MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL RURAL DA AMAZÔNIA - UFRA
DOUTORADO EM SAÚDE E PRODUÇÃO ANIMAL NA AMAZÔNIA

LUANE LOPES PINHEIRO

USO AUTÓLOGO DE CÉLULAS-TRONCO MESENQUIMAIS DE TECIDO ADIPOSO EM CÃES PORTADORES DE CINOMOSE COMO MODELO EXPERIMENTAL DE ESCLEROSE MÚLTIPLA

BELÉM
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Tese apresentada à Universidade Federal Rural da Amazônia, como parte das exigências do Curso de Doutorado em Saúde e Produção Animal na Amazônia: área de concentração Saúde e Produção Animal, para obtenção do título de Doutor.
Orientadora: Profª. Dra. Érika Renata Branco

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Aprovado em _____ de 2019.

BANCA EXAMINADORA

_____

Profª. Dra. Érika Renata Branco – Orientadora
UNIVERSIDADE FEDERAL RURAL DA AMAZÔNIA - UFRA

_____

Prof. Dr. Atanásio Serafim Vidane 1º Examinador
UNIVERSIDADE EDUARDO MONDLANE – MOÇAMBIQUE - ÁFRICA

_____

Prof. Dr. Edivaldo Herculano Corrêa de Oliveira– 2º Examinador
INSTITO EVANDRO CHAGAS - IEC

_____

Prof. Dr. André Marcelo Conceição Meneses – 3º Examinador
UNIVERSIDADE FEDERAL RURAL DA AMAZÔNIA - UFRA

_____

Prof. Dr. Ednaldo da Silva Filho – 4º Examinador
UNIVERSIDADE FEDERAL RURAL DA AMAZÔNIA - UFRA

_____

Prof. Dr. Carlos Eduardo Ambrósio - Suplente
UNIVERSIDADE DE SÃO PAULO – FMVZ/USP

PARECER DA COMISSÃO DE BIOÉTICA

UNIVERSIDADE FEDERAL RURAL DA AMAZÔNIA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o Projeto de Pesquisa, intitulado "*Terapia celular em modelo experimental de EM a partir de cães portadores de cinomose*", protocolo nº 053/2015 (CEUA) e 23084.012198/2015-81 (UFRA), sob a responsabilidade da **professora Érika Branco** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DEANIMAIS da Universidade Federal Rural da Amazônia, em reunião de 02/12/2015.

Vigência do projeto	Dezembro de 2015 a maio de 2016
Espécie/linhagem	Cão/SRD
Número de animais	20 animais
Peso/Idade	Até 10 anos
Sexo	Machos e fêmeas
Origem	Hospital Veterinário - UFRA

Belém, 10 de dezembro de 2015.

Profª Dra. Maria Cristina Manno
Coordenadora CEUA UFRA

Profª Drª Maria Cristina Manno
Membro Titular -CEUA/UFRA

À minha **família**, por todo amor, amparo e motivação.

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À **Universidade Federal Rural da Amazônia**

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À **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior**

Pela bolsa de estudo concedida.

À professora **Érika Renata Branco**

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Pelo apoio na realização desse estudo.

À **Danielli Martinelli**

Pela participação direta e dedicação na realização deste projeto.

Aos professores **Ana Rita de Lima**, **Leandro Nassar Coutinho** e **Ednaldo da Silva Filho**, e à **Carla Miranda**, **Raylene Uchôa**, **Marina Brito**, **Ana Tobelem**, **Raquel Fernandez** e **Luciana Pantoja**

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Aos **cães** e seus **tutores** que lutaram contra a cinomose

Pelo compromisso, dedicação e esforço minha eterna gratidão.

RESUMO

Diversos pesquisadores acreditam que algumas doenças que ocorrem naturalmente em animais de companhia podem refletir melhor as variações genéticas, ambientais e fisiológicas presentes na população humana. Visando convergir avanços paralelos, o reconhecimento de cães e gatos como modelos translacionais em estudos pré-clínicos na medicina regenerativa, levaria a avanços mais consistentes na pesquisa veterinária, promovendo ganhos acentuados de conhecimento e tecnologia. Devido às similaridades das alterações neuropatológicas da leucoencefalite desmielinizante induzida pelo vírus da cinomose com a esclerose múltipla em humanos, a cinomose em cães em fase neurológica tem representado uma das poucas ocorrências espontâneas para o estudo da patogênese da perda de mielina. Vários estudos têm sugerido a utilização de células-tronco mesenquimais como opção de tratamento para as doenças desmielinizantes e neurodegenerativas, assim, objetivou-se avaliar a segurança e eficácia do uso autólogo de células-tronco mesenquimais do tecido adiposo, em quatro cães que mantiveram sinais neurológicos após tratamento das manifestações sistêmicas da infecção pelo vírus da cinomose. Após três infusões de $1x10^7$ células pela via intra-arterial, os animais não expressaram mudança significativa quanto a locomoção, porém, três passaram de intensa mioclonia para moderada. Após um ano da terapia celular, os quatro cães passaram a se locomover de forma independente (Grau I e II), e em dois animais as mioclonias passaram para a condição leve. A utilização de células-tronco mesenquimais do tecido adiposo mostrou segurança em aplicações repetitivas sem efeitos adversos em curto prazo, e melhora na qualidade de vida de cães com sequela neurológica provocada pela leucoencefalite desmielinizante. Frente aos resultados encontrados, a toda literatura consultada, e ao histórico de comercialização de células-tronco na medicina veterinária brasileira, entendemos ser necessário revisar ensaios publicados com células-tronco derivadas do tecido adiposo, em doenças de animais de companhia com ocorrência espontânea, discutindo-se os desafios científicos da pesquisa na medicina regenerativa veterinária, e a fundamentação científica, ainda pouco clara, mediante o atual comércio celular, não controlado por agências reguladoras.

Palavras-chave: Células-tronco mesenquimais. Cinomose. Esclerose múltipla. Desmielinização. Terapia celular.

ABSTRACT

Several researchers believe that some diseases that occur naturally in companion animals may better reflect the genetic, environmental and physiological variations present in the human population. Aiming to converge parallel advances, the recognition of dogs and cats as translational preclinical models in regenerative medicine studies, would lead to more consistent advances in scientific research veterinary medicine, lags behind that human medicine in knowledge and technology. Due to the similarities of neurological changes of leukoencephalitis by distemper virus with a multiple sclerosis in humans, the distemper in dogs in the neurological phase was represented by one of the few spontaneous diseases for the study of the pathogenesis of myelin loss, associated with immune-mediated diseases. Several studies have suggested the use of mesenchymal stem cells as a treatment option for demyelinating and neurodegenerative diseases. Therefore, we investigated the use of adipose-tissue-derived stem cells in four dogs with neurological lesions caused by the distemper virus. After three infusions of $1x10^7$ cells by the intraarterial route, the animals did not demonstrate significant changes in their locomotive abilities. However, the intense myoclonus in three animals was reduced to a moderate level. At one year after the cell therapy, all four dogs started to move independently. In two animals, the myoclonic severity had become mild. It was concluded that the use of mesenchymal stem cells showed safety in repetitive applications, no adverse effects in the short term, and improve the quality of life of dogs with neurological sequelae caused by demyelinating leukoencephalitis. In view of the results found, the all consulted literature, and the historical of commercialization of stem cells in Brazilian Veterinary Medicine, we consider necessary to review the trials published with stem cells derived from adipose tissue in diseases of companion animals with spontaneous occurrence, discussing the scientific challenges of research in veterinary regenerative medicine, and the scientific basis, still unclear, for the current cellular trade, not controlled by regulatory agencies.

Keywords: Mesenchymal Stem Cells. Distemper vírus. Multiple Sclerosis. Demyelination. Cell therapy.

SUMÁRIO

CONTEXTUALIZAÇÃO

A cinomose é uma doença viral, multissistêmica, altamente contagiosa, que acomete carnívoros domésticos e selvagens, causada por um Morbillivirus da família Paramyxoviridae (DEEM et al., 2000). Para os cães domésticos o vírus da cinomose canina (CDV, do inglês Canine Distemper Vírus) é um dos mais importantes agentes infecciosos por sua característica altamente imunossupressora e pantrópico, representando cerca de 6% de todas as ocorrências na clínica de pequenos animais e uma das principais causas de mortalidade em cães (BEINEKE et al., 2009; MONTEIRO et al., 2010).

Considerada endêmica no Brasil, a cinomose afeta mais comumente filhotes, embora possa ocorrer em qualquer idade em animais não vacinados ou com histórico de vacinação incompleta, pois a exacerbação da doença depende da estirpe do vírus, idade e estado imunitário do animal no momento da infecção (BEINEKE et al., 2009). Uma vez manifestada, provoca um comprometimento duradouro das funções imunológicas celulares e humorais, tornando os animais susceptíveis a infecções oportunistas que resulta em uma variedade de formas clínicas, no qual a imunossupressão e o acometimento neurológico representam o principal agravante nesta espécie (KRAKOWKA et al., 1985; APPEL, 1987; BEINEKE et al., 2009; LEMPP et al., 2014).

O envolvimento do sistema nervoso central (SNC) representa uma complicação, que pode ocorrer em paralelo ou posteriormente a afecções a outros órgãos, por lesão direta induzida pelo vírus, ou lesão indireta e tardia devido à imunidade desenvolvida (FENNER, 2004; VANDEVELDE et al., 2005). Apresenta-se mais comumente como uma leucoencefalite desmielinizante (LD), caracterizada por lesão axonal, com um grau variável de desmielinização e inflamação mononuclear (ULRICH et al., 2014; LEMPP et al., 2014), no qual os sinais neurológicos refletem a localização do SNC afetado, sendo os mais evidentes, distúrbios de comportamento, convulsões, ataxia, paraplegia, tetraparesia, tetraplegia, disfunção de propriocepção, atrofia muscular, hiperestesia, mioclonia, déficits ou reflexos anormais e incontinência urinária (BEINEKE et al., 2009).

Apesar de décadas de pesquisa, nos últimos anos vários novos aspectos da neuropatogênese da LD têm sido descritos, a partir de investigações *in vivo* e *in vitro* com ênfase especial na interação axônio-mielina-glia (LEMMP et al., 2014). No processo agudo da cinomose, a replicação viral ocorre predominantemente em astrócitos e está associada à produção de fator de necrose tumoral alfa (TNF-α), o que coincide com o início da desmielinização (SEEHUSEN et al., 2007). Assim, o acometimento neuronal e a quebra da bainha de mielina são eventos não apenas associados à replicação viral, mas também mediados por citocinas produzidas localmente (BEINEKE et al., 2009). No processo crônico da cinomose, a progressão das lesões parece ser um evento imunopatológico, no qual o mecanismo imunológico e inflamatório estabelecido em fases posteriores, associado à tentativa de eliminação do vírus do SNC, induz a fase crônica da desmielinização (MORO et al., 2003; SEEHUSEN et al., 2007).

Devido às similaridades das alterações neuropatológicas, a leucoencefalite desmielinizante provocada pelo vírus da cinomose tem sido sugerida por pesquisadores como modelo de ocorrência natural para o estudo da patogênese da perda de mielina, associada a mecanismos imunomediados, tal como acontece na esclerose múltipla em humanos (KOESTNER, 1957; BAUMGÄRTNER; ALLDINGER, 2005; BEINEKE et al., 2009; LEMPP et al., 2014; ULRICH et al., 2014).

