UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

TRANSPLANTE DE LÂMINA PRÓPRIA OLFATÓRIA E RESPIRATÓRIA APÓS LESÃO MEDULAR EM RATOS: IMPLICAÇÕES SOBRE A RECUPERAÇÃO LOCOMOTORA, HIPERREFLEXIA E REGENERAÇÃO AXONAL

TESE DE DOUTORADO

LÍGIA ALINE CENTENARO

Porto Alegre
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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Neurociências, da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de Doutor em Neurociências.

Orientadora: Profa. Dra. Matilde Achaval Elena

Porto Alegre
2012

"Ao meu querido irmão, Alberto Frederico Centenaro, a quem nunca deixaremos de lembrar e amar."

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A vocês, *muito obrigada*!

"O único lugar aonde o sucesso vem antes do trabalho é no dicionário."

Albert Einstein

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LISTA DE ABREVIATURAS

5-HT	Serotonina
AMPc	Adenosina 3',5'-Monofosfato cíclico
ASIA	American Spinal Cord Injury Association
BDNF	Fator Neurotrófico Derivado do Encéfalo
BO	Bulbo Olfatório
Ca^{2+}	Íon Cálcio
CGRP	Peptídeo Relacionado ao Gene da Calcitonina
CNTF	Fator Neurotrófico Ciliar
EO	Epitélio Olfatório
GAP-43	Proteína Associada ao Crescimento 43
GDNF	Fator Neurotrófico Derivado de Células Gliais
GEO	Glia Embainhante Olfatória
GFAP	Proteína Glial Ácida Fibrilar
LP	Lâmina Propria
MAG	Glicoproteína Associada à Mielina
MNI	Motoneurônio Inferior
MNS	Motoneurônio Superior
NGF	Fator de Crescimento do Nervo
OMgP	Glicoproteína da Mielina de Oligodendrócitos
p75NGFR	Receptor de Neurotrofinas P75
SNC	Sistema Nervoso Central
VEGF	Fator de Crescimento Vascular Endotelial

Abreviaturas referentes aos artigos I e II

2WDC	Grupo submetido ao transplante de lâmina propria respiratória 2 semanas após a lesão
2WDT	Grupo submetido ao transplante de lâmina propria olfatória 2 semanas após a lesão
4WDC	Grupo submetido ao transplante de lâmina propria respiratória 4 semanas após a lesão

4WDT	Grupo submetido ao transplante de lâmina propria olfatória 4 semanas após a lesão
AC	Grupo submetido ao transplante de lâmina propria respiratória imediatamente
ANOVA	Análise de Variância
AT	Grupo submetido ao transplante de lâmina propria olfatória imediatamente
BB	Escala locomotora de Basso, Beattie e Bresnahan
CC	Cavidades Císticas
COBEA	Colégio Brasileiro de Experimentação Animal
DAB	Diaminobenzidina
FG	Fluorogold
H_2O_2	Peróxido de Hidrogênio
HBSS	Solução Salina Balanceada de Hank
LVe	Núcleo Vestibular Lateral
M1/M2	Córtex Motor Primário e Secundário
MdD/MdV	Campo Reticular Medular Ventral e Dorsal
OB	Bulbo Olfatório
OD	Densitometria Óptica
OEC	Células Gliais Embainhantes Olfatórias
OLP	Lâmina Própria Olfatória
PB	Tampão Fosfato
PBS	Tampão Fosfato Salino
PBS-TX	Solução de tampão fosfato salino e triton X-100
PnO/PnC	Núcleo Reticular Pontino Rostral e Caudal
Ra	Núcleo da Rafe
RLP	Lâmina Própria Respiratória
S1	Córtex Somatossensorial Primário
SCI	Lesão da Medula Espinal
SpVe	Núcleo Vestibular Espinal
VEGF	Fator de Crescimento Vascular Endotelial

RESUMO

Lesões medulares resultam em uma perda irreversível da função abaixo do sítio da lesão. Esses comprometimentos são permanentes e ocorrem devido à perda de neurônios localmente e também dos tratos axonais ascendentes e descendentes da medula espinal. Na tentativa de criar um ambiente favorável à regeneração dos axônios lesionados, células da glia embainhante olfatória (GEO) vêm sendo transplantadas como estratégia de tratamento em animais submetidos a diferentes modelos experimentais de lesões medulares. Entretanto, um consenso sobre o potencial terapêutico desse tipo de transplante celular ainda precisa ser estabelecido. O objetivo do presente trabalho foi verificar a eficácia do transplante de lâmina própria (LP) olfatória (que possui células da GEO) e de LP respiratória (desprovido de células da GEO), quando implantadas imediatamente, 2 ou 4 semanas após a realização da transecção da medula espinal. Doze semanas após a realização dos implantes, os animais que receberam LP olfatória e respiratória apresentaram uma melhora sutil na função motora dos membros posteriores. Além disso, o transplante de LP olfatória quando realizado imediatamente após a lesão reduziu a hiperatividade do reflexo de retirada, enquanto o implante desse tipo de tecido 4 semanas pós-lesão produziu uma discreta depressão dependente de frequência do reflexo de Hoffman (um análogo elétrico do reflexo monossináptico de estiramento). Nas diferentes janelas terapêuticas utilizadas, o transplante de ambos os tipos de LP produziu resultados comparáveis em relação à preservação do tecido medular, brotamento de neuritos e regeneração de fibras mielínicas no local da lesão, indicando que o tempo decorrido antes da realização dos transplantes não parece limitar os efeitos regenerativos. Todavia, as fibras mielínicas observadas no sítio da transecção nos animais que receberam LP olfatória 2 e 4 semanas pós-lesão possuíam menor área, diâmetro e espessura da bainha de mielina quando comparados aos animais que receberam LP respiratória nesses mesmos períodos. O transplante imediato de LP olfatória e respiratória também favoreceu o restabelecimento das conexões entre as fibras axonais lesionadas com núcleos do tronco encefálico e até mesmo com a região do córtex somatossensorial, como indicado pela presença de neurônios nessas regiões marcados positivamente com um marcador axonal retrógrado. Um número maior de fibras positivas para 5-HT foi observado no coto proximal dos grupos transplantados com ambos os tipos de LP em comparação às regiões da lesão e do coto caudal. Fibras positivas para CGRP estavam presentes em número considerável no local da lesão. A recuperação locomotora e a regeneração axonal no local da lesão foram limitadas e comparáveis entre os grupos transplantados nos diferentes tempos com LP olfatória e respiratória, sugerindo que esses resultados não estão exclusivamente relacionados à presença de células da GEO nos enxertos utilizados. Um melhor entendimento sobre o potencial restaurativo desse tipo de transplante é necessário a fim de justificar a aplicação dessa terapia em humanos.

Palavras-chave

Lesão medular; células gliais embainhantes olfatórias; recuperação funcional; hiperreflexia; regeneração axonal.

ABSTRACT

Spinal cord injury (SCI) results in an irreversible loss of function below the injury site. These permanent disabilities occur due to local neuronal death and loss of ascending and descending axons in the spinal cord. In attempt to create a favorable environment for the re-growth of injured axons, olfactory ensheathing cells (OECs) have been transplanted as a treatment strategy in animals submitted to different experimental models of SCI. However, a consensus on the efficacy of this cellular transplantation has yet to be reached. The main focus of the present study was explore the efficacy of olfactory lamina propria (OLP, graft containing OECs) or respiratory lamina propria (RLP, graft without OECs) when transplanted immediately, 2-week or 4-week after spinal cord transection. After 12 weeks of transplantation, animals with OLP and RLP grafts showed a subtle hindlimb motor improvement. Furthermore, the transplantation of OLP when performed immediately after injury reduced the withdrawal reflex over-responsiveness, while the implantation of this tissue 4 weeks post-injury produced a discrete frequency-dependent habituation of the Hoffman reflex (the electrical analogue of the classic tendon jerk reflex). In all therapeutic windows used, both lamina propria grafts produced comparable results for tissue sparing, fibers sprouting and re-growth of myelinated fibers at the lesion site, indicating that delayed transplantation approach does not seem to limit the regenerative effects. However, the myelinated fibers observed at the transection site of animals that received OLP 2 or 4 weeks after injury had a smaller myelinated fiber area, diameter and myelin sheath thickness when compared to those animals transplanted with RLP grafts in the same periods. The immediate transplantation of OLP and RLP also foster limited supraspinal axonal re-connection as shown by the presence of neurons stained by retrograde tracing in brainstem nuclei and in the somatosensory cortex. A larger number of 5-HT positive axons were found in the cranial stump of both lamina propria groups compared to the lesion and caudal regions. CGRP positive axons were present in considerable numbers at the SCI site. The locomotor recovery and axon reparative effects were limited and similar between groups transplanted at different times with OLP and RLP, suggesting that these results could not be exclusively related to OECs. In conclusion, a greater understanding of the restorative potential of these tissue grafts is necessary to strengthen the rationale for application of this treatment in humans.

Keywords

Spinal cord injury; olfactory ensheathing cells; functional recovery; hyperreflexia; axonal regeneration.

1 INTRODUÇÃO

A lesão medular é considerada uma das mais devastantes doenças encontradas na prática clínica (AO et al., 2007). Aproximadamente 2,5 milhões de pessoas vivem com lesão medular e estimativas recentes mostram que cerca de 130.000 novos casos são registrados ao ano. Homens em torno dos 30 anos são os mais acometidos, com a maior incidência dessa patologia ocorrendo aos 19 anos de idade (RICK HANSEN FOUNDATION, 2006). Devido aos crescentes avanços nos cuidados médicos emergenciais, a taxa de mortalidade dos pacientes com lesão medular é relativamente baixa (menor do que 5%) (AO et al., 2007). Além disso, a expectativa de vida desses indivíduos é considerável, com o tempo médio de sobrevida previsto para 38 anos após a lesão (McCOLL et al., 1997).

Lesões da medula espinal provocam um grande impacto na qualidade de vida dos pacientes, pois resultam em comprometimentos funcionais permanentes abaixo do nível da lesão. Esses déficits incluem a perda da sensação e do movimento, perda do controle vesical e intestinal, da função sexual, da regulação da temperatura e da pressão arterial (RAISMAN, 2007). Dessa forma, esses pacientes necessitam do sistema de saúde não somente durante a fase hospitalar inicial, mas também nos primeiros anos de vida pós-lesão (WYNDAELE e WYNDAELE, 2006). Ilustrações disso incluem as frequentes internações hospitalares decorrentes de complicações secundárias à lesão medular, como as infecções do trato urinário e úlceras de pressão, necessidade de serviços de assistência domiciliar e muitas vezes, auxílio psicológico (DRYDEN et al., 2004). Além disso, como a maioria dos pacientes sofre lesões medulares durante a sua idade média produtiva, eles passam a necessitar também da ajuda do sistema de previdência social (WYNDAELE e WYNDAELE, 2006). Os custos com cuidados clínicos durante longos períodos de tempo somam mais de dez milhões de dólares ao ano, gerando um importante impacto sócio-econômico (RICK HANSEN FOUNDATION, 2006).

1.1 Etiologia

Diversas condições patológicas podem afetar o funcionamento da medula espinal, incluindo desde a lenta evolução de doenças crônicas até insultos agudos ocasionados por traumas físicos ou acidentes vasculares (FREDERICKS e SALADIN, 1996). Lesões medulares de origem não-traumática estão frequentemente associadas a tumores, isquemia

vascular, doenças desmielinizantes, alterações degenerativas da coluna vertebral, infecções e doenças congênitas (McKINLEY, SEEL e HARDMAN, 1999; NEW, RAWICKI e BAILEY, 2002). No caso de lesões medulares ocasionadas por trauma, 38% dos casos ocorrem devido a acidentes automotivos, 26% devido a agressões com armas de fogo ou outros atos de violência, 22% devido a quedas (especialmente em pessoas idosas) e atividades recreacionais esportivas somam 7% (DOBKIN e HAVTON, 2004). Aproximadamente 50% das vítimas com lesão da medula espinal de origem traumática apresentam perda sensório-motora completa distalmente ao nível da lesão medular e menos de 5% desses pacientes terão um bom prognóstico funcional (DOBKIN e HAVTON, 2004).

1.2 Mecanismos Fisiopatológicos

A medula espinal é constituída por vias ascendentes, responsáveis pela condução das informações provenientes de receptores sensoriais e interneurônios ao encéfalo, e por vias descendentes, que enviam comandos motores e sinais modulatórios para a transmissão das informações sensitivas (PAXINOS, 2004) (Figura 1). Essa arquitetura normal da medula espinal pode ser perturbada por uma contusão, compressão, distração, laceração, transecção ou pela associação de um ou mais desses mecanismos de lesão (DUMONT et al., 2001). Consequentemente pode ocorrer à destruição da substância cinzenta dos cornos ventral e dorsal em um ou mais níveis espinais, associada à interrupção parcial ou completa dos tratos ascendentes e descendentes na substância branca. Os cotos distais dos axônios lesionados sofreram o processo de degeneração Walleriana. Além disso, raízes dorsais e ventrais podem sofrer avulsão, levando ao acometimento de outros segmentos medulares e aumento do tamanho da lesão (FREDERICKS e SALADIN, 1996; DOBKIN e HAVTON, 2004).

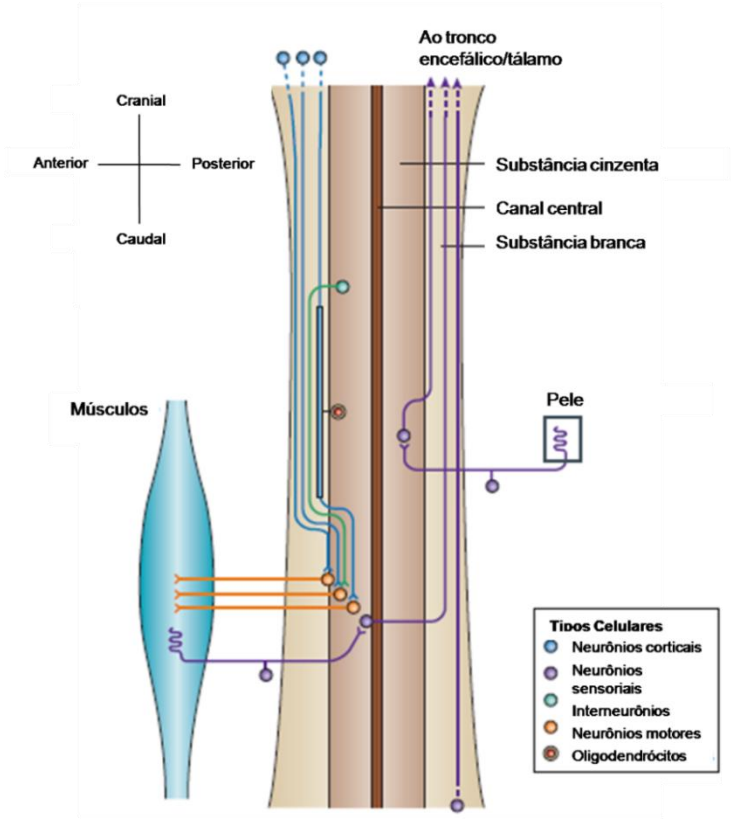

Figura 1 – Representação esquemática da medula espinal intacta em corte longitudinal. Neurônios sensoriais primários enviam seus axônios através do sistema nervoso periférico e, subsequentemente, projetam-se até regiões supra-espinais através dos axônios de neurônios de segunda ordem na substância branca da medula espinal. Axônios descendentes do córtex cerebral e tronco encefálico projetam-se para os neurônios motores na substância cinzenta da medula espinal que, por sua vez, enviam axônios através do sistema nervoso periférico para os órgãos alvo, incluindo os músculos. Oligodendrócitos são os responsáveis pela mielinização dos tratos axonais ascendentes e descendentes (Adaptado de THURET, MOON e GAGE, 2006).

O dano inicial à medula, também chamado de lesão primária, causa hemorragia, edema e morte necrótica das células residentes no sítio da lesão (OYINBO, 2011). Eventos secundários que incluem inflamação, respostas imunológicas, formação de radicais livres, excitotoxicidade e morte celular programada (apoptose) seguem-se aos efeitos agudos produzidos pela lesão (OYNBO, 2011). A resposta inflamatória pós-traumática é gerada principalmente por células da microglia ativadas, além de granulócitos, monócitos e linfócitos derivados do sangue que migraram através da barreira hemato-encefálica danificada (SCHWAB e BARTHOLDI, 1996). A isquemia no local da lesão provoca uma depleção metabólica que leva a uma despolarização da membrana dos neurônios e células gliais. Tal mudança no gradiente eletrolítico das células produz a ativação de canais de cálcio (Ca^{2+}) pré-sinápticos dependentes de voltagem e consequente liberação de aminoácidos excitatórios (glutamato, aspartato, homocisteína, etc.) para o espaço extracelular (LEE et al., 1999; SCHWAB et al., 2006). Espécies reativas de oxigênio também são produzidas no local da lesão e atuam como moléculas sinalizadoras que desencadeiam a progressão de ambos,

inflamação pós-traumática e apoptose (SCHWAB et al., 2006). Esses mecanismos de lesão secundários estão interligados em um ciclo de auto-propagação que pode estender-se de minutos até semanas após a ocorrência do evento primário, agravando a perda do tecido medular (OYINBO, 2011) (Figura 2).

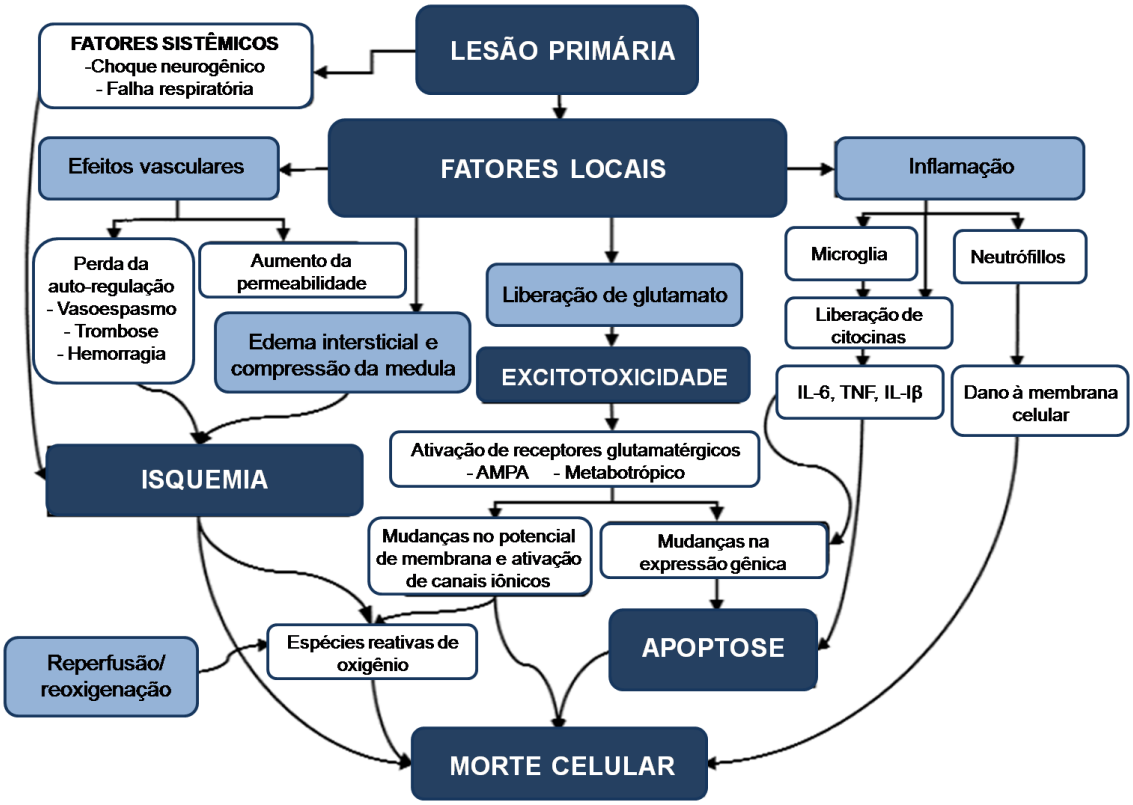

Figura 2 – Ilustração mostrando os mecanismos de lesão secundária subjacentes a uma lesão medular. O papel dos distúrbios vasculares, edema intersticial, compressão da medula, eventos excitotóxicos e inflamação são representados (Adaptado de DUMONT et al., 2001).

Como resultado final, ocorre a formação de cistos ou cavidades no sítio da lesão que é circundado por uma cicatriz glial. Astrócitos reativos e células microgliais ativadas são os componentes celulares mais encontrados nessa cicatriz que, além de produzir moléculas inibitórias à regeneração axonal, também forma uma barreira mecânica que impede o crescimento dos axônios (NIETO-SAMPEDRO, 2003; OUDEGA, 2007). Outras moléculas associadas à mielina como a NOGO-A, a glicoproteína associada à mielina (do inglês, *MAG*) e a glicoproteína da mielina de oligodendrócitos (do inglês, *OMgP*) também são liberadas no local da lesão e inibem a regeneração axonal (FILBIN, 2003; AO et al., 2007). Nesse contexto, os axônios falham em tornar-se parte integrante de novos ou antigos circuitos envolvidos na função sensório-motora (SILVER e MILLER, 2004; OUDEGA, 2007) (Figura 3).

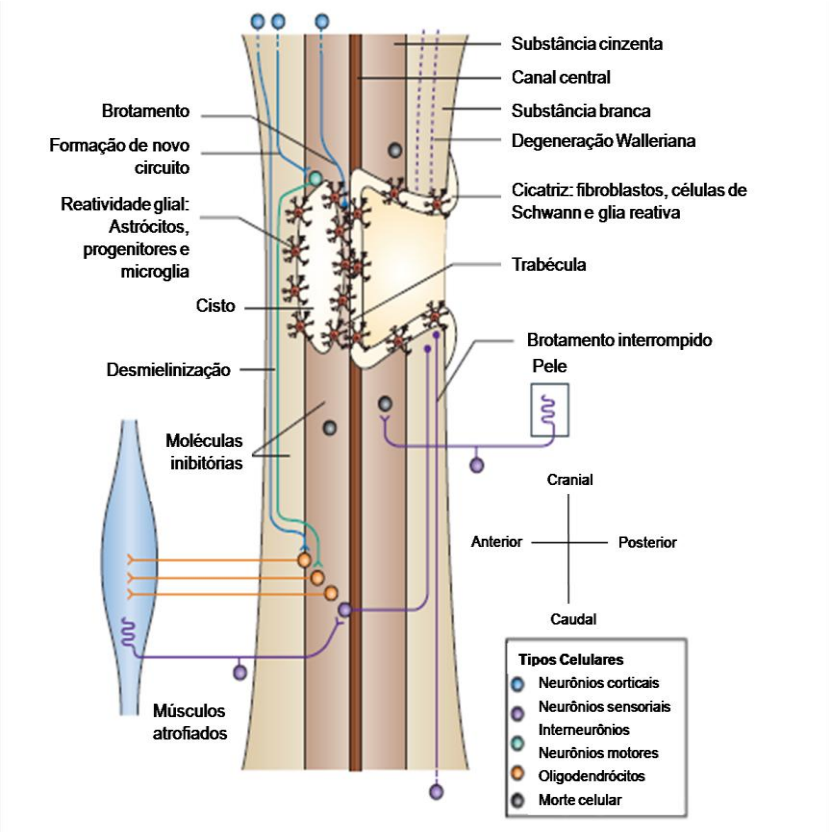

Figura 3 – Representação esquemática da medula espinal em corte longitudinal após uma lesão. Parte das células morre imediatamente, bem como progressivamente, após uma lesão medular. Uma cicatriz glial composta por astrócitos, microglia e células progenitoras desenvolve-se ao redor dos cistos e cavidades formadas no local da lesão. Debris celulares são fagocitados por macrófagos. Alguns axônios formam novos circuitos com neurônios motores por meio de interneurônios preservados. Muitos tratos axonais ascendentes e descendentes são interrompidos e falham em regenerar-se por longas distâncias. Células de Schwann do sistema nervoso periférico promovem discreta remielinização axonal, enquanto os músculos denervados sofrem atrofia muscular (Adaptado de THURET, MOON e GAGE, 2006).

1.3 Classificação dos Déficits Neurológicos

O grau de severidade das complicações decorrentes de uma lesão medular depende dos tratos axonais acometidos e da extensão de seu comprometimento (Figura 4). Segmentos da medula espinal inervam uma área da pele específica (dermátomo) e um grupo de músculos específicos (miótomo). Assim, uma avaliação precisa da sensibilidade e da força muscular nos diferentes níveis medulares determina o nível da lesão e dos comprometimentos funcionais associados (UMPHRED e CARLSON, 2007).

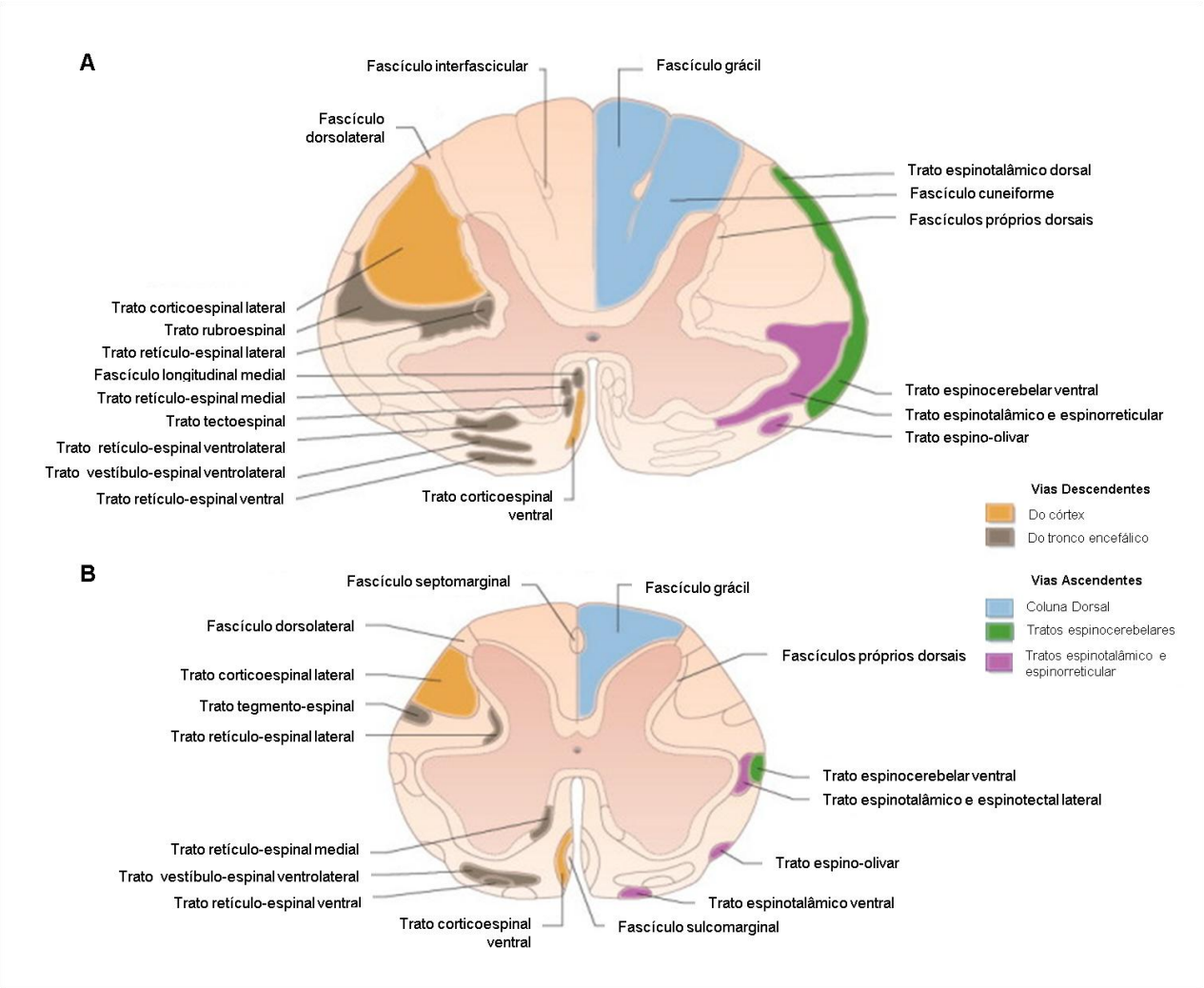

Figura 4 – Desenhos esquemáticos representando a posição aproximada dos tratos axonais aos níveis cervical (A) e lombar (B) da medula espinal em humanos (Adaptado de STANDRING, 2008).

