

UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
PROGRAMA DE PÓS GRADUAÇÃO EM MEDICINA VETERINÁRIA

HOMING DE CÉLULAS ESTROMAIS MESENQUIMAIS MULTIPOTENTES
CANINAS APÓS TRANSPLANTE PELA VIA EPIDURAL EM MODELO
EXPERIMENTAL DE LESÃO RAQUIMEDULAR AGUDA EM COELHOS

MARIANA CRISTINA RAMOS

Botucatu – SP

2020

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Dissertação apresentada junto ao
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Orientador: Prof.Dr. Rogério Martins Amorim

Coorientador: Prof. Dr. Matheus Bertanha

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RAMOS, M.C. ***Homing* de células estromais mesenquimais multipotentes caninas após transplante pela via epidural em modelo experimental de lesão raquimedular aguda em coelhos**. Botucatu, 2020. 60 p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

RESUMO

As células estromais mesenquimais multipotentes (CTM) têm sido apresentadas pela comunidade científica como uma alternativa promissora no tratamento de doenças inflamatórias, traumáticas, vasculares e degenerativas do sistema nervoso central devido a suas propriedades anti-inflamatórias, imunomoduladoras e neuroregenerativas. Um dos fatores que podem afetar a eficácia da terapia celular é a via de transplante utilizada. O objetivo deste estudo foi avaliar o *homing* de CTMs derivadas do tecido adiposo caninas (AdCTMs) transplantadas pela via epidural em modelo experimental de lesão raquimedular aguda em coelhos. A lesão raquimedular foi induzida experimentalmente em 8 coelhos por compressão do segmento medular T10, imediatamente após os animais foram submetidos ao transplante de AdCTMs caninas marcadas com nanocristais Qtracker 655[®] pela via epidural entre os espaços L7-S1. Sete dias após o transplante, o *homing* das AdCTMs caninas foi avaliado na medula espinhal, encéfalo, fígado, rins, baço e pulmão por meio de detecção de fluorescência em imageador *in vivo*. Os segmentos medulares L7-S1 e local de lesão foram analisados em microscopia confocal para a presença de AdCTMs marcadas. Constatou-se a presença de AdCTMs caninas em local de lesão na medula espinhal e segmento L7-S1, houve marcação em encéfalo, pulmão, fígado e rins, não houve marcação em baço. A presença de AdCTMs na medula espinhal comprova a capacidade destas células passarem pela dura mater e atingirem o local de lesão. O transplante epidural é viável e minimamente invasivo, sendo um bom candidato para o uso em terapia celular no contexto de doenças neurológicas.

Palavras-Chave: rastreabilidade celular, células estromais mesenquimais, transplante xenogênico, via epidural, trauma medular.

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ABSTRACT

Multipotent mesenchymal stromal cells (MSC) have been presented by the scientific community as a promising alternative in the treatment of inflammatory, traumatic, vascular and degenerative diseases of the central nervous system due to their anti-inflammatory, immunomodulatory and neuroregenerative properties. One of the aspects that can affect the effectiveness of the stem cell therapy is the transplantation route used. The aim of this study was to evaluate the homing of canine adipose derived MSCs (AdMSCs) transplanted via epidural injection in an experimental model of acute spinal cord injury in rabbits. New Zealand rabbits (n=8) were subjected to the spinal cord injury induced by compression of the 10th thoracic segment. Immediately after the surgical procedure, the animals were submitted to the transplantation of marked AdMSCs with quantum dots Qtracker 655® through epidural injection between the L7-S1 spaces. Seven days after the transplant, the brain, spinal cord, lungs, kidney, spleen and liver were collected and evaluated by fluorescence imaging. The spinal cord segments L7-S1 and thoracic injury site were evaluated by confocal microscopy to identify the presence of marked canine AdMSCs. Results shows that AdMSCs migrate to thoracic spinal cord segment, lungs, brain, liver and kidneys, no fluorescence was observed in spleen. The presence of canine AdMSCs in spinal cord indicates the capacity of these cells to overcome the dura mater and reach the injury site. The epidural delivery is feasible and minimally invasive and might be a good candidate for cell transplantation in the context of neurological diseases.

Keywords: stem cell tracking, xenotransplant, epidural transplantation, spinal cord injury, mesenchymal stromal cells.

CAPÍTULO 1

1. INTRODUÇÃO E JUSTIFICATIVA

As células estromais mesenquimais multipotentes (*Multipotent Mesenchymal Stromal Cells* -CTMs) têm atraído a atenção da comunidade científica devido a sua capacidade imunomoduladora e regenerativa, que as torna promissoras no tratamento de doenças inflamatórias, imunológicas, degenerativas, neurológicas, cardiovasculares, entre outras. Seu uso em lesões raquimedulares tem se mostrado benéfico por promover um efeito anti-inflamatório e reparador tecidual que pode levar a recuperação funcional motora e sensitiva em pacientes humanos e não humanos (DENG, 2006; BERNARDO; FIBBE, 2013; HOFFMAN; DOW, 2016).

A migração de células para o local da lesão é chamado de “*Homing*” e é um fator importante para a eficácia da terapia celular, uma vez que a sua ação é bastante ligada à capacidade das células em produzir fatores que atuam de maneira parácrina ou justácrina às células alvo, desta maneira, acredita-se que quanto mais próximo as CTMs estiverem do tecido lesado, melhores serão os resultados esperados (SOHNI; VERFAILLIE, 2013; HOFFMAN; DOW, 2016).

O uso de CTMs tem sido amplamente estudado no contexto das lesões raquimedulares e apresenta-se como uma alternativa terapêutica promissor. A via intratecal foi empregada em vários estudos envolvendo lesões inflamatórias do sistema nervoso central e lesões raquimedulares (SATAKE, 2004; BAKSHI et al., 2006; PAUL et al., 2009; YANG, 2018), demonstrando ter bons resultados relacionados à migração das células para o local lesionado e eficácia terapêutica (SATAKE, 2004; BAKSHI et al., 2006).

A via epidural foi pouco explorada até o momento, porém esta via tem grande potencial para ser usada em terapias celulares por ser pouco invasiva e pela dura mater ser suprida por arteríolas e vênulas meningeas que podem favorecer o *homing* das células transplantadas (LEE, et al., 2017). A busca

constante por técnicas pouco invasivas e eficazes para transplantes celulares estimula a experimentação de vias pouco exploradas. Recentemente a via intranasal foi avaliada no contexto de doenças cerebrais, indicando que as CTMs derivadas da geleia de Wharton do cordão umbilical humano migraram para o encéfalo de camundongos (GALEANO et al., 2018).

Buscar vias alternativas para transplantes celulares é importante, visto que outras vias utilizadas atualmente podem ocasionar maiores lesões para o paciente, como no caso de aplicações intralesionais, perilesionais, intratecais ou intraventriculares. A via intravenosa é muito utilizada em terapia celular, porém é sabido que as células podem ser aprisionadas em outros órgãos e não migrarem em quantidades suficientes para promoverem a regeneração do SNC (LEIBACHER ; HENSCHLER, 2016). O acesso epidural é muito utilizado para a administração de drogas anestésicas em casos de cirurgia abdominal, se mostrando eficiente para a distribuição destes medicamentos. É fácil de ser acessado e menos invasivo por não adentrar o espaço subaracnóide, limitando ainda mais o risco de lesão da medula espinhal.

Pensando em terapia celular no contexto clínico, a via epidural poderia ser uma alternativa ao transplante intratecal ou intravenoso caso seja comprovada que as células transplantadas pela via epidural sejam capazes de atravessar a dura mater e migrar para o local da lesão raquimedular.

O estudo das diferentes vias de transplante de CTM em lesões do sistema nervoso central é fundamental para o desenvolvimento de protocolos seguros e eficazes, antes desta biotecnologia ser aplicada na rotina clínica médico veterinária. Nosso grupo de pesquisa tem estudado a viabilidade, a segurança e a eficácia do transplante intratecal de CTM autóloga e alogênicas em equinos e caninos (MAIA et al., 2015; BARBERINI et al., 2018). Neste contexto, o estudo do *homing* das AdCTMs caninas após o transplante intratecal em modelo experimental de lesão raquimedular aguda em coelhos visa obter dados que suportem o desenvolvimento futuro de protocolos de terapia celular para o tratamento de enfermidades do sistema nervoso central em cães.

2. REVISÃO DE LITERATURA

2.1 Células estromais mesenquimais

As células tronco mesenquimais ou células estromais mesenquimais multipotentes são o tipo de célula-tronco somática mais estudado na atualidade, encontrando-se em todos os tecidos mesenquimais do organismo, podem ser isoladas principalmente da medula óssea, tecido adiposo, cordão umbilical, polpa de dente e anexos fetais (KOERNER et al., 2006; RICHARDSON et al., 2007; GATTEGNO-HO et al., 2012; LANGE-CONSIGLIO et al. 2013). Tem grande capacidade de se diferenciar em células linhagem mesodermal e também, apesar de ainda controverso, de se transdiferenciar em outras linhagens como as neuroectodermas (BYDLOWSKI et al., 2009; GATTEGNO-HO et al., 2012).