A esclerose múltipla (EM) é a mais frequente doença inflamatória desmielinizante e neurodegenerativa do SNC, responsável por incapacidade neurológica significativa em adultos jovens, entre 20 a 40 anos (PATANI; CHANDRAN, 2012). Estima-se o número de 35 mil pessoas com EM no Brasil e 2,3 milhões em todo o mundo, com prevalência na América do Norte e Europa, afetando duas vezes mais mulheres do que homens (MSIF, 2013).

Enfermidade complexa, a EM possui etiologia exata desconhecida, cujos dados epidemiológicos sugerem se tratar de uma doença autoimune, decorrente da interação entre fatores biológicos e ambientais, como: suscetibilidade genética, mecanismos autoimunes e infecções virais (SILVA; NASCIMENTO, 2014). Sua fisiopatologia também não é totalmente compreendida, sendo o principal evento representado por uma resposta aberrante das células do sistema imunitário à autoantígenos da mielina, resultando em inflamação, desmielinização e degeneração axonal (DULAMEA, 2015).

As lesões da EM ocorrem na substância branca do SNC, por destruição da bainha de mielina dos neurônios, gerando múltiplas áreas cicatriciais (escleroses), e

consequentemente uma deficiência na condução motora com diminuição ou bloqueio dos sinais nervosos, com manifestações clínicas variadas determinadas pela localização das lesões (CARDOSO, 2010). Incluem perda da força muscular, dormência, parestesias, coordenação alterada, paralisia aguda dos membros, perda aguda da visão (neurite óptica), disfunção cognitiva, perda da memória, fadiga, crises epilépticas, demência, distúrbios vesicais e intestinais, alterações de personalidade e labilidade emocional (CARDOSO, 2010; FINKELSZTEJN, 2014).

O tratamento para EM tem sido baseado em medicamentos imunomoduladores, que embora amenizem os sintomas e reduzam a frequência e a gravidade dos surtos, não interrompe a progressão contínua da neurodegeneração, cujo substrato patológico é a degeneração axonal (AULETTA et al., 2012; FINKELSZTEJN, 2014; ZHANG et al., 2014). Entretanto, vários estudos têm sugerido a utilização de células-tronco mesenquimais (CTMs) como opção de tratamento para as doenças desmielinizantes (FREEDMAN et al., 2010; RIVERA; AIGNER, 2012; DULAMEA, 2015).

Nesse contexto, a literatura dispõe de dados demonstrando que as CTMs transplantadas em modelos animais experimentalmente são capazes de reduzir a desmielinização, aumentar a neuroproteção e modular a inflamação (KASSIS et al., 2008; LANZA et al., 2009; RAFEI et al., 2009), e alguns ensaios clínicos têm demonstrado resultados preliminares promissores no uso de CTMs na esclerose múltipla (FREEDMAN et al., 2010; KARUSSIS et al., 2010; YAMOUT et al., 2010; BONAB et al., 2012; LLUFRIU et al., 2014; DULAMEA, 2015).

Para melhor compreensão, esclarecemos que as CTMs são consideradas uma linhagem de células-tronco somáticas responsáveis pela regeneração e manutenção de tecidos adultos (KEATING, 2012). Nas últimas décadas tornaram-se alvo de diversos estudos devido suas propriedades biológicas com possibilidades visionárias para o tratamento de uma variedade de doenças (CAPLAN, 2017).

Atualmente, a secreção de moléculas bioativas tem explicado o seu potencial terapêutico, e se tem mostrado que a produção de fatores tróficos e imunomoduladores são capazes de promover efeitos regenerativo, anti-inflamatório, angiogênico, anti-apoptótico, mitótico, anti-fibrótico, anti-bacteriano e anti-tumoral (CAPLAN; DENNIS, 2006; GNECCHI et al., 2008; CAPLAN; CORREA, 2011; BAGLIO et al., 2012; MEIRELES et al. 2009; TAO, et al., 2016, VIZOSO et al., 2017). Além disso, a identificação do secretoma das CTMs com efeitos parácrinos contribui fortemente ao

argumento que, sua função natural *in vivo*, é de atuar como sinalização para locais de lesão ou inflamação (CAPLAN, 2010; MAGUIRE, 2013; MADRIGAL et al., 2014). Entretanto, apesar de inúmeras pesquisas com qualidade e tecnologia cientifica, a natureza complexa das CTMs torna a sua caracterização desafiadora, e importantes aspectos permanecem mal definidos (RUSSELL et al., 2016; ASSONI et al., 2017; VISOZO et al., 2017; FITZSIMMONS et al., 2018).

Recentemente, Caplan (2017) considerou sua própria designação "células-tronco" mesenquimais (CAPLAN, 1991) cientificamente e terapeuticamente enganosa, sugerindo a mudança de nomenclatura para "células de sinalização medicamentosa" a fim de refletir com mais precisão o seu potencial terapêutico frente ao atual cenário mundial, de ensaios clínicos e comercialização não regulamentada de CTMs, no qual pacientes alimentam esperança de cura, por vezes, milagrosa.

Nesse contexto, na medicina humana os aspectos éticos e regulatórios da terapia com células-tronco são discutidos em todo o mundo e a maioria dos países possui alguma legislação de acordo com o tipo de produto celular, enquanto que na medicina veterinária a ausência de regulamentos permitiu que terapias com CTMs fossem, atualmente, oferecidas e comercializadas sobe altos custos para o tratamento de uma variedade de doenças de animais de companhia, embora não tenham atingidos padrões reconhecidos de evidencia em relação a eficácia e segurança (CYRANOSKI, 2013; DOMINICI et al., 2015; HOFFMAN, DOW, 2016; BORGES, 2018, FRANKLIN, 2018).

As recomendações atuais são direcionadas para a realização de estudos científicos bem conduzidos e controlados, que ofereçam dados de alta qualidade, somado à superação dos desafios associados à complexidade dos produtos baseados em células (BORGES, 2018; FRANKLIN, 2018). É valido considerar que na ciência veterinária esta configuração se torna difícil, entre outras razões, devido a limitações logísticas e econômicas associadas a tal desenho de estudo e falta de apoio à pesquisa (BAKKER et al., 2013).

Frente a isto, diversos pesquisadores acreditam que as doenças que ocorrem naturalmente em cães e gatos podem refletir melhor as variações genéticas, ambientais e fisiológicas presentes na população humana (CHRISTOPHER, 2015; HOFFMAN; DOW, 2016), e dessa forma, o reconhecimento destes como modelos translacionais pré-clínicos para a terapia baseada em células nos ensaios humanos, levariam a avanços

mais consistentes na pesquisa, convergindo avanços paralelos mediante uma colaboração multidisciplinar (CYRANOSKI, 2013; BAKKER et al., 2013; CHRISTOPHER, 2015; KOL et al., 2015; HOFFMAN; DOW, 2016; BEARDEN et al., 2017).

A cinomose canina representa uma das poucas ocorrências espontâneas em animais para o estudo da patogênese da perda de mielina (ULRICH et al., 2014), geralmente, os cães que sobrevivem a sintomatologia multissistêmica mantêm sequelas neurológicas incapacitantes, muitas vezes incompatíveis com a vida (SILVA et al., 2007). Dessa forma, novas terapias que possam modular as respostas imunes, promover a remielinização e mediar reparação do tecido neuronal danificado é um dos principais desafios no campo da medicina, tanto humana, quanto veterinária (LEMPP et al., 2014).

Em face ao exposto, inicialmente objetivamos avaliar o potencial terapêutico de CTMs autólogas derivadas do tecido adiposo, pela via intra-arterial, para recuperação de sequelas neurológicas em cães com leucoencefalite desmielinizante, induzida pelo vírus da cinomose, avaliando segurança em aplicações repetitivas, efeitos adversos à curto prazo e sinais de mudanças no quadro neurológico que possam demonstrar relevância clínica para pacientes humanos com esclerose múltipla.

Por fim, mediante aos resultados apresentados na vasta literatura consultada acerca da temática terapia celular, em animais de companhia, bem como a comercialização indiscriminada de células-tronco na medicina veterinária brasileira, sem a devida permissão e fiscalização dos órgãos competentes, entendemos ser necessário realizar uma revisão sobre o assunto, para formarmos nossa convicção quanto ao uso comercial destas células.

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ARTIGO I

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Mesenchymal stem cells in dogs with demyelinating leukoencephalitis as an experimental model of multiple sclerosis

Luane Lopes Pinheiro [a,*], Ana Rita de Lima [a], Danielli Martinelli Martins [a], Edivaldo Herculano C. de Oliveira [b], Michel Platini C. Souza [b], Carla Maria Figueiredo de Carvalho Miranda [c], Patrícia Cristina Baleeiro Beltrão-Braga [d], Fabiele Baldino Russo [d], Graciela Conceição Pignatari [d], Ednaldo da Silva Filho [a], Érika Branco [a]

[a] Institute of Animal Health and Production, Faculty of Veterinary Medicine, Federal Rural University of Amazonia, Belém, PA, Brazil
[b] Tissue Culture and Cytogenetics Laboratory of the Environment Sector, Instituto Evandro Chagas, Ananindeua, PA, Brazil
[c] Department of Surgery, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, SP, Brazil
[d] Institute of Biomedical Sciences, Department of Microbiology, University of São Paulo, São Paulo, SP, Brazil

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ABSTRACT

Researchers have used dogs with neurological sequelae caused by distemper as an experimental model for multiple sclerosis, owing to the similarities of the neuropathological changes between distemper virus-induced demyelinating leukoencephalitis and multiple sclerosis in humans. However, little is known about the role of mesenchymal stem cells in treating such clinical conditions. Therefore, we investigated the use of mesenchymal stem cells in four dogs with neurological lesions caused by the distemper virus. During the first year after cellular therapy, the animals did not demonstrate significant changes in their locomotive abilities. However, the intense (Grade V) myoclonus in three animals was reduced to a moderate (Grade IV) level. At one year after the mesenchymal stem cell infusions, three animals regained functional ambulation (Grade I), and all four dogs started to move independently (Grades I and II). In two animals, the myoclonic severity had become mild (Grade III). It was concluded that the use of mesenchymal stem cells could improve the quality of life of dogs with neurological sequelae caused by canine distemper, thus presenting hope for similar positive results in human patients with multiple sclerosis.

1. Introduction

Demyelinating leukoencephalitis is the major aggravating factor and cause of mortality from canine distemper [1]. It commonly represents the neurological stage of this disease, where inflammation of the central nervous system, demyelination, and axonal injury occur [1, 2]. However, similar to other morbilliviruses, the canine distemper virus behaves as a lymphotropic and immunosuppressive agent, rendering the animals highly susceptible to opportunistic infections and resulting in a variety of clinical forms that characterize distemper [3]. Dogs that survive this stage sustain disabling sequelae that are often incompatible with life [4]. Furthermore, owing to the morphological similarities to the neuropathological changes associated with human multiple sclerosis, canine distemper represents one of the few spontaneous occurrences in animals that can be applied as a model for the study of the pathogenesis of myelin loss [2, 3].