Lesões ao nível cervical determinam uma distribuição topográfica de déficits motores e/ou sensoriais que envolvem os membros superiores, o tronco e os membros inferiores, sendo denominada como tetraplegia ou quadriplegia (ILHA, 2011). A extensão do comprometimento nos membros superiores em um paciente com tetraplegia pode variar desde a discreta perda de movimentos de dedos no caso de uma lesão em nível de C8 ou a perda completa da função dos membros superiores a partir de lesões em C1 a C4 (UMPHRED e CARLSON, 2007). Por outro lado, o termo paraplegia refere-se ao comprometimento da função motora e/ou sensorial ocasionada por uma lesão da medula espinal em níveis torácico, lombar ou sacral. Na paraplegia, a função sensório-motora dos membros superiores é preservada e, dependendo do nível de lesão, o tronco e os membros inferiores são funcionalmente comprometidos (MAYNARD et al., 1997) (Figura 5). A Associação Americana de Lesões da Medula Espinal (*American Spinal Cord Injury Association, ASIA*)

desenvolveu uma escala que permite a realização de avaliações padronizadas do nível espinal acometido em diferentes pacientes. De acordo com essa escala, o termo "nível de lesão" refere-se ao segmento medular no qual a função permaneceu inalterada (UMPHRED e CARLSON, 2007).

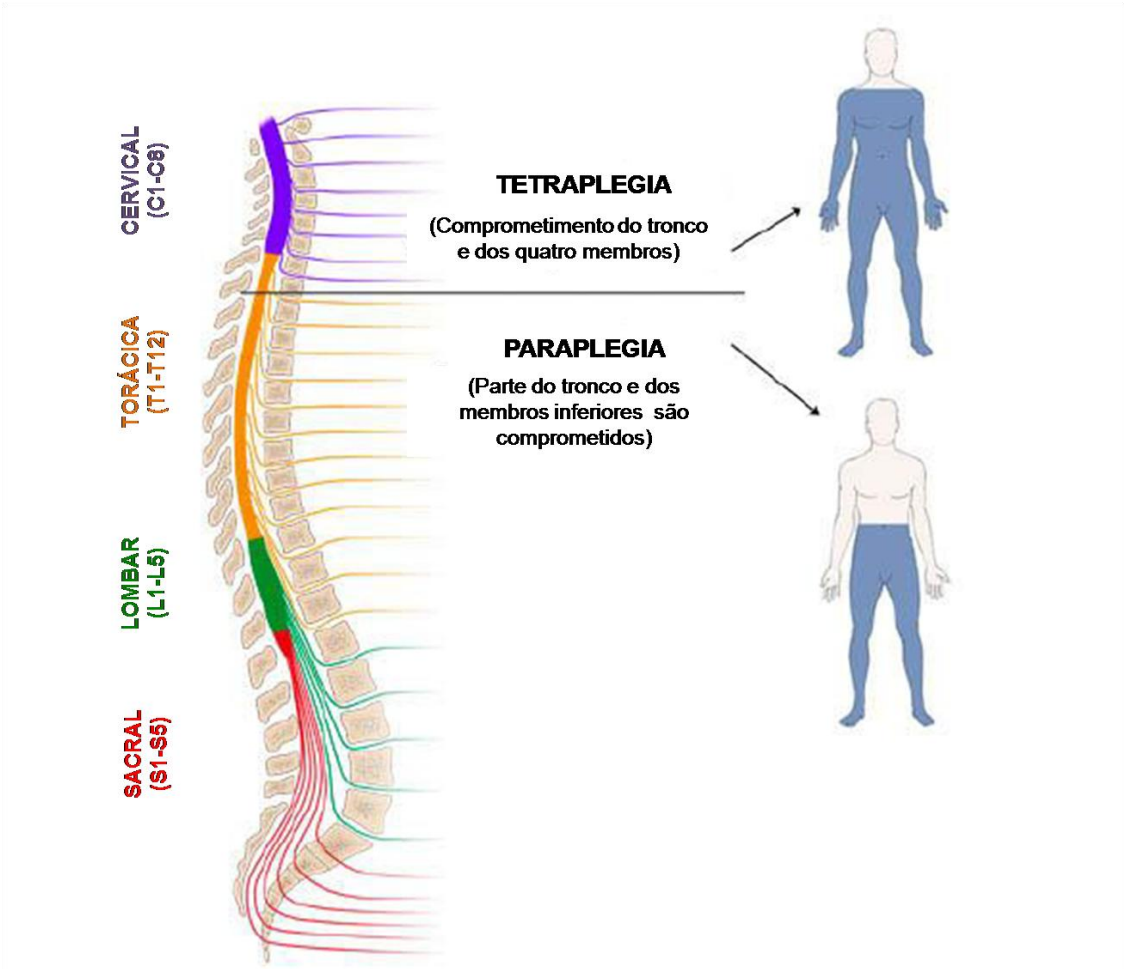

Figura 5 – Diferentes níveis de lesão e extensão dos déficits funcionais (paraplegia ou tetraplegia) observados em humanos após uma lesão medular (ADAPTADO de ILHA, 2011).

Em relação ao grau de extensão, as lesões da medula espinal podem ser definidas com sendo completas e incompletas funcionalmente. O termo lesão completa descreve a ausência de funções sensório-motoras abaixo do nível medular acometido, enquanto lesões incompletas referem-se à preservação parcial de algumas dessas funções (ILHA, 2011). A escala funcional da ASIA define o grau de perda neurológica em 4 escores: A – completa perda sensório-motora caudalmente ao nível da lesão medular, incluindo ausência de sensação sacral; B – não há função motora, mas a função sensorial está preservada caudalmente ao nível da lesão medular; C – alguma função motora está preservada, mas a maioria dos músculos caudalmente a lesão medular apresentam menos de três pontos em uma escala de cinco pontos para avaliação da força muscular; e D – a maioria dos músculos caudalmente a

lesão medular apresentam três ou mais pontos na escala de força (THURET, MOON e GAGE, 2006). Em pacientes com lesões completas, pode existir uma zona de preservação parcial, a qual identifica o segmento localizado mais caudalmente à lesão onde alguma função sensorial ou motora está preservada. Por exemplo, se a lesão de um paciente é classificada como C5, ASIA A, mas ele apresenta sensibilidade alterada ao nível de T1, a zona de preservação parcial da função sensorial será considerada T1 (UMPHRED e CARLSON, 2007).

Segundo dados epidemiológicos, o grau de perda neurológica mais frequente é a tetraplegia incompleta (34,1%), seguida pela paraplegia completa (23%), tetraplegia completa (18,3%) e paraplegia incompleta (18,5%) (RICK HANSEN FOUNDATION, 2006).

1.4 Alterações Neuromusculares

Ao nível espinal primariamente acometido pela lesão, os músculos apresentam uma paralisia flácida característica de lesões do motoneurônio inferior (MNI) e decorrente do dano à substância cinzenta da medula espinal nesse local. Entretanto, caudalmente ao local inicial da lesão, a paralisia caracteriza-se pela lesão do motoneurônio superior (MNS) com aumento do tônus muscular e hiperreflexia (KAKULAS, 2004).

Devido ao desuso muscular provocado pela paralisia, os músculos esqueléticos atrofiam-se em aproximadamente 30-60%, dependendo do tipo muscular e da gravidade do acometimento do tecido nervoso. Essa perda de trofismo muscular é acompanhada pela redução na capacidade máxima de geração de força e endurance desses músculos. Fibras musculares lentas oxidativas (tipo I) sofrem alterações metabólicas e passam a apresentar características histoquímicas de fibras glicolíticas rápidas (tipo II). Ocorre um aumento na fatigabilidade do tecido muscular e alterações em suas taxas de contração e relaxamento (QIN, BAUMAN e CARDOZO, 2010). Assim, durante os primeiros seis meses após lesões da medula espinal, a composição corporal se deteriora com perda acentuada do tecido muscular e ósseo, associada ao ganho de tecido adiposo (WILMET et al., 1995; CASTRO et al., 2000).

A espasticidade, um sinal da síndrome do MNS, é observada em 80% dos pacientes com lesões medulares. Tal sinal é definido como um aumento do reflexo tônico de estiramento (tônus muscular) dependente da velocidade e exacerbação das respostas reflexas (REESE et al., 2005). A espasticidade também se caracteriza pela ativação de um conjunto de

unidades motoras sem o devido controle consciente, levando a produção de movimentos que não são habitualmente desempenhados. Dessa forma, quando um indivíduo com lesão medular incompleta tenta realizar um movimento, pouca ou nenhuma ativação pode ser observada nos músculos primariamente envolvidos com a ação desejada, enquanto outros músculos podem ser ativados de maneira errônea. Em alguns casos, músculos de ambos os lados do corpo podem ser ativados quando o paciente tenta mover apenas um de seus membros. Essas respostas anormais podem ocorrer quando um indivíduo não está tentando mover nenhum determinado grupo muscular ou articulação, isto é, a espasticidade ocorre espontaneamente (ROY e EDGERTON, 2012).

A exacerbação das respostas reflexas, também chamada de hiperreflexia, é um dos componentes da espasticidade que emerge ao longo do tempo. Assim, imediatamente após a uma lesão da medula espinal existe um período chamado de "choque medular", caracterizado pela perda dos reflexos tendinosos abaixo do nível de lesão, paralisia e flacidez muscular (YATES et al., 2008). Semanas depois, varias respostas reflexas tornam-se exacerbadas, incluindo o reflexo de retirada e os reflexos tendinosos (ELBASIOUNY et al., 2010). A fisiopatologia da hiperreflexia é desconhecida, embora vários mecanismos como a perda de inibição pré-sináptica (SCHINDLER-IVENS E SHIELDS, 2000), aumento no número de receptores pós-sinápticos ou na excitabilidade desses receptores (LITTLE et al., 1999), mudanças na excitabilidade dos motoneurônios (HECKMANN, GORASSINI e BENNETT, 2005), aumento nas aferências sinápticas e brotamento terminal (JANKOWSKA e HAMMAR, 2002) possam estar envolvidos.

Uma forma de se avaliar qualitativamente a hiperreflexia no âmbito experimental baseia-se na observação dos reflexos flexores de retirada (do inglês, *withdrawal reflexes*). Esses reflexos são mediados por fibras nervosas amielínicas nociceptivas C e fibras mielinizadas de pequeno calibre A delta, que agem sobre os motoneurônios. Fisiologicamente, os reflexos flexores de retirada limitam-se aos músculos responsáveis pela retirada de uma parte do corpo que ocasionalmente entra em contato com um estímulo lesivo. Entretanto, após uma lesão medular esses reflexos tornam-se difusos e hiperativos (WOLPAW e TENNISSEN, 2001). Outra ferramenta utilizada para se avaliar as mudanças nas funções reflexas é o registro eletromiográfico do reflexo de Hoffmann ou reflexo H, um análogo elétrico do reflexo monossináptico de estiramento (MISIASZEK, 2003; YABLON e STOKIC, 2004). O reflexo H é elicitado através da aplicação de um estímulo elétrico no nervo periférico que inerva o músculo no qual o reflexo será gravado. Um único estímulo gera uma onda M de curta latência, resultante da estimulação direta dos motoneurônios que

inervam esse músculo, e de uma onda H de maior latência, que é uma medida da atividade dos motoneurônios α ativados pelos aferentes Ia (GOZARIU et al., 1998). Em indivíduos normais, a amplitude dessa onda H diminui com a aplicação de estímulos de maior freqüência, enquanto a onda M permanece constante (LEE et al., 2005). Todavia, em pacientes com lesões da medula espinal essa diminuição na amplitude da onda H torna-se menos sensível a variações na frequência do estímulo, resultando em um aumento na razão H/M.

1.5 Modelos Animais

Estudos experimentais utilizando modelos animais são considerados um passo indispensável antes da aplicação clínica de uma determinada estratégia de tratamento, pois permitem a observação de suas repercussões *in vivo*. Devido a esse fato, os modelos animais de lesão medular estão cada vez mais sofisticados em sua reprodutibilidade e as análises das consequências sensoriais, motoras e vegetativas após a aplicação de uma intervenção tem se tornado mais precisas (MARQUES et al., 2009). Muitos laboratórios utilizam ratos como animais experimentais, devido à facilidade em sua manutenção e manejo. Além disso, esses animais são priorizados devido à similaridade entre as suas respostas histológicas frente a lesões do sistema nervoso central (SNC) quando comparadas aquelas observadas em outros mamíferos, incluindo os humanos (SANTOS-BENITO, MUÑOZ-QUILES e RAMÓN-CUETO, 2006).

Em relação aos diferentes modelos de lesões medulares adotados na prática experimental, podemos destacar três que são amplamente utilizados: compressão, contusão e transecção. Lesões por compressão são induzidas através da utilização de fórceps, clip de aneurisma modificado ou pela colocação de um determinado peso sobre a medula espinal exposta (FEHLINGS e TATOR, 1995). Também existem estudos nos quais a compressão é feita através da insuflação de um balão que foi previamente implantado no espaço subdural (MARTIN et al., 1992; ROSENZWEIG e MCDONALD, 2004).

Por outro lado, as lesões por contusão são realizadas através da aplicação de um impacto sobre a medula espinal exposta, utilizando equipamentos como o "*New York University impactor*" e o "*Infinite Horizons impactor*" (SCHEFF et al., 2003). Recentemente foi desenvolvido um aparato que utiliza grampos vertebrais para permitir a realização de uma lesão da medula espinal por contusão associada a luxação vertebral e distração em ratos (CHOO et al., 2009). Apesar de reproduzirem mais fidedignamente as lesões encontradas em

humanos, os métodos de contusão da medula frequentemente dificultam a interpretação dos resultados funcionais e morfológicos obtidos devido à plasticidade das fibras intactas (STEWARD, ZHENG e TESSIER-LAVIGNE, 2003; BAREYRE et al., 2004).

Modelos de transecção envolvem a abertura da dura-máter e secção de parte ou de toda a extensão da medula espinal, podendo ou não ser associados à sucção do tecido medular em casos onde porções da medula espinal precisam ser removidas (ROSENZWEIG e MCDONALD, 2004). Pesquisas com foco sobre a regeneração axonal utilizam principalmente a transecção completa da medula espinal como modelo experimental, pois permitirem ao experimentador um controle exato sobre a localização e a extensão da lesão (TAKAMI et al., 2002; FOUAD et al., 2005; CHOO et al., 2009). Assim, para que a eficácia de uma terapia seja determinada após uma transecção medular, as fibras axonais precisam atravessar o local da lesão, reinervar funcionalmente seus alvos e produzir uma adequada recuperação funcional. Todavia, as dificuldades em manter os ratos saudáveis após o emprego desse tipo de lesão têm persuadido muitos pesquisadores a testar seus paradigmas apenas durante curtos períodos de tempo pós-lesão (SANTOS-BENITO et al., 2006).

É importante salientar que a escolha do modelo experimental de lesão medular deve estar de acordo com as questões endereçadas em cada pesquisa, uma vez que os diferentes tipos de lesão disponíveis fornecem informações complementares a respeito do reparo da medula espinal (ROSENZWEIG e MCDONALD, 2004).

1.6 Estratégias de Tratamento

Até pouco tempo atrás, o tratamento após lesões medulares era baseado apenas na prestação de cuidados essenciais e prevenção de possíveis complicações a longo prazo. Essa estratégia era considerada adequada, uma vez que tinha como base o conhecimento científico dos anos 90 que postulava não haver reparo no tecido nervoso (RICK HANSEN FOUNDATION, 2006). Entretanto, sabe-se atualmente que existe uma capacidade intrínseca limitada de reparo após lesões do SNC, e que esse potencial regenerativo pode ser incrementado se um ambiente favorável ao crescimento axonal for disponibilizado (RAMÓN Y CAJAL, DE FELIPE e JONES, 1991; SANTOS-BENITO, 2006).

Inúmeras pesquisas vêm sendo desenvolvidas na busca de terapias que forneçam aos neurônios lesionados as condições necessárias para sua sobrevivência e regeneração axonal (SANTOS-BENITO, 2006). Esses procedimentos terapêuticos incluem: 1)

administração de fatores de crescimento (BLESCH e TUSZYNSKI, 2003; ZHANG et al., 2009); 2) bloqueio de moléculas inibitórias ao crescimento axonal (KIM et al., 2004; STEWARD et al., 2008) e inibidores da cicatriz glial (SCHIWY et al., 2009); 3) agentes farmacológicos como mediadores inflamatórios (LÓPEZ-VALES et al., 2010), fármacos que elevam a adenosina 3',5'-monofosfato cíclico (AMPc) (BRETZNE et al., 2010) e condroitinase (FOUAD et al., 2005); além de 4) diversos tipos de transplantes celulares, como as células de Schwann (TAKAMI et al., 2002; LAVDAS et al., 2010), macrófagos (BOMSTEIN et al., 2003), tanicitos (PRIETO, CHAUVET e ALONSO, 2000), células ependimárias (KITADA et al., 2001), precursores de oligodendrócitos (BAMBAKIDIS et al., 2004), pontes de nervos periféricos (CÔTE, AMIN e HOULE, 2011), células tronco de diferentes fontes (MARQUES et al., 2010; MOTHE et al., 2011), suportes artificiais (DEUMENS et al., 2006) e células gliais embainhantes olfatórias (RAMÓN-CUETO et al., 2000; LÓPEZ-VALES et al., 2006; LI et al., 2011; TAKEOKA et al., 2011). Na tentativa de potencializar a regeneração axonal e a recuperação funcional após lesões da medula espinal, essas estratégias de tratamento citadas acima também vêm sendo testadas em associação (FOUAD et al., 2009; GARCÍA-ALÍAS et al., 2011).

1.6.1 Células da glia embainhante olfatória (GEO)

O sistema olfatório de mamíferos possui a habilidade única de renovar sua população neuronal continuamente durante a vida adulta (GRAZIADEI e GRAZIADEI, 1979; AU e ROSKAMS, 2003). Esse sistema é constituído pela mucosa olfatória perifericamente e pelo bulbo olfatório (BO) e córtex olfatório a nível central. Mais especificamente, a mucosa olfatória é formada pelo epitélio olfatório (EO) e pela lâmina propria (LP), sendo responsável pela detecção de moléculas odoríferas presentes no ambiente. Os potenciais de ação gerados nos neurônios sensoriais olfatórios que se encontram nessa mucosa são transmitidos centralmente ao BO e subsequentemente ao córtex olfatório, permitindo a percepção de um determinado odor (GOMEZ e CELII, 2008). Devido a sua função e localização, a mucosa olfatória pode entrar em contato com substâncias tóxicas, ficando vulnerável a lesões físicas e químicas (FARBMAN, 1990). Assim, estima-se que a cada 4 ou 6 semanas os neurônios sensoriais olfatórios de roedores morrem e suas conexões com o BO são perdidas (CALOF et al., 1989). É com base nesse fato que a mucosa olfatória precisa renovar constantemente seus neurônios sensoriais ao longo do tempo (LINDSAY, RIDDEL e BARNETT, 2010).

Uma diferença notável entre as regiões do sistema olfatório e de outras regiões do SNC pouco permissivas ao crescimento axonal reside em seus respectivos tipos de células gliais (RAMÓN-CUETO e AVILA, 1998). Além de astrócitos, microglia e oligodendrócitos, o sistema olfatório possui um tipo diferenciado de células gliais, a chamada GEO (DOUCETTE, 1990; BARNETT, HUTCHINS e NOBLE, 1993). Golgi e Blañes foram os primeiros histologistas a descreverem a existência da GEO, encontrada na LP olfatória, no nervo olfatório e nas camadas mais externas (glomerulares) do BO (DOUCETTE, 1991; FRANSSEN, BREE, VERHAAGEN, 2007; GUÉROUT et al., 2010) (Figuras 6 e 7). Esse tipo de célula glial envolve os axônios sensoriais olfatórios desde a LP até o BO, fornecendo a aos axônios que estão se regenerando um suporte mecânico, trófico e evitando que entrem em contato com moléculas inibitórias presentes no ambiente extracelular (VALVERDE e LÓPEZ-MASCARAQUE, 1991; RAMÓN-CUETO e AVILA, 1998). Nesse contexto, as células da GEO são uma população única de células gliais que continuamente envolve os axônios desde a periferia até o SNC.

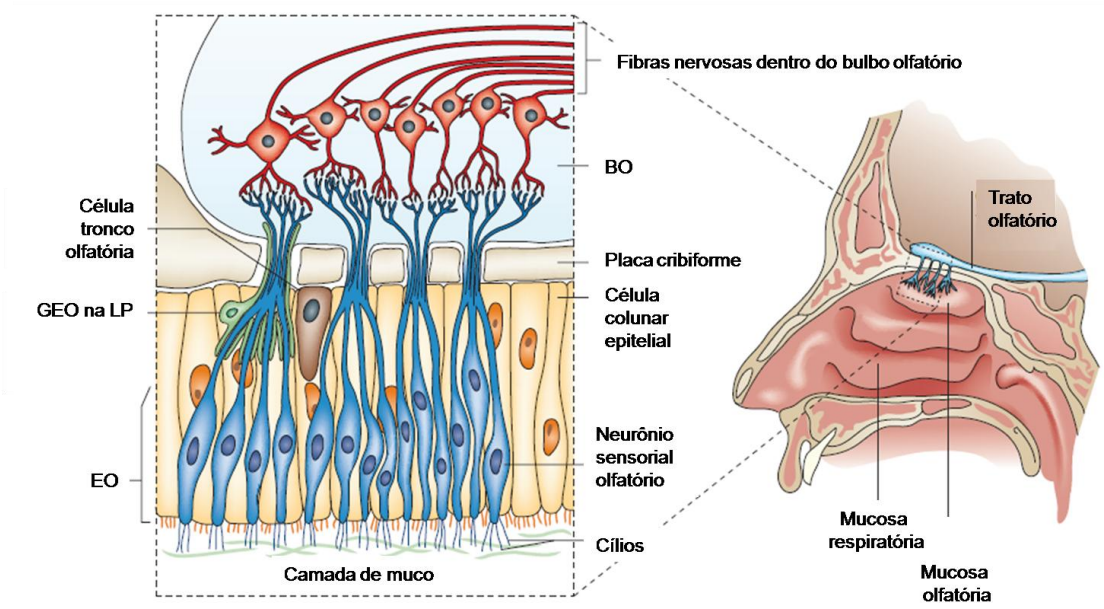

Figura 6 - Visão esquemática de uma secção sagital de parte dos componentes do sistema olfatório em humanos (direita) e detalhamento das estruturas que se encontram próximas à placa cribiforme (maior aumento, esquerda). Na base do epitélio olfatório (EO) são encontradas células tronco que dão origem a novos neurônios. Os axônios desses neurônios recém-formados são envolvidos pelas células da glia embainhante olfatória (GEO) desde a lâmina própria (LP) até chegarem ao bulbo olfatório (BO) e restabelecerem as conexões que haviam sido perdidas (Adaptado de THURET, MOON e GAGE, 2006).

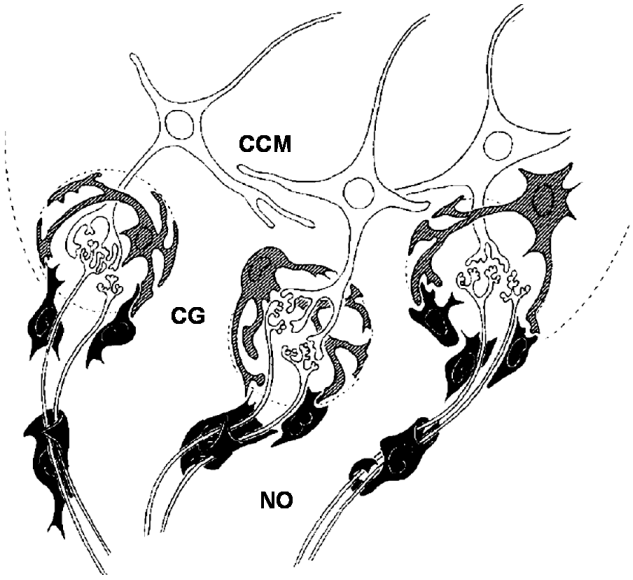

Figura 7 – Neurônios olfatórios estendem seus axônios desde o epitélio olfatório até estabelecerem sinapses na camada de células mitrais (CCM) do bulbo olfatório. As células da glia embainhante olfatória envolvem os fascículos de axônios no nervo olfatório (NO) e contribuem com os astrócitos centrais na delimitação do glomérulo olfatório na camada glomerular (CG). Células da glia embainhante olfatória são representadas em preto; astrócitos estão sombreados em cinza e os axônios dos neurônios olfatórios estão delineados em preto (Adaptado de VALVERDE, SANTACANA e HEREDIA, 1992).

Devido às importantes funções desempenhadas pelas células da GEO na regeneração axonal que ocorre continuamente no sistema olfatório, vários estudos vêm sendo realizados utilizando o transplante desse tipo de células em diferentes modelos animais de lesão medular (RAMÓN-CUETO et al., 1998; 2000). O mecanismo exato pelo qual o transplante de células da GEO poderia contribuir para o reparo do tecido medular após uma lesão ainda não está completamente elucidado. Entretanto, estudos prévios mostram que essas células quando implantadas no sítio da lesão reduzem a cicatriz glial (LU et al., 2006), facilitam o crescimento axonal na interface transplante/tecido medular intacto (LI, LI e RAISMAN, 2005), reduzem da expressão de proteoglicanos (GARCÍA-ALIÁS et al., 2004), promovem angiogênese (RICHTER et al., 2005), e remielinização (SASAKI et al., 2006), além de liberarem vários fatores de crescimento, como o fator de crescimento neural (do inglês, *NGF*), o fator neurotrófico derivado do encéfalo (*BDNF*), o fator neurotrófico derivado de células gliais (*GDNF*) e o fator neurotrófico ciliar (*CNTF*) (LIPSON et al., 2003).