Estudos reportaram a capacidade das CTMs em se diferenciar a partir da indução *in vitro* e também *in vivo* em células com aparência de neurônios que expressam marcadores típicos de células neuronais adultas, sugerindo que essas células podem abandonar o comprometimento com sua linhagem celular (HOFSTETTER et al., 2002; DENG et al., 2006). Estas células apresentam propriedades progenitoras aliadas à habilidade de regular a resposta imunológica, influenciando tanto as respostas imunológicas inatas quanto adaptativas (BERNARDO;FIBBE, 2013; URDZÍKOVÁ, 2014; ANKRUM, 2014).

As CTMs exibem um potencial anti-inflamatório e imunomodulatório através da interação célula-célula (com células imunes como linfócitos B e T, células *natural killers* e células dendríticas), pela secreção de fatores solúveis no ambiente local (prostaglandina E2 (PGE2), interleucina-4 (IL-4), IL-6, IL-10,

fator de transformação do crescimento β (TGF- β), fator de crescimento hepático (HGF), interferon gama (IFN- γ), entre outros, também induzem tolerância imune aos transplantes alogênicos (JONES;MCTAGGART, 2008).

Para que haja uniformidade nos estudos com células estromais e afim de que os resultados possam ser comparados, a Sociedade Internacional de Terapia Celular (ISCT) propôs critérios mínimos para considerar as células humanas como células estromais mesenquimais multipotentes. É necessário que estas sejam aderentes ao plástico quando mantidas em condições de cultura e devem expressar, na citometria de fluxo, os marcadores de superfície (*cluster designation- CD*) CD105 (SH2), CD 73 (SH3/4), CD 90 (THY-1) em 95% da população de células, serem negativas para CD34 (marcador de todas as células hematopoéticas), CD11b (marcador de célula imune), CD14, CD19, CD45, CD79 α , MHC classe II e antígeno leucocitário humano HLA-DR (aceitando-se no máximo 2% da população positiva) devem ainda se diferenciar em condições de cultura em linhagens condrogênicas, osteogênicas e adipogênicas (DOMINICI et al., 2006).

2.2. Terapia com CTM em animais domésticos

Relatou-se o uso de CTMs em modelos animais e em ensaios pré-clínicos para uma grande variedade de enfermidades como doenças cardiopulmonares, doenças osteomusculares, reparação de fraturas, osteoartrites, osteonecrose, tendinites, doença do disco intervertebral, doenças neurológicas como a meningoencefalomielite de causa desconhecida em cães, esclerose múltipla em humanos e lesões raquimedulares, além de doenças autoimunes, renais e dermatológicas (HOFFMAN; DOW, 2016, MURPHY; MONCIVAIS; CAPLAN, 2013).

No estudo da eficiência do transplante celular em felinos naturalmente acometidos por doença renal crônica, utilizou-se CTMs provenientes da membrana amniótica, obtendo-se resultados positivos quanto a melhora da

função renal pela diminuição dos valores de creatinina e ureia (VIDANE et al., 2017).

Em cães, CTMs derivadas do tecido adiposo foram usadas em animais naturalmente acometidos por doença inflamatória intestinal. Os animais receberam uma única dose intravenosa de 2×10^6 células por kilo, sendo a resposta a terapia avaliada com base em dois índices clínicos específicos para doença intestinal inflamatória (*Clinical Inflammatory Bowel Disease Activity Index* (CIBDAI) e *Canine Chronic Enteropathy Clinical Activity Index* (CCECAI)), além da normalização dos valores de proteína C-reativa, albumina, folato e cobalamina. Observou-se melhora significativa dos indicadores após seis semanas do tratamento (PEREZ-MERINO et al., 2015).

No estudo da doença inflamatória não infecciosa do sistema neurológico de cães, utilizou-se a terapia celular com CTMs autólogas provenientes da medula óssea pelas vias intratecal + intra-arterial ou intratecal + intravenosa. Os autores observaram melhora do quadro neurológico, principalmente quanto a dor cervical, e não foram relatadas reações adversas ao transplante (ZEIRA et al., 2015).

No contexto de doença do disco intervertebral (IVDD), BACH e colaboradores em 2019 demonstraram melhores resultados no pós-operatório quando a cirurgia de descompressão medular foi associada ao transplante celular epidural de células derivada do tecido adiposo quando comparado ao grupo tratado apenas com a cirurgia de descompressão.

2.3. Vias de transplante no contexto das doenças do sistema nervoso

Objetivando produzir o melhor resultado possível com a terapia celular é necessário estabelecer a melhor via de transplante que garanta uma boa distribuição das CTMs, inclusive em casos em que não é possível submeter o paciente a um procedimento cirúrgico e anestésico (PAUL et al, 2009; YANG,

2018). Já foram utilizadas as vias intravenosa, intra-arterial, intratecal, epidural, intra-articular, intralesional, intraventricular, intraperitoneal e intranasal (SATAKE, 2004; CARRADE et al., 2011; MOTHE et al., 2011; SOHNI;VERFAILLIE, 2013; HOFFMAN;DOW, 2016; FONSECA et al 2017; GALEANO et al., 2018; YANG, 2018; BACH et al., 2019).

Especula-se que a melhor via seria aquela mais próxima do tecido comprometido, garantindo uma maior quantidade de células próximas a lesão. Todavia, a inoculação direta das CTMs no local da lesão pode acarretar maior dano tecidual, além de submeter as células a um ambiente hostil, levando a morte celular precoce (SATAKE, 2004).

Nas lesões em sistema nervoso central (SNC), a administração de CTMs pela via intratecal é uma alternativa interessante por proporcionar que mais células se concentrem no espaço sub aracnóide, tornando a terapia celular mais eficaz e segura (SATAKE, 2004; MOTHE et al., 2011). O acesso é obtido pela punção nas cisternas magna ou lombo-sacra, aonde as CTMs são administradas diretamente no espaço subaracnóide.

Já a via epidural consiste na inoculação das células acima da dura mater, não adentrando o espaço sub aracnóide. O acesso pode ser feito pela introdução da agulha no espaço L7-S1, em cães, gatos e coelhos por exemplo.

Em um modelo experimental de lesão raquimedular em ratos compararam-se as vias intravenosa e intratecal pela punção entre vértebras lombares. A via intratecal possibilitou que mais células se distribuíssem para o local de lesão, melhor regeneração tecidual e menor resposta imunológica local do que nos animais que receberam o transplante por via intravenosa, mostrando superioridade da via intratecal em relação a via intravenosa (PAUL et al., 2009).

Quando uma via sistêmica, como a via intravenosa, é utilizada, existem dúvidas se as células transplantadas são capazes de exercer o *homing* celular para o local da lesão em quantidade suficiente para promover efeito terapêutico, ou se acabam aprisionadas em capilares sanguíneos pulmonares dentre outros, podendo ocasionar oclusão de microvasos (WALCZAK et al

2008). Quando exercem efeito terapêutico, é necessário entender se atuam de forma endócrina, parácrina ou justácrina. Para responder perguntas como essa, estudos que visam rastrear as células transplantadas são necessários (SOHNI ; VERFAILLIE, 2013).

Em modelos animais em que se utilizou a via intravenosa para o transplante de CTM, foi observado que as células se alojam principalmente nos pulmões. Esse sequestro para o pulmão pode ser explicado pela grande dimensão das CTM em comparação com o diâmetro dos capilares pulmonares, resultando em aprisionamento nos capilares de parte das células transplantadas (WALCZAK et al., 2008; LEIBACHER ; HENSCHLER, 2016). Para contornar o problema foi proposto, previamente ao transplante celular, o uso de vasos dilatadores como nitroprussiato de sódio (GAO et al., 2001; SCHREPFER et al., 2007).

2.4. Transplante celular xenogênico

A ausência de expressão de MHC-II em CTMs é possivelmente um dos maiores responsáveis pela capacidade de tais células evadirem o sistema imunológico do hospedeiro e exercer sua capacidade imunossupressora. Tal função é mediada em MSCs humanas pela PGE2 (CUI et al., 2007). Por estas características, as MSCs já foram muito utilizadas em transplantes alogênicos e xenogênicos em modelos experimentais com ratos, camundongos, primatas e cães (LIN; LIN ;LUE, 2012). Como no caso do transplante de CTMs derivadas do tecido adiposo humano em modelo de lesão raquimedular em ratos (VILLANOVA JUNIOR et al., 2020).

Autores publicaram respostas satisfatórias em relação a aceitação das células por parte dos hospedeiros em transplantes xenogênicos. Em estudo com coelhos imunocompetentes, células-tronco derivadas da medula óssea de ratos sobreviveram e promoveram bom crescimento ósseo na coluna vertebral lombar dos coelhos (KIM et al., 2008).