Multiple sclerosis, a complex human disease with unknown etiology and pathophysiology, manifests primarily as a result of an aberrant response of the immune system cells to the autoantigens of the myelin sheath of neurons. This condition results in multiple areas of scarring (sclerosis) and is also characterized by inflammation, demyelination, and axonal degeneration, which occur in canine distemper as well [5]. Because multiple sclerosis and similar degenerative myelopathy in domesticated animals require treatments that aim to recover the myelin sheath and repair the damaged neuronal tissue, their therapy and cure remain major challenges in both the human and veterinary medical fields [1].

Several research groups have investigated the therapeutic use of mesenchymal stem cells (MSCs) for demyelinating diseases [5, 6, 7, 8]. Some results have suggested that MSCs could promote endogenous repair

* Corresponding author.
E-mail address: luanelopespinheiro@gmail.com (L.L. Pinheiro).

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and exert positive immunomodulatory effects to reduce demyelination, increase neuroprotection, modulate inflammation, and promote the differentiation of neural MSCs into oligodendrocytes (myelin-producing cells in the central nervous system) [9]. In addition, some clinical trials have shown promising results in the use of MSCs in multiple sclerosis [5, 10, 11, 12, 13, 14].

MSCs, which are considered a somatic stem cell line, are present in the perivascular regions of adult tissues that are responsible for cell regeneration and homeostasis [15]. These cells have already been isolated from a variety of tissues (e.g., bone marrow, umbilical cord blood, skin, dental pulp, etc.), among which adipose tissue stands out as a common source owing to a higher rate of isolation and yield [16]. Thus, the present study aimed to evaluate the therapeutic potential of MSCs in inducing the recovery from neurological sequelae in dogs naturally affected by demyelinating leukoencephalitis, assessing signs of neurological changes that may represent hope for human patients with multiple sclerosis.

2. Materials and methods

The dogs used in this study were from the Medical Clinic Sector of the Veterinary Hospital "Prof. Mário Dias Teixeira" at the Federal Rural University of Amazonia (UFRA). The animal protocol was approved by the UFRA Committee on Ethics in the Use of Animals (Protocol No. 053/2015).

2.1. Treatment protocol and evaluation parameters

We selected four dogs (designated c1, c2, c3, and c4) with evident signs of demyelinating leukoencephalitis. The diagnosis of distemper was confirmed from the clinical signs and through laboratory tests. After treatment of the multisystemic clinical symptomatology, neurological sequelae compatible with those caused by the disease still remained, however, without alterations in laboratory tests, including the polymerase chain reaction (PCR) - negative for the distemper virus.

The dogs were given a complete neurological examination, which consisted of evaluations of their mental state, locomotion, cranial nerves, postural reactions, spinal reflexes, sensory perception, and muscle tone. For analytical purposes, the neurological record was rated as 0 for absence, 1 for decrease, 2 for normality, and 3 for increase of the evaluated signal. Two neurological scales that were created by Santos [17] were used for evaluating the sequelae of distemper. One scale was for locomotion, with the following grades: (I) functional ambulation; (II) ataxic animal – walks with incoordination; (III) tetraparetic animal – stays in station, but does not get up; (IV) tetraparetic animal – does not stay in station or stand up; and (V) tetraplegic animal – without deep pain and with signs of Grade IV. The other scale was for myoclonus, with the following grades: (I) absent; (II) only at moments of agitation; (III) present – mild; (IV) present – moderate; and (V) present – intense.

The MSCs were extracted from the flank adipose tissue of each canine patient of this study through enzymatic digestion according to the protocol of Zuk et al. [18]. Three separate doses of 1×10^7 cells at passages P3 or P4 were injected into the dogs through the femoral artery at 30-day intervals, and monthly neurological examinations before each application as well as one final evaluation one year later were carried out.

2.2. Clinical conditions of the selected animals

Prior to MSC treatment, all animals were conscious and the neurological changes at the first visit were related to locomotion, postural reactions, spinal reflexes, muscle tone, and myoclonus. c1 presented with monoparesis of the right pelvic limb, with decreased conscious proprioception and hypertonia of this limb, besides motor incoordination and spontaneous falls. c2 presented with monoparesis of the right pelvic limb, with decreased conscious proprioception, patellar hyperreflexia, and hypertonia of this limb, as well as motor incoordination and spontaneous falls. c3 presented with functional deambulation, without changes in the neurological examination. c4 presented with tetraparesis, the absence of conscious proprioception, and hypertonia in the four limbs, as well as cervical stiffness. The four dogs had myoclonus of several muscular groups, with a noticeably greater incidence in the masticatory muscles. The myoclonus was classified as intense for c1, c2, and c4 and moderate for c3.

2.3. MSC cultivation, cryopreservation, and phenotype analysis

After isolation, the MSCs were maintained in cultures at 37 °C with 5% CO_2 in growth media complete (Dulbecco's modified Eagle's medium, with 20% fetal bovine serum), with a medium change every 2–3 days. The cultures were cryopreserved at the P0 and P1 passages. After thawing, the viability of the cells at each passage was tested using the trypan blue exclusion dye (0.4%) test (Sigma, USA). For intra-arterial administration, the MSCs were thawed and maintained in culture for an average of 7 days for the expansion needed to reach the determined amount of cells (1×10^7 cells).

For phenotype analysis by immunofluorescence, the cells were plated and incubated with primary anti-CD105 (1:25), anti-CD34 (1: 100), and anti-CD45 (1: 100) antibodies from Abcam (USA), and goat anti-CD73 (1:25) and anti-vimentin (1:25) antibodies from Santa Cruz Biotechnology (USA). Following further processing, they were analyzed under a Nikon 80i fluorescence microscope.

For phenotype analysis by flow cytometry, the cells were first incubated with the primary antibodies (CD105, CD73, CD90, CD34, CD45, and CD79) for 45 min at 4 °C. After washing in phosphate-buffered saline, they were incubated with phycoerythrin- or fluorescein isothiocyanate-conjugated secondary antibodies for 30 min. Following this, 10,000 events were acquired on the FACSCalibur flow cytometer and FlowJo software was used to analyze the data obtained.

2.4. Gene expression by RT-qPCR

Total RNA was extracted using TRIzol Reagent (Life Technologies, USA) and reverse transcribed into cDNA using SuperScript III (Invitrogen, USA), following the manufacturers' protocols. The cDNA was then subjected to quantitative PCR (qPCR) using SYBR Green Supermix (Bio-Rad, USA). Each sample was run in triplicate. Primers for specific genes were synthesized using Primer3 software (v. 0.4.0) or were available from the Harvard Primer Bank online. The conditions of the PCR cycles were as follows: 30 s at 95 °C, 30 s at 95 °C, 30 s at 60 °C, and 45 s at 72 °C for 50 cycles. Melting curve analysis was then conducted to verify the amplification specificity. All analyses were done by absolute quantification, with the levels of the target genes normalized to that of the *GAPDH* gene as a reference control, using standard curves.

2.5. MSC differentiation potential

To determine the osteogenic differentiation potential of the MSCs, 5×10^3 MSCs/mL were cultured in osteogenic differentiation induction medium (STEMPRO Osteogenesis Kit; Gibco, USA), with a change of the medium on every other day for 14 days, according to the manufacturer's recommendations. The cells were then stained with 2% Alizarin Red S (Sigma-Aldrich, USA) for 5 min.

For observation of their adipogenic differentiation potential, 1×10^4 MSCs/mL were cultured in induction medium (STEMPRO Adipogenesis Kit, Gibco, USA), with a change of the medium on every other day for 14 days. The cells were then evaluated by staining with 1.25% Oil Red O (Sigma-Aldrich, USA) for 5 min.

For determination of their chondrogenic differentiation potential, the MSCs were cultured in a conical tube at high cell density (5×10^7 cells/mL) in a micromass system. After centrifugation and disposal of the maintenance medium, chondrogenic differentiation induction medium (STEMPRO Chondrogenesis Kit; Gibco, USA) was added, followed by

homogenization and further centrifugation. The tube was maintained in an oven at 37 °C with 5% CO_2, with change of the differentiation medium on every other day for 21 days, following the manufacturer's recommendations. For fixation of the micromass cells, 4% paraformaldehyde was added. The cells were then dehydrated in serial dilutions of ethanol, embedded in paraffin blocks, and further processed according to routine histological protocols. The blocks were cut into 5-μm-thickness sections that were then stained with 1% Alcian Blue solution for 10 min.

2.6. Chromosomal stability analysis

The numerical chromosomal stability of cells of the P4, P6, and P8 passages was analyzed. To obtain the cells at metaphase, 100 μL of 0.016% colchicine solution (Gibco, USA) was added to 5 mL of the culture and the cells were kept in a 37 °C oven for 1 h. The cells were then dissociated with trypsin and transferred to a conical tube for centrifugation at 556 *g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in a 0.075 M hypotonic solution (KCl) and kept in a 37 °C oven for 10 min. Subsequently, Carnoy's fixative solution (methanol:acetic acid, 3:1) was added, and the mixture was homogenized and centrifuged; this process was repeated two more times. Finally, the pellet was resuspended in 2 mL of the fixative and the cells were stored under 6 °C refrigeration. For visualization of the chromosomes, slides containing the cells were stained for 3 min with 10% Wright solution diluted in phosphate buffer (pH 6.8), and 15 metaphase cells of each passage were analyzed under a Leica DM1000 optical microscope. Images of the best metaphase cells were captured using the GenASIs platform (Applied Spectral Imaging, USA), which is also used for karyotyping.

2.7. Statistical analysis

Descriptive statistics were used as appropriate. The Friedman test at 5% significance was applied to determine the median treatment effect for the locomotion and myoclonus scores before and after three infusions and after one year.

3. Results

After 24 h, it was possible to observe the adherence of some of the MSCs to the plastic surface of the culture flask, and the cell confluency reached 80% after 48 h (Fig. 1A, B). After thawing, the cells propagated rapidly, maintaining a fibroblastic morphology in all the passages analyzed (P1–P8) (Fig. 1C), with a mean viability of 94.7% (Fig. 2A). The mean viability of the infused cells was 97.5% (P3) and 98.5% (P4).

In the immunocytochemical evaluation, the analyzed samples were positive for the mesenchymal labels CD105, CD73, and vimentin, and negative for the hematopoietic cell markers CD34 and CD45 (Fig. 1D–I). In the flow cytometric analysis, the cells showed positivity for the CD105, CD73, and CD90 markers, whereas the CD79, CD34, and CD45 hematopoietic cell markers were undetectable (Fig. 2B).