A maioria dos estudos envolvendo o transplante de GEO após lesões medulares utiliza como fonte de obtenção dessas células as camadas mais externas do BO. Todavia, essa estrutura é de difícil acesso em humanos, com risco considerável de danos colaterais (FRANKLIN, 2002). O uso de células da GEO purificadas a partir da LP ou de amostras desse tecido como um todo facilitaria a realização de transplantes autólogos em pacientes, uma vez que essa estrutura reveste bilateralmente o septo nasal em sua região mais posterior e

é mais acessível em humanos e roedores (LU et al., 2002; AU e ROSKAMS, 2003) (Figura 8). É importante salientar que a lâmina própria respiratória também reveste bilateralmente o septo nasal em sua porção dorso-anterior e possui uma composição celular semelhante à LP olfatória, contendo feixes axonais do nervo olfatório, fibras do nervo trigeminal, células de Schwann, endotélio, fibroblastos intersticiais e células imunes residentes. Entretanto, a lâmina própria respiratória é desprovida de células da GEO (MACKAY-SIM e ST JOHN, 2011).

Células da GEO derivadas da LP olfatória diferem em muitos aspectos daquelas encontradas no BO (YAMAMOTO et al., 2009; GUÉROUT et al., 2010). Por exemplo, a GEO derivada da LP olfatória expressa vários antígenos que não são observados nas células do BO. Além disso, as propriedades regenerativas dessas diferentes fontes de células também parecem ser distintas quando transplantadas no local da lesão medular (RICHTER et al., 2005). Um maior entendimento sobre as diferenças existentes entre as células da GEO encontradas na LP e no BO é necessário antes de estabelecermos qual dessas fontes de obtenção seria mais adequada para o transplante após lesões medulares.

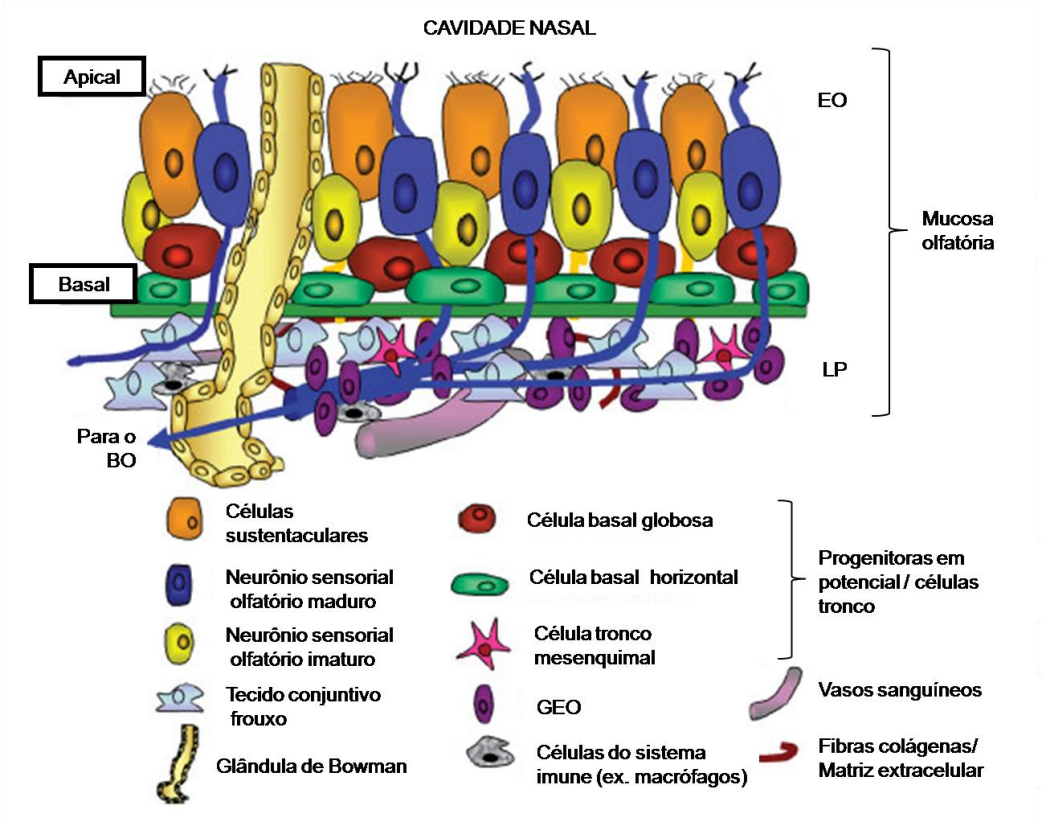

Figura 8 – Composição celular da mucosa olfatória. A mucosa olfatória é constituída pelo epitélio olfatório (EO) e pela lâmina própria (LP). O EO contém os neurônios sensoriais olfatórios que detectam o odor, células sustentaculares (células de suporte não neuronais), glândulas de Bowman e seus ductos, além de células tronco (células basais globosas e células basais horizontais). A LP é composta pelas células gliais embainhantes olfatórias (GEO) que envolvem os fascículos axonais provenientes do EO e também por tecido conjuntivo frouxo, que é formado por fibroblastos, macrófagos, pericitos, células endoteliais, músculo liso (ao redor dos

vasos sanguíneos) e células de Schwann (que envolvem os axônios que inervam vasos sanguíneos). Acredita-se também que existam células tronco mesenquimais residindo na LP (Adaptado de LINDSAY, RIDDELL e BARNETT, 2005).

Outro aspecto relevante em relação à utilização de células da GEO é a janela terapêutica ideal para sua aplicação. Na maioria dos estudos experimentais essas células gliais são transplantadas imediatamente, ou seja, logo após a realização dos diferentes paradigmas de lesão da medula espinal. Intervenções aplicadas agudamente podem reduzir o processo inflamatório e hemorragia, diminuindo a liberação de metabólitos citotóxicos (DEUMENS et al., 2006). Entretanto, a alta concentração das substâncias nocivas liberadas por esses processos patológicos pode afetar a sobrevivência e a proliferação das células transplantadas no local da lesão medular (FITCH et al., 1999). Alguns estudos sugerem que a aplicação de uma terapia após um curto período da ocorrência do efeito lesivo (fase subaguda), seria uma abordagem mais favorável para promover a regeneração axonal e recuperação funcional (COUMANS et al., 2001). Além disso, do ponto de vista clínico, o transplante autólogo de células da GEO somente poderia ser viabilizado somente depois de decorrido um determinado período de tempo pós-lesão (LÓPEZ-VALES et al., 2006). Por outro lado, como a vasta maioria dos pacientes possui lesões medulares consideradas crônicas, estudos também vêm realizando o transplante de células da GEO em uma fase mais tardia (HOULE e TESSLER, 2003). Lesões crônicas são consideradas mais estáveis e estratégias de tratamento empregadas nesse período são vantajosas na medida em que não interferem sobre uma possível recuperação espontânea.

Um consenso sobre a eficácia do transplante de células da GEO após lesões da medula espinal também precisa ser melhor estabelecido (TETZLAFF et al., 2011). Alguns estudos realizados previamente mostram resultados funcionais e morfológicos promissores com a utilização do transplante de células da GEO (KUBASAK et al., 2008; LU et al., 2001; 2002; RAMÓN-CUETO et al., 1998; 2000). Em contrapartida, outras pesquisas não encontram resultados positivos ou mostram benefícios muito limitados após a utilização desse tipo de transplante celular (BOYD et al., 2004; GUEST et al., 2008; STEWARD et al., 2006). Por exemplo, Lu e colaboradores (2002) utilizando o transplante de LP olfatória 4 semanas após a realização de uma transecção completa da medula espinal, encontraram resultados positivos sobre a função locomotora e regeneração de fibras serotoninérgicas. Por outro lado, um estudo subsequente empregando o mesmo paradigma de lesão e de transplante não encontrou evidências de recuperação funcional ou regeneração axonal (STEWARD et al., 2006). Além disso, casos de autotomia também são vistos em alguns trabalhos utilizando

células da GEO, restando dúvidas sobre o surgimento de dor neuropática após a utilização desse tipo de transplante (RICHTER et al., 2005; GUEST et al., 2008; TETZLAFF et al., 2011). Portanto, apesar dessa terapia celular ser considerada promissora, cuidados devem ser tomados quanto à aplicação indiscriminada da GEO para o tratamento de lesões medulares (TETZLAFF et al., 2011).

2 JUSTIFICATIVA E HIPÓTESE

Até o presente momento, não existe cura para pacientes com lesões da medula espinal (THURET, MOON e GAGE, 2006). Entretanto, devido aos crescentes avanços da medicina, um número cada vez maior de indivíduos sobrevive após a ocorrência desse tipo de lesão (AO et al., 2007). A expectativa de vida desses pacientes é considerável, embora importantes complicações estejam associadas, como por exemplo, paralisia muscular, espasticidade, perda sensorial, dor, úlceras de pressão, infecções do trato urinário, entre outras (DRYDEN et al., 2004).

Durante muito tempo, o mau prognóstico relacionado à recuperação da função em pacientes com lesões da medula espinal fez com que a comunidade e governos focassem seus esforços apenas na promoção da atenção básica e redução das complicações associadas (RICK HANSEN FOUNDATION, 2006). Todavia, a constatação de que axônios podem regenerar-se mediante a criação de um ambiente favorável ao seu crescimento, intensificou a busca de terapias que favoreçam o restabelecimento dos circuitos axonais lesionados e, consequentemente, restaurem as funções comprometidas após lesões da medula espinal (LÓPEZ-VALES et al., 2006). O transplante de células da GEO, um tipo celular que medeia à regeneração axonal no sistema olfatório, vem sendo testado no âmbito experimental como estratégia terapêutica após diferentes modelos de lesão medular (TETZLAFF et al., 2011). Porém, o uso dessa terapia permanece bastante controverso, uma vez que parte das pesquisas mostram resultados funcionais e morfológicos promissores, enquanto outros estudos não conseguem reproduzir esses achados (RAMÓN-CUETO et al., 1998; 2000; LU et al., 2001; 2002; TAKAMI et al., 2002; STEWARD et al., 2006). Neste contexto, o presente trabalho justifica-se por acrescentar informações sobre os efeitos, mecanismo de ação e janela terapêutica ideal para a aplicação do transplante de células da GEO em animais com transecção completa da medula espinal. Este trabalho auxiliará na elucidação do real potencial regenerativo desse tipo de intervenção.

Quanto à hipótese do trabalho, acredita-se que o modelo de lesão adotado produzirá a secção completa dos tratos axonais ascendentes e descendentes da medula espinal, causando uma paralisia dos membros posteriores dos animais e hiperreflexia abaixo do nível da lesão. O transplante de LP olfatória em uma das janelas terapêuticas testadas promoverá uma recuperação considerável da locomoção e a normalização das respostas reflexas nos animais lesionados. Tal melhora será obtida através do crescimento axonal no sítio da lesão, estimulado pela presença de células da GEO. Por outro lado, postula-se que o transplante de

LP respiratória, um tecido de composição semelhante à LP olfatória que não possui as células gliais em questão, promoverá pouca ou nenhuma regeneração axonal. Assim, a recuperação funcional dos animais transplantados com LP respiratória nos mesmos períodos pós-lesão será limitada pela ausência das células da GEO nesses implantes.

3 OBJETIVOS

3.1 Objetivo Geral

Este estudo teve como objetivo geral avaliar os efeitos do transplante de LP olfatória (que contém células da GEO) ou respiratória (que não contém células da GEO) quando realizado imediatamente, 2 e 4 semanas pós-lesão, sobre a regeneração axonal e recuperação funcional de ratos com transecção completa da medula espinal ao nível de T8-T9.

3.2 Objetivos Específicos

(i) Avaliar a possível recuperação da locomoção em ratos submetidos à transecção da medula espinal e ao transplante de LP olfatória ou respiratória em diferentes janelas temporais (Artigo I);

(ii) Verificar os efeitos do transplante de LP olfatória ou respiratória quando aplicado em diferentes janelas temporais sobre a hiperatividade das funções reflexas em ratos com transecção da medula espinal, utilizando avaliações do reflexo de retirada e do reflexo H (Artigo II);

(iii) Identificar a presença de células da GEO nos implantes de LP olfatória antes e após seu transplante em ratos com transecção da medula espinal, empregando a técnica de imunoistoquímica para marcadores específicos dessas células (proteína glial ácida fibrilar – GFAP, receptor de neurotrofina p75 – p75NGFR e S-100) (Artigo I);

(iv) Estimar a quantidade de tecido nervoso preservado no local da transecção medular após o transplante de LP olfatória e respiratória nas diferentes janelas temporais, bem como avaliar a presença de remodelamento neurítico nessa região, utilizando marcações para GFAP e proteína associada ao crescimento 43 (GAP-43), respectivamente (Artigo I);

(v) Delimitar o local da transecção medular em ratos submetidos ao transplante de LP olfatória e respiratória nas diferentes janelas temporais após transecção da medula espinal e avaliar a regeneração de axônios positivos para serotonina (5-HT) e peptídeo relacionado ao gene de calcitonina (CGRP) no epicentro da lesão e nos cotos proximal e distal (Artigo I);

(vi) Verificar o possível restabelecimento das conexões entre os tratos axonais lesionados e regiões supra-espinais após o transplante em diferentes janelas temporais de LP olfatória e respiratória em ratos com transecção da medula espinal, por meio da

contagem de neurônios corados com um marcador axonal retrógrado (*Fluorogold*) em núcleos do tronco encefálico e córtex cerebral (Artigo I);

(vii) Avaliar a regeneração de fibras mielínicas em diferentes regiões da lesão medular (epicentro, 1 e 2 mm caudalmente) após o transplante de LP olfatória e respiratória nas diferentes janelas temporais empregadas, além de analisar as características morfológicas dessas fibras (área e diâmetro da fibra, espessura da bainha de mielina e diâmetro axonal) (Artigo II).

4 RESULTADOS

4.1 Artigo I

A primeira parte dos resultados foi publicada no periódico *Brain Research*, sob a forma de um artigo intitulado "*Olfactory and respiratory lamina propria transplantation after spinal cord transection in rats: Effects on functional recovery and axonal regeneration*". O referido artigo segue-se abaixo.

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BRAIN RESEARCH

Research Report

Olfactory and respiratory lamina propria transplantation after spinal cord transection in rats: Effects on functional recovery and axonal regeneration

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ABSTRACT

Spinal cord injury (SCI) has very poor clinical prospects, resulting in irreversible loss of function below the injury site. Although applied in clinical trials, olfactory ensheathing cells transplantation (OEC) derived from lamina propria (OLP) is still a controversial repair strategy. The present study explored the efficacy of OLP or respiratory lamina propria (RLP) transplantation and the optimum period after SCI for application of this potential therapy. Adult male rats were submitted to spinal cord transection and underwent acute, 2-week or 4-week post-injury transplantation with pieces of OLP (containing OECs) or RLP (without OECs). After grafting, animals with OLP and RLP showed discrete and similar hindlimb motor improvement, with comparable spinal cord tissue sparing and sprouting in the lesion area. Acute transplantation of OLP and RLP seems to foster limited supraspinal axonal regeneration as shown by the presence of neurons stained by retrograde tracing in the brainstem nuclei. A larger number of 5-HT positive fibers were found in the cranial stump of the OLP and RLP groups compared to the lesion and caudal regions. Calcitonin gene-related peptide fibers were present in considerable numbers at the SCI site in both types of transplantation. Our results failed to verify differences between acute, 2-week and 4-week delayed transplantation of OLP and RLP, suggesting that the limited functional and axon reparative effects observed could not be exclusively related to OECs. A greater understanding of the effects of these tissue grafts is necessary to strengthen the rationale for application of this treatment in humans.

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Abbreviations: 2WDC, 2-Week Delayed Control; 2WDT, 2-Week Delayed Treated; 4WDC, 4-Week Delayed Control; 4WDT, 4-Week Delayed Treated; AC, Acute Control; AT, Acute Treated; BBB, Basso, Beattie and Bresnahan Scale; FG, Fluorogold; OEC, Olfactory Ensheathing Cells; OLP, Olfactory Lamina Propria; RLP, Respiratory Lamina Propria; SCI, Spinal Cord Injury

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1. Introduction

Spinal cord injury (SCI) results in loss of central control of motor, sensorial, and autonomic functions below the site of injury (van den Berg et al., 2010). Despite the application of neuroprotective treatments, such as methylprednisolone or interleukin-10, the clinical prospects for spinal cord lesions are currently very poor (Fitch and Silver, 2008; Takami et al., 2002b). Functional disabilities occur due to local neuronal death and loss of ascending and descending axons in the spinal cord, either by direct trauma or secondary damage (Hausmann, 2003; Ramer et al., 2005).The hostile environment produced by glial scarring, the presence of inhibitory molecules associated with oligodendrocyte myelin and inadequate neurotrophin supply are responsible for impaired regeneration of severed axons after SCI (Franssen et al., 2007).

In attempts to provide a cellular milieu appropriate for axonal regrowth and the restoration of lost neural circuits, several primary cell types have been used in transplantations into the SCI site (Kwon and Tetzlaff, 2001; Tetzlaff et al., 2011). The olfactory system has attracted considerable interest as a promising source of cells for transplantation after SCI, because of its capacity for lifelong regeneration (Lindsay et al., 2010). The main focus of attention in the olfactory tissue has been a unique type of glia, known as the olfactory ensheathing cells (OECs) (Doucette, 1991; Raisman, 2001; Ramón-Cueto and Muñoz-Quiles, 2011). These cells reside within the two main regions of the olfactory axis: peripherally, in the lamina propria and centrally, along the nerve fiber layer of the olfactory bulb (OB) (Au and Roskams, 2003). The OECs are responsible for maintaining an environment which favors neurite outgrowth and the creation of new functional synapses in the central nervous system (Au and Roskams, 2003; Franssen et al., 2007).

Due to their supposed axon regenerative properties, OECs have been extensively studied in animal models of SCI. Although some research has shown locomotor and axonal regeneration improvements, a consensus on the efficacy of this cellular transplantation and mode of action has yet to be reached (Barnett and Riddell, 2007; Boyd et al., 2004; Franssen et al., 2008; Kubasak et al., 2008; Raisman and Li, 2007; Ramón-Cueto and Avila, 1998; Ramón-Cueto et al., 1998, 2000; Tetzlaff et al., 2011). The source of OECs for transplantation into injured spinal cord is also subject of debate (Richter et al., 2005). However, the use of olfactory lamina propria (OLP) grafts, which is a more accessible source of OECs in humans, could enable a safer approach for autologous transplantation (Bianco et al., 2004; Féron et al., 1998; Franklin, 2002).

The devastating prognosis associated with the social and economic impacts, has led to increased efforts to find therapies that provide functional recovery for people who undergo severe SCI (Blight, 2002; van den Berg et al., 2010). According to previous studies, the use of OLP transplantation is a promising, though controversial, repair strategy (Lu et al., 2001, 2002; Steward et al., 2006). In the present study we hypothesized that the OECs present in OLP grafts could create a favorable glial environment that would favor neurite and axonal outgrowth after thoracic spinal cord transection in rats. Thus, OLP transplantation could produce higher levels of hindlimb motor recovery when compared to respiratory lamina propria (RLP), which is a graft devoid of OECs. Additionally, we tested the efficacy of OLP transplantation in three different therapeutic windows (acutely, 2 weeks and 4 weeks post-injury), since another key aspect in the translation of this therapy to clinical practice is their potential to produce axonal regeneration even when transplantation is delayed after SCI.

2. Results

2.1. *Hindlimb motor function*

Fig. 1 illustrates average Basso, Beattie, and Bresnahan scale scores (BBB) before and across the post-injury survival interval for the experimental groups. Prior to the injury (naive test), no differences were observed in the average of inter-group BBB scores and the animals showed normal locomotor activity (scored as 21). By contrast, at 5 days post-injury (test 1) there was a complete flaccid paralysis of both hindlimbs movements in most animals and BBB scores were 0.21±0.09 for the acute control group (AC), 0.23±0.16 for the acute treated group (AT), 0.18±0.09 for the 2-week delayed control group (2WDC), 0.21±0.09 for the 2-week delayed treated group (2WDT), 0.16±0.09 for the 4-week control group (4WDC), 0.41±0.37 for the 4-week treated group (4WDT) (mean±SEM). Instead of the slight recovery of motor skills observed from 20 days after SCI to the end of this study, there were no differences between the average BBB scores obtained at any time point comparing acute, 2-week or 4-week OLP transplanted groups with their respective RLP control groups (one-way repeated measures ANOVA; acute groups $F_{(1,20)}=0.13$, $p>0.05$; 2-week delayed groups $F_{(1,22)}=1.66$, $p>0.05$; 4-week delayed groups $F_{(1,22)}=0.11$, $p>0.05$). In the last functional test, the BBB scores were 3.5±0.9 for the AC group; 2.7±0.5 for the AT group; 2.6±0.4 for the 2WDC group; 3.0±0.6 for the 2WDT group; 2.6±0.6 for the 4WDC group and 2.0±0.4 for the 4WDT group. No differences were found when data from the last functional test were compared between all the studied groups (one-way ANOVA $F_{(5,65)}=0.57$, $p>0.05$).

2.2. *Spinal tissue sparing and sprouting*

Analysis of the glial fibrillary acidic protein (GFAP) immunoreactive sections revealed a variation in the morphology of the lesion sites among the experimental groups: some rats displayed transparent cavities that separated their spinal cord stumps, while others contained smaller cavities. The preserved tissue area, determined by the presence of healthy looking cells and GFAP immunoreactivity, was measured to quantify the repair effects produced by OLP or RLP transplantation. Although no significant differences were found between the groups (one-way ANOVA $F_{(5,21)}=0.75$, $p>0.05$), the AT and 4WDT groups presented higher levels of spinal tissue sparing (488.7±101.1; 613.2±77.1, respectively) when compared to their respective controls, the AC and 4WDC groups (303.1±77.3; 414.8±96.4, respectively). The 2-week delayed transplantation of both lamina propria grafts seems to promote similar spinal tissue sparing levels (450.9±123.2; 478.6±120.9 respectively) (Fig. 2A).

The presence of sprouting axons was indicated by growth associated protein-43 (GAP-43) immunoreactivity at the SCI site of the groups (AC—0.1±0.0; AT—0.2±0.0; 2WDC—0.1±0.0; 2WDT—0.1±0.0; 4WDC—0.1±0.0; 4WDT—0.2±0.0). The

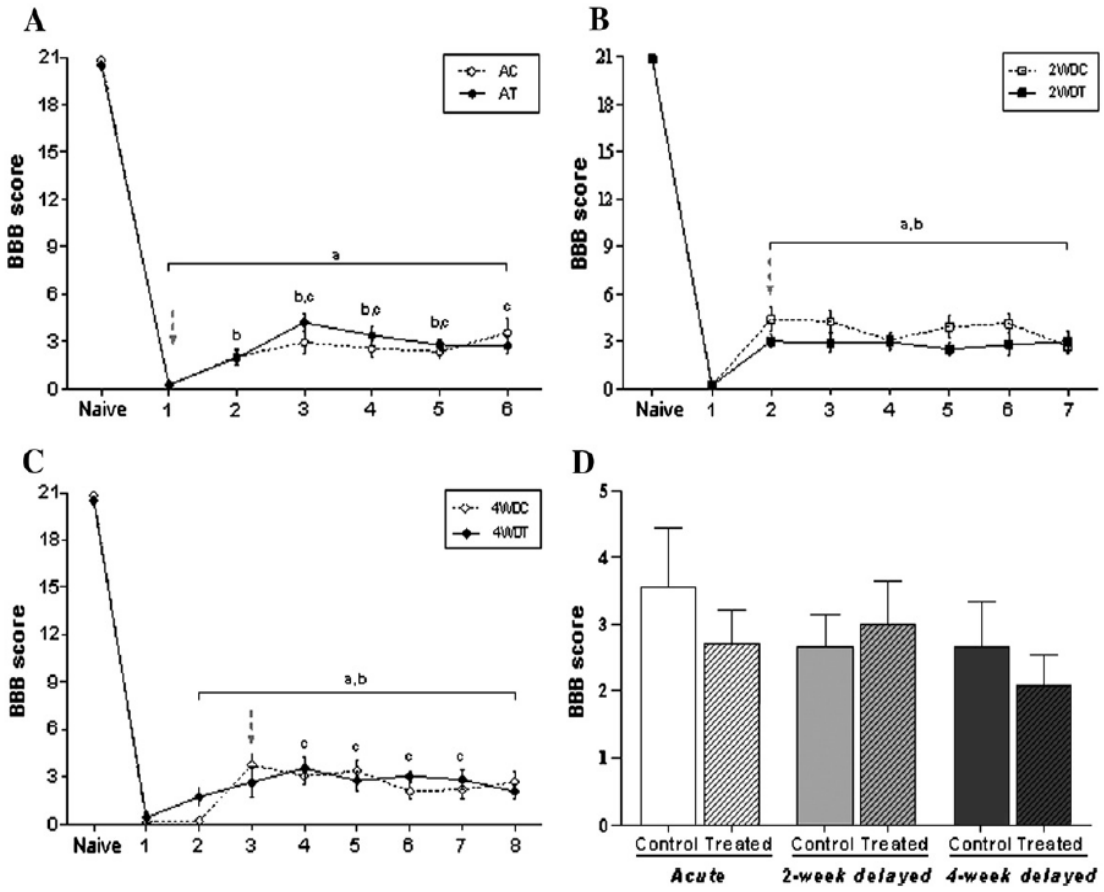


Fig. 1 – (A) BBB scores before (naive) and postoperatively at days 5, 20, 35, 50, 65, 80 (1–6 tests) after spinal cord transection and acute OLP or RLP transplantation. a—$p<0.001$ for AC and AT groups compared to naive; b—$p<0.05$ for AC group compared to test 1; c—$p<0.05$ for AT compared to test 1. (B) BBB scores before (naive) and postoperatively at days 5, 20, 35, 50, 65, 80, 95 (1–7 tests) after spinal cord transection and 2-week delayed OLP or RLP transplantation. a—$p<0.001$ for 2WDC and 2WDT groups compared to naive; b—$p<0.01$ for 2WDC and $p<0.05$ for 2WDT compared to test 1. (C) BBB scores before (naive) and postoperatively at days 5, 20, 35, 50, 65, 80, 95, 110 (1–8 tests) after spinal cord transection and 4-week delayed OLP or RLP transplantation. a—$p<0.001$ for 4WDC and 4WDT groups compared to naive; b—$p<0.05$ for 4WDC compared to test 1; c—$p<0.05$ for 4WDC group compared to test 1. (D) BBB final scores of each experimental group after spinal cord transection and 42 days after OLP or RLP transplantation at different times. No differences were found when all experimental groups were compared ($p>0.05$). Arrows indicate the time at which the experimental groups underwent OLP or RLP transplantation. Abbreviations: 2WDC—2-Week Delayed Control; 2WDT—2-Week Delayed Treated; 4WDC—4-Week Delayed Control; 4WDT—4-Week Delayed Treated; AC—Acute Control; AT—Acute Treated; BBB—Basso, Beattie, and Bresnahan scale; OLP—Olfactory Lamina Propria; RLP—Respiratory Lamina Propria.

optical densitometry for this protein showed no differences when comparing acute, 2-week or 4-week OLP transplanted groups with their respective RLP controls (one-way ANOVA $F_{(5,25)}=0.64$, $p>0.05$) (Figs. 2B, C, D).

