo e testar a hipótese sugerida no Capitulo 2 sobre sua participação no controle do trofismo muscular dependente da atividade após LME;

- Realizar um estudo ultraestrutural no local da lesão/transplante de GEO para verificar a presença de fibras mielínicas, bem como a interação morfológica entre as células GEO e as fibras em regeneração.
- Realizar uma marcação imunoistoquímica da proteína glial fibrilar ácida (GFAP) no local da lesão. Esta imunomarcação nos permitirá delimitar precisamente as bordas da lesão e realizar a quantificação do volume de tecido lesionado. Estas análises já estão em andamento e pretendemos juntar estes resultados ao artigo do Capítulo 3 antes de submetê-lo à publicação.
- Estudar as alterações morfológicas e bioquímicas nos segmentos lombares dos animais com LME, transplantados com GEO e submetidos ao treino de marcha, para mostrar a plasticidade induzida pelo estímulo físico na ME. As amostras para a realização deste estudo encontram-se guardadas à -80°C em freezer e serão processadas no decorrer deste ano. Estes resultados darão origem a um novo artigo.

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