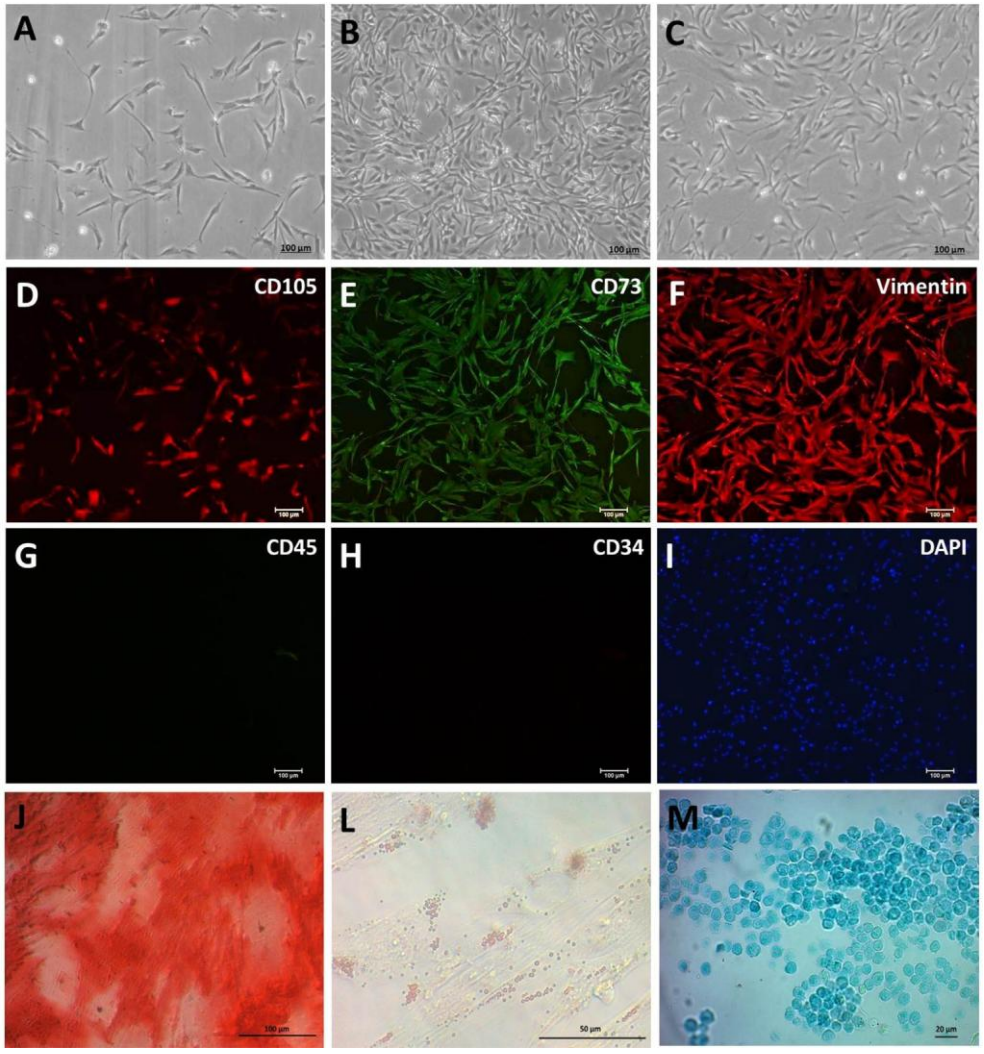

Fig. 1. A, B, and C: Photomicrography of the canine mesenchymal stem cell (MSC) culture after two days of isolation, after six days at cell confluence, and at the P1 passage after thawing, respectively. Scale bar: 100 μm (5×). D–I: Immunocytochemical characterization of MSCs, with positive labeling for the mesenchymal markers CD105, CD73, and vimentin, and negative labeling for the hematopoietic markers CD45 and CD34, as evidenced by the DAPI-stained cell nucleus. Scale bar: 100 μm. J: Osteogenic differentiation, demonstrated by Alizarin Red coloration of the extracellular calcium matrix. L: Adipogenic differentiation, demonstrated by Oil Red O coloration of the lipid droplets. M: Chondrogenic differentiation, demonstrated by Alcian Blue staining of the proteoglycans. Scale bar: 100 μm (5×).

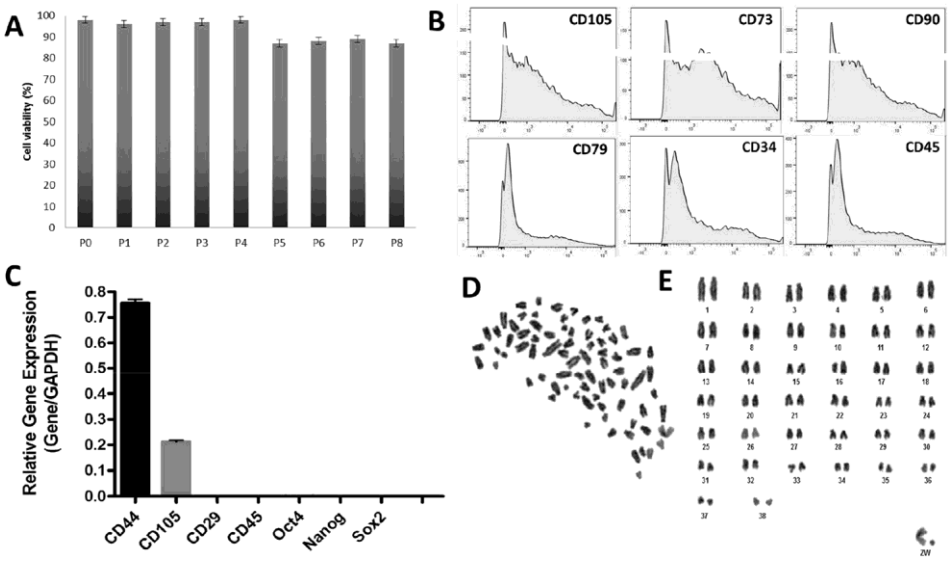

Fig. 2. A: Trypan blue dye exclusion test of cell viability in passages P1–P8. B: Phenotype analysis by flow cytometry of mesenchymal stem cells (MSCs) positive for markers CD105, CD73, and CD90, and negative for CD79, CD34, and CD45. C: RT-qPCR analysis of MSCs with CD44 and CD105 expression, and absence of CD29, CD45, *Oct4*, *Nanog*, and *Sox2* expression. D: Canine MSCs at the end of metaphase. E: Karyotype of the canine MSCs, showing 2n ¼ 78.

As determined by RT-qPCR, the analyzed samples did not express pluripotency-related genes (*Nanog*, *Oct4*, and *Sox2*), as expected. However, they showed CD44 and CD105 expression (mesenchymal cell markers) and had no expression of CD29 and CD45 (hematopoietic cell markers) (Fig. 2C).

With regard to the cell differentiation processes, the MSC culture produced an extracellular calcium matrix in the osteogenic differentiation medium, revealing cells with osteogenic characteristics (Fig. 1J). In the adipogenic differentiation medium, the cells had a rounded shape and accumulation of lipid droplets in their cytoplasm, indicative of adipogenic differentiation (Fig. 1L). Histologically, the micromass that had formed under the induction of the chondrogenic differentiation medium showed rounded cells surrounded by a glycosaminoglycan matrix (Fig. 1M).

The numerical chromosomal stability of the MSC cultures was successfully demonstrated. Cells in the analyzed passages (P4, P6, and P8) maintained the diploid number of 78 chromosomes for the domestic dog (Fig. 2D, E).

In the evaluation after the first autologous infusion of MSCs, the c1 and c4 animals presented changes in the neurological examinations when compared with the initial examination and at the time of admission of the canine patients to the study. That is, c1 presented normal locomotion of the right pelvic limb and the absence of both motor incoordination when walking and spontaneous falls. The c2 and c3 animals did not present changes in the neurological analysis, whereas the c4 tetraparetic animal showed the absence of cervical rigidity, being able to support its head, which alleviated the initial difficulty in eating and allowed self-feeding without the help of the owner.

After the second MSC infusion, although the c2 animal maintained paresis and hypertonia of the right pelvic limb, it presented with normal conscious proprioception and normal patellar reflex of this limb. Despite that it still had motor incoordination while walking, the animal showed improvement in relation to spontaneous falls.

The c1 and c3 animals did not present any changes in this second neurological examination. In contrast, although the c4 animal remained tetraparetic, it presented with normal muscle tone in all limbs and normal conscious proprioception in the thoracic limbs, and maintained decreased proprioception in the pelvic limbs. In the neurological examination after the third MSC infusion, no changes were observed in relation to the previous evaluations in all four patients.

At one year after the last MSC infusion, the c1 and c3 dogs did not present changes in their neurological examination. The c2 animal presented with functional ambulation without incoordination and without hypertonia of the right pelvic limb. The c4 animal presented with normal proprioception of the pelvic limbs, and started to stay in station and to walk with considerable incoordination.

With regard to the myoclonus, improvement occurred after the first infusion of MSCs in the c1 animal and after the third administration in the c2 and c4 animals. During the treatment, the c3 animal showed no changes in its myoclonus scale. After one year, the myoclonus of animals c1 and c3 had decreased to a mild intensity (Grade III), whereas that of animals c2 and c4 remained at a moderate degree (Table 1).

On the basis of the neurological evaluation scale for distemper [12], we can conclude that the use of MSCs was successful when the animal was at Grade I for locomotion and Grade I, II, or III for myoclonus. The three infusions of MSCs at 30-day intervals were successful in one animal only in terms of locomotive improvement, whereas they were successful in changing the myoclonus from Grade V (intense) to Grade IV (moderate) in three animals. However, in the evaluation at one year post treatment, three animals regained functional ambulation (Grade I), and all four animals were able to move independently (Grades I and II). Moreover, two animals presented with Grade III (mild) myoclonus.

Finally, the Friedman test was applied to compare the median treatment effect in the group for the locomotive and myoclonic degrees. The results revealed that there were no significant differences before and after the three infusions, and before and after one year of treatment (Fig. 3).

4. Discussion

We emphasize that depending on the virulence of the virus strain, and the age and immune status of the dog, distemper can be fatal in many cases [2]. This justifies the low number of animals used in this study, since we aimed to select only dogs with sequelae of neurological lesions (demyelinating leukoencephalitis) caused by the distemper virus, all of which had already been treated conservatively and conventionally but did not show recovery of their motor integrity. The dogs of this study did not present with multisystemic clinical symptoms and had no changes in their laboratory test findings, such blood counts and negative RT-qPCR for the virus, in accord with the animals recommended by Gebara et al. [19] and Nelson and Couto [20].

Table 1
Evolution of the neurological conditions in dogs following mesenchymal stem cell therapy.