2.5. *Homing* celular

Homing pode ser definido como o processo de transmigração das CTMs pelo endotélio vascular seguindo um gradiente de fatores quimiotáticos liberados por certos tecidos (KARP ; TEO, 2009).

Entender os processos que levam as células mesenquimais a trafegar pelo organismo e atingir um local inflamado e que necessita de reparação tecidual é de grande importância para que a terapia celular se torne mais acessível na prática clínica, sendo possível escolher vias de transplantes menos invasivas e mais eficazes (SOHNI ; VERFAILLIE, 2013).

Pouco se sabe sobre os exatos mecanismos que as CTMs usam para atingir o tecido alvo, porém muitos fatores de crescimento e citocinas já foram identificadas como sendo atuantes no processo, como VEGF-A, HGF, TGF- β 1, TNF- α , SDF-1 α , IL-6, IL-8, IGF 1, e receptores, moléculas de adesão e metaloproteinases como CXCL-12, CCL-2, CCL-3, CCR4, CXCR4, VCAM, ICAM (RUSTER et al., 2006; FOX et al., 2007; ZHANG et al., 2008, LEIBACHER; HENSCHLER, 2016).

Alguns fatores influenciadores para a migração celular foram apontados, como a idade do indivíduo doador das células e quantidade de receptores quimiotáticos do paciente que recebe as células (KARP;TEO,2009).

A confluência em cultura celular exerce um papel importante, de acordo com DE BECKER et al. (2007), uma cultura totalmente confluenta (100%) teve piores resultados em relação ao *homing* para a medula óssea do que culturas menos confluentes (50%), acredita-se que as células em culturas muito confluentes possam perder capacidade de migração transendotelial pelo aumento da TIMP-3 (Inibidor tecidual de metaloproteinase 3), inibidor de metaloproteinase (MMP).

CTM liberam enzimas e proteases como Metaloproteinase- 2 (MMP-2), Metaloproteinase da matriz de membrana tipo 1 (MT1-MMP), TIMP-2 que degradam a membrana basal do endotélio vascular e matrix extracelular, o que

possibilita que estas células migrem em direção a fatores quimiotáticos (RIES et al., 2007).

De maneira semelhante, culturas em alta passagem apresentaram piores resultados em relação a capacidade de migração celular. No estudo de ROMBOUITS e PLOEMACHER (2003) em modelo murino onde estudou-se o *homing* para a medula óssea, células recém isoladas apresentaram melhor migração para a medula óssea e baço em relação a células em maior tempo de cultura. Especulou-se que o maior tempo em cultura influenciou na quantidade de receptores de citocinas e moléculas de adesão que promovem o *homing* celular, como a diminuição de SH3, molécula de adesão intercelular (ICAM-1) e integrina $\beta 1$ que já foi descrito em CTM primárias humanas (WAGERS et al., 2002).

A interação entre selectina e integrina $\beta 1$ (componente da molécula de adesão VLA-4) expressas nas CTMs participam no processo de rolamento promovendo firme adesão da célula ao endotélio, passo anterior e primordial para a transmigração pelo endotélio vascular (RUSTER et al., 2006).

Para que ocorra a migração celular para o tecido alvo um conjunto de receptores específicos, moléculas de adesão celular e interação com as células endoteliais são necessários. Os tecidos em condições inflamatórias liberam fatores que estimulam a migração celular por entre as células endoteliais. Proteínas de adesão como integrinas são expressas na membrana plasmática das CTMs e estão envolvidas com a adesão nas proteínas da matriz extracelular como colágeno, fibronectina e laminina (FOX et al., 2007).

Adiciona-se que, em células expandidas em cultura há pouca presença de CXCR-4, receptor quimiotático do fator derivado de célula-tronco (SDF-1) (SHI et al., 2007), este governa o rolamento das células circulantes no endotélio vascular sendo assim de grande importância para o processo de homing (POTAPOVA et al., 2008).

O local de isolamento, propriedades do meio de cultura e métodos de cultivo, como recriação do ambiente hipóxico, onde naturalmente residem as CTM, parecem estimular a motilidade das células por regular positivamente a

presença de metaloproteinases comparado com ambientes normóxicos (ANNABI et al., 2003).

Entender os mecanismos de agregação celular ativos e passivos possivelmente ajudará a melhorar o entendimento da migração. Células que migram ativamente por métodos semelhantes a leucócitos se achatam e se espalham ao se preparar para realizar a transmigração pelo endotélio vascular, reduzindo a chance de bloqueio vascular. Já as células que são sequestradas passivamente alteram o fluxo sanguíneo local e podem ocasionar oclusão de microvasos (DIACOVO et al., 1996; WALCZAK et al., 2008)

No estudo de KRAITCHMAN et al. (2005) foi observado que CTMs transplantadas pela via intravenosa foram inicialmente encontradas em grandes quantidades nos pulmões, porém nas próximas mensurações (24 horas e entre o 3º e o 7º dia) houve a mobilização com aumento gradual da quantidade dessas células para o tecido cardíaco infartado, o que endossa a importância de avaliar o momento transcorrido desde o transplante até a procura pelas células no tecido alvo uma vez que se trata de um fluxo dinâmico.

Neste contexto, técnicas que possibilitam o acompanhamento *in vivo* das células é de grande importância, uma vez que análises no mesmo animal em diferentes momentos reduz o número de animais utilizados nos projetos, atendendo recomendações de comitês de ética na experimentação animal.

Aparelhos utilizados para rastrear células *in vivo* são: Câmaras para captura de imagens bioluminescentes ou fluorescentes *in vivo* ou *ex vivo*, aparelhos de ressonância magnética e SPECT/CT para visualizar células marcadas com material paramagnético, ou substância radioativa como ¹¹¹In oxine (TANG et al., 2003; KRAITCHMAN et al., 2005; HSIAO et al., 2007; SONG ; KU, 2007; WALCZAK et al., 2008).

Apesar de existir correlação entre o maior número de células transplantadas e maior quantidade de células detectadas no tecido alvo, estudos como o de DOS SANTOS RAMALHO et al. (2018) mostraram que há um limite em que aumentando-se a quantidade de células não há benefício

funcional ou melhor *homing* celular para determinado tecido estudado. Três doses de transplante de MSCs (8×10^4 , 8×10^5 ou 8×10^6) foram testadas pela via intraperitoneal em modelo murino de lesão raquimedular, o melhor desempenho quanto a melhoria funcional e migração das células para a injúria tecidual foi com a dose de 8×10^5 , e não na maior dose, de 8×10^6 .

O local em que as células são transplantadas, influencia no caminho percorrido pelas CTMs até atingir o seu alvo. Diversos trabalhos já demonstraram que as células transplantadas por vias sistêmicas (intravenosa, intraperitoneal, intrarterial e intracardíaca) são sequestradas passivamente principalmente em capilares e arteríolas pulmonares, hepáticos e esplênicos (BARBASH et al., 2003; SACKSTEIN et al., 2008, WALCZAK et al., 2008; KARP ; TEO, 2009).

Dessas vias sistêmicas, as vias intrarterial e intracardíaca parecem diminuir o aprisionamento das CTMs nesses órgãos, infere-se que estudos para determinar vias de transplante de células que possibilitem maior mobilidade destas para seu órgão alvo, sem que haja o sequestro para pulmão, fígado ou baço são de grande importância para a aplicação clínica da terapia celular (WALCZAK et al., 2008).

2.6. *Homing* celular nos modelos de lesão raquimedular

As lesões raquimedulares têm sido um modelo de estudo para a terapia celular uma vez que as propriedades anti-inflamatórias e regenerativas das CTM podem ser uma alternativa promissora para esta condição debilitante tanto para pacientes humanos quanto para os animais domésticos.

Estudos com CTM em lesões traumáticas raquimedulares experimentais foram realizados em ratos, camundongos, primatas e coelhos, demonstrando regeneração tecidual e melhora da locomoção dos animais (HOFSTETTER et al., 2002; SATAKE, 2004; DENG et al., 2006; MOTHE et al., 2011; HOFFMAN;DOW, 2016; FONSECA et al., 2017). Essas pesquisas em modelos

experimentais avaliaram a recuperação funcional dos membros acometidos e/ou a migração das células para o local da lesão.

Em modelo murino de lesão raquimedular por contusão unilateral nos espaços intervertebrais das vértebras torácicas T9 e T10, foi possível comprovar que CTMs alogênicas derivadas da medula óssea e marcadas com proteína verde fluorescente (GFP) migraram para o local de lesão quando transplantadas pela via intratecal (subaracnóide) lombar. Observou-se ainda nesse estudo que as células não só penetraram o parênquima medular lesado como também se diferenciaram-se em células neurais imaturas. A agregação celular foi maior após sete dias do transplante, diminuindo em número de células observadas após quatro semanas (SATAKE, 2004).