2.3. *Retrograde tracer labeled cells*

Few fluorogold (FG) positive neurons were found in the brain areas of the animals that received OLP and RLP transplantation (Table 1). The observed labeled cell bodies were comparable in size and appeared in clusters, as previously described by Vavrek et al. (2007).

The primary and secondary motor cortices (M1/M2) did not exhibit labeled neurons in any groups. Only one animal, from the AC group, was found to have FG-positive neurons in the primary somatosensory cortex (S1). In the brainstem, animals from the AC group showed labeled neurons in the spinal vestibular nucleus (SpVe), lateral vestibular nucleus (LVe), caudal and rostral pontine reticular nuclei (PnO/PnC) and animals from the AT group exhibited FG-positive neurons in the dorsal and ventral medullary reticular fields (MdD/MdV), raphe nuclei (Ra), SpVe, LVe and PnO/PnC nuclei. In the 2-week delayed groups, FG-labeled neurons were observed in the LVe nuclei of the 2WDC group and in the PnO/PnC of the 2WDT group. The 4WDC group exhibited few FG-positive neurons in the LVe nuclei, while no labeled neurons were observed in any studied areas of the 4WDT group (Table 1).

2.4. *Serotonin (5-HT) and calcitonin gene-related peptide (CGRP) pattern fibers in caudal, rostral and lesion areas*

Animals transplanted with both types of lamina propria had most evident 5-HT immunostained fibers in the rostral stump of longitudinal spinal cord sections (AC—0.9±0.2; AT

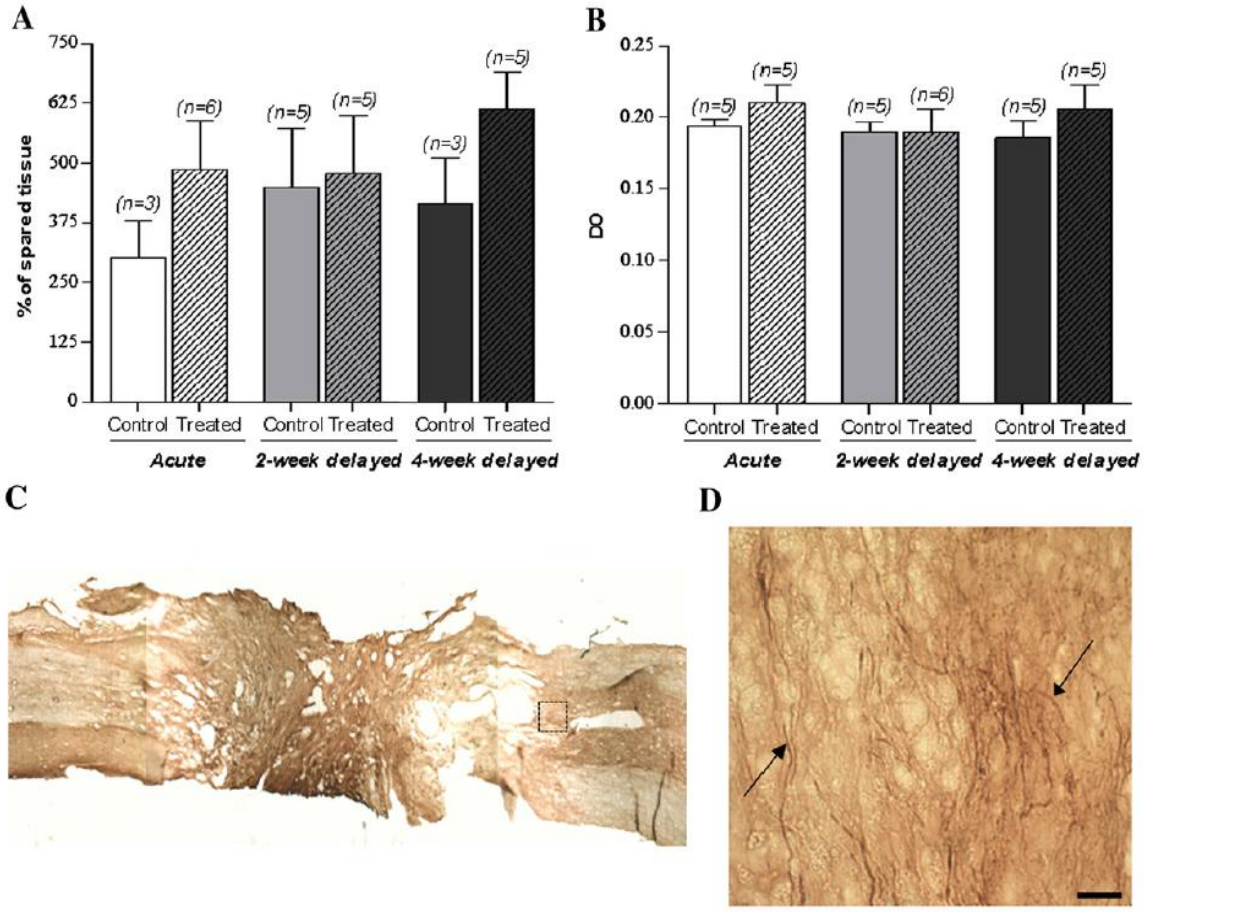


Fig. 2 – (A) Spinal cord spared tissue immunostained with GFAP in animals submitted to spinal cord transection and olfactory or respiratory lamina propria transplantation. No differences were found between groups ($p>0.05$). (B) GAP-43 immunoreactivity in the lesion region of injured rats transplanted with both types of lamina propria. No differences were found between groups ($p>0.05$). (C) Representative longitudinal spinal cord section immunostained by GAP-43, showing the lesion site. (D) Higher magnification of the highlighted box, showing GAP-43 positive sprouting axons (arrows). Magnification—100×, scale bar—100 μm. Abbreviations: GAP-43—Growth Associated Protein-43; GFAP—Glial Fibrillary Acidic Protein.

—1.0±0.5; 2WDC—0.5±0.3; 2WDT—0.4±0.0; 4WDC—0.7±0.2; 4WDT—0.6±0.0). A GFAP negative region delineated the injured area in the spinal cord and, in most animals, 5-HT fibers did not extend beyond the vicinity of the lesion border (AC—0.00±0.0; AT—0.01±0.0; 2WDC—0.00±0.0; 2WDT—0.01±0.0; 4WDC—0.03±0.0; 4WDT—0.02±0.0). Moreover, in the majority of slices analyzed, no 5-HT labeled axons were found in the caudal stump (AC—0.00±0.0; AT—0.00±0.0; 2WDC—0.01±0.0; 2WDT—0.01±0.0; 4WDC—0.00±0.0; 4WDT—0.00±0.0) (Figs. 3, 4). No differences were detected in the 5-HT immunoreactivity of the rostral, lesion and caudal regions of spinal cord when all groups were compared (Kruskal–Wallis $p=0.37$; $p=0.73$; $p=0.34$, respectively) (Fig. 6).

As expected, ascending sensory fibers immunostained by CGRP were detected in the caudal stump of the experimental groups (AC—1.27±0.3; AT—1.08±0.3; 2WDC—1.42±0.6; 2WDT—1.42±0.8; 4WDC—0.87±0.2; 4WDT—1.10±0.2). There were considerable levels of CGRP fibers in the lesion region (AC—1.71±0.4; AT—1.37±0.3; 2WDC—0.88±0.2; 2WDT—1.19±0.1; 4WDC—0.85±0.2; 4WDT—1.75±0.5), showing that both OLP and RLP transplantation stimulated the growth/sprouting of CGRP fibers in animals submitted to SCI. In the rostral stump, CGRP immunoreactive fibers were also detected in all groups, but particularly in the AT and 2WDC groups (AC—1.56±0.9; AT—3.58±2.1; 2WDC—4.10±3.0; 2WDT—1.40±0.6; 4WDC—1.79±0.9; 4WDT—1.29±0.5) (Figs. 3, 5). No differences in the rostral, lesion and caudal regions of the spinal cord were observed comparing the groups (Kruskal–Wallis $p=0.97$; $p=0.25$; $p=0.90$, respectively) (Fig. 6).

3. Discussion

The hindlimb motor recovery and axonal growth-promoting effects of OECs, OLP and olfactory mucosa have been studied in different models of spinal cord lesions (see Table 2). In the present study, we have investigated and compared the restorative efficiency of OLP and RLP transplants, in three different therapeutic windows (acutely, 2-week and 4-week delayed), after a complete thoracic spinal cord transection in adult rats. By the twelfth week after transplantation, animals with OLP or RLP showed a discrete and similar hindlimb motor improvement. All transplants produced comparable results for spinal cord tissue sparing and sprouting, evaluated using GFAP and GAP-43

Table 1 – Number and location of fluorogold retrogradely stained cells in the individual rats ($n=3$)

	MdV/ MdD	Ra	SpVe	LVe	LC	PnC/ PnO	M1/ M2	S1
Acute control	0	0	6	25	0	16	0	0
	0	0	0	7	0	0	0	32
	0	0	0	0	0	0	0	0
Acute treated	15	0	3	16	0	4	0	0
	4	5	12	19	0	7	0	0
	0	0	0	0	0	12	0	0
2 weeks delayed	0	0	0	3	0	0	0	0
control	0	0	0	0	0	0	0	0
	0	0	0	6	0	0	0	0
2 weeks delayed	0	0	0	0	0	0	0	0
treated	0	0	0	0	0	31	0	0
	0	0	0	0	0	0	0	0
4 weeks delayed	0	0	0	0	0	0	0	0
control	0	0	0	6	0	0	0	0
	0	0	0	0	0	0	0	0
4 weeks delayed	0	0	0	0	0	0	0	0
treated	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0

Note the absence of labeling cells in *LC* and M1/M2. MdV/MdD—Ventral and Dorsal Medullary Reticular Fields; PnO/PnC—Caudal and Rostral Pontine Reticular Nuclei; Ra—Raphe Nuclei; SpVe—Spinal Vesribular Nucleus; LVe—Lateral Vestibular Nucleus, LC—*Locus Coeruleus*; M1/M2—Primary and Secondary Motor Cortex; S1—Primary Somatosensory Cortex.

immunohistochemistry. Acute transplantation of OLP and RLP seems to foster some limited supraspinal axonal regeneration, as indicated by the presence of cells stained by retrograde tracing in brainstem nuclei. However, retrogradely labeled cells in cortical areas were only observed following acute RLP transplantation. A larger number of 5-HT positive fibers were found in the cranial stump of the OLP and RLP groups compared to the lesion and caudal regions analyzed. CGRP fibers were present in considerable number at the SCI site in both transplantation types.

Although the mechanisms underlying the regenerative properties of OECs in the SCI site are not completely elucidated, reduction of glial scarring (Lu et al., 2006), facilitation of axon re-entry into the host–graft interface (Li et al., 2005), reduction of proteoglycan expression (García-Alías et al., 2004), angiogenesis (Richter et al., 2005), remyelination (Sasaki et al., 2006) and growth-factors release (Lipson et al., 2003) are considered the main benefits of this cell transplantation (Tetzlaff et al., 2011). We were able to detect the presence of OECs in the lamina propria before and after grafting in the transection site, but the limitations of our study were the lack of the OECs quantification and the inability to investigate the possible migratory properties of these cells after transplantation. Nevertheless, some aspects of OECs behavior after transplantation have been previously documented. In an olfactory nerve injury, OECs were seen to remain at the lesion site forming a conduit that can guide regenerating nerve axons, analogously to Schwann cells after a peripheral nerve injury (Li et al., 2005; Williams et al., 2004). After a cervical spinal cord injury model, Lu et al. (2006) failed to demonstrate any unique migratory properties of OECs, concluding that these cells probably spread due to pressure at the injection site,

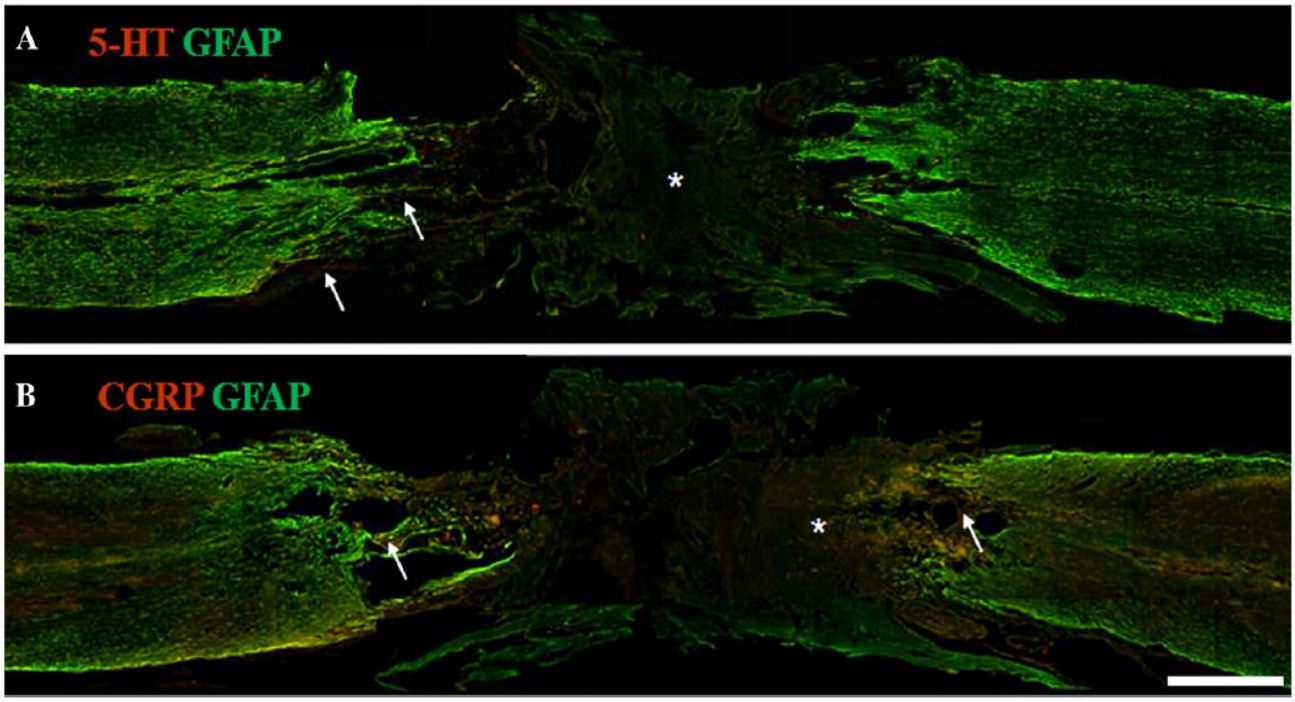


Fig. 3 – 5-HT and CGRP fiber regeneration in a representative animal with moderate cavitation at the lesion site, approximately 18 weeks after spinal cord transection. (A) There are 5-HT fibers surrounding the margin of the transection site in the rostral stump (arrows), but the majority of these sprouts do not continue in the GFAP-negative lesion area (asterisk). 5-HT fibers are not seen in the caudal stump. (B) CGRP fibers are found surrounding the border of the transection site in both stumps (arrows) and penetrating the GFAP-negative lesion area (asterisk). Rostral is to the left. Photomontages were made from digital images with magnification of 100×, 2.2 μm of optical stack thickness and 11 confocal planes. Pixel size—638.92×638.92 μm. Scale bar—500 μm. Abbreviations: 5-HT—Serotonin; CGRP—Calcitonin gene-related peptide; GFAP—Glial Fibrillary Acidic Protein.

without active migration. On the other hand, Richter et al. (2005) showed a superior migratory ability of OECs derived from lamina propria when compared to OECs derived from OB after crush of spinal cord dorsolateral funiculus at the C3–C4 level. Thus, the migratory capacity of these cells after transplantation into different injury sites is still controversial.

OLP transplants have the dual advantage of providing a physical bridge that could mechanically favor axonal sprouting as well as being a reservoir of OECs (Lu et al., 2002). RLP provides the same bridging function and shares many of the cell types with OLP (olfactory nerve bundles, trigeminal nerve fibers, Schwann cells, endothelium, interstitial fibroblasts and tissue resident immune cells) (Mackay-Sim and St John, 2011). These shared cells present in RLP may have been responsible for the hindlimb motor improvement and the CGRP regeneration observed at the lesion site (Lindsay et al., 2010). On the other hand, the restoration of a cell continuum alone within the spinal cord may have largely contributed to the results found with both transplant types. According to this latter hypothesis, animals in which 4 mm were removed from spinal cord and with a matrigel only-bridge showed BBB scores comparable to those observed in the RLP groups. In the animals transplanted with matrigel, myelinated axons were exhibited in the injury site, with 5-HT positive fibers crossing the lesion and penetrating the caudal stump (Fouad et al., 2005). In another similar study, alginate-based capillary gels were inserted after transection of the dorsal column at the C3 level. Similarly, a robust growth of coerulospinal projections and GAP-43 positive fibers was shown within the hydrogel (Prang et al., 2006). However, animals submitted to spinal cord transection and injections of culture medium only (without any bridge at the lesion), also obtained BBB scores that were very close to those observed with our OLP/RLP grafts. Many GAP-43-immunoreactive axons were found in the stumps of these culture-medium-injected group and some CGRP-positive axons invaded the lesion epicenter (López-Vales et al., 2006). In the present study, a lesion-only control group was not included in order to avoid the use of a large number of animals. Moreover, animals without any type of transplantation would not develop the immune responses present in the other groups submitted to heterologous tissue transplantation. More studies are required to verify whether comparable outcomes reported in this study could be found in either untreated or matrigel-only bridge groups, in order to elucidate the possible positive effects exerted by cells other than OECs present in the RLP after spinal cord transection.

Previous studies have emphasized the importance of an appropriate post-injury period for repair after SCI (Schiwy et al., 2009; Takami et al., 2002a). Most experimental studies only performed OECs or tissue transplants acutely (Guest et al., 2008; Kubasak et al., 2008; Lu et al., 2001; Ramón-Cueto and Avila, 1998; Ramón-Cueto et al., 2000). However, transplantation of purified OECs or lamina propria after SCI in humans implies delayed grafting (Tetzlaff et al., 2011). The release of cytotoxic metabolites derived from hemorrhage and/or inflammation could be prevented by more acute interventions, but this procedure could be harmful to the transplanted cells at an intermediate stage (Deumens et al., 2006; Martin et al., 1996). Lu et al. (2001, 2002) showed a great improvement in hindlimb motor function, spinal reflex and enhanced regeneration of raphespinal fibers with OLP transplantation immediately or 4 weeks after spinal cord transection. On the other hand, Steward et al. (2006) failed to find evidence of functional recovery and showed only limited regeneration of raphespinal axons after spinal cord transection and 4-week delayed OLP transplantation. In our study, functional recovery, tissue sparing and axon sprouting/regeneration outcomes were comparable between animals with OLP or RLP grafts, uninfluenced by the different transplantation times (acute, 2 weeks or 4 weeks post-injury). Raphespinal axons rarely extended beyond the lesion border, but CGRP fibers were evident in the center of the lesion after both types of transplants. Thus, the optimal time-window for cellular or tissue transplantation continues to be ill-defined, but this parameter does not seem to limit the effects obtained from the grafts. In addition, as CGRP axon regeneration may be related to nociception transmission, interventions that favor axonal regeneration after SCI must be controlled to ensure that appropriate rather than inappropriate connections are restored (Richter et al., 2005).

Despite current controversy in animal studies, clinical trials using cultured OECs from lamina propria or olfactory mucosa grafts have been made in chronically injured humans (Chhabra et al., 2009; Féron et al., 2005; Lima et al., 2006, 2010; Mackay-Sim et al., 2008). There is still divergence regarding the functional results and, moreover, the procedures used in some of these studies were not administered according to formal clinical trial protocols (Mackay-Sim and St John, 2011).

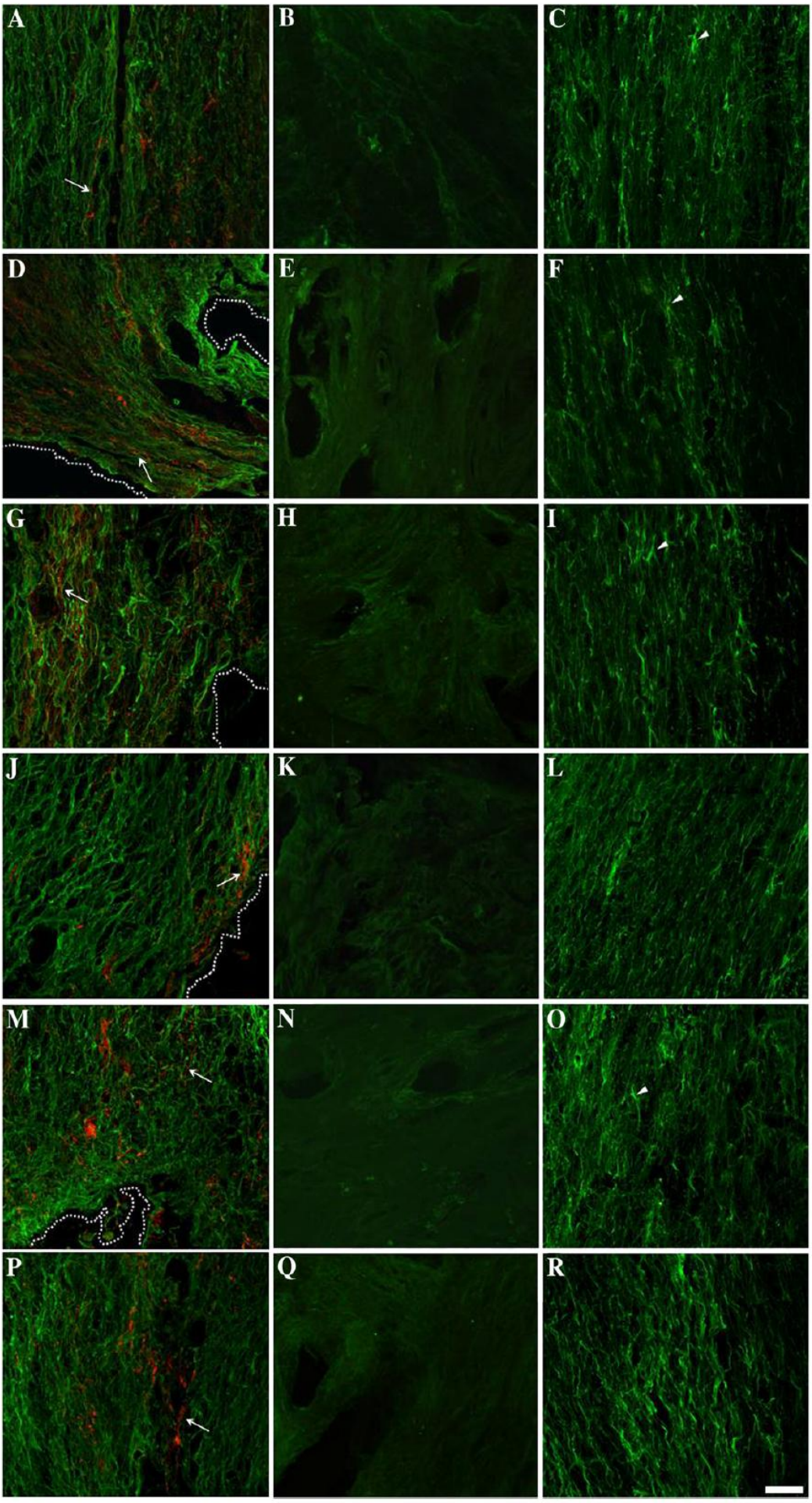
3.1. *Conclusions*

Acute, 2-week or 4-week delayed OLP and RLP transplantation produced a discrete functional recovery over time and comparable CGRP fiber sprouting in the lesion site, but failed to produce regeneration of raphespinal descendent fibers. OECs are only one cell type found in olfactory mucosa, which is a tissue of considerable cellular complexity. This is particularly relevant when clinical trials involving the transplantation of these tissue samples in a complex injury such as the damaged central nervous system are conducted (Lindsay et al., 2010). A better understanding of the effects of OLP and RLP transplantation in SCI animal models is necessary in order to strengthen the rationale for the application of this treatment in humans. Additionally, cells transplants combined with other therapies, such as the administration of MAG, OMP and NOGO-A inhibitors, growth-factors, and/or treadmill step training may increase the possible beneficial results after spinal cord injury.

4. Experimental procedure

4.1. *General*

Experimental procedures were approved by the Research Ethics Committee of the Universidade Federal do Rio Grande do Sul (No. 2007892). The animals were cared for in accordance with Arouca Brazilian law (11794/2008) and the National Institute of Health's Guidelines for Care and Use of Laboratory Animals (publication No. 85-23, revised 1985). The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animal handling recommendations of the Brazilian Society for Neurosciences and the International Brain Research Organization were also followed.

A total of 108 male *Wistar* rats (local breeding colony), 280–380 g in body weight, and 13 weeks old were used in this study. Groups of two or three animals were maintained in standard plexigas boxes (46×24×15 cm), under 12:12 h light/dark cycle, in a temperature controlled environment (20±2 °C) with food and water *ad libitum*. The animals were tested during the light phase of the photo cycle.

Initially, animals were separated in experimental animals (n=72) and lamina propria donors (n=36). Experimental animals (n=72) were again randomly divided into six groups: (1) *AC*—rats submitted to RLP transplantation, immediately after spinal cord transection (n=11); (2) *AT*—rats submitted to OLP transplantation, immediately after spinal cord transection (n=12); (3) *2WDC*—rats submitted to RLP transplantation, two weeks after spinal cord transection (n=12); (4) *2WDT*—rats submitted to OLP transplantation, two weeks after spinal cord transection (n=12); (5) *4WDC*—rats submitted to RLP transplantation, four weeks after spinal cord transection (n=12); (6) *4WDT*—rats submitted to OLP transplantation, four weeks after spinal cord transection (n=12). All efforts were made to minimize the number of animals studied and their suffering. Thus, similarly to a previous studies, a lesion-only group (i.e., without any type of transplantation) was not included (Lu et al., 2001, 2002; Steward et al., 2006).

4.2. *Spinal cord transection*

For spinal cord transection procedure, animals were anesthetized using pentobarbital (40 mg/kg, i.p., Cristália, São Paulo—SP, Brazil) and maintained on a heating pad. The hair overlying the area of interest was shaved and the skin was cleaned. A midline incision in the thoracic area was made and muscle/connective tissues were dissected to expose the T8-T9 vertebrae. After a laminectomy, the spinal cord was transected at two levels using microscissors (approximately 2–3 mm apart). The segment between these incisions was removed, leaving a gap (Fig. 7A, left). To ensure completeness of the lesion, the spinal cord stumps were lifted, placed back into the vertebral canal and a curved needle was passed through the lateral extension of vertebral canal at lesion center (Ilha et al., 2011; Ramón-Cueto et al., 2000). A piece of hemostatic sponge (Technew, São Paulo—SP, Brazil) was placed on the transection site, and then muscles, connective tissue and skin were sutured. The animals were gently warmed until recovery.