	Evaluation	Clinical signs Pre-infusion	30 days after 1st infusion	30 days after 2nd infusion	30 days after 3rd infusion	1 year after infusions
C1	Locomotion	Monoparesia TM-R (II)	Normal (I)	Normal (I)	Normal (I)	Normal (I)
	Myoclonus	Intense (V)	Intense (V)	Moderate (IV)	Moderate (IV)	Mild (III)
	Muscle tone	3 PL-R	2 PL-R	2 PL-R	2 PL-R	2 PL-R
	Proprioception	1 PL-R	2 PL-R	2 PL-R	2 PL-R	2 PL-R
C2	Locomotion	Monoparesia TM-R (II)	Monoparesia TM-R (II)	Monoparesia TM-R (II)	Monoparesia TM-R (II)	Normal (I)
	Myoclonus	Intense (V)	Intense (V)	Intense (V)	Moderate (IV)	Moderate (IV)
	Muscle tone	3 PL-R	3 PL-R	3 PL-R	3 PL-R	2 PL-R
	Proprioception	1 PL-R	1 PL-R	2 PL-R	2 PL-R	2 PL-R
	Patellar reflex	3 PL-R	3 PL-R	2 PL-R	2 PL-R	2 PL-R
C3	Locomotion	Normal (I)	Normal (I)	Normal (I)	Normal (I)	Normal (I)
	Myoclonus	Moderate (IV)	Moderate (IV)	Moderate (IV)	Moderate (IV)	Mild (III)
C4	Locomotion	Tetraparesia (IV)	Tetraparesia (IV)	Tetraparesia (IV)	Tetraparesia (IV)	Ataxia (II)
	Myoclonus	Intense (V)	Intense (V)	Intense (V)	Moderate (IV)	Moderate (IV)
	Muscle tone	3 TL-L 3 TL-R	3 TL-L 3 TL-R	2 TL-L 2 TL-R	2 TL-L 2 TL-R	2 TL-L 2 TL-R
		3 PL-L 3 PL-R	3 PL-L 3 PL-R	2 PL-L 2 PL-R	2 PL-L 2 PL-R	2 PL-L 2 PL-R
	Proprioception	0 TL-L 0 TL-R	0 TL-L 0 TL-D	2 TL-L 2 TL-R	2 TL-L 2 TL-R	2 TL-L 2 TL-R
		0 PL-L 0 PL-R	0 PL-L 0 PL-R	1 PL-L 1 PL-R	1 PL-L 1 PL-R	2 PL-L 2 PL-R

TL ¼ thoracic limb; PL ¼ pelvic limb; L ¼ left; R ¼ right; 0 ¼ absent; 1 ¼ decreased; 2 ¼ normal; 3 ¼ increased.

In this context, the literature highlights that the most evident signs of demyelinating leukoencephalitis in distemper are behavioral disorders, convulsions, ataxia, tetraparesis, tetraplegia, proprioception and cranial nerve dysfunctions, muscular atrophy, hyperesthesia, myoclonus, deficits or abnormal reflexes in the spine, and urinary incontinence, regardless of the evolution phase of the disease [3]. The neurological changes observed in the animals selected for this study were related to locomotion (ataxia and monoparesis), postural reactions (absent or diminished proprioception), spinal reflexes (hyperesthesia), muscle tone (hypertonia), and myoclonus of various muscle groups, in accord with the literature [3, 20]. Moreover, myoclonus is the most common sign of this condition, being present without other neurological signs [21], as was the case in one of the selected animals. Demyelinating leukoencephalitis, the neurological phase of distemper, has been suggested as a suitable naturally occurring model for the study of the pathogenesis of myelin loss associated with immune-mediated mechanisms, such as that which occurs in multiple sclerosis [1, 2, 22, 23]. Multiple sclerosis causes a deficiency in motor conduction, with a decrease or blockage of the nerve signals that control muscle coordination, strength, sensitivity, and vision [24]. In addition, when considering an experimental model, researchers search for an attractive species for translational studies, such as dogs, since they are large, long-living, and genetically diverse, and share many biochemical and physiological similarities with humans [25].

Unfortunately, there is still no known cure for multiple sclerosis [6]. However, the literature has encouraging data suggesting the use of MSCs as a treatment option for demyelinating diseases, as was demonstrated in rodent models of multiple sclerosis where MSCs elicited strong antioxidative and neuroprotective effects resulting from the release of anti-apoptotic molecules and neurotrophins, which led to an improvement in the clinical evolution of the disease and reductions of both demyelination and axonal loss [5, 26]. However, the mechanisms underlying the therapeutic effects are still unknown and may involve one or more of the following possibilities, according to Rivera and Aigner [26]: transdifferentiation of MSCs in mature neurons and/or functional oligodendrocytes (plasticity); immunoregulatory effect on host-derived immunoreactive cells (immunomodulation); protective effect on the survival of damaged neurons and/or oligodendrocytes (neuroprotection); and induction of the differentiation and maturation of neural precursor cells or oligodendrocyte progenitor cells present at the lesion site (remyelination).

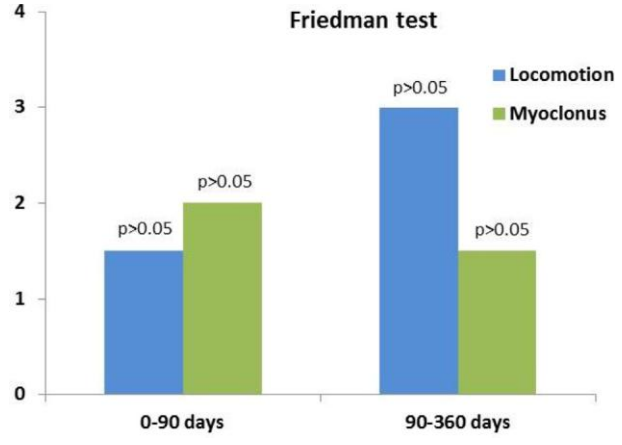

Fig. 3. Friedman test at 5% significance, indicating no significant differences between the degrees of locomotion and myoclonus in the group before and after three infusions, and before and after one year of therapy.

In the case of dogs, most studies use adipose tissue as the source of stem cells, where they are collected by non-invasive procedures such as liposuction or lipectomy, as was done in this study [27]. The protocol for the isolation and culture of MSCs from this tissue has been described by several groups [13, 27, 28], and meets the International Society for Cell Therapy recommendations regarding the characterization of MSCs, which establish a minimum of three criteria: adherence to the plastic surface in culture; expression of surface antigen markers (CD73, CD90, and CD105) and absence of hematopoietic cell markers (CD11b, CD14, CD19, CD29a, CD34, CD45, and HLA-DE); and differentiation into at least three lineages [29]. In the present study, after the enzymatic isolation of adipose tissue, the canine MSCs adhered to the plastic surface of the culture bottle, with a fibroblastoid morphology; maintained chromosomal integrity up to the last analyzed passage (P8); presented potential for osteogenic, adipogenic, and chondrogenic differentiation; and were phenotypically and functionally similar to human and canine MSCs from other previous studies [13, 27, 28, 30].

On the basis of rodent assays, as mentioned above, and considering the advantages of using the dog as an experimental model, Pinheiro et al. [31] evaluated the use of MSCs derived from the fetal olfactory epithelium of dogs and delivered intravenously in animals with acute distemper associated with symptomatic therapy, but obtained negative results in relation to the improvement of systemic and neurological clinical signs. In a randomized study, Brito [32] evaluated the use of bone marrow MSCs, delivered intravenously into dogs, in the neurological phase of distemper with motor signals that affected ambulation, and obtained a positive result in relation to the control group. Recently, Monteiro [33] assessed the effects of allogenic adipose tissue-derived MSCs on neurological abnormalities in dogs in the chronic phase of distemper, and observed that 13 of the 30 animals had a reduced neurological scale score, albeit most remained tetraparetic. Despite promising results after a single administration of

MSCs, both Brito [32] and Monteiro [33] reported a poor health status of some dogs during the study, which may be related to the acute stage of clinical symptomatology, which was different from that of our dogs. Similar to our study, Gonçalves et al. [34] recently used dogs with neurological sequelae only after treatment of the multisystemic symptoms of distemper, and performed three intravenous infusions of adipose tissue-derived MSCs, but theirs were allogenic. In that study, based on the numerical scale proposed by the authors for evaluation, attenuation of the clinical signs was observed after 15 days and was maintained throughout the 180 days of observation, but with statistical differences only for the urinary incontinence and fecal incontinence variables.

In our present uncontrolled clinical trial, the autologous use of adipose tissue-derived MSCs by the intra-arterial route on every 30 days, totaling three infusions, resulted in improvement of the neurological status of the animals, with one dog regaining functional ambulation and achieving a reduction of the myoclonic intensity to Grade IV (moderate). However, consistent improvements were observed in the evaluation after one year post treatment, where three animals regained functional ambulation (Grade I), all animals moved independently (Grades I and II), and two animals presented Grade III myoclonus (mild).

The route of MSC administration is an important variable that can define the success of a transplant by interfering directly with the efficient delivery of cells to the site of interest [35]. The venous system, being the least invasive route, has been the one most often used. However, in addition to a lack of knowledge regarding the actual cellular concentration required to reach the desired lesion area, studies have shown that MSCs accumulate rapidly in the lungs, spleen, and liver after administration [35, 36]. However, by bypassing the initial uptake by the lungs, administration through the arterial system results in a greater availability of cells to ischemic sites, but may lead to a greater probability of microvascular occlusions [37]. In our study, no side effects related to short-term and long-term intra-arterial MSC administrations were observed, and choice for the femoral artery was considered due to easier access compared to the carotid artery or intrathecal route.

We believe that the indiscriminate commercialization of stem cells as a form of "treatment" of various diseases (including for the recovery of canine distemper sequelae) is unacceptable in both veterinary medicine, since it cannot be stated categorically that this therapy does in fact lead to the healing of patients. This is a current concern in many countries owing to the lack of regulations and control for the clinical use of stem cells in veterinary medicine, allowing for the increasing offer of the service by private companies and resulting in the implementation of therapies that lack proven effectiveness either *in vitro* or in preclinical animal studies [38, 39]. The US Food and Drug Administration's Center for Veterinary Medicine was the only legislative body to formally publish specific definitions and recommendations for stem cell use through guidelines [40], where cell-based products must follow the same legal requirements that apply to other animal drugs, forcing the industry to prove efficacy and manufacturing quality and safety prior to commercialization [38].

Despite the promising results regarding the alleviation of the severity of the disabling lesions of demyelinating leukoencephalitis caused by distemper (considered irreversible and often incompatible with animal life), our findings are considered limited because of the small sample size, and future studies should involve a greater number of animals. In addition, both *in vivo* and *in vitro* studies should be performed to determine the mechanisms underlying the therapeutic effects of MSCs in dog with neurological sequelae of distemper. In this context, the technology of induced pluripotent stem cells, from genetically modified and reprogrammed adult cells [41], would be a powerful tool in basic research, tissue differentiation research, and disease modeling, as well as being promising for future clinical applications.

5. Conclusions

Our results indicate that the strategy of three intra-arterial infusions of 1×10^7 MSCs, with a 30-day interval in between administrations, appears to be safe in dogs with demyelinating leukoencephalitis caused by the distemper virus, and presents moderate efficacy for the rehabilitation of neurological signs after recovery from a multisystemic infection, with considerable improvements in the neurological status of the animals after one year of cell therapy. However, further extensive investigations are needed for a better understanding of the mechanism of action of these MSCs on the injured nervous tissue and the time of recovery, in future studies that include a larger number of animals, placebo group and investigation of other routes of administration.

Declarations

Author contribution statement

Luane Lopes Pinheiro: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Ana Rita de Lima: Analyzed and interpreted the data.

Danielli Martinelli Martins, Michel Platini C. Souza, Carla Maria Figueiredo de Carvalho Miranda: Performed the experiments.

Edivaldo Herculano C. de Oliveira, Patrícia Cristina Baleeiro Beltrão-Braga, Fabiele Baldino Russo, Graciela Conceição Pignatari, Ednaldo da Silva Filho: Contributed reagents, materials, analysis tools or data.

Érika Branco: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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ARTIGO II

Stem Cell Reviews and Reports
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Is Stem Cell Commerce in Small Animal Therapies Scientifically and Morally Justified?