Em outro modelo experimental murino de acidente vascular cerebral foi possível inferir que as células mesenquimais derivadas da medula óssea de ratos transplantadas pela via intravenosa foram atraídas para as áreas isquêmicas e que o endotélio vascular cerebral apresentou-se pró-aderente após o evento isquêmico. Este aspecto favoreceu o recrutamento das MSCs CD44 positivas como ligante de integrinas e selectinas do endotélio vascular cerebral no local lesionado (YILMAZ et al., 2011; LEIBACHER; HENSCHLER, 2016).

Também no estudo de ratos com encefalomielite auto-imune experimental (EAE), foi possível observar através de bioluminescência o acúmulo de AdCTMs em focos de inflamação no SNC após 16 e 30 dias do transplante intravenoso. Os autores demonstraram o papel das alfas-4 integrinas na migração da AdCTM alogênicas para o tecido nervoso, contudo não foi possível demonstrar o número de células que migraram para o local da lesão (CONSTANTIN et al., 2009; LEIBACHER; HENSCHLER, 2016).

Nos trabalhos cujo foco era o *homing* das CTMs em doenças neurológicas isquêmicas e pró-inflamatórias, em sua maioria estudados em modelos experimentais murinos de EAE ou acidente vascular isquêmico, notou-se presença de células no local da lesão três dias após o transplante intravenoso, em menor número dentro de três dias a três meses e raramente

reportou-se a persistência das células por mais de três meses. Conclui-se então que o *homing* é um processo transitório. Os autores ainda afirmam que devido as tecnologias disponíveis para rastrear as células não é possível determinar se estas migram para seu alvo como células intactas (LEIBACHER ;HENSCHLER, 2016).

Na maior parte dos trabalhos revisados optou-se por rastrear as células transplantadas entre o sétimo e 14º dia após o transplante, devido a hipótese de que as células transplantadas levam algum tempo para migrarem e serem encontradas em grande quantidade no local de lesão (HOFSTETTER et al., 2002; SATAKE, 2004; DENG et al., 2006; NISHIMURA et al., 2013; MOTHE et al., 2013; URDZÍKOVÁ, 2014). Observou-se ainda que mesmo quando há morte das MSCs é observado melhora funcional e melhora da regeneração tecidual, provavelmente devido a redução da inflamação, modificação do microambiente e prevenção de apoptose (NISHIMURA et al., 2013; URDZÍKOVÁ et al 2014; YANG et al, 2018).

3. HIPÓTESE

O transplante de AdCTMs caninas pela via epidural em modelo experimental de lesão raquimedular compressiva aguda em coelhos possibilita o *homing* celular para o local da lesão.

4. OBJETIVOS

4.1. OBJETIVOS GERAIS

Avaliar o *homing* das AdCTMs caninas transplantadas pela via epidural em modelo experimental de lesão raquimedular compressiva aguda em coelhos.

4.2. OBJETIVOS ESPECÍFICOS

- Cultivar e marcar as AdCTMs caninas *in vitro* com quantum dots QTracker 655®;
- Estabelecer o modelo de lesão raquimedular compressiva em coelhos por inflação do balão de cateter de embolectomia tipo FOGARTY no espaço epidural conforme adaptação de VANICKÝ et al., 2001;
- Avaliar a capacidade das AdCTMs caninas migrarem para a medula espinhal até o local da lesão (T10) a partir do local de transplante (L7-S1) e para o encéfalo, fígado, rim, baço e pulmão, por meio da detecção da fluorescência em imageador e por microscopia confocal;

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Homing of canine multipotent mesenchymal stromal cells after epidural transplant in a rabbit model of compressive spinal cord injury

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Abstract

Multipotent mesenchymal stromal cells (MSCs) have been presented by the scientific community as a promising alternative for the treatment of inflammatory, traumatic, vascular, and degenerative diseases of the central nervous system due to their anti-inflammatory, immunomodulatory, and neuroregenerative properties. One of the aspects that can affect the effectiveness of stem cell therapy is the transplantation route used. The aim of this study was to evaluate the homing of canine adipose derived MSCs (AdMSCs) transplanted via epidural injection in an experimental rabbit model of compressive spinal cord injury. New Zealand rabbits (n=8) were subjected to spinal cord injury induced by compression of the 10th thoracic segment. Immediately after the surgical procedure, the animals were subjected to the transplantation of marked AdMSCs with quantum dots through epidural injection between the L7–S1 spaces. Seven days after the transplant, the brain, spinal cord, lungs, kidney, spleen, and liver were collected and evaluated by bioluminescence imaging. The spinal cord segments L7–S1 and the thoracic injury site were evaluated by confocal microscopy to identify the presence of marked canine AdMSCs. Results showed that AdMSCs migrated to the thoracic spinal cord segment, lungs, brain, liver, and kidneys, and no bioluminescence was observed in the spleen. The presence of canine AdMSCs in the spinal cord indicates the capacity of these cells to overcome the dura mater and reach the transplantation site. Epidural delivery is feasible and minimally invasive and might be a good candidate for cell transplantation in the context of neurological diseases.

Introduction

Multipotent mesenchymal stromal/stem cells (MSCs) are a promising approach for the treatment of inflammatory, degenerative, neurological, and cardiovascular diseases due to their immunomodulatory and regenerative potential. The use of MSCs has been widely studied in spinal cord injury models and in preclinical trials, with good results reported in terms of morphological and functional improvement after traumatic, ischemic, and inflammatory injuries [1-7].

Homing is the process by which MSCs reach the target site for transmigration across the endothelium [8]. This process has been considered crucial for the effectiveness of stem cell therapy, since the mechanism of action of MSCs is presumably linked to their ability to produce factors that act in a paracrine or justacrine manner to the target cell. Thus, the closer the MSCs are to the injured tissue, the better the expected results [9, 10,11,12]. Little is known about the exact mechanisms that MSC utilize to reach the target tissue; however, many growth factors and cytokines, including VEGF-A, HGF, TGF- β 1, TNF- α , SDF-1 α , IL-6, IL-8, and IGF 1, and receptors, adhesion molecules, and metalloproteinases, such as CXCL-12, CCL-2, CCL-3, CCR4, CXCR4, VCAM, and ICAM, have already been identified as being active in the process [13-16].

The data are still scarce and controversial regarding the fate of MSCs after cell transplantation through different routes. When the intravenous route is used, following systemic distribution of some cells and their subsequent disappearance, a large concentration of cells is observed in the lung [17-19]. In order to circumvent the trapping of cells in pulmonary capillaries and encourage more cells to migrate to the injured spinal cord, many researchers have used transplants close to the injury site through intralesional, intrathecal, or interventricular injections [20,21].

However, direct transplantation into the injured spinal cord adds additional risk to the procedure, distributes the cells in a hostile environment, and is potentially clinically unfeasible because it necessitates subjecting the

patient to anesthetic and surgical procedures [22]. Therefore, transplantation pathways that ensure that cells are delivered close to the lesion site and that favor them crossing the blood-brain barrier are essential [3, 14, 23].

Epidural injection is an alternative for MSC transplantation for neurological diseases because it is less invasive and has already been extensively studied in relation to drug delivery in anesthesia and chronic pain control, providing good distribution of the drugs [24]. However, the systemic absorption of drugs administered by this route occurs through diffusion through epidural fat and ligaments. Locally, the drugs spread through the spinal meninges, reaching the subarachnoid space and the cerebrospinal fluid (CSF), where they are distributed throughout the spinal cord and brain using the cerebrospinal fluid flow. The greatest barrier to the local diffusion of these drugs is the arachnoid mater [25,26].

In this context, the aim of this study was to evaluate canine adipose derived MSCs (AdMSC) homing after epidural transplantation in a rabbit model of compressive spinal cord injury. It is necessary to evaluate the applicability and effectiveness of transplanted cells and to promote the homing of MSCs to a neurological system injury site before this technique can be widely used in the clinical context of neurological diseases, such as spinal cord injuries. We hypothesized that canine AdMSCs transplanted by the epidural route would be able to distribute themselves both systemically and locally and reach the spinal cord injury site.

Materials and Methods

Experimental design

This study was approved by the Ethics Committee on Animal Experimentation, previously established by the Brazilian College of Animal Experimentation (COBEA), as stated in the protocol CEUA-FMB-UNESP, nº 1286/2019. Eight male and female rabbits from the Botucatu Genetic Group from the Central Animal Farm of UNESP (São Paulo State University) - Campus

Botucatu, São Paulo, weighing 2.5. 4.0 kg and approximately 6 months of age were used. The animals were kept in individual cages with dimensions of 55 × 55 × 55 cm and suspended 80 cm from the floor. The room temperature was maintained at 20 °C with 12-h light cycles. Animals received a commercial diet (Nutricoechos, Purina Nutrimentos Ltd., Viana, ES) and water ad libitum during the study period. During the three-day postoperative period, the animals also received green leaves (kale) and were kept under the care of specialized technicians.