4.3. *Post-operative care*

Animals received the analgesic Dimorph (morphine sulfate, s.c., 0.08–0.16 mg/kg, Cristália, São Paulo—SP, Brazil) twice a day, during the first 4 days post-injury. Rats were also treated prophylactically with Baytril (Enrofloxacin, s.c., 2.5 mg/kg, Bayer, São Paulo—SP, Brazil) to prevent urinary tract infections for 14 days. Bladders were manually expressed twice a day until it was no longer distended and palpable, indicating that the animal had developed an automatic bladder voidance reflex (15–20 days). Animals were daily monitored for infections and general health throughout the post-injury survival period. Animals did not exhibit autophagia during the experimental period.

4.4. *Dissection and lamina propria preparation*

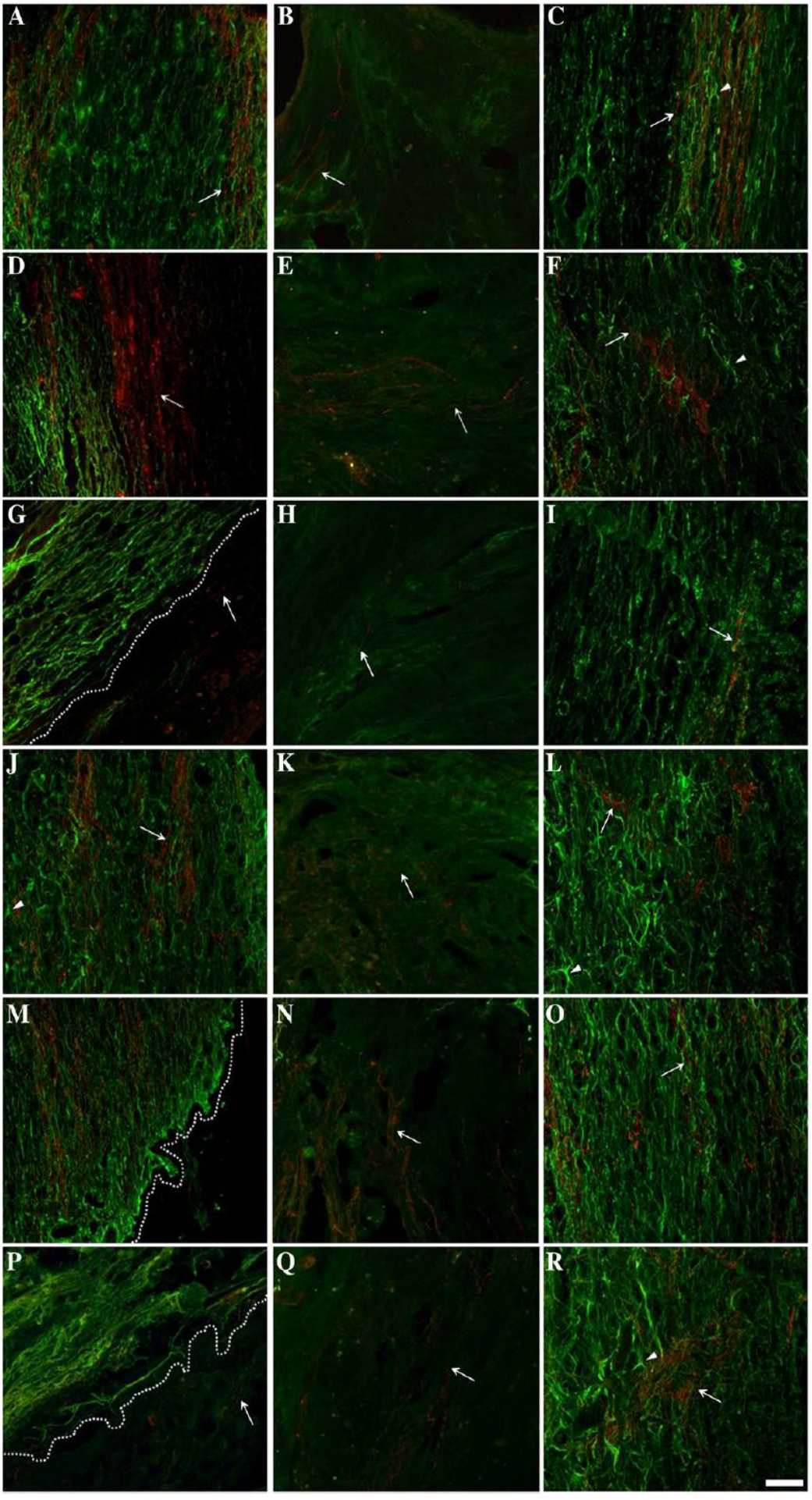
OLP and RLP were dissected according to the method described by Steward et al. (2006). Donors male *Wistar* rats (n=36), 280–380 g in body weight and 13 weeks old, were decapitated. The head was bisected just off the midline in such a way as to allow visualization of the nasal septum and OB. The nasal septum was removed using microscissors and placed in a Petri dish containing Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F12 tissue culture media (DMEN/F12, Sigma-Aldrich, USA).

Olfactory mucosa bilaterally lines the posterior part of the nasal septum and its lamina propria contains OECs. Fig. 8A shows a coronal section of the olfactory mucosa, with the olfactory epithelium and OECs in lamina propria. These fusiform glial cells were identified by their immunoreactivity for p75 neurotrophin receptor (rabbit anti-p75NTR, 1:300, Sigma-Aldrich, USA, N3908), S-100 (rabbit anti-S-100, 1:600, Sigma-Aldrich, USA, S2644) and GFAP in low intensity (mouse anti-GFAP, 1:400, Sigma-Aldrich, USA, G3893) (Ramer et al., 2004; Ramón-Cueto and Avila, 1998).

Respiratory mucosa is thinner than olfactory mucosa and bilaterally covers the dorso-anterior part of the nasal septum. As shown in Fig. 8B, RLP is devoid of OECs. However, p75, S-100 and GFAP markers alone are not exclusive to these glial cells and the staining observed in RLP could be related to the presence of Schwann cells from the trigeminal nerve (Mackay-Sim and St John, 2011).

Using a scalpel, two similar sized pieces of olfactory or respiratory mucosa were dissected from the donor's nasal septum and immediately placed in ice-cold DMEN/F12. In the respiratory tissue dissection, the vomeronasal nerve was avoided. Olfactory and respiratory tissues were separately in-

Fig. 4 – Longitudinal spinal cord sections, double-stained for 5-HT (red) and GFAP (green) at approximately 18 weeks post-injury. AC group (A–C), AT group (D–F), 2WDC group (G–I), 2WDT group (J–L), 4WDC group (M–O) and 4WDT group (P–R). Left—rostral stump (dashed lines represents the lesion border); Center—lesion epicenter (GFAP negative); Right—caudal stump. The majority of 5-HT axons were located in rostral non-injured areas and stopped at the border of the scar. Note the absence of 5-HT axon immunoreactivity in the lesion epicenter and caudal stump. Arrows indicate 5-HT fibers. Arrowheads point to astrocytic cell body. Magnification—40×. Optical stack thickness—0.69 μm. Pixel size—397×397 μm. Scale bar—50 μm. Abbreviations: 2WDC—2-Week Delayed Control; 2WDT—2-Week Delayed Treated; 4WDC—4-Week Delayed Control; 4WDT—4-Week Delayed Treated; 5-HT—Serotonin; AC—Acute Control; AT—Acute Treated; GFAP—Glial Fibrillary Acidic Protein.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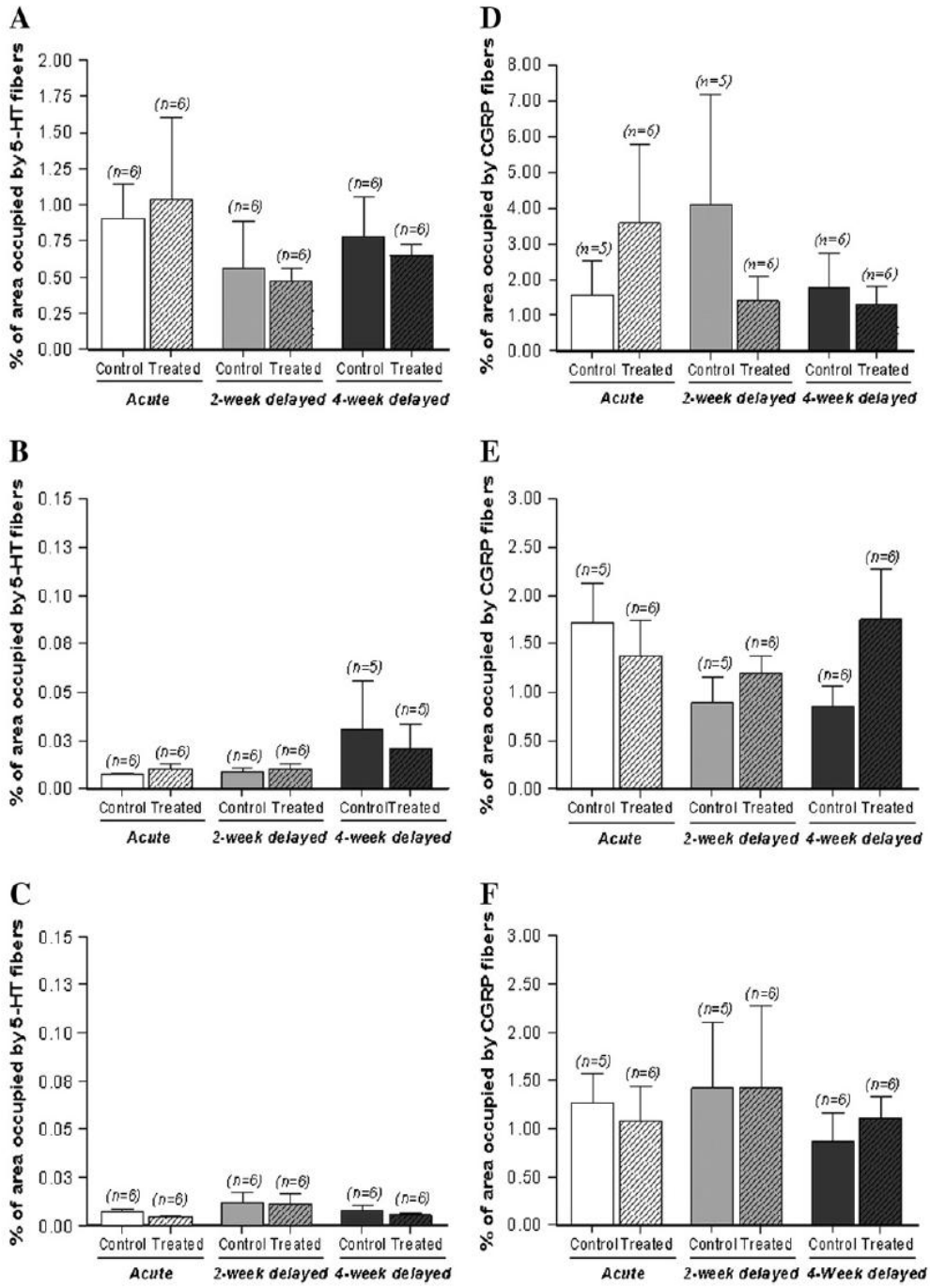


Fig. 6 – Quantitative data of 5-HT (A–C) and CGRP (D–E) axon profiles in rostral (above), lesion (center) and caudal (below) regions of spinal cord at approximately 18 weeks post-injury. There were no statistical differences between groups transplanted with olfactory or respiratory lamina propria in the studied fibers ($p > 0.05$). Abbreviations: 5-HT—Serotonin; CGRP—Calcitonin gene-related peptide.

cubated in 2.4 units/mL dispase II solution (Sigma-Aldrich, Germany, D4693) at 37 °C. After enzymatic digestion, both types of lamina propria samples were carefully separated from the epithelium using a micro-spatula under a dissection microscope and then cut into small pieces (approximately 3–4 mm^2 for grafting). Then, the tissue was rinsed with Hank's

Fig. 5 – Longitudinal spinal cord sections, double-stained for CGRP (red) and GFAP (green) at approximately 18 weeks post-injury. AC group (A–C), AT group (D–F), 2WDC group (G–I), 2WDT group (J–L), 4WDC group (M–O) and 4WDT group (P–R). Left—rostral stump (dashed lines represents the lesion border); Center—lesion epicenter (GFAP negative); Right—caudal stump. Note the presence of CGRP axon immunoreactivity in the lesion epicenter, rostral and caudal stumps. Arrows indicate CGRP fibers. Arrowheads point to astrocytic cell body. Magnification—40×. Optical stack thickness—0.69 μm. Pixel size—397 × 397 μm. Scale bar—50 μm. Abbreviations: 2WDC—2-Week Delayed Control; 2WDT—2-Week Delayed Treated; 4WDC—4-Week Delayed Control; 4WDT—4-Week Delayed Treated; AC—Acute Control; AT—Acute Treated; CGRP—Calcitonin gene-related peptide; GFAP—Glial Fibrillary Acidic Protein.

Buffered Salt Solution (HBSS, Sigma-Aldrich, Brazil) and placed in iced DMEM/F12 until transplantation into the host.

4.5. *Transplantation of OLP and RLP*

The acute animal groups were transplanted immediately after spinal cord transection with RLP (AC group) or OLP (AT group). The other animal groups received RLP and OLP grafts 2 weeks post-SCI (2WDC and 2WDT groups, respectively) and 4 weeks post-SCI (4WDC and 4WDT groups, respectively). For this procedure, rats were re-anesthetized (as described above) and the original incision was re-opened. Scar tissue was removed and the gap between the rostral and caudal stumps was filled with pieces of respiratory (2WDC and 4WDC groups) or olfactory (2WDT and 4WDT groups) lamina propria (Fig. 7A, right). A piece of hemostatic sponge (Hemospon, Technew, São Paulo—SP, Brazil) was placed over the transplantation site to ensure blood homeostasis. Again, muscle and skin layers were sutured and post-operatory care was maintained as previously described.

Approximately 18 weeks after spinal cord injury, the viability of grafted tissue was demonstrated by the presence of fusiform-shaped OECs immunoreactive for p75NTR, S-100 and GFAP at the site of spinal cord transection (Fig. 8C). RLP control grafts continued to be devoid of OECs, as confirmed by the lack of cells expressing the three markers used in the lesion area (Fig. 8D).

4.6. *Behavioral assessment*

Hindlimb motor function was assessed using the BBB locomotor rating (Basso et al., 1996). This scale is qualitative, widely used and designated to assess the functional recovery of hindlimbs after lesions in thoracic spinal cord. The score of this scale ranges from 0 (no hindlimb movement) to 21 (normal movement of the hindlimbs). In this study, BBB assessment was accomplished preoperatively (naive) and postoperatively after the SCI (at days 5, 20, 35, 50, 65, 80 post-injury for the AC and AT groups; at days 5, 20, 35, 50, 65, 80, 95 post-injury for the 2WDC and 2WDT groups; and at days 5, 20, 35, 50, 65, 80, 95, 110 post-injury for the 4WDC and 4WDT groups). For each test, rats were placed in an open-field (60×30×40 cm) for 5 min. The test session was recorded with a video camera (Sony Handycam DCR-SR88, São Paulo—SP, Brazil) to allow later analysis by a blinded observer. The scores of the left and right hindlimbs were averaged and taken as the BBB score of each animal.

4.7. *Retrograde tracer injection*

At the end of behavioral analysis, rats were anesthetized as described above. An incision was made at the T12 vertebrae level to expose the spinal cord below the SCI site. After a laminectomy, FG retrograde tracer (2% dextran tetramethylenerhodamine, Biotium Inc., Hayward—CA, USA) was injected using a stereotaxic apparatus (Insight, Ribeirão Preto—SP, Brazil) coupled to a 1 μL Hamilton syringe (Hamilton Company, Reno—NV, USA). Three injections of FG (0.05 μL, 1 min duration each) were made at midline (0.5, 0.8 and 1.5 mm deep) and 1 mm laterally (0.5, 0.8 and 1.2 mm deep) in each side of this spinal cord level (Steward et al., 2006). Post-operatory care was done as previously described.

4.8. *Tissue preparation and immunohistochemistry*

One week after the retrograde tracing injections, rats received an overdose of pentobarbital (100 mg/kg body weight, i.p., Cristália, São Paulo—SP, Brazil) and were transcardially perfused with saline solution and buffered 4% paraformaldehyde (pH 7.4) using a peristaltic pump (30 mL/min, Milan Equipamentos Científicos, Colombo—PR, Brazil). Brain, brainstem and thoracic spinal cord with approximately 2-cm long (including the lesion site) were removed, post-fixed in the same fixative solution and cryoprotected in 15% and 30% sucrose in phosphate buffer saline (PBS). Prior to embedding in Tissue-Tek, spinal cord samples were photographed with a digital camera (Sony Cyber-Shot DSC-S950, São Paulo—SP, Brazil) on a dark background to provide morphological visualization of the injury site (Fig. 7B). After this, samples were quickly frozen in isopentane (Merck, Germany) cooled in liquid nitrogen and stored at −80 °C.

Primary somatosensory cortex, primary and secondary motor cortex and the entire brainstem were serially sliced (200 μm thick, 150 μm apart) using a cryostat (CM1850, Leica, São Paulo—SP, Brazil) to allow retrograde tracer visualization. These sections were mounted on gelatin-coated glass slides, covered with aqueous mounting medium (FluorSave, Calbiochem, Darmstadt, Germany) and coverslips. The entire spinal cord samples were longitudinally cut (25 μm), in a series of 5 slides per animal with 7–8 sections per slide. Two slides per animal were used to perform immunohistochemistry by the peroxidase method (Sternberger, 1979). Initially, sections were washed in PBS, followed by a 30 min period with 3% hydrogen peroxide (H_2O_2). After several washes in PBS, sections were pre-incubated in 1% albumin solution with 0.4% triton X-100 (PBS-Tx). Then, slices were incubated for 48 h at 4 °C in either GFAP (rabbit anti-GFAP, 1:200, DAKO Denmark A/S, Denmark, Z0334) or GAP-43 antibodies (mouse anti-GAP-43, 1:500, Santa Cruz Biotechnology Inc., USA, SC33705). Sections were rinsed in PBS-Tx and re-incubated in goat anti-rabbit IgG (1:100, Sigma-Aldrich, USA, R2004) or goat anti-mouse IgG (1:100, Sigma-Aldrich, USA, M8642) for 2 h. Following PBS washes, slices were placed in peroxidase anti-peroxidase (1:500, Sigma-Aldrich, USA, P1291) for 1 h and 30 min. The immunohistochemical reaction was developed by incubating the slices in a medium containing 0.06% 3,3 diaminobenzidine (DAB, Sigma-Aldrich, USA, D5637) and then in the same solution containing 1 μM of 3% H_2O_2 per mL of DAB medium for 10 min each. Finally, slices were rinsed with PBS, dehydrated with ethanol, cleared with xylene and covered with Permount and coverslips. Control sections were prepared by omitting the primary antibody and replacing it with PBS.

In double staining protocols, fibre tracts were stained using the following antibodies: rabbit anti-serotonin (1:5000, Sigma-Aldrich, USA, S5545) for serotonergic axons in the spinal cord coming from raphe nuclei; and rabbit anti-CGRP (1:1500, courtesy of Dr. Rodrigo, Instituto Cajal, Spain) as a marker for ascending sensory neurons. Fibrous scar borders were defined using immunoreactivity to GFAP (mouse anti-GFAP, 1:400, Sigma-Aldrich, USA, G3893). The protocol consisted of

Table 2 – Studies performed with several types of olfactory ensheathing cells transplantation in different spinal cord injury models.

Reference and injury model	Species/strain	Graft properties	Transplantation time	Behavioral results	Histological results	Survival
Takami et al. (2002a) –Moderate contusion at T9 level	Adult female Fisher rats	–OB OECs from adult female Fischer rats –SCs from adult female Fischer rats –Combination of OECs/SCs	–7 days post—injury	–No improvements of BBB score in OEC group –Increase in BBB scores at 8–11 weeks post-injury in SCs only group (10.8 to 11.8)	–Less cavitation and more sparing in grafted groups –Less intense GFAP and CSPG staining in OEC—only grafts versus SCs –Higher number of propriospinal and brainstem axons reached long distances beyond the grafted area with SCs and SC/OEC grafts but not with OEC only –Corticospinal fibers terminate closer to the lesion epicenter in grafted animals.	–12 weeks
Barakat et al. (2005) –Moderate contusion at T9 level	Adult female Fischer rats	–OB OECs from adult female Fischer rats –SCs from adult female Fisher rats	–8 weeks post-injury	–SCs but not OECs resulted in increased BBB scores (Control: 8.5; SC group: 10.2; OEC group: 8.5) –SCs but not OECs resulted in small improvements in base of support and hindpaw rotation	–8–11 weeks after transplantation, SCs survive better than OECs –NF positive axons where observed in OEC transplantation –SC transplants, but not OEC, contained significantly more CGRP and 5-HT positive axons –CGRP fibers arise primarily from DRGs adjacent to the lesion –SCs did not promote corticospinal fibers growth	–19 weeks
Pearse et al. (2007) –Moderate contusion at T8 level	Adult females Fischer rats	–OB OECs from adult female Fischer rats –SCs from adult Fischer rats –FBs from adult Fischer rats	–7 days post-injury	–At 9 weeks, only SC+OEC injection group increased BBB scores (12.3) vs. FBs transplant (9.8) and injury controls (10.7) –No transplant reduced gridwalk errors or reduced base of support and stride length	–Cell survival decreased to a low level by 3 weeks post-transplantation, especially when injected at transection site –Migration of OEC—only was not observed –At later times, significant host SCs infiltration was shown –No sensory or supraspinal axon growth into transplants –Host axons were associated with or ensheathed by transplanted glia –Numerous myelinated axons were found within regions of grafted SCs but no OECs	3 days or 3, 9 and 28 weeks
Richter et al. (2005) –Crush of dorsolateral funiculus at C3–C4 level	Adult male Sprague Dawley rats	–OB OECs of P5 mice expressing eGFP –LP OECs of P5 mice expressing eGFP	–Immediately post injury	–Not reported	–OEC survival was higher when transplanted in both stumps –Both OEC types reduced lesion and cavity formation, increased angiogenesis, endogenous Schwann cell infiltration and axonal sprouting –LP–OECs increased outgrowth of axonal subpopulations but also increased autotomy	–1 or 28 days

(continued on next page)

Table 2 (continued)

Reference and injury model	Species/strain	Graft properties	Transplantation time	Behavioral results	Histological results	Survival
Lu et al. (2006) –C4 spinal cord dorsal column wire knife lesion	Adult female Fischer rats	–OECs from olfactory mucosa of postnatal day 5 Fischer rats –Bone stromal cells of adult female Fischer rats FBs of adult female Fischer rats	–Immediately post-injury	–Not reported	–OECs failed to support bridging of corticospinal axons –The "bridging" tract of OECs formed within 1 h of cell injection, increasing the possibility that cells passively spread –NF positive axons penetrate into the lesion site dependently or independently from regions with OEC tracts SCs infiltrated in OEC grafts of lesion cavity and associate with penetrating axons –OECs proliferated in injection sites, cell tracts and lesion sites, indication that these cells can also accumulate through proliferation	–1, 3, 12 or 24 h –3, 7, 9 days PI – 4 weeks
Ramón-Cueto et al. (1998) –Complete transection at T9 level (4 mm gap)	Adult female Fischer rats	–OECs from adult Fischer rats and SCs cells from adult Fischer rats within a channel with Matrigel	–Immediately post-injury	–Not reported	–Numerous NF, GAP-43, CGRP, and 5-HT positive fibers traversed both interfaces of the cord with the channel filled with SCs and OECs stumps injection –5-HT axons extended long distances along the connective tissue outside of the channels to the caudal spinal cord –Ascending propriospinal axons were seen caudally to the graft –OECs integrated and migrated through host and graft tissue	–6 weeks
López-Vales et al. (2007) –Complete transection at T8 level	Adult female Sprague Dawley rats	–OB OECs from P22-P23 Sprague Dawley rats	–Immediately post-injury –30 min post-injury – 7 days post-injury	–OECs reduced hindlimb hyperreflexia and increased BBB scores (Control: 0–2; Acute OEC group: 4,4; Delayed OEC group: 3,7) –OECs transplants recovered MEP	–OECs produced axonal regeneration of raphespinal, coerulospinal and corticospinal axons within the caudial stump –Expression of GFAP and NG2 was reduced in perilesional cord segments in transplanted animals	–36 weeks
López-Vales et al. (2007) –Complete transection at T8 level	Adult female Sprague Dawley rats	–OB OECs from P22-P23 Sprague Dawley rats	–45 days post-injury	–OECs increased BBB scores (Control: 0.92; OEC group: 2.5) –Limited recovery of MEP with OEC vs. no recovery in control	–No significant amounts of corticospinal and 5-HT positive axon growth through the lesion site and into the caudal spinal cord from transplanted animals –Some 5-HT axons extended long distances through the gray matter –Delayed transplantation of OECs failed to reduce astrogliosis	–20 weeks
Lu et al. (2001) –Complete transection at T10 level (1–2 mm gap)	Adult female Sprague Dawley rats	–OLP from adult Sprague Dawley rats –LP OECs from adult Sprague Dawley rats	–Immediately post-injury	–Both OEC types increased BBB scores (Control: 0–2; OEC group: 6–8) –OECs recovered spinal reflex circutry (assessed using the	–Nerve fibers passed through the transection site in OLP transplanted animals –5-HT positive fibers were found distal to the transection site –Retrograde labeling of brainstem raphe and gigantocellularis neurons were observed,	–10 weeks

				rate-sensitive depression of the H-reflex)	indicating regeneration of descending pathways in OLP transplanted animals	
Lu et al. (2001) –Complete transection at T10 level	Adult female Sprague Dawley rats	–OLP from adult female Sprague Dawley rats –RLP from adult female Sprague Dawley rats	–4 weeks post-injury	–OLP significantly increased BBB scores (OLP group: 4.3; RLP group: 1.0)	–5-HT positive axons were observed caudal to the transection site in OLP transplanted animals	–10 weeks for histology and 14 weeks for behavior
Steward et al. (2006) –Complete transection at T10 level	Adult female Sprague Dawley rats	–OLP from adult female Sprague Dawley rats –RLP from adult female Sprague Dawley rats	–4 weeks post-injury	–No significant differences in BBB scores between groups at any time point –No differences in bladder retention of urine	–FG injection caudal to the lesion did not reveal evidence of regeneration of descending axons across transection site –Few 5-HT positive axons extended in both lamina propria transplants; –Few 5-HT positive axons were also found caudal to the injury in 2 animals that received OLP and in one animal that received RLP	–10 weeks
Present study –Complete transection at T8–T9 level (1–2 mm gap)	Adult male Wistar rats	–OLP from adult male Wistar rats –RLP from adult male Wistar rats	–Immediately post-injury –2 weeks post-injury –4 weeks post-injury	–No significant differences in BBB scores between groups at 42 days post-transplantation (AC: 3.5; AT: 2.7; 2WDC: 2.6; 2WDT: 3.0 4WDC: 2.6; 4WDT: 2.0)	–All transplants produced comparable results for spinal cord tissue sparing and sprouting evaluated using GFAP and GAP-43 staining –Acute transplantation of OLP and RLP seems to foster some limited supraspinal axonal regeneration as observed with FG tracing –A higher number of 5-HT positive fibers was found in the cranial stump of OLP and RLP groups compared to the lesion and caudal regions; CGRP fibers were present in considerable number at the SCI site in both types of transplantation	–Approximately 18 weeks

Abbreviations: 2WDC-2-Week Delayed Control; 2WDT—2-Week Delayed Treated; 4WDC—4-Week Delayed Control; 4WDT—4-Week Delayed Treated; 5-HT—Serotonin; AC—Acute Control; AT—Acute Treated; BBB—Basso, Beattie, and Bresnahan Scale; CGRP—Calcitonin Gene-Related Peptide; CSPG—Chondroitin Sulfate Protooglycan; DRGs—Dorsal Root Ganglia; eGFP—Green Fluorescent Protein-expressing; FBs—Fibroblasts; FG—Fluorogold; GAP-43—Growth Associated Protein-43; GFAP—Glial Fibrillary Acidic Protein; LP OECs—Olfactory Ensheathing Cells from Lamina Propria; MEP—Motor Evoked Potentials; NF—Neurofilament; OB OECs—Olfactory Ensheathing Cells from Olfactory Bulb; OEC—Olfactory Ensheating Cells; OLP—Olfactory Lamina Propria; RLP—Respiratory Lamina Propria; SCs—Schwann Cells; WGA–HRP—Agglutinin–Horseradish Peroxidase.