Luane Lopes Pinheiro[1] · Ana Rita de Lima[1] · Érika Branco[1]

Abstract
The lack of clear regulations for the use of veterinary stem cells has triggered the commercialization of unproven experimental therapies for companion animal diseases. Adult stem cells have complex biological characteristics that are directly related to the therapeutic application, but several questions remain to be answered. In order to regulate the use of these cells, well-conducted, controlled scientific studies that generate high-quality data should be performed, in order to assess the efficacy and safety of the intended treatment. This paper discusses the scientific challenges of mesenchymal stem cell therapy in veterinary regenerative medicine, and reviews published trials of adipose-tissue-derived stem cells in companion animal diseases that spontaneously occur.

Keywords Adipose-derived stem cell (ASC) · Cat · Cell therapy · Dog · Small animal

Introduction

Cell therapy in veterinary medicine is a reality. Several companies around the world have been offering the use of autologous or allogeneic mesenchymal stem cells (MSCs) as a treatment option for various diseases that affect companion and competition animals, indicating the commercial potential of this innovative technology in the current bioeconomic climate [1, 2].

Adipose-derived stem cells (ASCs) are the most common source of commercialized cells because they are easily harvested and a large number of cells can be obtained in a single procedure, either by liposuction, biopsy, or minor surgery [3, 4]. The therapies offered are made using ASCs or stromal vascular cells from adipose tissue that are expanded in culture and stored in cell banks [5].

In most countries, stem cell commerce is not controlled by regulatory agencies. In 2015, the Veterinary Medicine Center of the US Food and Drug Administration (FDA) published specific definitions and recommendations for cell-based veterinary products, and stated that MSCs should follow the same regulations as are applied to drugs, so safety, efficacy, and product quality must be proven by well-conducted research [6, 7].

In addition to a lack of regulation, and although the exact characterization, mechanism of action, and definitive efficacy of MSCs remain uncertain, cellular therapy in veterinary medicine is performed under high public demand, which is reinforced by positive anecdotal reports; however, these reports are based on a small number of patients without adequate controls, or studies involving case series or case reports [8]. Therefore, most of the clinical literature on the use of MSCs for animal therapy are studies that do not meet the gold standard of evidence-based medicine through randomized controlled trials [9, 10]. In veterinary science, conducting such studies is difficult because of logistical and economic limitations associated with such study designs, limited sample sizes, and inter-species variability [9].

This paper discusses the scientific challenges of MSC therapy in regenerative veterinary medicine, and reviews published trials of ASCs in companion animal treatments for spontaneously occurring diseases.

Mesenchymal Stem Cells or Medical Signaling Cells?

In the 1960s, Friedenstein et al. [11] identified bone marrow cells with osteogenic potential, because a fibroblast-like subpopulation adhered to the culture plastic and exhibited the

✉ Luane Lopes Pinheiro
luanelopespinheiro@gmail.com

[1] Institute of Animal Health and Production, Federal Rural University of Amazônia, Av. Perimetral, 2501, Belém, Pará 66.077-830, Brazil

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ability to expand into a colony forming unit. Later, Owen [12] proposed that non-hematopoietic stem cells are present in bone marrow, and Caplan [13] named them mesenchymal stem cells, based on their multipotent mesodermal capacity. Mesenchymal stem cells in almost all adult tissues are responsible for the maintenance and recovery of tissues against disease and injury [14].

Stem cells are classically defined as undifferentiated cells with a capacity for differentiation and self-renewal [15], and adult stem cells could be used in tissue engineering as an alternative to embryonic stem cells [16].

Mesenchymal stem cells have been isolated from a variety of tissues [reviewed in 17, 18], and characterized based on the minimum criteria established by the International Society of Cell Therapy (ISCT). Mesenchymal stem cells exhibit good proliferation capacity as adherent cells in culture systems, differential trilineage (osteogenic, chondrogenic, and adipogenic) potential, and both positive and negative specific phenotypic expression for a panel of cell-surface markers [19].

Several studies have suggested that in vitro ectodermal and endodermal potentials could make mesenchymal stem cells promising options for the treatment of a variety of diseases [17, 20]. In addition, mesenchymal stem cells have immune privileges because they do not express human leukocyte antigen complex (or major histocompatibility complex) class II surface antigens, and CD40, CD80, and CD86 costimulatory molecules avoid immunological surveillance and T cell recognition, making them candidates for autologous as well as allogeneic therapy [21, 22].

However, the lack of a definitive mesenchymal stem cell marker confounds the interpretation of these studies [23]. The search for the origin of mesenchymal stem cells has found similarities between the expression of cell-surface markers and isolated vascular pericytes [24, 25], and pericytes correspond to MSCs in vivo and are the source of cultured MSCs, which could explain their possible isolation from almost all adult tissues [26].

Moreover, the in vivo capacity of MSCs remains unclear, and the secretion of bioactive molecules highlights the therapeutic potential of mesenchymal stem cells in the tissue repair process [27]. Translational models have shown that the production of trophic and immunomodulatory factors can have regenerative, anti-inflammatory, angiogenic, anti-apoptotic, mitotic, anti-fibrotic, anti-bacterial, and anti-tumor effects [18, 28–34]. The mesenchymal stem cell secretome has paracrine effects, which strongly suggests that their natural function in vivo are as signaling cells at sites of injury or inflammation [35–37].

Twenty-six years after being named “mesenchymal stem cells”, Caplan [27] stated that the nomenclature was scientifically and therapeutically misleading and proposed that they be called “medicinal signaling cells” instead, in order to more accurately reflect their therapeutic potential. Caplan’s [27] concern is that calling them mesenchymal stem cells infers tissue repair through the differentiation and replacement of damaged cells and results in the unregulated marketing of MSC, which patients sometimes think is miraculous.

Although Caplan’s [27] nomenclature is extremely important for human regenerative medicine and veterinary medicine, this review will maintain the nomenclature of “mesenchymal stem cell, MSC”, in accordance with the ISCT and studies published in the field of regenerative medicine for companion animals.

Characterization of Companion Animal MSCs: Adipose Tissue

Despite numerous studies using high-quality scientific technology, the heterogeneity of human MSC populations makes definitive cell characterization inherently challenging [38]. Although MSCs that are isolated from different tissues share the same characteristics, they differ in their therapeutic potential [39]. It is highly probable that the MSC secretome varies with anatomical location, and which tissue source may be suitable for a particular clinical situation has not yet been established [18].

Since the first record of adherent cells obtained from the stromal vascular fraction (SVF)-cultivation of lipo-sucked adipose tissue having multipotent potential [40], this cell population has become one of the most studied in the field of regenerative medicine, with numerous studies describing its biological aspects, its possible in vitro pluripotency, and its use with translational models [reviewed by 20, 41, 42].

Initially named “processed liposuction cells”, the Zuk et al. [40] method of obtaining them is based on the enzymatic digestion of the adipose tissue extracellular matrix for the isolation of the SVF. A heterogeneous population of red blood cells, fibroblasts, endothelial cells, smooth muscle cells, pericytes, and preadipocytes is maintained under conditions suitable for eliminating contaminating cells and obtaining a consistent adherent layer. In order to provide a consensus among the variety of terminologies published for this population of cells, the term “adipose-derived stem cells” (ASCs) was adopted at the 2nd International Federation for Adipose Therapeutics and Science Annual Conference, which is frequently used [20].

Interest in adipose tissue as a source of MSCs for possible clinical applications is based on easy access to tissue and an abundance of isolated cells, in comparison with bone marrow and other sources [4, 43–46].

Several studies that have characterized the ASCs of different species have been published in the last two decades, in accordance with the minimum criteria proposed for human MSCs [3, 47–58].

The first studies in dogs and cats focused on cell morphology and proliferation, flow cytometry, and trilineage differentiation, in order to reflect the early human literature on the subject and the criteria established by the ISCT [3, 47, 52, 59, 60]. However, adherence to a culture flask is the only universal criterion for application between species [38]. Being able to express cell-surface markers with a human-cell-based panel and a small pool of commercially available reagents capable of cross-reacting with canine and feline species has been challenging [61]. In addition, several studies have included protocol modifications for the demonstration of in vitro multipotency due to the different responses of canine and feline ASCs to traditional protocols for differentiation induction, particularly chondrogenic [3, 59, 62–65].

By shifting the focus to MSC non-progenitor functions, some studies have established a more detailed profile of the cell-surface markers, gene expression, and immunomodulatory capacity of ASCs in dogs and cats [38, 61, 66–73]. However, cellular aspects that are directly related to the therapeutic application remain poorly defined, and, as in the human literature, it is difficult to compare studies due to differences in isolation, culture, reagents, and measurements [61].

A consensus among research protocols, better manufacturing consistency, and improved biological characterization (including immunomodulatory potential and secretory capacity analysis) would improve our understanding of the impact of each factor on the safety and efficacy of veterinary ASCs in clinical trials [8].

Regulation of Cell-Based Therapy in Regenerative Veterinary Medicine

The ethical and regulatory aspects of human stem cell therapy are important and most countries have some bioethical legislation, depending on the type of cellular product used [10]. For cell-based therapies, many countries have used existing regulatory frameworks for conventional pharmaceuticals, while others have developed new rules for biological products or have adapted rules for drugs and medical devices for cell therapies [19].

In contrast, in regenerative veterinary medicine, the lack of clear regulations for the use of animal stem cells for clinical purposes has resulted in private veterinary clinics offering treatment without adequate research into the efficacy of cell therapy against several diseases [74–76].

The only legislative institution to date to formally publish specific definitions and recommendations for cell-based veterinary products in response to increased clinical use in animals is the Veterinary Medicine Center of the FDA. Here, products containing cell material based on stem cells or whole blood derivatives are called animal-cell-based products, which meet the legal definition of a "drug" as they are intended to treat, control, or prevent a disease or other condition [6]. Therefore, for the FDA, the same legal and regulatory requirements that apply to other animal drugs also apply to products based on animal cells, and before they can be legally marketed they must be reviewed and approved, and experimental data should be analyzed to ensure that the product is safe, effective, and of high quality [10, 76]. However, this is not entirely clear, because the FDA specifically states that the document issued provides guidance and "does not establish legally binding obligations."

For regulatory purposes, the FDA does not define specific in vitro or in vivo models that are necessary or generate experimental data on animal species. Instead, the focus is on clinical trials that use naturally sick animals, under the tutelage of proprietors, in duly registered trials at the agency [7]. These recommendations may support regulatory agencies in other countries to require private companies to perform well-conducted and controlled scientific studies with high-quality data in order to elucidate the efficacy and safety of cell treatments [10].

In Brazil, the use of stem cells in human medicine is still exhaustively discussed and closely monitored by the National Agency of Sanitary Surveillance based on Law N° 11.105, known as the Biosafety Law, which authorizes the use of embryonic and adult stem cells in research projects. In veterinary medicine, there is no regulation or supervision by the authorities responsible, namely the Ministry of Agriculture, Livestock, and Supply (MAPA) and the Federal Council of Veterinary Medicine (CFMV), resulting in the uncontrolled commercialization of stem cells.

It is worth noting that according to the Veterinarian's Code of Ethics (CFMV Resolution N° 1138/16), it is forbidden for veterinarians to divulge information on professional subjects in a sensationalist, promotional, untruthful, or scientifically unproven way, or to prescribe medicines without registration with a competent authority. In addition, following the Good Practices in Clinical Research [77], experimental therapies in veterinary medicine should be restricted to use in clinical trials within defined study protocols.