Canine AdMSCs immunophenotyping and multipotentiality assays

Cells were obtained from the adipose tissue of healthy dogs undergoing elective surgeries. The adipose tissue samples were stored in Hank's Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA, USA) and divided into fragments (1-2 mm³) to be digested with collagenase type 1A (Sigma-Aldrich, St Louis, MO, USA). Cells were kept at 37 °C and stirred every 10 min for 1–2 h. After neutralizing the enzyme with 10% fetal bovine serum (FBS) (Sigma-Aldrich), the material was filtered and centrifuged to separate the cell pellet. The cells obtained were divided into 25 cm² bottles with 6 ml of medium and incubated at 37 °C in a humid atmosphere containing 95% air and 5% CO₂.

The culture medium was prepared with Dulbecco's Modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12 Gibco; Thermo Fisher Scientific, Grand Island, NY, USA), 10% FBS, 1% penicillin/streptomycin, and 1.2 % amphotericin (Sigma-Aldrich), and changed after the first 48 h and then every 3 to 4 days.

The cells were trypsinized with 0.25% trypsin, (Gibco; Thermo Fisher Scientific) when they reached 80% confluence. As soon as the cell population was isolated in the third passage, the MSCs were characterized by multipotentiality tests and immunophenotypic labeling. The cell samples used were previously characterized.

Canine cells were evaluated for surface markers in reactions containing species-specific antibodies for rats that included: CD 90-PERCP (BD Pharmingen, San Diego, CA, USA), CD 45-PE (BD), CD 106-PE (Abcam, Cambridge, MA, USA), CD11b-APC (Abcam), and CD 34-FITC (Biorbyt, St Louis, MO, USA). Canine MSCs were analyzed on a BD FACSCalibur flow cytometer (Becton Dickinson Company, San Jose, USA).

Cell suspensions at a concentration of 1×10^6 cells diluted in 100 μ L of saline were used, and conjugated antibodies were added to the solution according to the manufacturer's recommendations. After the analysis, the data were evaluated using Cell Quest Pro software (Becton Dickinson Company).

In order to confirm multipotentiality, cultured cells were induced to differentiate into adipogenic, chondrogenic, and osteogenic cells *in vitro* using StemPro adipogenesis®, chondrogenesis®, and osteogenesis® differentiation sets (Gibco; Thermo Fisher Scientific) following the manufacturer's recommendations. Passage two to six were used for *in vivo* experiments.

Canine AdMSC labeling with quantum dots

Prior to cell transplantation, cells were trypsinized and marked with a Qtracker 655® (Cell Labeling Kit, Molecular Probes, OR, USA, cat code: Q25029) according to the manufacturer's recommendations. The labeled cells were resuspended in 0.3 ml of HBSS at the time of transplantation.

Spinal cord injury model

As an anesthetic protocol, the animals were administered pre-anesthetic medications of 2 mg/kg midazolam (Cristalia, Itapira, SP, Brazil) and 30 mg/kg ketamine (Vetnil, Louveira, SP, Brazil) intramuscularly. Anesthetic induction was performed with isoflurane (Cristalia, Itapira, SP, Brazil) in a facemask until tracheal intubation, and maintenance with isoflurane and inhaled oxygen.

Venous access was obtained by catheterization of the marginal ear vein to maintain fluid therapy with lactated Ringer's solution, analgesic bolus, and infusion of analgesic solution containing 10 ug/kg/h fentanyl citrate (fentanyl, Janssen-Cilag Farmacêutica, SP, Brazil), 0.3 mg/kg/h ketamine (Vetnil, Louveira, SP, Brazil) and 3 mg/kg/h lidocaine (EMS, Hortolândia, SP, Brazil).

The animals were kept in a prone position for trichotomy and antisepsis of the entire dorsal region, followed by the surgical procedure for dorsal laminectomy between 8th thoracic vertebra (T8) and 9th thoracic vertebra (T9). After obtaining a good spinal exposure for the insertion of a Fogarty 3 French catheter (Edwards Lifesciences, CA, USA) and careful advancement through the epidural space to 10th thoracic segment (T10), the balloon was inflated with 1 cm³ of air for 10 s as described by Vanický et al. in 2001 [27], enough time to cause a spinal cord injury, then the balloon was deflated and removed from the spinal canal.

The animals received 0.5 mg/kg meloxicam (Ouro Fino, Cotia, SP, Brazil) subcutaneously once daily for 3 days, 50 mg/kg dipyrone (Halex Istar Indústria Farmacêutica Ltd, GO, Brazil) every 12 h for 5 days, and 30 mg/kg gabapentin every 12 h until the end of the procedure. Enrofloxacin (União Química, São Paulo, SP, Brazil) was also administered at 5 mg/kg once daily for 5 days.

Canine AdMSCs epidural transplantation

The transplant was performed immediately after spinal cord injury under the same anesthetic protocol described for the surgical procedure. After trichotomy and local antisepsis, the space between the L7 and S1 vertebrae was punctured. The correct needle positioning was verified by the non-observation of CSF in the needle tube, and the aspiration test of the physiological solution was dropped by negative pressure. The syringe was attached to the needle with the cells in suspension and the application was performed slowly and continuously with 1.5×10^6 cells per rabbit suspended in 0.3 ml of HBSS.

Collection and preparation of tissue samples

Rabbits were euthanized with an overdose of 50 mg/mL thiopental (Thiopentax, Cristalia, Itapira, SP, Brazil) seven days after the surgical procedure + transplantation and were immediately perfused intracardially with physiological solution followed by 4% paraformaldehyde buffer. The spinal cord, brain, kidneys, liver, spleen, and lungs of each animal were dissected. The organs were stored at 4 °C in 4% paraformaldehyde until analyses.

MSCs tracking

Ex-vivo fluorescence detection

The nanocrystal-labeled MSCs were screened in the spinal cord, brain, kidneys, liver, spleen, and lungs seven days after the epidural transplant. The imager fluorescence detection method (FX PRO *in vivo* imager, Bruker, TX, USA) was used. For imaging, the values of the 610 excitation wavelength and 700 emission wavelength were used with an exposure time of 5 s. The organs (*ex-vivo*) to be imaged were arranged side by side and were analyzed for fluorescence positivity or negativity compared to the same negative control organ, thus avoiding the possibility of autofluorescence. Organs that showed more fluorescence than the negative control were considered positive; those equal to the negative control were considered negative.

Spinal cord (n=8), brain (n=7), liver (n=7), lung (n=7), spleen (n=6), and kidney (n=7) were imaged. The same spinal cords were screened for the presence of AdMSCs QTracker 655[®] labeled at two different sites: the lesion site (T10) and transplantation site (L7–S1 spinal cord segment).

Confocal microscopy evaluation

After imaging, the spinal cord tissue was prepared for analysis using confocal microscopy. The fragments of the segments corresponding to the T10 lesion site and cell transplant site (L7–S1) were stored overnight in 10% and 20% sucrose solutions, sequentially, for cryopreservation. They were frozen in liquid nitrogen and n-hexane to be sectioned longitudinally and transversely using a cryostat to obtain 10 µm fragments. The samples were stored in a –80 °C freezer until use. After cleavage of the fragments into 10 µm thick cross sections, the slides were processed and marked with 4, 6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA), and analyzed using confocal microscopy (TCS, SP8, Leica®, Wetzlar, Germany).

For each of the eight animals analyzed by confocal microscopy, two spinal cord cuts were made: one in the L7-S1 portion and the other at the T10 (lesion site). Each slide was analyzed in 3 different fields so that to determine the average amount of fluorescence present. The amount of fluorescence in the confocal microscopy images was analyzed using the ImageJ program, (Java, 1.51k version, <https://imagej.nih.gov/ij/download.html>) .

Histological analyses

Cuts of spinal cords, brains, lungs, livers and kidneys were made to perform morphological analyses by hematoxylin and eosin (HE) staining. The slides were analyzed qualitatively for the presence of hemorrhage and inflammation.

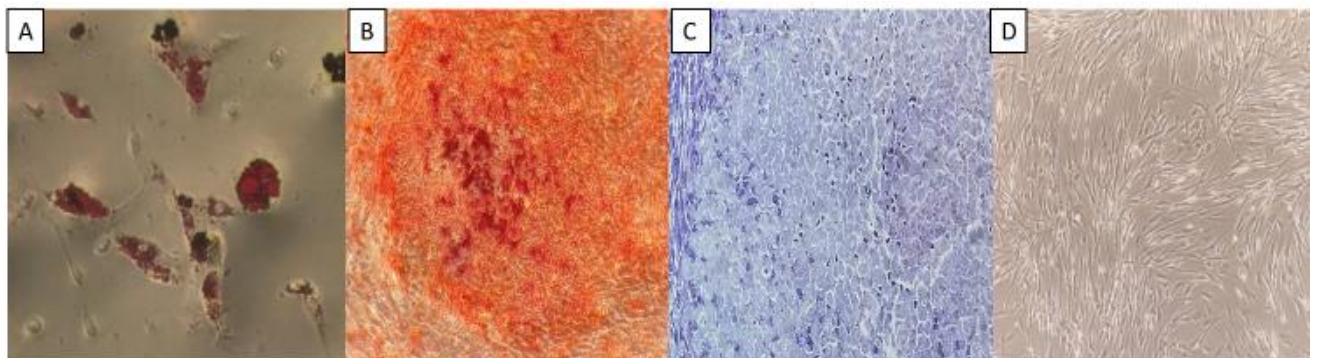
Statistical analysis

The integrated pixel density variable was assessed in the spinal cords for normality using statistical tests (Shapiro-Wilk), descriptive statistics, and graphic analysis. The t-test was performed for unpaired samples. The level of significance assumed was equivalent to $p < 0.05$. The analysis was performed using software (GraphPad Prism version 8 for Mac, San Diego, CA, USA).