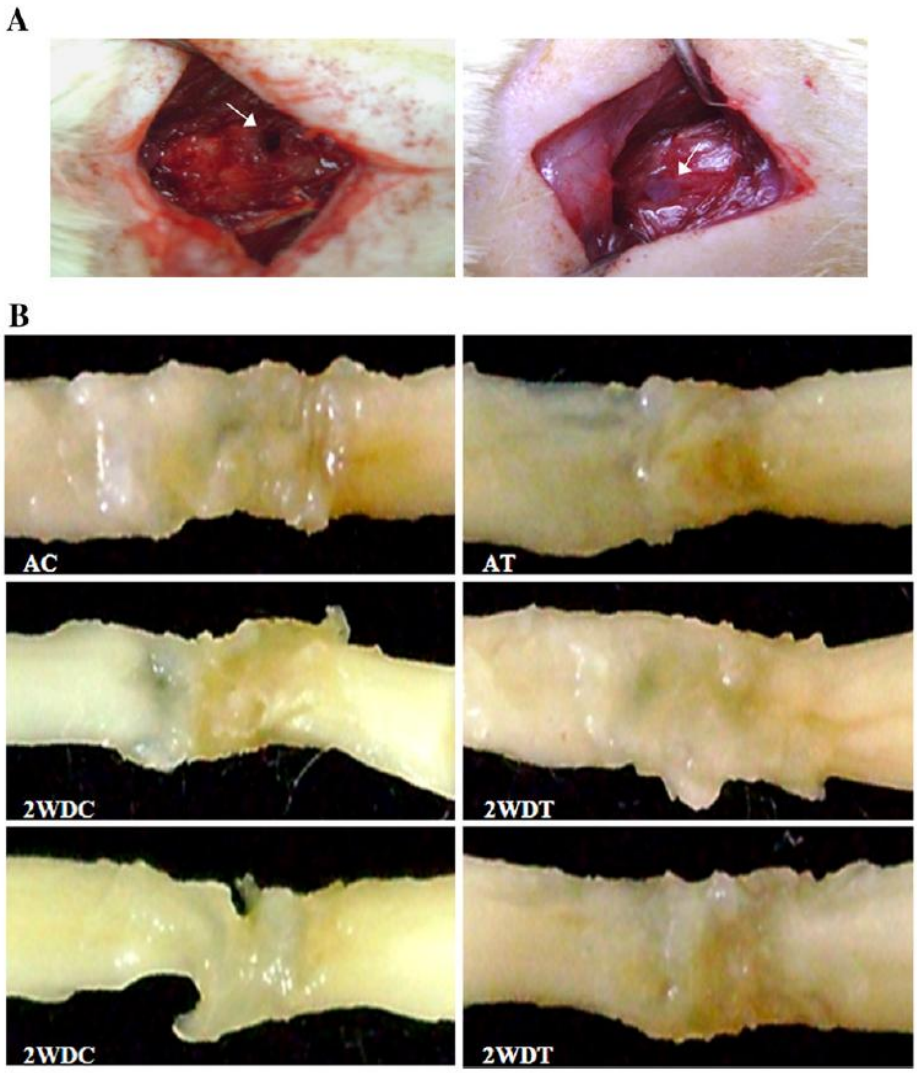


Fig. 7 – (A) Illustration of the cavity formed as a result of the spinal cord injury procedure (left, arrow) and appearance of the transplanted olfactory or respiratory lamina propria in the lesion gap (right, arrow). (B) Macroscopic view of the spinal cords from different animal groups. Note that the transection site is filled with transplanted tissue and these transplants bridge a gap between the rostral (left) and caudal segments.

washing the sections with PBS, followed by permeabilization with 0.25% PBS-Tx. After this, sections were blocked in 1% albumin for 30 min. Incubation with the first antibodies was carried out in 1% albumin in PBS-Tx at 4 °C for 48 h. Following PBS washes, sections were incubated in secondary antibodies anti-mouse Alexa 488 (1:500, Molecular Probes, Invitrogen, USA, A10680) and anti-rabbit Alexa 555 (1:500, Molecular Probes, Invitrogen, USA, A21428). The slides were covered with aqueous mounting medium (FluorSave, Calbiochem, Darmstadt, Germany) and coverslips.

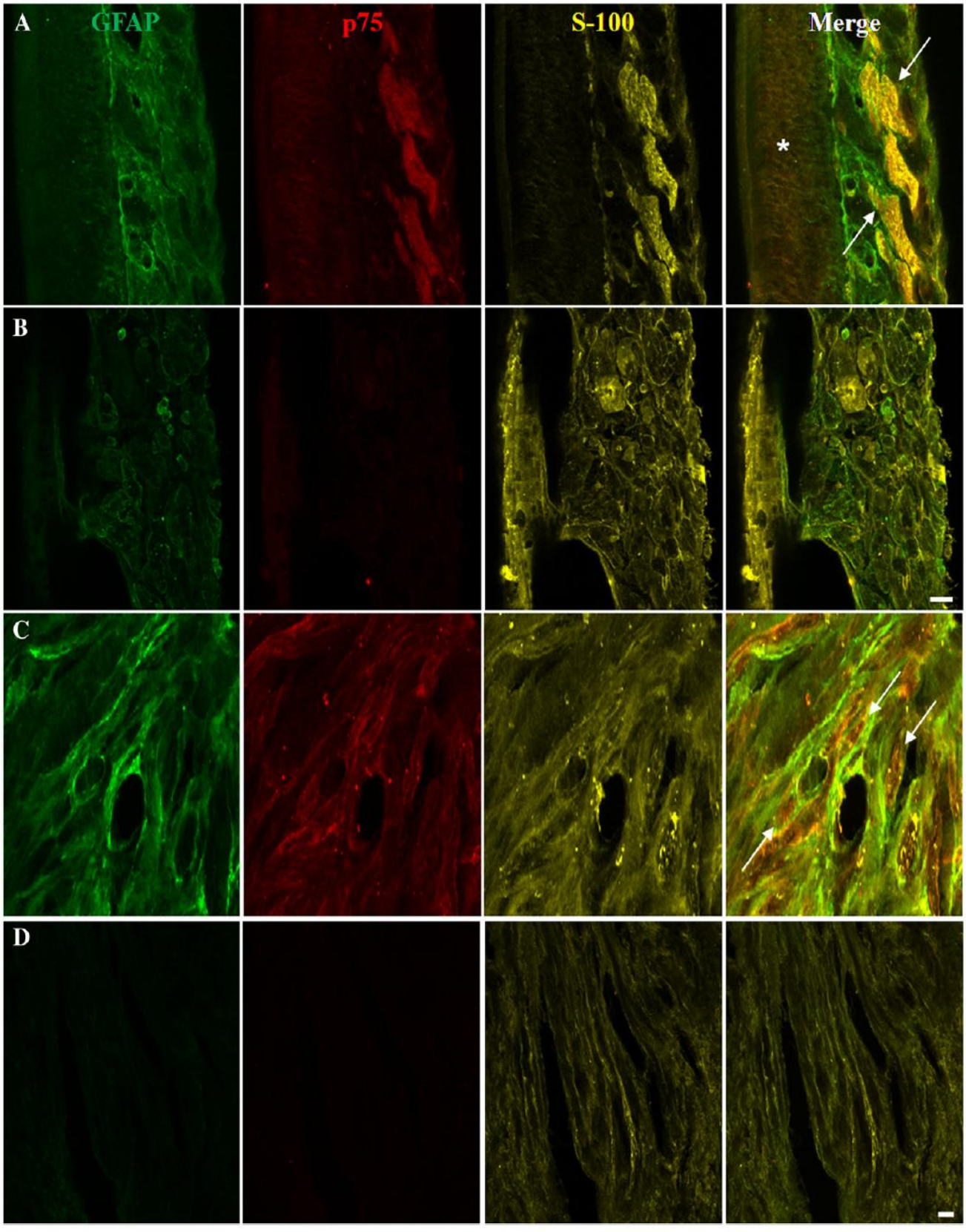
4.9. *Image analysis*

4.9.1. *Retrograde tracing assessment*

Sections from the entire brainstem and sensorimotor cortex (n=3 per group) were visualized in serial stack images

Fig. 8 – (A) Olfactory and (B) respiratory mucosa dissected from nasal septum of adult rat, immunostained by GFAP (first column), p75 (second column) and S-100 (third column). Olfactory ensheathing cells were identified by immunoreactivity for the three markers used (arrows). Respiratory lamina propria is devoid of olfactory ensheathing cells. Asterisks indicate olfactory epithelium. Magnification—600× (water objective). Pixel size—265×265 μm (A—0.57 μm optical stack thickness and 44 confocal planes; B—1.0 μm optical stack thickness and 21 confocal planes). Scale bar—20 μm. (C) Olfactory and (D) respiratory lamina propria at the site of spinal cord transection, immunostained by GFAP (first column), p75 (second column) and S-100 (third column). Olfactory ensheathing cells maintained their fusiform aspect and continued to express the same three markers observed before transplantation (arrows). Respiratory lamina propria remains devoid of olfactory ensheathing cells. Magnification—400×. Pixel size—497×497 μm (C—0.76 μm optical stack thickness and 21 confocal planes; D—0.1 μm optical stack thickness and 14 confocal planes). Scale bar—20 μm. Abbreviations: GFAP—Glial Fibrillary Acidic Protein, p75—p75 Neurotrophin Receptor.

(11.96 μm thick, 16–17 serial stack images per slice) obtained with an Olympus confocal FV-1000. Plan-Apochromat 10× objective lens were used (Numerical aperture - NA 0.30) and the pinhole was set in automatic mode. The *FG signal* (blue) was visualized with a wide band ultraviolet excitation filter (Excitation—331 nm, Emission—418 nm). Images were made using a photomultiplier detector and all pictures were analyzed with Image J Software 1.42q (Wayne Rasband, National Institutes of Health, USA). The total number of FG labeled neurons in the propriospinal and selected supraspinal regions, i.e., MdV/MdD (5 slices per animal on average), PnO/PnC (7 slices per animal on average), Ra (including the raphe pallidus, raphe obscurus, raphe magnus—20 slices per animal on average), SpVe (7 slices per animal on average), LVe (6



slices per animal on average), *locus coeruleus* (LC—4 slices per animal on average), M1/M2 (25 slices per animal on average) and S1 (20 slices per animal on average) was counted bilaterally by a blinded observer (Iannotti et al., 2004; Xu et al., 1995). Axons from these nuclei project to the thoracolumbar spinal cord and play important roles in locomotor function (Holstege and Kuypers, 1987; Iannotti et al., 2004; Kim et al., 2002). The location and number of FG-stained cellular bodies were determined from each section using an overlaid grid and a stereotaxic atlas (Paxinos and Watson, 1998).

4.9.2. *Spinal tissue sparing and GAP-43 optical densitometry*

Images of diaminobenzidine-stained spinal cord sections (20×) were taken using a Nikon Microscope Optiphot-2 (Japan) coupled to a CMOS camera (518 CU, Micrometrics) and analyzed with Image J Software 1.42q. Subsequently, digital RGB (24-bit) images with resolution of 254×254 DPI were converted to grayscale (8-bit) and corrected for unequal illumination (shading correction). All lightning conditions and magnifications were held constant.

To evaluate spinal tissue sparing, pictures of GFAP-immunostained spinal cord sections were captured with the lesion-part in the center. Samples with no continuity between rostral and caudal stumps were discarded from this analysis. After standardized background corrections, black-and-white 8-bit images were thresholded and tissue area fractions measured in each section. Since not all sections of the whole spinal could be used for analysis, volume and total area values of spinal cord tissue sparing could not be obtained. On average, 5 images were analyzed from each rat and a mean of spared tissue was calculated for experimental group (6 animals each group) (adapted from Kubasak et al., 2008).

Images of GAP-43 immunohistochemistry were also obtained from the injured part of the spinal cord. After standardized background corrections, a mask of each spinal cord section image was created using an auto-threshold tool from Image J, hence avoiding vacuolization and interrupted tissue integrity. Thereafter, optical densities (OD) of the images were measured from whole injury regions within the area of interest, i.e., the mask itself. OD was calculated using the following formula:

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

Where "OD" is the optical density; "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 6–16 images were analyzed from each rat and 6 animals were analyzed per group.

4.9.3. *Axon profile quantification*

5-HT and CGRP fiber populations were also identified using a Nikon Microscope Optiphot-2 (Japan) with a green excitation filter for the Alexa 555 signal (G-2A, Excitation—510/560). Double-labeling with GFAP antibody was used to delineate the fibrous scar borders and the signal for Alexa 488 was detected using a blue excitation filter (B-2A, Excitation—450/490). Pictures with resolution of 254×254 DPI, were taken at magnification of 200× using a CMOS camera (518 CU, Micrometrics) and analyzed with Image J Software 1.42q. The total area occupied by 5-HT or CGRP axons was determined separately in the rostral, lesion and caudal regions, throughout the width of the tissue sections. To assess 5-HT fibers, pictures were taken of the rostral stump (in the region with abundant visible astrocytes), the central part of the lesion (approximately) and near the scar border of the caudal stump. Analogously, images of CGRP fibers were taken of the caudal stump (in the region with abundant visible astrocytes), in the central part of the lesion (approximately) and near the scar border of the rostral stump. All images (on average, 19 pictures per spinal cord region in each animal, 6 animals per group) were turned into binary (black and white) and a constant threshold value was used to measure the total percentage area (%) occupied by axon fibers.

4.10. *Statistical analysis*

Data were expressed as means±SEM. Open field locomotor scores were analyzed between groups using analysis of variance (ANOVA) with *time* as the repeated measure. When there were statistically significant F values ($p \leq 0.05$), Bonferroni's *post hoc* tests were conducted by comparing OLP transplantation with the corresponding RLP group. Regarding assessment of spinal tissue sparing and regional optical densitometry, all groups were analyzed using one-way ANOVA followed by Bonferroni's *post hoc* test. The Kruskal–Wallis test was used for axon profile data (5-HT or CGRP). Values were run on SPSS 11.5 (Statistical Package for the Social Sciences, Inc., USA).

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4.2 Artigo II

A segunda parte dos resultados está submetida ao periódico *Journal of Neurotrauma,* sob a forma de um artigo intitulado "*Implications of olfactory lamina propria transplantation on hyperreflexia and myelinated fiber regeneration in rats with complete spinal cord transection*". O referido artigo segue-se abaixo.

Journal of Neurotrauma

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Implications of olfactory lamina propria transplantation on hyperreflexia and myelinated fiber regeneration in rats with complete spinal cord transection

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Keywords:	spinal cord injury, TRANSPLANTATION, axonal regeneration, LOCOMOTOR FUNCTION

IMPLICATIONS OF OLFACTORY LAMINA PROPRIA TRANSPLANTATION ON HYPERREFLEXIA AND MYELINATED FIBER REGENERATION IN RATS WITH COMPLETE SPINAL CORD TRANSECTION

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Running title: Spinal cord injury and olfactory lamina propria

Table of Contents title: Olfactory lamina propria transplantation in rats with spinal cord injury

Abstract

Olfactory ensheathing cells (OECs) have been transplanted after several models of spinal cord injury (SCI) in attempt to create a favorable environment for the re-growth of injured axons. However, a consensus on the efficacy of this cellular transplantation has yet to be reached. In order to explore the possible restorative properties of such grafts, the present study investigated the effects of olfactory lamina propria (OLP) transplantation on hyperreflexia and myelinated fiber regeneration in adult rats with complete spinal cord transection. The efficacy of OLP (graft containing OECs) and respiratory lamina propria (RLP, graft without OECs) were tested at different post-injury times (immediately, 2-week and 4-week delayed), to establish the optimum period for transplantation. The transplantation of OLP immediately and 4-week delayed, respectively, reduced withdrawal reflex over-responsiveness and produced discrete frequency-dependent habituation of the H reflex. However, animals transplanted with OLP 2 or 4 weeks after injury exhibit smaller myelin sheath thickness, myelinated fiber area and diameter at the lesion site compared to their respective RLP groups. In the therapeutic windows used, both lamina propria grafts produced comparable results for the myelinated fiber density and for the estimated total number of myelinated fibers at the lesion site, indicating that delayed transplantation approach does not seem to limit the regenerative effects. Despite the ongoing clinical use of OECs, it is important to emphasize the need for more experimental studies to clarify the exact nature of the repair capacity of these grafts in the treatment of SCI.

Key words: Spinal cord injury, Olfactory ensheathing cells, Delayed transplantation, Hyperreflexia, Myelinated fiber regeneration.

Introduction

Hyperreflexia is one of the numerous deficits observed following spinal cord injury (SCI) that limit functional recovery, since the segmentary control of gait is modulated by several spinal reflexes (Valero-Cabré and Navarro, 2001). During normal movement, polysynaptic ipsilateral and contralateral withdrawal reflexes have a role as inductors of the central pattern generator of locomotion, whereas the stretch reflex is important in the control of muscle contraction and interjoint coordination (Abelew et al., 2000; Carrier et al., 1997; Levin and Feldman, 1994). The pathophysiology of the reflex over-responsiveness resulting from SCI is related to the lack of presynaptic inhibition, the control of which is believed to depend on the regeneration of the axonal pathways at the injury site (Hultborn, 2006; Yates et al., 2008a,b,c).

It is known that after SCI, axons do not spontaneously restore the lost neural circuits, but retain the ability to regrow if provided with an appropriate cellular milieu (Coumans et al., 2001; López-Vales et al., 2006). The failure of axons to regenerate at the SCI site contrasts with the renewal capacity of the olfactory system, which is provided with a particular type of glial cell named olfactory ensheathing cells (OECs) (Richter et al., 2005; Zigova et al., 1992). These glial cells support the replacement of olfactory sensory neurons, guiding and ensheathing the newly formed axons from the olfactory lamina propria (OLP) until they establish functional connections at the olfactory bulb (OB) (Guérout et al., 2010; Lu et al., 2006; Ramer et al., 2004).

OECs have been extensively studied and transplanted after several models of SCI, seeking to create a favorable environment for regeneration of injured axons (see Tetzlaff et al., 2011). The OB has been the preferred source of cells, although cells from the OLP could facilitate self-transplantation (Bianco et al., 2004; Lu et al., 2001). Some previous experimental studies have shown promising morphological and functional results (Kubasak et al., 2008; Lu et al., 2001, 2002; Ramón-Cueto et al., 1998, 2000), while others found only limited or no effects (Boyd et al., 2004; Guest et al., 2008; Steward et al., 2006). In addition, we recently reported a failure of olfactory lamina propria (OLP) grafts to recover motor function and serotonergic axon tracts, thus placing the restorative properties of such cells in doubt (Centenaro et al., 2011).

In order to describe alternative parameters that could demonstrate the possible repair capacities of OECs, the current study sought to assess the effects of OLP transplantation on hyperreflexia and myelinated fiber regeneration in adult rats with spinal

cord transection at the thoracic level. The initial hypothesis was that OECs present in the OLP grafts could favor axonal growth at the lesion site and, consequently, restore the presynaptic inhibition in the reflex circuits. Thus, the OLP transplanted groups would exhibit a decrease in hyperreflexia below the injury site when compared to the control groups that received respiratory lamina propria (RLP), a graft devoid of OECs. In our experimental approach, the efficacy of OLP and RLP transplantation was tested at different post-injury times (acutely, 2-week and 4-week delayed), based on previous studies that suggest an optimal window of opportunity for repair post-injury (López-Vales et al., 2007; Takami et al., 2002).

Experimental Procedure

General

Experimental procedures were approved by the Research Ethics Committee of the Universidade Federal do Rio Grande do Sul (Nr. 2007892). The animals were cared for in accordance with Arouca Brazilian law (11794/2008) and the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals (publication no.85-23, revised 1985). The animal handling recommendations from the Colégio Brasileiro de Experimentação Animal (COBEA) and the International Brain Research Organization were also followed.

A total of 108 male *Wistar* rats from a local breeding colony, weighing 280-380 g and aged 13 weeks, were separated into experimental animals (n=72) and lamina propria donors ($n=36$). These animals were maintained in groups of two or three in standard plexigas boxes (46 x 24 x 15 cm), under 12:12 h light/dark cycle, in a temperature controlled environment (20 ± 2 ºC) with food and water *ad libitum*. All tests were conducted during the light phase of the photo cycle.

Experimental animals ($n=72$) were again randomly divided into six groups: (1) Acute control group (AC) – rats submitted to RLP transplantation immediately after spinal cord transection ($n=11$); (2) Acute treated group (AT) – rats submitted to OLP transplantation immediately after spinal cord transection ($n=12$); (3) 2-week delayed control group (2WDC) – rats submitted to RLP transplantation two weeks after spinal cord transection ($n=12$); (4) 2-week delayed treated (2WDT) – rats submitted to OLP transplantation two weeks after spinal cord transection ($n=12$); (5) 4-week delayed control group (4WDC) – rats submitted to RLP transplantation four weeks after spinal cord transection ($n=12$); (6) 4-week delayed treated group (4WDC) – rats submitted to OLP transplantation four weeks after spinal cord transection ($n=12$). Similarly to previous studies, a lesion-only group (i.e., without any type

of transplantation) was not included in the present study in order to minimize the number of animals used and their suffering (Centenaro et al., 2011; Lu et al., 2001, 2002; Steward et al., 2006).

Spinal cord transection and post-operative care

Firstly, animals were adequately anesthetized using pentobarbital (40 mg/kg, i.p., Thiopentax®, Cristália, São Paulo, SP, Brazil) and maintained on a heated pad. The hair overlying the animal´s back in the area of interest was shaved and the skin was cleaned. A midline incision in the thoracic area was made and muscle/connective tissues were dissected to expose the T8-T9 vertebrae. After a laminectomy, the exposed spinal cord was transected at two levels using microscissors (approximately 2-3 mm apart) and the segment between these incisions was removed, leaving a gap. A curved needle was passed through the lateral extension of vertebral canal at lesion area to ensure that a complete transection had been made (Ilha et al., 2011; Ramón-Cueto et al., 2000). A piece of hemostatic sponge (Hemospon®, Technew, São Paulo, SP, Brazil) was placed on the transection site and the surgical wounds were sutured. Then, the animals returned to their home cages that were gently warmed until recovery from surgery.

Regarding post-operative care, the morphine sulfate (0.08-0.16 mg/kg, s.c., twice a day, Dimorph®, Cristália, São Paulo, SP, Brazil) was administered as analgesic during 4 days post-injury and the antibiotic enrofloxacin (2.5 mg/kg, s.c., once a day, Baytril®, Bayer, São Paulo, SP, Brazil) was used to prevent urinary tract infections, for 14 days. Bladders were manually expressed at least twice a day (15 - 20 days) until the bladder was no longer distended and palpable, indicating that the animal had developed an automatic bladder voidance reflex. Throughout the post-injury survival period, animals were monitored daily for infections and general health. No animals exhibited autophagia during the experimental period.

Lamina propria dissection and transplantation

The olfactory mucosa bilaterally lines the posterior part of the nasal septum and its lamina propria contains OECs. By contrast, the respiratory mucosa bilaterally lines the dorso-anterior part of the nasal septum and lacks OECs (to view images, see Centenaro et al., 2011). Olfactory and respiratory mucosa samples were obtained from donors rats (*n=36*), which had been decapitated and had the head bisected just off the midline to allow the visualization of the nasal septum and the OB. The donor´s nasal septum was removed and two

similar sized pieces of both mucosa types were dissected and immediately placed in ice-cold Dulbecco´s Modified Eagle Medium/ Ham´s Nutrient Mixture F12 tissue culture medium (DMEN/F12, Sigma-Aldrich, Steinheim, Germany). Thereafter, the mucosa pieces were incubated in 2.4 units/mL dispase II solution (Sigma-Aldrich, Steinheim, Germany) at 37 °C. After enzymatic digestion, OLP and RLP samples were carefully separated from the mucosa using a micro-spatula under a dissection microscope (Wild, Gais, Switzerland) and then cut into small pieces (approximately 2-3 mm^2 for grafting). Afterwards, both lamina propria samples was rinsed with Hank's Buffered Salt Solution (HBSS, Sigma-Aldrich, Steinheim, Germany) and placed in iced DMEM/F12 until transplantation into the host spinal cord (Steward et al., 2006).

The acute animal groups were transplanted with RLP (AC group) or OLP (AT group) within 15 minutes following spinal cord transection. The other animal groups received RLP and OLP grafts 2 weeks (2WDC and 2WDT groups, respectively) and 4 weeks post-SCI (4WDC and 4WDT groups, respectively). For these delayed transplants, rats were re-anesthetized (as described above) and the original incision was re-opened. Scar tissue was removed and the gap between the rostral and caudal stumps was filled with pieces of RLP or OLP, according to the experimental group. A piece of hemostatic sponge (Hemospon®, Technew, São Paulo, SP, Brazil) was placed over the transplantation site to ensure blood homeostasis. Again, muscle and skin layers were sutured and post-operatory care was maintained as previously described.

Approximately eighteen weeks after SCI, the viability of the grafted tissue was demonstrated by the presence of fusiform-shaped OECs immunoreactive for p75 neurotrophin receptor (p75NTR), S-100 and glial fibrillary acidic protein (GFAP) at the spinal cord lesion site. RLP control grafts continued to be devoid of OECs, as confirmed by the lack of cells expressing these three markers in the lesion area (to view images, see Centenaro et al., 2011).

Spinal reflexes assessment

The withdrawal reflex was elicited prior to injury (naive) and at days 5, 20, 35, 50, 65, 80 post-SCI for the AC and AT groups, at days 5, 20, 35, 50, 65, 80, 95 post-SCI for the 2WDC and 2WDT groups, and at days 5, 20, 35, 50, 65, 80, 95, 110 post-SCI for the 4WDC and 4WDT groups. In each test, animals were handled in such a way that their hindlimbs remained suspended. The skin between the 1st and the 2nd toes of both right and left hindlimbs were briefly pinched with a thin forceps. The mechanical nociceptive stimulus applied was sufficient in strength and duration to elicit the reflex response, but not to cause

any type of injury. Each testing section was recorded with a video camera (Sony Handycam DCR-SR88, São Paulo, SP, Brazil) to allow later analysis. A blinded examiner observed each reflex response and attributed the score "0" to no withdrawal response, "1" to weak withdrawal response (slight flexion of the ankle), "2" to normal withdrawal response (flexion of the ankle and knee, moving the limb away from the noxious stimulus), "3" to hyperactive withdrawal reflex (excessive flexion of the ankle and knee, moving the limb away from the noxious stimulus) and "4" to clonus response (repeated flexion of the ankle and knee, moving the limb away from the noxious stimulus) (adapted from Gale et al., 1985 and Ilha et al., 2011). The average value from both hindlimbs was calculated and taken as the animal's score.