In human medicine, there is great concern that the popularization of unproven cell therapies may adversely affect the legitimate development of evidence-based cellular therapies, and that direct marketing to consumers may weaken the regulatory instruments that are designed to protect patients from physical injury and financial exploitation [78]. Therefore, the use of therapies that are of unproven efficacy should be strictly supervised, instead of unethically promoting products of doubtful clinical efficacy and with possible unknown long-term risks [79].

ASC-Based Therapy in Companion Animals

Companion animals (pets) are treated as family members, and because of the long-term treatment of chronic diseases, the life

expectancies of dogs and cats have increased [75]. However, many diseases in veterinary medicine are non-responsive to conventional treatments, and regenerative therapy using cell-based products is seen as a promising alternative [8].

In the last decade, the number of private laboratories that market ASCs has increased considerably. There is a dearth of data on small animals, with only a few pilot trials, low-quality veterinary clinical trials, and/or pre-clinical trials as translational models for human medicine conducted (Table 1). Of the 22 studies selected from 2008 to 2018, 17 were on dogs (allogeneic ASCs = 10, autologous ASCs = 5, SVF = 1, SVF versus allogeneic ASCs = 1) and 5 were on cats (allogeneic ASCs = 4 and autologous ASCs = 1), and the aims and quality of the studies varied widely, with most (17/22) being observational, without any control or randomization.

Controlled, randomized clinical trials play a crucial role in generating the best scientific data on the efficacy and safety of therapeutic interventions, and are now classified in terms of the benefits and risks of a potential medicine and are required to obtain marketing authorization [101].

FDA regulatory guidelines concern the safety of veterinary MSCs used in clinical trials and evaluations of tumorigenicity and immunogenicity, donor selection criteria, adventitious agent transmission, long-term safety, safety of repetitive applications, cell survival, biodistribution, and ectopic tissue formation [6]. Most of the studies selected had only the minimum ISCT criteria, and only four evaluated other parameters, such as karyotype [86, 93, 96], biodistribution [89], and immunomodulatory potential [93, 96].

In allogeneic transplant trials, donors have only been described as being in good health, and only three studies have included careful health evaluations [85–87]. Adipose tissue can be subcutaneous or visceral abdominal, and obtaining ASCs involves using different culture conditions, duration, and preparation techniques, which makes it impossible to compare studies of the same product, i.e., manufacturing consistency.

The routes of administration used in the studies were systemic intravenous (8/22) or directly at the site of interest (14/22) (Table 1). Adverse effects reported after intravenous applications were related to allergies [87], and those at the site of application were related to pain [82] and swelling [85]. Adverse effects are undesirable effects that occur during or after a therapeutic intervention, whereas adverse events are events that may occur during the period of treatment with a drug but without necessarily indicating a causal relationship, and should be identified during the clinical stages of a trial of a new product [102]. Only one trial has reported all adverse events occurring during the stipulated follow-up time (test group, $n = 6$; control group, $n = 9$) [86].

The lengths of the tests ranged from 1 to 24 months, with many being 6 months long (10/22). ASC therapy for chronic diseases that are unresponsive to conventional treatments is an alternative to long-term anti-inflammatory and immunosuppressive treatments and their side effects [103]. In order to approve any class of drug, short-term safety is not required by regulatory agencies; however, the undesirable effects of biological products are greater, given the important immunological processes that are derived from the treatment [104]. In addition, the underlying mechanism of action of MSCs is not fully understood, and may influence multiple physiological processes [61].

Dosages vary considerably among trials. For example, the number of cells applied by the intra-articular route for canine osteoarthritis ranges from 4 to 30×10^6 cells. Five trials that used the intravenous route applied dosages that were based on the animals' weights [92, 93, 95, 99, 100]. Conducting pre-clinical trials by experts in the field of the disease under investigation is a key preliminary step for clinical trials, and without them, the number of cells that are commercially used in veterinary patients is entirely empirical.

Methods of comparing treatment efficacy between trials include measuring physiological parameters, conducting specific tests and examinations, and using analogue visual scales and numerical scoring scales for quality of life. Evaluations scored by the animal's owner are recognized by regulatory agencies as having important advantages, because the stress of animals in an unknown clinical environment may mask clinical signs, so observations in the family environment may provide a better evaluation of some parameters [86]. However, well-conducted trials with blind evaluations are crucial [105], because owner perception can be affected by a desire for improvement, and by participating in the study. Only two of the trials were randomized with a placebo control [86, 99].

MSCs have been used for a long time in veterinary orthopedics, which has the largest number of publications of ASC studies that have investigated canine osteoarthritis (OA) (9/22) (Table 1). The main focus has been to investigate disease-modifying effects by inducing short-term cartilaginous regeneration and anti-inflammatory effects, reducing the constant need for pain medication and its side effects [76]. Trials have been conducted on dogs with OA on the hips [80–83], elbows [60, 84, 85], and both [86, 87], with different degrees of joint involvement, ages, weights, and breeds. All analyzed the effects of a single dose of cells, including SVF and allogeneic or autologous ASCs, alone or in association with other biological products, such as platelet-rich plasma or hyaluronic acid. The majority (8/9) used the intra-articular route, but one study applied the cells at acupuncture points [83] and another used the intravenous route in cases of more than one joint affected [87].

Although the results of all OA studies have indicated a positive therapeutic benefit with no clear regenerative effects, the duration of the beneficial effect varied considerably (from 90 to 360 days), according to the follow-up time. However,

Table 1 Adipose-derived stem cell (ASC) treatments of companion animals with spontaneously occurring diseases

Disease	Species	Study design	N	Treatment	N° cells	Administration route	Evaluation	Administration route	Result	Author
OA of coxofemoral joint	Canine	Controlled, blinded	13	Autologous ASCs + PRGF-Endoret ($n = 8$) or control group ($n = 5$)	15×10^6	IA	Force platform analysis	Evaluation on days 30, 90, and 180	Mean values of PVF and VI significantly improved in the 6 months post-treatment in the OA group, resulting in reduced lameness	[80]
OA of coxofemoral joint	Canine	Controlled, blinded	15	Autologous ASCs ($n = 10$) or control group ($n = 5$)	15×10^6	IA	Force platform analysis	Evaluation on days 30, 90, and 180	Mean values of PVF and VI significantly improved within the first 3 months post-treatment in the OA group. After this, the effect decreased to initial values	[81]
OA of coxofemoral joint	Canine	Randomized, blinded, parallel group	39	Autologous ASCs ($n = 18$) or autologous PRP ($n = 17$)	30×10^6	IA	Pain Assessment (AVS scale); owners' satisfaction questionnaire	Evaluation on days 30, 90, and 180	Improvement observed in both groups: Significant differences at 3 months favoring PRP and 6 months favoring ASCs; no change in the degree of OA of each dog; adverse effects reported: pain post-injection ($n = 2$)	[82]
OA of coxofemoral joint	Canine	Open, baseline, parallel group	9	Autologous SVF ($n = 4$) or allogeneic ASCs ($n = 5$)	$2–5 \times 10^6$ Autologous SVF $2–8 \times 10^5$ Allogeneic ASCs	Three acupuncture points	Subjective assessment (worse, no modification, or improvement) by a veterinarian: range of motion, lameness, and pain on manipulation	Evaluation on days 7, 15, and 30	Improvement observed after 7 days in all dogs treated with SVF, and after 15 days in all dogs treated with ASCs; effects maintained for 30 days	[83]
OA of humeroradial joints	Canine	Open, baseline	14	Autologous SVF	$4–5 \times 10^6$	IA	Numeric rating scale for lameness, pain on manipulation, range of motion, and functional disability by a veterinarian; standard questionnaire adapted from CODI by owners	Evaluation on days 30, 60, 90, and 180	Improvement in grades evaluated in the orthopedic examination: 30% functional disability, 40% lameness, 15% pain. Owners' score: improvement of 30% up to 180 days	[84]
OA of humeroradial joints	Canine	Open, baseline	4	Autologous ASCs + PRP ($n = 2$) or autologous ASCs + HA ($n = 2$)	$3–5 \times 10^6$	IA	Orthopedic examination (not described) by veterinarian	Evaluation on day 30	Improvement observed in claudication and pain in the manipulation of joints in all animals. No comparison between groups	[82]
OA of humeroradial joints	Canine	Open, baseline	30 (39-art.)	Allogeneic ASCs + HA	12×10^6	IA	Quality of life scored by the owner; biopsy before and after 12 months	Evaluation on days 90, 180, and 360	Functional improvement in claudication (31/39) without use of medication for 12 months; indications	[85]

Table 1 (continued)

Disease	Species	Study design	N	Treatment	N° cells	Administration route	Evaluation	Administration route	Result	Author
									of cartilaginous regeneration by biopsy; adverse effects reported: swelling after application ($n = 2$)	
OA in various joints (hips, stifles, and shoulders)	Canine	Randomized, blinded, and placebo--controlled	74	Allogeneic ASCs ($n = 36$) or placebo--controlled group ($n = 38$)	12×10^6	IA	Client-Specific Outcome Measurement (CSOM), veterinarian checks pain on manipulation and global score, owner checks global score.	Evaluation on days 30 and 60	CSOM, pain on manipulation, and the global score statistically improved in treated dogs compared to placebo dogs; at 60 days, there were no statistical differences between groups; adverse events reported: test group ($n = 6$) and control group ($n = 9$)	[86]
OA in various joints (hips, stifles, and shoulders)	Canine	Open, baseline	203	Allogeneic ASCs	?	IA ($n = 128$), or IV ($n = 65$), or IA and IV ($n = 10$)	Quality of life scale (pain, mobility, and functional disability) by veterinarian	Evaluation on day 70	Functional improvement in 85% of the animals; more favorable results in young dogs (<5 years) IA route. Adverse events reported: allergy after IV application ($n = 2$)	[87]
Thoracolumbar disc disease with no deep pain	Canine	Randomized, parallel group	34	Allogeneic ASCs + decompression surgery ($n = 9$) or decompression surgery ($n = 25$)	10×10^6	Local application	Subjective neurological scale	Evaluation on day 180	Success rate of the ASC group (77.8%, 7/9) was significantly higher than that of the surgery group (52%, 13/25)	[88]
Chronic spinal cord injury (≥6 months)	Canine	Open, baseline	6	Allogeneic ASCs	10×10^6	Lesion localization, X-ray-guided	Olby scale	Evaluation on days 28, 56, 84, and 112	Changes in the Olby scale were obtained in three dogs after 8 weeks: two recovered only degrees of joint movement and one regained walking after 16 weeks	[89]
Keratoconjunctivitis sicca	Canine	Open, baseline	12	Allogeneic ASCs	8×10^6	Around the lacrimal glands	Schirmer tear test; ocular parameters scored: ocular discharge, conjunctival hyperemia, and corneal changes	Evaluation on days 90, 180, and 270	Average Schirmer tear test values and all clinical signs showed a statistically significant change	[90]
Keratoconjunctivitis sicca	Canine	Open, baseline	15	Allogeneic ASCs	1×10^6	Directly into lacrimal glands (dorsal	Schirmer tear test; ocular parameters scored: ocular discharge,	Evaluation on days 7, 14, 21, and 28,	Statistically significant increase in the Schirmer tear test, and ocular surface	[91]