Results

Canine AdMSCs showed mesenchymal fate and trilineage differentiation potential

The immunophenotype profile presented by the cells confirmed their mesenchymal origin. Immunophenotypic analysis demonstrated positive expression for CD 90 (99,91%) and absence of expression for hematopoietic antigens CD45 (2.08%), CD34 (6.01%), CD 106 (9.14%), and CD 11b (2.67%). The induction of *in vitro* differentiation of adipogenic, chondrogenic, and osteogenic lines was positive (Figure 1). For adipogenic differentiation, lipid droplet deposition within the cell cytoplasm was observed when stained with Oil Red O stain. For osteogenic differentiation, all samples cultured in the induction medium demonstrated intense extracellular deposition of stained calcium in Alizarin Red S. In chondrogenic differentiation, the micromass was observed by staining with toluidine blue.



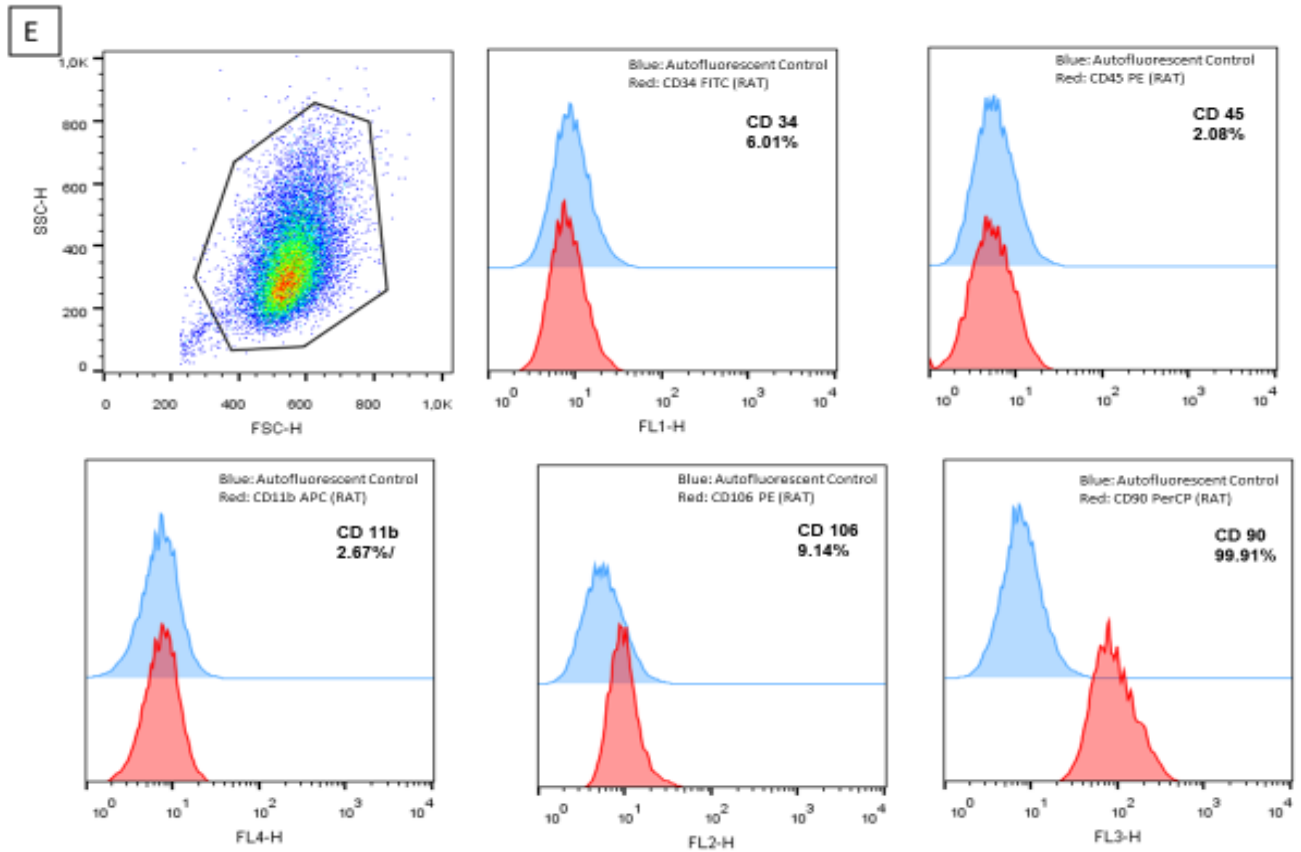


Figure 1. Potential for differentiation of canine AdMSCs in adipogenic (A), osteogenic (B) and chondrogenic (C) lines, 20x magnification. Note the deposition of lipid droplets within the cell cytoplasm stained with Oil Red O. (A). Extracellular calcium deposition stained in Alizarin Red S (B). Formation of micromass stained by toluidine blue (C). AdMSCs at 20x magnification, 3rd passage. Note the fibroblastoid morphology and adherence to plastic (D) and Immunophenotypic analysis (E).

Clinical and histological evaluation confirm the experimentally induced spinal cord injury

The technique used, adapted from VANICK et al [27], was effective to induce myelopathy in all animals, characterized by paraparesis with motor and proprioceptive deficits of the pelvic limbs. The animals showed no deficit in urinary function. However, the clinical follow-up and/or functional recovery of the neurological deficits was not within the scope of this work.

Morphological evaluation of the T10 spinal cord segment revealed inflammatory changes compatible with the compressive injury caused, such as myelomalacia, congestion, and glitter cells were observed (Figure 2A, 2B, 2C).

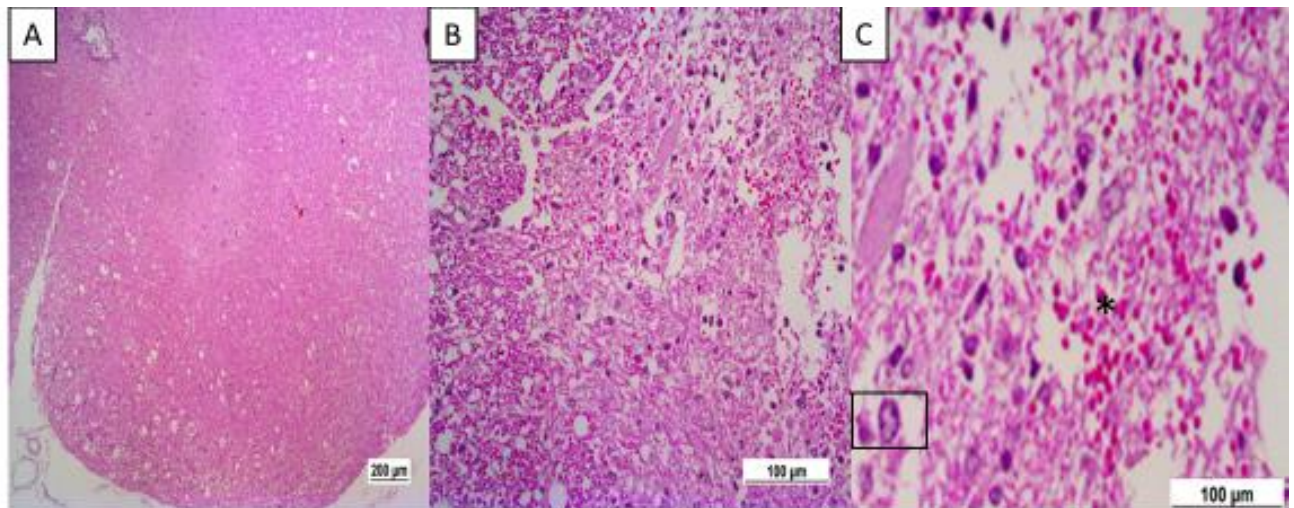


Figure 2. Histological evaluation of the ventral horn of the spinal cord (T10 segment) seven days after experimental injury (A, B and C). Note the frequent spheroids and vacuolation of white matter (A), discrete satellitosis and central chromatolysis (B). Note the frequent spheroid and vacuolization of white matter, central chromatolysis, extensive area of hemorrhagic necrosis (malacia) (asterisk), mainly in gray matter with moderate glitter cells (Square)(C), 100 µm scale.