The Hoffman reflex (H reflex) was assessed sixteen weeks after the spinal cord transection. For this procedure, the rats were re-anesthetized as described above and placed prone. After trichotomy and skin cleaning, 5 incisions of approximately 5 mm were made to permit the adequate positioning of monopolar needle electrodes (Neurobase, Campo Grande, MS, Brazil). For stimulation of the sciatic nerve trajectory, two electrodes were positioned (1 cm apart) close to the nerve trunk. Another two electrodes were inserted into the gastrocnemius muscle belly (1 cm apart), to allow electrophysiological recording. One ground electrode was inserted in the gluteus muscle belly (Broetto Cunha et al., 2011). All evaluations were performed using a two-channel electromyograph (Neuro-Mep-Micro, Ivanovo, Russia), linked to a computer (Acer, San Jose, CA, USA) equipped with Neuro-Mep.net software (Ivanovo, Russia). Stimulation of the sciatic nerve produced two responses, an early M-wave produced by direct activation of motoneuronal axons and a later H-reflex, produced by the activation of the muscle afferents which establish synapse on gastrocnemius motoneurons. In order to establish the threshold and maximal response levels, the H-reflex was first tested in 5 trials at a frequency of 0.2 Hz. Thereafter, stimulations at 1, 5 and 10 Hz were performed. The change in the response at various frequencies was calculated as the percentage of the response at 0.2 Hz in order to determine depression of the H-reflex as a function of stimulation frequency (Reese et al., 2005; Yates et al., 2008a,c).

Histological and morphometric study

Ten days after the H reflex assessment, animals received an overdose of pentobarbital (100 mg/kg body weight, i.p., Thiopentax®, Cristália, São Paulo, SP, Brazil) and were transcardially perfused with saline and a fixative solution of 2.5% glutaraldehyde (Merk, Darmstadt, Germany), 4% paraformaldehyde (Synth, Diadema, SP, Brazil) and 0.1 M phosphate buffer (pH 7.4; PB), using a peristaltic pump (30 mL/min, Milan Equipamentos

Científicos, Colombo, PR, Brazil). After this, the spinal cord lesion site were dissected, sectioned at the center and the distal part of these samples were postfixed in 1% OsO4 (Sigma chemicals Co, Saint Louis, Mo, USA) in PB, dehydrated in a graded series of alcohol and propylene oxide (Acros, NJ, USA), embedded in resin (Durcupan ACM ®, Fluka, Buchs, Werdenberg, Switzerland), and polymerized at 60°C (Hermel et al., 2006; Ilha et al., 2008). Transverse-semithin sections (1µm) were serially sliced using an ultramicrotome (Leica, Wetzlar, Hesse, Germany) at three regions: in the lesion epicenter, 1 and 2 mm caudally. Finally, the sections were stained with 1% toluidine blue (Merk, Darmstadt, Bundesland, Germany) in 1% tetraborate (Ecibra, Santo Amaro, SP, Brazil).

Digital images of the spinal cord sections (1000x initially and further amplification of 50x for analysis) were acquired using an optical microscope (Optiphot-2®, Nikon, Tokyo, Japan) coupled to a CMOS camera (Micrometrics 518 CU ®, Accu-Scope Inc., Commack, NY, USA) and Image Pro Plus Software 6.0 (Media Cybernetics Inc., Bethesda, MD, USA). For each rat, 8 images were taken at lesion epicenter, 1 and 2 mm caudally, totalizing 24 images per animal. The first two images were obtained of the most lateral regions of the section and used to determine the fiber density and estimate the total number of fibers (see below), while the others were taken randomically in regions with a higher number of myelinated axons fibers. The average number of fibers analyzed per animal group in the three spinal cord regions was 2280.

The morphometric measurements were calculated in both large and small myelinated fibers and included the following parameters: (1) myelinated fiber density (number of fibers/mm^2); (2) estimated total number of myelinated fibers in the section (number of fibers/mm^2); (3) average myelinated fiber area (μm^2); (4) average myelinated fiber diameter (µm); (5) average axon diameter (µm) and (6) average myelin sheath thickness (µm) (Ilha et al., 2008; Michailov et al., 2004). The myelinated fiber density was determined by the ratio of the myelinated fibers/total area of interest analyzed (1109.26 μm^2), while the estimate of the total myelinated fibers in the section was made multiplying the myelinated fiber density by the total area of the spinal cord section. The average myelin sheath thickness was examined using the measurement tools in Image Pro Plus software. The areas were estimated using a pointcounting technique (using grids with point density of 1 point per 0.936 μm^2 and the following equation (Da Silva et al., 2007; Hermel et al., 2006):

$$\hat{A}=\Sigma p.a/p$$

Where: $\hat{A}$ is the area, Σp is the sum of points, and a/p is the area/point value (0.936 μm^2).

To estimate the fiber and axon diameters, the area of each individual fiber was measured and the value obtained was converted to the diameter of a circle having an equivalent area.

Statistical Analysis

Withdrawal and H reflex data were expressed as mean ± SEM (standard error of the mean) and analyzed using analysis of variance (ANOVA) with *time* and *frequency* as the repeated measures, respectively. When statistically significant differences were found, Bonferroni's *post hoc* tests were conducted by comparing OLP transplantation with the corresponding RLP group. Because the morphological measurements failed to assume the Gaussian distribution, they were expressed as median ± interquartile range. The statistical analysis was performed using the non-parametric Kruskal-Wallis test followed by Dunn´s multiple comparison test. The differences were considered significant when $p \leq 0.05$.Values were run on SPSS 11.5 (Statistical Package for the Social Sciences Inc., Armonk, NY, USA).

Results

Withdrawal reflex

Figure 1 illustrates the average withdrawal reflex scores before and across the post-injury survival interval for the experimental groups. Prior to the injury (naive test), animals exhibited normal reflex activity (scores around 2). A hyperreflexive withdrawal response (scored as 3 or 4) was usually observed as from test 2 (20 days post-injury).

In the AC group, the higher scores in the withdrawal reflex response persisted until the last test session (test 6) (mean±SEM; 3.04±0.28) when compared to the naive test (1.59±0.18). By contrast, the AT group showed no differences in tests 5 (2.83±0.30) and 6 (2.90±0.38), compared to the naive test (1.91±0.05). No differences were found between the AC and AT groups in all test sessions (one-way repeated measures ANOVA; $F_{(1,21)}=0.27$, $p>0.05$) (Figure 1A).

In relation to the 2WDT group, lower withdrawal reflex scores were found in tests 2, 3 and 4 when compared to the 2WDC group (one-way repeated measures ANOVA; $F_{(1,22)}=16.00$, $p<0.05$). In the last assessment (test 7), both animal groups exhibited similar levels of hyperreflexia, showing significantly higher scores (3.62±0.12 and 3.70±0.14 for 2WDC and 2WDT, respectively) compared to the naive test (1.83±0.07 and 1.41±0.12 for 2WDC and 2WDT, respectively) (Figure 1B).

Animal groups transplanted 4 weeks post-injury also showed a hyperactive withdrawal reflex in the last test session (test 8) (3.66±0.17 and 3.62±0.13 for 4WDC and 4WDT, respectively) in contrast to their scores observed in the naive test (1.75±0.16 and 1.83±0.14 for 4WDC and 4WDT, respectively). No differences were found between the 4WDC and 4WDT groups in all test sessions (one-way repeated measures ANOVA; $F_{(1,22)}$=0.42, p>0.05) (Figure 1C).

H reflex

Analysis of the H reflex amplitude at increased low-frequencies (1, 5, and 10 Hz) showed no differences between the groups transplanted with OLP or RLP, either acutely or 2 weeks post-SCI (one-way repeated measures ANOVA; $F_{(1,8)}$=0.33, p>0.05; $F_{(1,7)}$=1.08, p>0.05, respectively). Only the 4WDT group showed lower H reflex amplitude in the frequencies studied, when compared to the 4WDC group (one-way repeated measures ANOVA; $F_{(1,7)}$=59.30, p<0.05) (Figure 2A-C).

Morphometric analysis

The myelinated fiber area, the myelinated fiber diameter and the myelin sheath thickness were smaller in the 2WDT and the 4WDT groups compared to their respective controls, the 2WDC and the 4WDC groups (Kruskal-Wallis one-way ANOVA; p<0.0001) (Figures 3, 4). No significant differences were observed in relation to the parameters cited above in the acutely transplanted groups (Figures 3, 4). A smaller axon diameter was found in the 2WDT group compared to the 2WDC group (Kruskal-Wallis one-way ANOVA; p<0.0001) (Figures 3, 4).

In the myelinated fiber density analysis, no differences were found between the OLP groups transplanted acutely, 2-week or 4-week delayed and their respective RLP groups at the lesion epicenter, 1 and 2 mm caudally (Kruskal-Wallis one-way ANOVA; p>0.05) (Figure 5). In the ANOVA analysis, there was a difference in the estimated total number of myelinated fibers between the groups, but this difference was not maintained in the post-hoc test (Kruskal-Wallis one-way ANOVA; p=0.04) (Figure 5).

Discussion

The present study was designed to investigate the therapeutic potential of OECs by performing OLP transplantation in adult rats with a complete thoracic spinal cord

transection, at three different post-injury times (acutely, 2-week and 4-week delayed). In the last test, the withdrawal reflex over-responsiveness of the animals that received acute transplantation of OLP returned to the scores observed prior to the lesion. There was a discrete low frequency-dependent habituation of H reflex in the animals transplanted 4-weeks after injury with OLP, but this group exhibited a smaller myelin sheath thickness, myelinated fiber area and diameter at the lesion site compared to the respective RLP control group. Animals transplanted 2-weeks delayed with OLP also showed smaller myelin sheath thickness, axon diameter, myelinated fiber area and diameter when compared to the RLP control group. In the therapeutic windows used, both lamina propria grafts produced comparable results for the myelinated fiber density and estimated total number of myelinated fibers at the lesion site, demonstrating that a delayed transplantation approach does not seem to limit the regenerative effects.

SCI results in the loss of motor function control mechanisms conveyed by the interrupted axonal pathways. In the first lesion phase, known as "spinal shock", humans and animals exhibit muscle paralysis, hypotonia and loss of tendon reflexes below the level of injury (Valero-Cabré et al., 2004). After weeks or months a spastic syndrome develops, leading to an exacerbation of tendon jerks, increased muscle tone and muscle spasms (Hiersemenzel et al., 2000). In the present study, withdrawal reflex responses increased at around 20 days post-injury in all the experimental groups, demonstrating the time-dependent development of hyperreflexia in spinalized rats. A recovery of the withdrawal reflex to the scores observed prior to the injury was only seen in the group transplanted acutely with OLP grafts.

Previous studies have shown that low frequency-dependent depression of the H reflex also takes weeks to develop after SCI (Reese et al., 2005). The H reflex is the electrical analogue of the monosynaptic stretch reflex, commonly used in the clinical setting (Misiaszek, 2003; Yablon and Stokic, 2004). Low-intensity electrical stimulation of the afferent nerve generates a short latency M-wave that results from direct stimulation of the motor axons innervating the muscle, and a long-latency H-wave, which is a measure of the α-motoneurons activated by Ia afferents (Gozariu et al., 1998). In normal animals, the H-wave amplitude decreases with the use of increased low stimulation frequencies (between 1 and 10 Hz) (Lee et al., 2005; Yates et al., 2008a). However, as cited above, the decrease in the H-wave amplitude becomes less sensitive to stimulus frequency weeks after SCI, resulting in a lack of H reflex frequency habituation (Lee et al., 2005; Thompson et al., 1992). The 4-week

delayed transplantation of OLP restored the H-reflex frequency-dependent depression, in contrast to the animals that received RLP grafts at the same time after injury.

Since the hyperreflexia is linked to the loss of presynaptic inhibition in Ia afferents, we postulated that the positive results on the reflex function of animals with acute and 4-week delayed transplantation of OLP were due to axonal regrowth at the lesion site promoted by the presence of OECs in these grafts (Reese et al., 2005; Skinner et al., 1996). However, the morphological data did not support this hypothesis. RLP transplantation produced similar effects to those of OLP grafts in terms of fiber regeneration, as shown by the comparable values for the estimated total number of myelinated fibers and the myelinated fiber density at the lesion. Centenaro et al. (2011) showed similar CGRP axon regeneration at the transection site and serotonergic axon sprouting in the rostral stump of animals transplanted with OLP and RLP acutely, 2-weeks and 4 weeks delayed. On the other hand, Lu et al. (2001, 2002), using acute or 4-week delayed lamina propria grafts into the transected spinal cord, demonstrated evidence of descending axon regeneration across the injury only in OLP transplanted animals.

It is important to note that both lamina propria transplant types used in the present study function as a "bridge" across the lesion gap, composed of similar cells types (olfactory nerve bundles, trigeminal nerve fibers, Schwann cells, endothelium, interstitial fibroblasts and tissue resident immune cells) that could enable axonal regeneration (Centenaro et al., 2011). A considerable number of myelinated and serotonergic axons were reported in matrigel-only grafts transplanted acutely after spinal cord transection (Fouad et al., 2005). Deumens et al. (2006) also demonstrate that the biomatrix implantation itself stimulates ingrowth of neurofilament positive fibers after spinal cord dorsal hemissection. Thus, regeneration of severed fibers across lesion gaps may not only require a growth-stimulating source, but also a physical substrate to promote the connection between the spinal cord stumps.

Beyond the absence of differences in relation to the number of regenerated fibers, the groups that received 2-week and 4-week delayed transplantation of OLP exhibited myelinated fibers with significant smaller areas and diameters, as well as a reduced myelin sheath thickness at the lesion site. No differences were found in these above-cited parameters in the group acutely treated with OLP. Therefore, is it clear that the number and the morphometric characteristics of those regenerated fibers do not appear to correlate with the observed decrease in over-responsiveness in the withdrawal and H reflexes. To our knowledge, this is the first study to analyze morphometric differences between the fibers found at the injury site of animals transplanted with lamina propria grafts.

Our morphological results suggest the existence of mechanisms other than the restoration of presynaptic inhibition are involved in the decreased hyperreflexia produced by OLP grafts. Similarly, López-Vales et al. (2007) demonstrated that OECs from OB when transplanted 45 days after spinal cord transection were able to modulate the caudal stump excitability independently of axonal regeneration. Although not addressed in this study, the reorganization or preservation of local circuits below the lesion site after OLP transplantation could be related to the observed reflex function improvements. OECs are reported to express a variety of neurotrophic factors, including nerve growth factor *(*NGF), *brain*-derived neurotrophic *factor* (BDNF), glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), as well as increase neoangiogenesis and up-regulate vascular endothelial growth factor (VEGF) expression (Lipson et al., 2003; López-Vales et al., 2004; Woodhall et al., 2001; Zhu et al., 2010). These glial cells could provide adequate nutritional and trophic support for axons in the process of regenerating and those remaining below the injury site.

Concluding remarks

In summary, our data provide evidence that acute and 4-week delayed OLP transplantation in spinalized rats produce a discrete improvement in reflex function below the injury level. The mechanism by which this graft reduced hyperreflexia was not directly related to the axonal regeneration at the lesion site, since OLP and RLP transplantation produced comparable myelinated fiber regrowth. OLP grafts could enable autologous transplantation of OECs in humans after SCI, but this source of cells has only recently been tested for its ability to promote recovery after lesion (Féron et al., 1998; Franklin, 2002). Because some of these studies have questioned the locomotor and axonal improvements produced by OLP grafts, the present study aimed to explore alternative parameters and mechanisms that could reveal the effects of OEC transplantation (Centenaro et al., 2011; Steward et al., 2006). Therefore, despite the ongoing clinical use of lamina propria-derived OECs and olfactory mucosa (Chhabra et al., 2009; Dobkin et al., 2006; Féron et al., 2005; Lima et al., 2006, 2010; Mackay-Sim et al., 2008), it is important to emphasize the need for more experimental studies to clarify the exact nature of the repair capacity of these grafts in the SCI treatment (Lindsay et al., 2010; Mackay-Sim and St John, 2011).

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Author Disclosure Statement

No competing financial interests exist.

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Figure Legends

Figure 1. (**A**) Withdrawal reflex scores before (naive) and postoperatively at days 5, 20, 35, 50, 65, 80 (1-6 tests) after spinal cord transection and *acute* RLP or OLP transplantation. **[a]**$p<0.05$ for AC groups compared to naive test. **[b]**$p<0.05$ for AT groups compared to naive test. **[c]**$p<0.05$ for AC groups compared to test 1. **[d]**$p<0.05$ for AT groups compared to test 1. (**B**) Withdrawal reflex scores before (naive) and postoperatively at days 5, 20, 35, 50, 65, 80, 95 (1-7 tests) after spinal cord transection and *2-week delayed* RLP or OLP transplantation. **[a,b]**$p<0.05$ for 2WDC and 2WDT groups compared to naive test and to test 1, respectively. **[c]**$p<0.05$ for 2WDT groups compared to test 6. **[d]**$p<0.05$ for 2WDT groups compared to test 7. *$p<0.05$ for 2WDC compared to 2WDT group. (**C**) Withdrawal reflex scores before (naive) and postoperatively at days 5, 20, 35, 50, 65, 80, 95, 110 (1-8 tests) after spinal cord transection and *4-week delayed* RLP or OLP transplantation. **[a]**$p<0.05$ for 4WDC and 4WDT groups compared to naive test. **[b]**$p<0.05$ for 4WDC group compared to test 1. **[c]**$p<0.05$ for 4WDT groups compared to test 1. Arrows indicate the time at which the experimental groups underwent OLP or RLP transplantation. Abbreviations: 2WDC – 2-Week Delayed Control; 2WDT – 2-Week Delayed Treated; 4WDC – 4-Week Delayed Control; 4WDT – 4-Week Delayed Treated; AC – Acute Control; AT – Acute Treated; OLP – Olfactory Lamina Propria; RLP – Respiratory Lamina Propria.

Figure 2. H reflex at increased low-frequencies (0.2, 1, 5 and 10 Hz) in animals with (**A**) *acute*, (**B**) *2-week* and (**C**) *4-week delayed* transplantation of respiratory or olfactory lamina propria. Assessments were performed 17 weeks after spinal cord transection. $^{*}p<0.05$ compared to 4WDC group. Abbreviations: 2WDC – 2-Week Delayed Control; 2WDT – 2-Week Delayed Treated; 4WDC – 4-Week Delayed Control; 4WDT – 4-Week Delayed Treated; AC – Acute Control; AT – Acute Treated.

Figure 3. Spinal cord lesion epicenter of animals submitted to RLP and OLP transplantation. In the therapeutic windows used, both transplant types produced comparable myelinated fiber regeneration. All panels show toluidine blue-stained, 1-µm-thick plastic sections. (**A**, **B**) *Acute* RLP and OLP transplanted animals, respectively. (**C**, **D**) *2-week delayed* RLP and OLP transplanted animals, respectively. (**E**, **F**) *4-week delayed* RLP and OLP transplanted animals, respectively. Arrows indicate myelinated fibers. Asterisks point to degenerative debris. Scale Bar – 10 µm. Abbreviations: OLP – Olfactory Lamina Propria; RLP – Respiratory Lamina Propria; CC – Cystic Cavities.

Figure 4. (**A**) Myelinated fiber area, (**B**) myelinated fiber diameter, (**C**) axon diameter and (**D**) myelin sheath thickness at three regions of spinal cord lesion (epicenter, 1 mm and 2 mm caudally) in animals with RLP or OLP grafts. (**A**, **B**, **D**) $^{*}p<0.001$ for 2WDC and 4WDC compared to 2WDT and 4WDT, respectively. (**C**) $^{*}p<0.001$ for 2WDC compared to 2WDT. Abbreviations: 2WDC – 2-Week Delayed Control; 2WDT – 2-Week Delayed Treated; 4WDC – 4-Week Delayed Control; 4WDT – 4-Week Delayed Treated; AC – Acute Control; AT – Acute Treated; OLP – Olfactory Lamina Propria; RLP – Respiratory Lamina Propria.

Figure 5. (**A**) Myelinated fiber density and (**B**) estimated total number of myelinated fibers at three regions of spinal cord lesion (epicenter, 1 mm and 2 mm caudally) in animals with RLP or OLP grafts. There were no statistical differences between groups transplanted with respiratory or olfactory lamina propria in the different therapeutic windows ($p>0.05$). Abbreviations: 2WDC – 2-Week Delayed Control; 2WDT – 2-Week Delayed Treated; 4WDC – 4-Week Delayed Control; 4WDT – 4-Week Delayed Treated; AC – Acute Control; AT – Acute Treated; OLP – Olfactory Lamina Propria; RLP – Respiratory Lamina Propria.

Figure 1

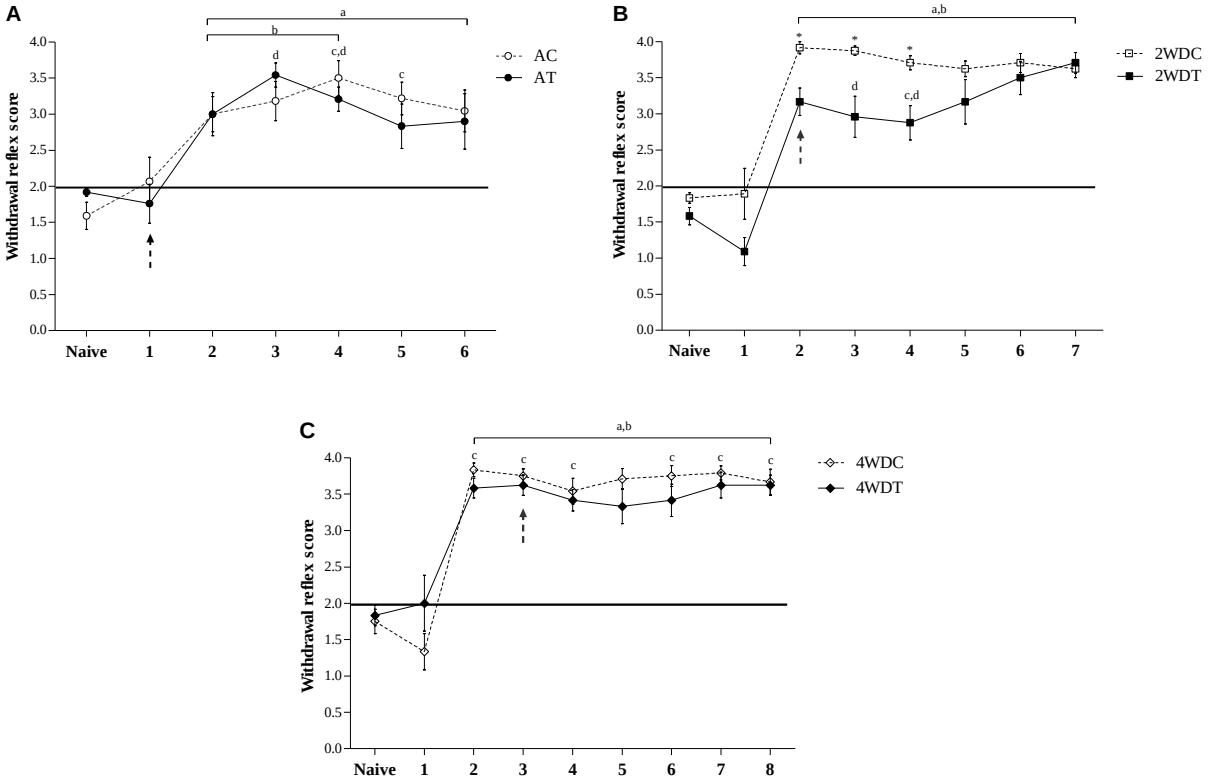

A
B
C
Withdrawal reflex score
AC
AT
2WDC
2WDT
4WDC
4WDT
Naive
a
b
a,b
c,d
d
c
*

Figure 2

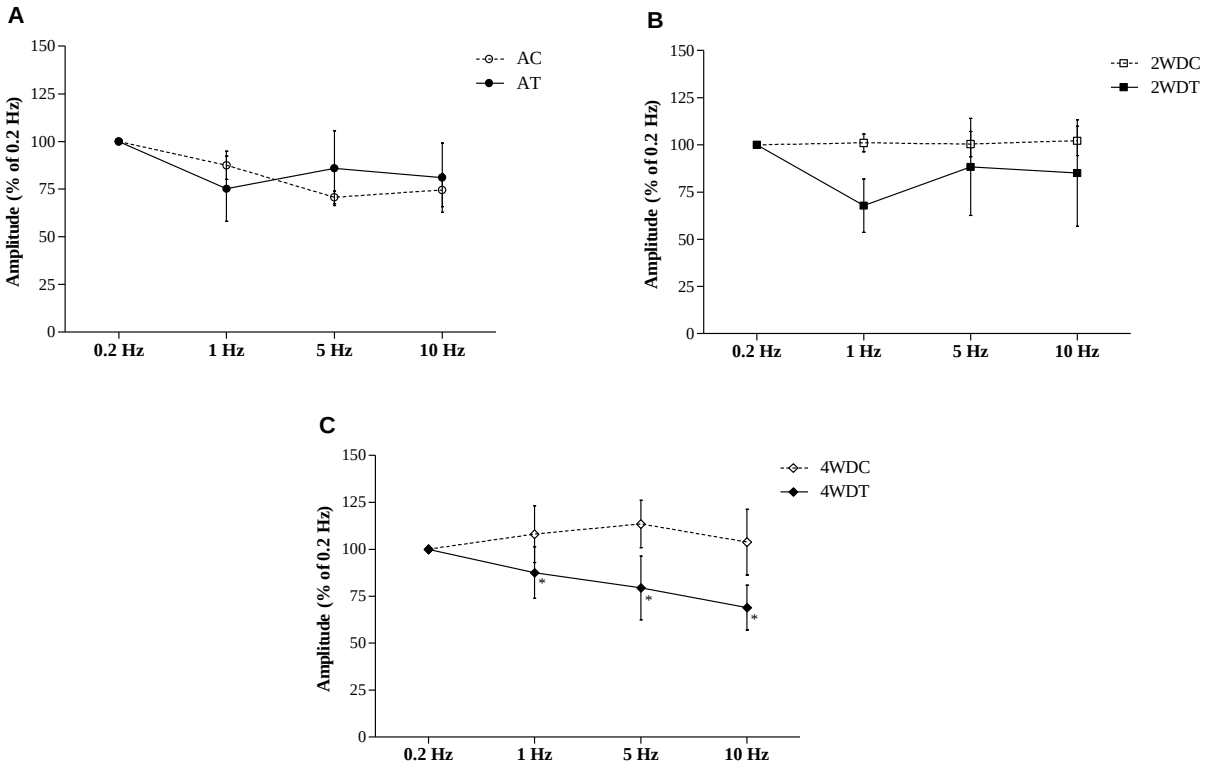

A
Amplitude (% of 0.2 Hz)
150
125
100
75
50
25
0
0.2 Hz
1 Hz
5 Hz
10 Hz
AC
AT
B
Amplitude (% of 0.2 Hz)
2WDC
2WDT
C
Amplitude (% of 0.2 Hz)
4WDC
4WDT
*