Canine AdMSCs migrated systemically and to central nervous system after epidural transplantation

Ex-vivo fluorescence detection imaging

Organs in the imaging analyzer were positive for the fluorescent canine AdMSCs in the liver (n=6/7), spinal cord (n=2/8), brain (n=6/7), kidney (n=6/7), and lung (n=4/7), and fluorescence was not observed in any of the imaged

spleens (n=6) (Figure 3). Two out of eight spinal cords were positive for canine AdMSCs (Figure 4). Of these two animals, one was positive at the spinal cord injury site and the other was positive from the proximity of the T10 injury site to the cervical segment. Brains were positive in six of seven imaged animals, corresponding to 85.7%, and the livers and kidneys were positive in six of the seven animals tested (85.7%). The lungs, on the other hand, showed 57.14% positivity, with four organs positive out of seven imaged (Figure 3).

Despite the low positivity of spinal cords in the imaging analyzer, confocal microscopy was used to confirm the presence of Qtracker-labeled MSCs in all imaged spinal cords. Confocal microscopy was also performed on two brain samples. It was possible to confirm the presence of these cells in the brain parenchyma, as seen in the imaging analyzer.

The results of fluorescence imaging of the organs combined with those of confocal microscopy in the present study demonstrate the ability of canine AdMSCs to distribute systemically from epidural transplantation between the L7–S1 vertebrae and to reach the nervous tissue.

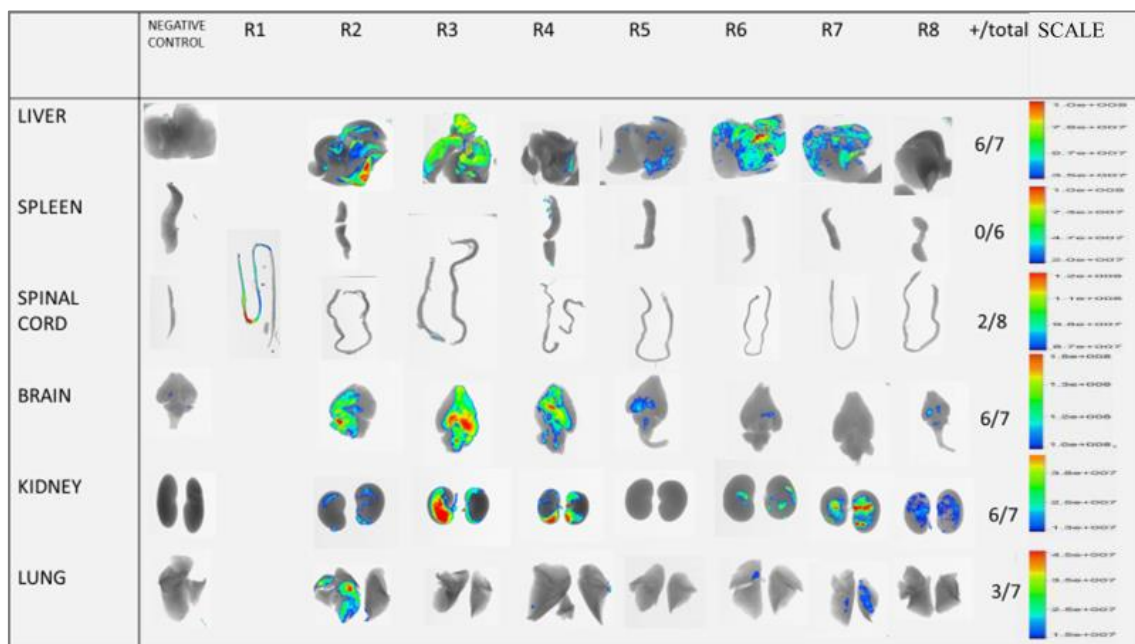


Figure 3. Ex-vivo fluorescence detection imaging of the spinal cord, brain, liver, kidneys, and lungs. On the left, negative control organs, in the middle, organs

from rabbits 1 to rabbit 8. Organs that were equal to the negative control were considered negative and those with more fluorescence than their negative control were considered positive. The intensity scale graded from 8.7×10^7 to 1.2×10^8 photons/second/mm² for spinal cord, from 1.0×10^8 to 1.5×10^8 photons/second/mm² for brain, from 3.5×10^7 to 1.0×10^8 photons/second/mm² for liver, from 2.0×10^7 to 1.0×10^8 photons/second/mm² for spleen, from $1, 5 \times 10^7$ up to 4.5×10^7 photons / second / mm² for lungs, and from 1.3×10^7 up to 5.0×10^7 photons/second/mm² for kidneys.

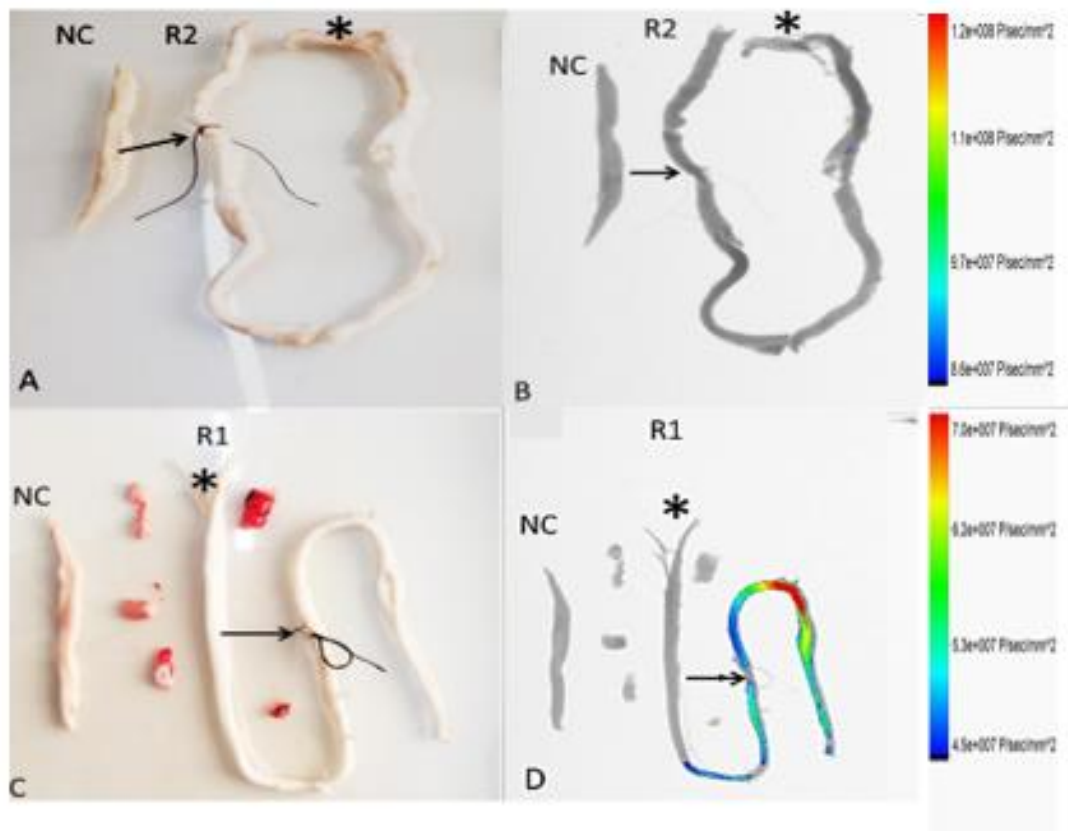


Figure 4. Ex-vivo fluorescence detection imaging of the spinal cord. Figures A and C show the setup of spinal cords from rabbits number one (R1) and two (R2) and the negative spinal cord controls (NC) before imaged. Figures B shows a negative fluorescence detection in the spinal cord of the rabbit R2, while Figure D shows a positive fluorescence detection in the spinal cord of the rabbit R1. The arrows indicate the site of experimental spinal cord injury (10th

thoracic segment) and the asterisks indicate the end of the spinal cord (cauda equina), corresponding with the site of the canine AdMSCs transplantation by the epidural route.

Confocal microscopy

Through confocal microscopy, it was possible to verify the presence of cells marked with nanocrystals in spinal cord tissue both at the application site in L7-S1 and at the injury site in T10 in all of the animals studied.

Animal number one was not positive in fluorescence imaging at the transplantation site, but MSC markings in this region were observed by confocal microscopy. Animal number 3 was positive in fluorescence only at the injury site, but by confocal microscopy, marked cells were seen both at the application site and at the injury site (Figures 5). The other spinal cords were negative in the bioluminescence chamber but positive for the presence of Qtracker® at the injury site and also in the L7–S1 segment by confocal microscopy.