Figure 3

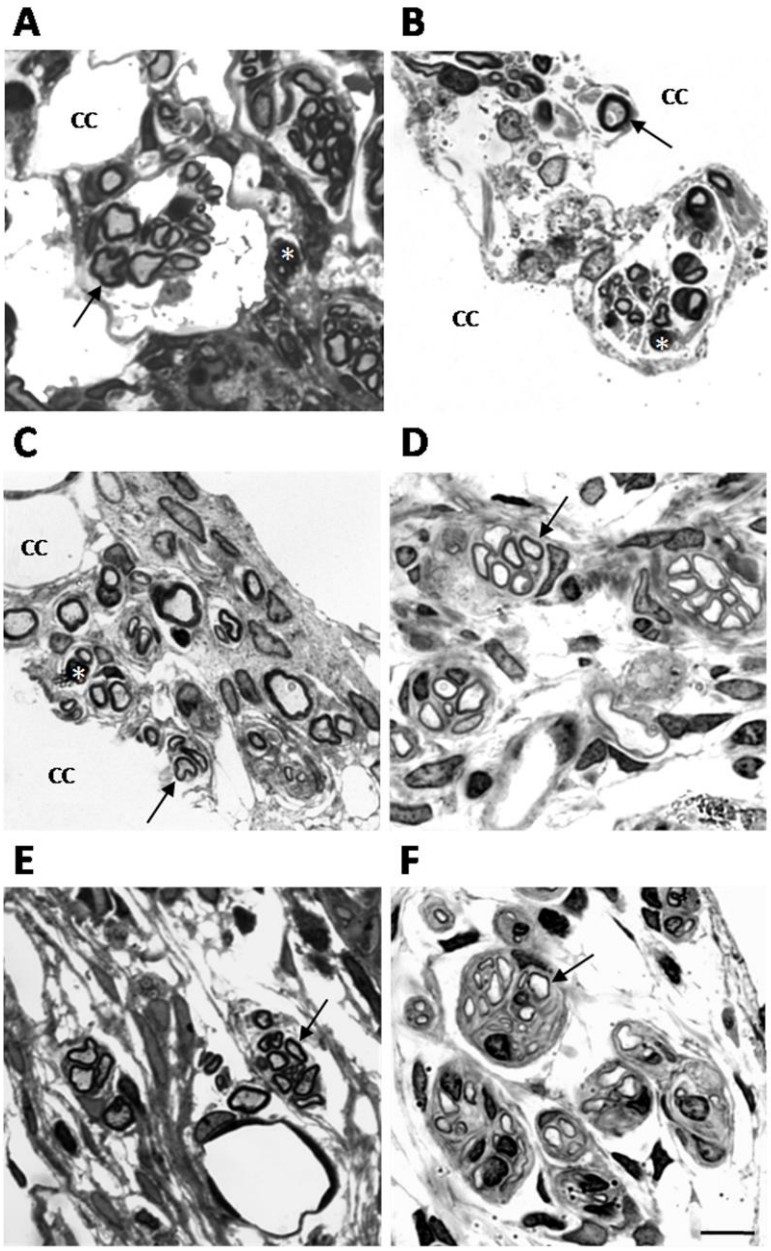


Figure 4

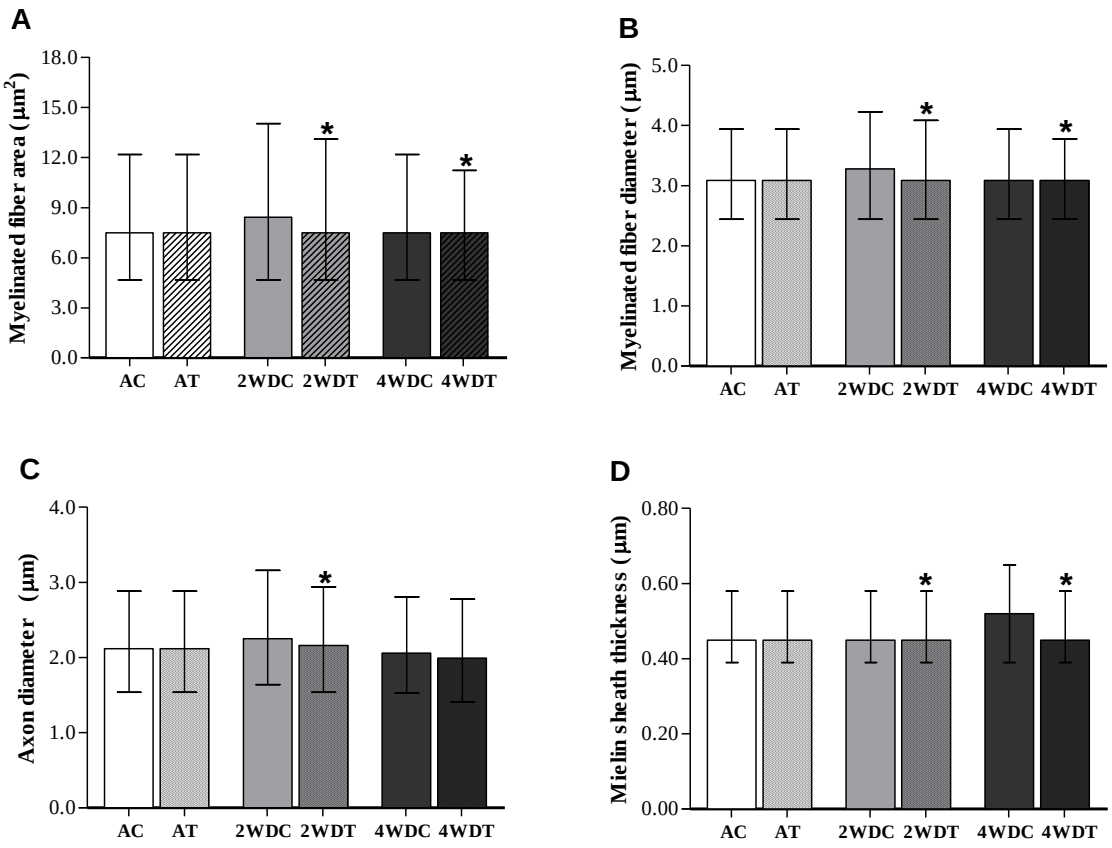
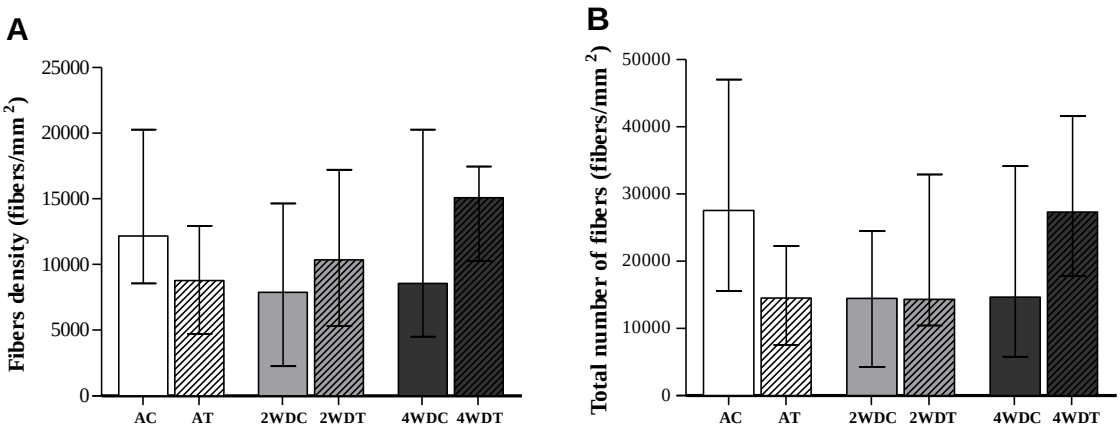

A
Myelinated fiber area (μm²)
18.0
15.0
12.0
9.0
6.0
3.0
0.0
AC AT 2WDC 2WDT 4WDC 4WDT
B
Myelinated fiber diameter (μm)
5.0
4.0
3.0
2.0
1.0
0.0
AC AT 2WDC 2WDT 4WDC 4WDT
C
Axon diameter (μm)
4.0
3.0
2.0
1.0
0.0
AC AT 2WDC 2WDT 4WDC 4WDT
D
Mielin sheath thickness (μm)
0.80
0.60
0.40
0.20
0.00
AC AT 2WDC 2WDT 4WDC 4WDT

Figure 5


A
Fibers density (fibers/mm 2)
25000
20000
15000
10000
5000
0
AC
AT
2WDC
2WDT
4WDC
4WDT
B
Total number of fibers (fibers/mm 2)
50000
40000
30000
20000
10000
0
AC
AT
2WDC
2WDT
4WDC
4WDT

5 DISCUSSÃO

O presente estudo buscou investigar o potencial terapêutico das células da GEO através da realização do implante de LP olfatória imediatamente, 2 ou 4 semanas após a transecção completa da medula espinal em ratos machos adultos. Os efeitos do transplante de LP olfatória foram devidamente comparados com implantes de LP respiratória, um tecido semelhante em sua composição celular, mas desprovido de células da GEO. As análises comportamentais e histológicas foram especificamente voltadas para a verificação de uma possível recuperação funcional e regeneração axonal mediadas pelo emprego dessa estratégia de tratamento.

5.1 Recuperação funcional e regeneração de fibras mielínicas

A maioria dos eventos traumáticos em humanos resulta de uma combinação de forças contusivas e compressivas sobre a medula espinal, as quais frequentemente não determinam uma secção completa dessa estrutura. Entretanto, no presente estudo o modelo de transecção completa da medula espinal foi adotado tendo em vista que esse tipo de lesão produz uma importante disfunção sensório-motora abaixo do nível da lesão, com paralisia e hiperreflexia dos membros posteriores (ZHANG et al., 2007). Quando realizada de maneira adequada, a transecção medular interrompe completamente as projeções dos centros superiores de controle motor para os circuitos neuronais que se encontram caudalmente à lesão. Dessa forma, esse modelo de lesão medular facilita a interpretação dos resultados funcionais obtidos, uma vez que exclui a ocorrência de uma possível recuperação pela plasticidade de fibras intactas (STEWARD, ZHENG e TESSIER-LAVIGNE, 2003; BAREYRE et al., 2004). A transecção da medula espinal também permite uma maior acurácia na diferenciação entre regeneração axonal e brotamento de axônios não lesionados, o que seria difícil de ser estabelecido utilizando modelos que mimetizam mais fielmente as lesões medulares encontradas na prática clínica.

Neste estudo os animais apresentaram uma paralisia flácida associada à perda da função motora de ambos os membros posteriores logo após a realização da transecção medular. Em torno de 20 dias após a lesão, uma resposta exacerbada do reflexo de retirada desenvolveu-se e, no mesmo período, uma melhora sutil na movimentação dos membros posteriores foi observada em todos os grupos experimentais. Essa discreta recuperação locomotora foi semelhante nos grupos transplantados com ambos os tipos de LP até o final do

protocolo experimental, sugerindo que tal melhora funcional não estava diretamente relacionado à presença de células da GEO nos implantes. Steward e colaboradores (2006) também não encontraram evidências de recuperação da função motora utilizando o transplante de LP olfatória ou respiratória 4 semanas após a transecção da medula espinal. Além disso, injeções de células da GEO provenientes do BO não melhoraram o desempenho locomotor de ratos submetidos a um modelo de contusão moderada da medula espinal (TAKAMI et al., 2002).

Por outro lado, uma redução na hiperatividade do reflexo de retirada foi verificada nos animais que receberam o transplante de LP olfatória imediatamente após a lesão da medula espinal. No último teste funcional realizado (12 semanas pós-lesão), esses animais obtiveram escores semelhantes àqueles observados previamente à transecção medular. O transplante de LP olfatória realizado 4 semanas pós-lesão também produziu uma habituação dependente de frequência do reflexo H, uma resposta observada em animais intactos. Assim, o período de tempo entre a ocorrência da transecção medular e o emprego desse tipo de transplante celular mostrou-se um fator relevante quanto aos efeitos produzidos por essa terapia sobre as funções reflexas. De acordo com esse achado, Lu e colaboradores (2001; 2002) mostraram uma melhora na função reflexa através do implante de LP imediatamente ou 4 semanas após a lesão medular. Entretanto, esses estudos citados acima também mostraram uma melhora considerável da locomoção através do transplante de LP olfatória, resultado que não foi encontrado no presente trabalho.

Os achados morfológicos referentes à regeneração de fibras mielínicas no local da lesão sugerem que existam outros mecanismos, além da restauração da inibição pré-sináptica, envolvidos na redução da hiperreflexia observada nos animais com implantes de LP olfatória. Tal hipótese baseia-se no fato de que o transplante de ambos os tipos de LP permitiram um crescimento comparável de fibras mielinizadas na região da lesão, como demonstrado pela densidade de fibras mielinizadas e a estimativa do número total dessas fibras. Da mesma forma, López-Vales e colaboradores (2007) mostraram que células da GEO provenientes do BO, quando transplantadas 45 dias pós-lesão, modulavam a excitabilidade do coto caudal da medula espinal independentemente de regeneração axonal. Além da diminuição da inibição pré-sináptica, outras mudanças fisiológicas que podem estar envolvidas na hiperreflexia incluem a hiperexcitabilidade e/ou mudanças nas propriedades intrínsecas dos motoneurônios α, redução na depressão da transmissão de fibras Ia, crescimento sináptico e alterações na morfologia de motoneurônios α (REESE et al., 2005). Assim, é possível que o transplante de LP olfatória tenha sido capaz de produzir uma reorganização ou a preservação dos

motoneurônios α dos circuitos neuronais abaixo do sítio da lesão e, consequentemente, uma redução na hiperatividade das funções reflexas seria observada apenas com o uso desse tipo específico de implante. Estudos prévios mostram que células da GEO além de guiarem axônios olfatórios em regeneração, também expressam uma variedade de fatores neurotróficos, aumentam a angiogênese e causam uma *up-regulation* da expressão do fator de crescimento vascular endotelial (do inglês, VEGF) (LÓPEZ-VALES et al., 2004; ZHU et al., 2010). Assim, as células gliais em questão presentes na LP olfatória podem ter fornecido um suporte nutricional e trófico adequado para os neurônios que estavam abaixo do nível da lesão medular e que sobreviveram ao evento lesivo, não estando diretamente relacionadas à regeneração axonal no local da lesão.

A criação um substrato físico entre os dois cotos medulares através do implante de ambos os tipos de LP pareceu ser de vital importância para o crescimento axonal no local da lesão. Em um estudo anterior, animais que receberam apenas o implante de matrigel (um extrato de proteínas da membrana basal que forma um gel) também apresentaram um número considerável de fibras mielinizadas no sítio da transecção medular (FOUAD et al., 2005). Por outro lado, a realização dos transplantes de LP em diferentes janelas temporais não limitou a regeneração axonal, mesmo quando essa estratégia de tratamento foi aplicada mais tardiamente (4 semanas após a lesão medular). Entretanto, é importante salientar que os animais transplantados com LP olfatória 2 e 4 semanas após a lesão da medula espinal possuíam fibras mielínicas de menor área, diâmetro e espessura da bainha de mielina quando comparadas àquelas observadas nos animais implantados com LP respiratória nos mesmos períodos. Portanto, a presença de axônios mielinizados na região da lesão medular deve ser um achado interpretado com cautela, uma vez que essas fibras também devem apresentar uma morfologia adequada para que o retorno de sua funcionalidade ocorra.

5.2 Regeneração de tratos axonais específicos e o restabelecimento de suas conexões com estruturas encefálicas

Um número considerável de fibras descendentes serotoninérgicas foi observado no coto medular rostral dos animais transplantados com LP olfatória e respiratória nas diferentes janelas temporais utilizadas em comparação às regiões do epicentro da lesão e do coto caudal. A maioria desses axônios não se estendia além das proximidades da borda da lesão. De acordo com esses achados, fibras imunorreativas para 5-HT foram evidentes apenas

nas regiões do coto proximal de animais submetidos a uma transecção da medula espinal e ao transplante tardio (30 dias pós-lesão) de LP olfatória e respiratória. Embora axônios serotoninérgicos fossem vistos atravessando a cicatriz glial, nenhum deles alcançava o coto distal (STEWARD et al., 2006).

Fibras ascendentes positivas para CGRP foram encontradas no sítio da lesão e nos cotos rostral e caudal da medula espinal, revelando que ambos os tipos de LP foram capazes de estimular o crescimento/brotamento desses axônios quando transplantados em diferentes tempos pós-lesão. De acordo com nosso conhecimento, esse é o primeiro estudo que buscou verificar a influência do transplante de LP olfatória e respiratória sobre a regeneração de axônios positivos para CGRP. Tais fibras axonais também foram encontradas no coto distal e proximal de animais submetidos ao transplante de células da GEO provenientes do BO, que foram realizados imediatamente ou 7 dias após a transecção da medula espinal (LÓPEZ-VALES et al., 2006). No entanto, uma vez que fibras CGRP positivas respondem a estímulos nociceptivos, intervenções que favorecem a regeneração axonal após lesões medulares devem ser controladas para assegurar que as conexões a serem restabelecidas produzam uma melhora funcional, sem desenvolver outras complicações secundárias. Finalmente, é importante salientar que os resultados referentes à regeneração das fibras positivas para 5-HT e CGRP encontrados neste estudo não foram influenciados pelos diferentes tempos no qual o transplante de LP foi realizado.

Apenas uma pequena parte dos axônios regenerados após o transplante de ambos os tipos de LP conseguiram restabelecer suas conexões no córtex somatossensorial primário (S1) e em núcleos específicos do tronco encefálico. Esse resultado foi baseado na observação de que nessas regiões encefálicas haviam poucas células coradas com um marcador axonal retrógrado, que havia sido injetado na região lombar da medula espinal dos animais experimentais. Mais uma vez, a restauração de um contínuo de células entre os cotos medulares por meio dos implantes de ambos os tipos de LP, podem ter favorecido o crescimento axonal desse número limitado de fibras por longas distâncias. Entretanto, esses resultados vão de encontro aos achados de um estudo realizado anteriormente, no qual uma considerável marcação retrógrada foi observada no núcleo magno da rafe apenas nos animais que receberam o transplante imediato de LP olfatória, sem evidências dessa marcação após o implante de LP respiratória (LU et al., 2001). Essa divergência pode estar relacionada com a gravidade da lesão empregada, pois no presente estudo aproximadamente 1-2 mm do tecido medular foi retirado no local da lesão. No estudo de Lu e colaboradores foi realizada apenas uma secção completa da medula espinal, sem a remoção de parte dessa estrutura.

5.3 Preservação do tecido nervoso e brotamento neurítico no local da lesão

Estudos prévios já demonstraram que injeções de células da GEO provenientes do BO limitam a formação de cavidades e cistos no local da transecção medular através da redução na degeneração tecidual e da morte axonal retrógrada (RAMÓN-CUETO et al., 2000; KUBASAK et al., 2008). Além disso, quando injetadas no local da lesão e em ambos os cotos medulares, essas mesmas células do BO aumentam o crescimento de fibras positivas para GAP-43 (ANDREWS e STELZNER, 2007). No presente estudo os transplantes de LP olfatória realizados imediatamente, 2 ou 4 semanas após a lesão medular não promoveram uma maior preservação do tecido nervoso e brotamento neurítico quando comparados aos implantes de LP respiratória. Essa divergência entre os resultados pode estar relacionada com a fonte de células da GEO utilizada nos implantes. Entretanto, Richter e colaboradores (2005) mostraram uma redução na área de lesão do funículo dorsolateral da medula usando o transplante de células da GEO purificadas a partir da LP, mas esse resultado não foi verificado através do emprego de células provenientes do BO. Mais estudos são necessários para que sejam estabelecidas as diferenças entre as células da GEO obtidas do BO e da LP olfatória (YAMAMOTO et al., 2009; GUÉROUT et al., 2010).

5.4 Células da GEO nos implantes de LP

As células da GEO presentes na LP olfatória foram especificamente identificadas por sua imunorreatividade para p75NTR, S-100, GFAP e também pelo seu aspecto fusiforme. A co-expressão periférica desses marcadores antigênicos é restrita às células que se encontram em torno dos feixes de neurônios sensoriais olfatórios, sendo útil na distinção da GEO de outras células presentes na LP olfatória (AU e ROSKAMS, 2003). Tais células também foram verificadas expressando os mesmos marcadores imunoistoquímicos no sítio da lesão, em torno de 18 semanas após a transecção medular. A LP respiratória manteve-se desprovida de células da GEO antes e após seu implante.

No presente estudo não foi realizada uma investigação do comportamento desse tipo de célula glial após o seu transplante no local da lesão medular. Um estudo prévio mostrou que as células da GEO purificadas da LP olfatória, possuíam uma habilidade migratória superior quando comparadas àquelas derivadas do BO, após seu implante em um modelo de lesão por esmagamento da medula espinal (RICHTER et al., 2005). Por outro lado,

em um paradigma de lesão do nervo olfatório, tais células permaneceram como um conduto para o crescimento dos axônios em regeneração, de maneira semelhante às células de Schwann em lesões nervosas periféricas (WILLIAMS et al., 2004; LI, LI e RAISMAN, 2005). Lu e colaboradores (2006) afirmaram que as células da GEO derivadas da mucosa olfatória não migravam ativamente quando transplantadas após um modelo de lesão medular, apenas se espalhavam de acordo com a pressão no exercida no local da injeção. Embora essa seja uma das limitações do presente estudo, o mecanismo exato pelo qual essas células gliais atuam quando implantadas em diferentes modelos de lesões medulares precisa ser melhor esclarecido.

5.5 Aplicação clínica das células da GEO

Apesar de todas as significativas controvérsias encontradas na literatura envolvendo o uso terapêutico de células da GEO, testes clínicos utilizando o transplante desse tipo de células provenientes da LP olfatória vêm sendo realizados em pacientes com lesões crônicas da medula espinal (LIMA et al., 2006; 2010; MACKAY-SIM et al., 2008; CHHABRA et al., 2009). Células da GEO purificadas de biópsias nasais foram injetadas em três pacientes paraplégicos e não foram relatadas evidências da formação de cistos ou de outras complicações relacionadas a esse procedimento no primeiro ano após sua realização (FÉRON et al., 2005). Em contrapartida, também não foram identificadas mudanças funcionais significativas 3 anos após esse implante celular (MACKAY-SIM et al., 2008).

Utilizando uma abordagem diferente, Lima e colaboradores (2006; 2010) mostraram que o transplante autólogo de mucosa olfatória era uma terapia viável, além de relatarem uma melhora funcional em 11 dos 20 indivíduos com lesões medulares crônicas que haviam sido transplantados. Utilizando a mesma estratégia terapêutica, um trabalho subsequente não mostrou nenhuma melhora nos parâmetros neurológicos, eletrofisiológicos e urodinâmicos dos 5 pacientes analisados (CHAABRA et al., 2009). Além disso, exames de ressonância magnética revelaram o aparecimento de siringomielia (cisto ou siringe na medula espinal que se expande continuamente) em um desses pacientes e aumento na extensão da mielomalácia (“amolecimento” da medula espinal devido à necrose isquêmica ou hemorrágica) em 4 dos pacientes (CHAABRA et al., 2009).

Por fim, fica evidente a necessidade da realização de pesquisas futuras que determinem a fonte ideal para a obtenção das células da GEO e esclareçam detalhadamente

seus mecanismos de ação após o transplante em modelos animais de lesões medulares. Além disso, a utilização de terapias adicionais como a administração de fatores de crescimento, antagonistas de moléculas inibitórias ao crescimento axonal e programas de reabilitação específicos podem potencializar os benefícios desse transplante celular após lesões da medula espinal. Essas questões devem ser endereçadas primeiramente no âmbito experimental a fim de justificar ou não a utilização desse tratamento em testes clínicos formais.

6 CONCLUSÕES E PERSPECTIVAS

Os resultados obtidos nessa tese nos permitem concluir que:

- Transplantes de LP olfatória e respiratória em diferentes janelas terapêuticas produziram uma discreta melhora na função locomotora de animais com transecção completa da medula espinal ao nível torácico;
- Uma redução na hiperatividade do reflexo de retirada foi observada nos animais que receberam transplante LP olfatória imediatamente após a lesão. Além disso, o transplante de LP olfatória realizado 4 semanas após a transecção da medula espinal foi capaz de restabelecer a depressão dependente de frequência do reflexo H em comparação ao implante de LP respiratória realizado no mesmo período de tempo;
- A regeneração de fibras mielinizadas no epicentro da lesão, 1 e 2 mm caudalmente foi semelhante nos animais que receberam ambos os tipos de LP nos diferentes períodos após a lesão medular. Entretanto, essas fibras mielínicas possuíam uma menor área, diâmetro e espessura da bainha de mielina nos animais transplantados com LP olfatória 2 e 4 semanas pós-lesão;
- Uma pequena parte das fibras axonais lesionadas restabeleceram suas conexões no tronco encefálico e até mesmo no córtex S1 após o transplante de LP olfatória e respiratória, como indicado pela presença de células positivas para o marcador axonal retrógrado nessas regiões;
- Um aumento no número de fibras descendentes serotoninérgicas foi observado no coto proximal dos animais transplantados com LP olfatória e respiratória, mas essas fibras não se estenderam além das proximidades da borda da lesão. Fibras aferentes positivas para CGRP foram encontradas em número considerável no sítio da lesão e nos cotos rostral e caudal da medula espinal, revelando que ambos os tipos de implantes quando aplicados nos diferentes tempos pós-lesão foram capazes de estimular o crescimento/brotamento desse subtipo de fibras;
- O período de tempo decorrido antes do transplante de LP olfatória e respiratória não limitou a regeneração axonal no local da transecção medular, mas apenas o transplante de LP olfatória realizado imediatamente e 4 semanas pós-lesão produziu uma normalização das respostas reflexas abaixo do nível da lesão;
- Mais estudos são necessários para que o papel desempenhado pelas células da GEO presentes nos implantes de LP olfatória seja estabelecido e também para verificar se a

utilização de outros tratamentos associadas a esse tipo de transplante podem incrementar a locomoção e regeneração axonal de animais com lesões da medula espinal antes da utilização dessa terapia em estudos envolvendo humanos.

Colocamos como perspectivas futuras:

- Caracterizar as atividades das células da GEO presentes na LP após seu implante no sítio da lesão medular, verificando sua possível capacidade migratória através da cicatriz glial e interação com axônios em regeneração;
- Verificar os efeitos do transplante de LP olfatória sobre os motoneurônios da região lombar da medula espinal, liberação de fatores neurotróficos e atividade da enzima NA^{+}-K^{+}-ATPase que podem estar relacionados com a melhora da função reflexa observada nos animais submetidos à transecção da medula espinal;
- Avaliar os efeitos do transplante de células da GEO purificadas da LP olfatória quando injetadas nos cotos proximal e distal da medula espinal em associação com a aplicação de terapias complementares como o implante de biomatrizes no local da lesão e o treinamento de marcha em animais com transecção da medula espinal.

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