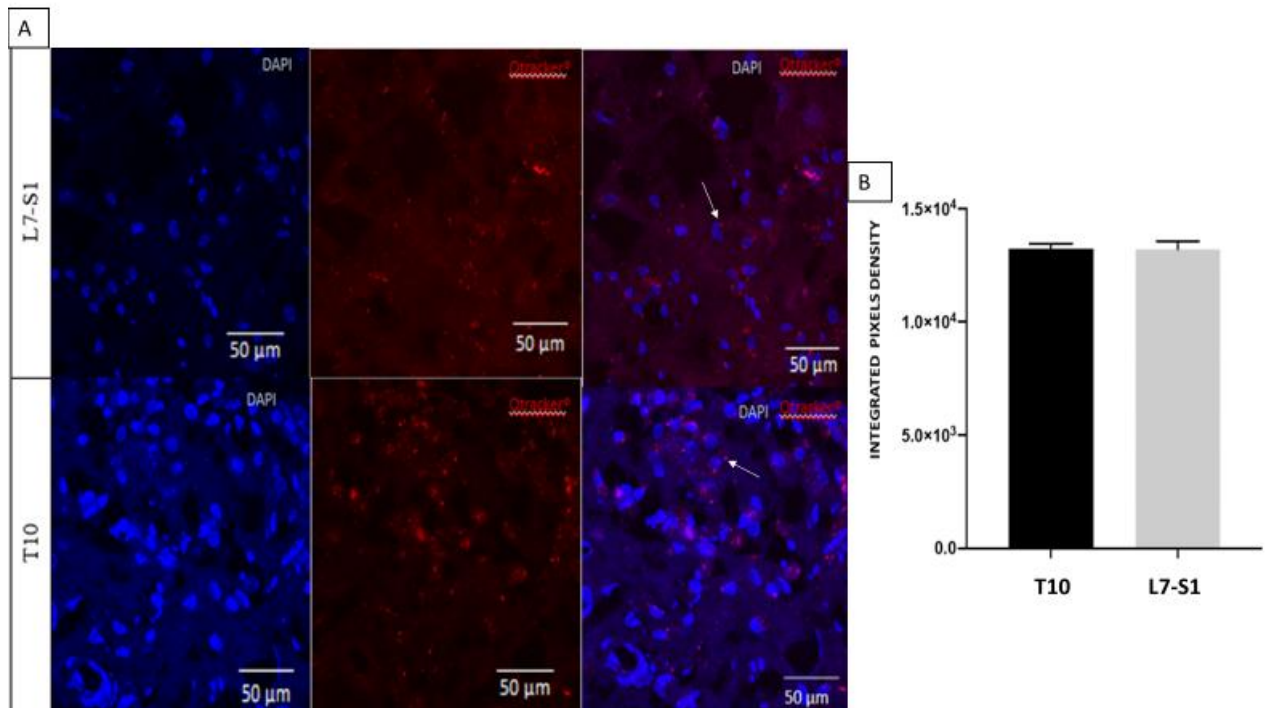


Figure 5. Confocal microscopy images of the L7–S1 spinal cord segment (site of the transplant) and of the T10 spinal cord segment (site of experimental injury) (A). Note the presence of canine AdMSCs labelled with Qtracker 655® at the L7-S1 and T10 spinal cord segments (arrows, merged images DAPI + Qtracker). Quantitative analysis of the fluorescence intensity of canine AdMSCs labelled with Qtracker 655® at the L7-S1 and T10 spinal cord segments seven days after transplantation.

Discussion

Although the mechanisms that govern the cellular homing of MSCs to the injury site are not known, it is known that tissue damage induces the local production of chemokines that attract MSCs to the injury site through previously explored mechanisms [8]. In this study, we induced spinal cord injury using the arterial embolectomy catheter compression model and evaluated the ability of

canine AdMSCs to migrate to the damaged spinal cord segment and their distribution to other organs. Previous studies have demonstrated the ability of MSCs to migrate to the spinal cord tissue following intrathecal transplantation [3,20,21].

However, the epidural route via lumbar puncture in the context of transplantation of MSCs is still poorly studied. Bach et al. [6] showed good results for ambulatory improvement of dogs after epidural transplantation of AdMSC, but the transplantation was performed at the trans-operative injury site in spinal cord decompression surgery. To our knowledge, the fate of AdMSCs via the epidural route in the L7-S1 intervertebral space has not yet been explored.

In the present study, Qtracker 655 nanocrystals were used as a marker of canine AdMSCs because of their ease of internalization in the cell cytoplasm and low cytotoxicity [28]. Good results regarding the traceability of MSCs using the nanocrystal Qtracker have already been reported for bone marrow-derived MSCs in a tendonitis model in horses [29]. An ex-vivo imager fluorescence detection was used to investigate the presence of fluorescence from nanocrystal labeled canine AdMSCs. Imaging by detecting fluorescence has been presented as a good option for tracking mesenchymal stem cells. The technique demonstrated a good relationship between the amount of fluorescence detected and the number of cells [30].

The small amount of spinal cord markings by the ex-vivo imager fluorescence detection can be explained by the use of the epidural transplant route and possible difficulty of MSCs in crossing the dura mater and reaching the spinal cord tissue.

On the other hands there was fluorescence detection in kidney (6/7), lung (3/7), liver (6/7), and brain (6/7). These may have gone through the process of active migration to target tissues other than the injury site at T10, or they may have gained blood flow through vessels in the epidural region and sequestered in capillaries and venules. This phenomenon is well described for the lung when the intravenous route is used [19, 30].

Through confocal microscopy, it was possible to verify the presence of canine AdMSCs marked in the spinal cord tissue in all animals, both at the application site and at the injury site (Figure 5).

The results of fluorescence imaging of the organs combined with those of confocal microscopy in the present study demonstrate the ability of AdMSCs to distribute systemically from puncture and epidural transplantation between the L7–S1 vertebrae to reach the nervous tissue. The canine AdMSCs transplanted by epidural route may have been distributed between the meninges up to the CSF, and thus distributed throughout the spinal cord, as well as through blood circulation.

Additionally, we also showed that canine AdMSCs transplanted via epidural route were distributed systemically to the kidneys, liver, lung and brain. The systemic distribution can be explained by the diffusion of cells through the epidural fat, vessels, and local arteries. The systemic distribution of drugs after epidural injection is well described mainly in the context of the use of anesthetics for surgery or analgesia [25].

Surprisingly, a large number of animals were marked in the brain. Six of the seven brains were positive, the presence of labeled cells was confirmed by confocal microscopy. These findings may corroborate the migration of MSCs to the brain and justify the use of this route in cell transplants in clinical cases of brain disorders.

The time elapsed from the transplant to tracking in the target tissue is important. In our study, we evaluated cell migration seven days after transplantation. In addition, we also performed an experiment in rabbits that underwent a previously described surgical procedure and cell transplantation with rabbit AdMSCs via the epidural route and sacrificed three days after the procedure. The same organs were investigated in this animal, and the presence of markings only in the lungs was verified by fluorescence imaging, corroborating the possibility that the cells were transiently homing to the lung.

However, through confocal microscopy, markings compatible with the Qtracker in the spinal cord of this animal were visualized (data not shown).

The literature varies regarding the time it takes the cells to reach the target organ. MOTHE et al.[3] screened neural progenitor cells derived from the spinal cord and MSCs derived from bone marrow after intrathecal transplantation by lumbar puncture in a rat spinal cord injury model, and found a greater number of cells transplanted into the spinal cord at 27 days compared to 13 and 17 days. In our study, the cells were tracked at 7 days post-transplantation. Further studies should be conducted to explore the potential for cell migration at other time points, between 1 and 30 days after transplantation.

It would also be interesting to evaluate cell migration without the production of tissue injury as compared to the results found in the present study. In rats, the sequestration of MSCs in pulmonary capillaries has been demonstrated, probably due to the large size of the cells in relation to the capillaries [19] and their low deformation capacity [17].

In a study by GAO et al.[16], immediately after the intrarterial, intravenous, or intraperitoneal transplantation of MSCs marked with ^{111}In -oxine, only lung marking was observed. After 48h, intense liver staining and less lung staining were observed compared to immediately after transplantation. The kidneys and spleen also showed radioactivity within 48h. In our study, we did not observe spleen marking, but we did have positive results in the kidneys and livers.

The fact that cell transplantation occurred shortly after the surgical procedure and, therefore, tissue injury, makes the microenvironment hostile for transplanted cells [22]. It is important to note that, due to the methods used in the present study, it is not possible to determine whether the marked cell is alive or dead, mainly because it is a xenotransplant. In the case of canine AdMSC death and consequent phagocytosis by mononucleated cells, the Qtracker in the cytoplasm of the MSC can mark the defense cells and the perceived fluorescence is due to the migration of mononuclear cells.

Conclusions

AdMSCs are able to migrate from the epidural space and reach target organs such as the injured spinal cord and brain; therefore, the epidural route is an alternative for cell transplantation via systemic routes, such as the intravenous or intrarterial route, in the context of inflammatory diseases of the nervous system. It is feasible and safe delivery route because it does not require an invasive surgical procedure to perform cell transplantation, and sedation is sufficient to perform the technique. Further studies need to be conducted to assess the potential for functional recovery, neuro-regenerative roles, and safety of the technique before the procedure is used routinely in a clinical setting.

Conflicts of Interest

The authors declare no conflicts of interest

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