Table 1 (continued)

Disease	Species	Study design	N	Treatment	N° cells	Administration route	Evaluation	Administration route	Result	Author
						and third eyelid)	conjunctival hyperemia, and corneal changes	as well as 6 and 12 months (eight dogs)	improvements were found in all eyes studied, except for severe cases	
Atopic dermatitis	Canine	Open, baseline	5	Autologous ASCs ($n = 5$)	1.3×10^6 kg	IV	CADESI-03; Pruritus visual analogue scale	Evaluation at weeks 2–3, 6–8, and 10–12	No significant changes	[92]
Atopic dermatitis	Canine	Open, baseline	26	Allogeneic ASCs	1.5×10^6 kg	IV	CADESI-04; Pruritus visual analogue scale; owner's global assessment score	Evaluation on days 7, 30, 90, and 180	Pruritus and CADESI-04 scores decreased significantly after 1 week and month of treatment, respectively, and remained stable for 6 months	[93]
Dilated cardiomyopathy	Canine	Open, baseline, pilot study	15	Allogeneic ASCs transduced with AAV2 ($n = 15$)	10×10^6	IV retrograde coronary	Electrocardiogram, echocardiogram and Holter	Evaluation at months 3, 6, 8, 12, 18, and 24	No significant improvements in the evaluated parameters and progression of the disease; no advantage according to recently published survival data in similarly affected dogs	[94]
Inflammatory bowel disease	Canine	Open, baseline	11	Allogeneic ASCs	2×10^6 kg	IV	Scale CIBDAI and CCECAI; clinical parameters (C-reactive protein, albumin, folate, and cobalamin)	Evaluation on day 42	Clinical remission for nine treated dogs	[95]
Feline eosinophilic keratitis	Feline	Open, baseline, pilot study	5	Allogeneic ASCs, two applications (60 days apart)	1×10^6	Subconjunctival (around the ocular surface lesion)	Ophthalmic examination	Evaluation at months 1, 3, 6, and 11	Significant resolution of corneal and conjunctival lesions at 6 months (3/5)	[96]
Refractory chronic gingivostomatitis	Feline	Open, baseline	7	Autologous ASCs, two applications (30 days apart)	20×10^6	IV	SDAI scoring; oral biopsies	Evaluation at months 1, 3, and 6	Complete clinical remission ($n = 3$) or substantial clinical improvement ($n = 2$). Two cats did not respond to treatment	[97]
Refractory chronic gingivostomatitis	Feline	Open, baseline	7	Allogeneic ASCs, two applications (30 days apart)	20×10^6	IV	SDAI scoring; oral biopsies	Evaluation at months 1, 3, and 6	Complete clinical remission ($n = 2$) or substantial clinical improvement ($n = 2$). Three cats did not respond to treatment	[98]
Chronic enteropathy	Feline	Randomized, placebo--controlled	11	Allogeneic ASCs ($n = 7$). Two applications (14 days apart)	2×10^6 kg	IV	Clinical parameters (fPLI, fTLI, folate, and cobalamin); owners'	Evaluation at days 14, 30, and 60	Significant improvement or complete resolution of clinical signs in 5/7 treated	[99]

Table 1 (continued)

Disease	Species	Study design	N	Treatment	N° cells	Administration route	Evaluation	Administration route	Result	Author
				or placebo-- controlled group ($n = 4$)			questionnaire and fecal consistency scale		cats and 0/4 placebo cats after 2 months	
Chronic kidney disease	Feline	Randomized, placebo-- controlled	8	Allogeneic ASCs ($n = 4$). Three applications (14 days apart) or placebo-- controlled group ($n = 4$)	2×10^6 kg	IV	Clinical parameters (UPC, creatinine, complete blood count, biochemical profile, and urine analysis)	Evaluation at months 2, 4, 6, and 8	No significant changes in clinical parameters	[100]

AAV2 Adeno-Associated Virus 2 Ttyrosine Mutant, *AO* Osteoarthritis, *CADESI* Canine Atopic Dermatitis Extent and Severity Index (version 03 or 04), *CCECAI* Canine Chronic Enteropathy Clinical Activity Index, *CIBDAI* Clinical Inflammatory Bowel Disease Activity Index, *CODI* Cincinnati Orthopedic Disability Index, *ASCs* Adipose Stem Cells, *AVS* Analogic Visual Scale, *fPLI* Feline Pancreatic Lipase Immunoreactivity, *fTLI* Feline Trypsinlike Immunoreactivity, *HA* Hyaluronic Acid, *IA* Intraarticular, *IV* intravenous, *VI* Vertical Impulse, *PVF* Peak Vertical Force, *PRGC* Plasma Rich in Growth Factors, *PRP* Platelet Rich Plasma, *SDAI* Stomatitis Disease Activity Index Scoring System, *UPC* Urine Protein-to-Creatinine

the only trial that has been performed in accordance with the requirements for double-blind randomized regulation reported that at 60 days, there were no statistical differences in the effect of allogeneic ASCs between the test group and the placebo group [86]. Considering the variation in the number of cells used and the different methods applied, there is a need for studies on the duration of the beneficial effects of allogeneic and autologous ASCs and intra-articular SVF without interference from tissue sampling, in order to further evaluate therapy efficacy and feasibility for canine OA.

A large proportion of the trials (9/22) were conducted based on the immunomodulatory and anti-inflammatory properties of MSCs in patients that were unresponsive to currently available treatments for diseases of great clinical importance in small animals (Table 1). For ophthalmologic diseases, allogeneic transplantation into the lacrimal glands was performed in three tests: two in dogs with varying degrees of keratoconjunctivitis sicca [90, 91] and one in cats with eosinophilic keratitis [96].

Using intravenous systemic infusion, the effects of ASCs were evaluated in two trials on feline chronic gingivostomatitis using autologous ASCs [97] and allogeneic ASCs [98], in treat inflammatory bowel disease in dogs using autologous ASCs [95], in treat feline chronic enteropathy using allogeneic ASCs [99], and in two trials on canine atopic dermatitis using autologous ASCs [92] and allogeneic ASCs [93]. Other trials (4/22) investigated debilitating conditions and diseases responsible for morbidity and mortality, which in experimental models have shown promising results [106], and because of the importance for human medicine, reach higher visibility for hopeful owners. These trials evaluated the transplantation of allogeneic ASCs in dogs with severe chronic spinal cord injury by intralesional percutaneous application guided by digital x-ray [89], in dogs with intervertebral disc disease combined with decompressive surgery [88], in dogs with dilated cardiomyopathy by retrograde coronary infusion [94], and in cats with chronic kidney disease by the intravenous route [100].

Overall, the results of the above-mentioned trials are clinically relevant, and are of great importance for ASC research in pet diseases. However, many studies lack quantitative data for analysis [96], had inconclusive results [97, 98], insufficient data [92, 94], were too short [88–91, 93, 95, 99], or were well-conducted placebo-controlled trials with an insufficient amount of data obtained [100]. Therefore, the scientific basis of the potential and efficacy of treatments for different diseases is unclear. There is a lack of understanding of the mechanism of action that supports their clinical use, and poor standardization of preparation methods that ensure the quality and consistency of the cellular product. High-quality, carefully conducted trials should be conducted by veterinary specialists in specific diseases, in order to gather reliable evidence of the safety and efficacy of the product for use in canine and feline patients.

Challenges and Expectations of Cell-Based Therapy in Regenerative Veterinary Medicine

Trials that have used MSCs against spontaneously occurring pet diseases raise questions that preclude meaningful conclusions regarding the efficacy and safety of therapy. Many of these studies lack crucial aspects such as statistical power, blind groups, and appropriately sized controls, with no progression beyond pilot data; therefore, the results should be interpreted with caution [10, 76].

These trials also reflect the scientific challenges faced in MSC research regarding product variability, which makes efficacy difficult to assess. These include variations in material and manufacturing processes (method, culture time, and cryopreservation), a lack of species-specific reagents, variable tissue sources and donors, and a lack of biological characterization [8, 9, 75].

From a regulatory perspective, collecting data in well-conducted trials will overcome the challenges associated with product variability, and ideally, the standardization of preparation methods will allow for more direct comparisons between trials [10]. Setting standards and recommendations for products based on animal cells, such as the guidelines established by the FDA in the US, will reinforce the need for evidence of the efficacy and safety of the product prior to commercialization. Therefore, the challenges faced by manufacturers and researchers in maintaining the consistency of product manufacturing will impact our understanding of cell biology, and drive the development of safe and effective regenerative therapies [8, 10].

Scientific research in veterinary medicine lags behind that in human medicine [75]. The clinical investigation of new therapies is divided into successive and staggered phases with increasing levels of complexity and exposure, in which potency testing, for example, is a regulatory requirement for advanced clinical trials [107]. This is crucial for elucidating whether the cellular product has the intended effect at a specific dose, which ensures manufacturing consistency and the delivery of an effective product [108].

However, due to the complex nature of cell-based products, standardization with engineering and manufacturing is a challenge that needs to be overcome, as recognized by regulators [76]. In addition, analyzing a single effector pathway for a positive clinical finding may be misleading because MSCs have several properties that are induced by different mechanisms of action, which depend upon the microenvironment in which they are to be inserted and the receptor immune status [109, 110]. With the recognition of such challenges, rigorously designed and monitored clinical studies should be performed as a means of gathering reliable evidence regarding the safety and efficacy of cell-based products [78].

However, veterinary clinical research still faces limited funding and a lack of support, so researchers have increasingly recognized the important role that companion animals play as translational preclinical models that are relevant to cell-based therapy in humans [9, 61, 74, 75, 89, 111]. Naturally occurring diseases in dogs and cats may reflect genetic, environmental, and physiological variations present in the human population, and natural complexity would counteract the tide of reductionism of "one molecule, one target" [75, 111]. A multidisciplinary approach through collaboration among scientists, physicians, and veterinarians to promote the use of pet diseases as translational models would lead to consistent advances [103]. Another practical solution would be the formation of alliances between veterinary practitioners and veterinary researchers, which would promote treatment being provided in a predefined manner and results that could be analyzed, in contrast to the current trend, in which many treatments of animals under the custody of their owners are not recorded [10].

Conclusions

Without regulation, MSC therapies are currently offered and marketed at high cost for the treatment of a variety of pet diseases, despite the fact that there is little evidence of their efficacy and safety. In human medicine, the ISCT is concerned that the popularization of unproven cell therapies could harm patients and negatively affect the legitimate development of evidence-based therapy, and warns that although there is a long history of biomedical advances, there is also a long history of abuse and profits from the sale of unproven medical interventions [78]. Without a response from regulatory bodies, such concerns extend to veterinary medicine. It is up to the regulatory and oversight institutions of each nation to proceed with the scientific development of MSC therapy. Until then, veterinarians should carry out their ethical and moral duty and help owners tutors make informed decisions, and ensure that they understand the risks of any unproven experimental therapy.

In conclusion, there is a disregard for the lives of diseased animals when stem cells are indiscriminately marketed with the promise of a cure or an effective improvement in the symptoms. All of this could be avoided if the following questions were answered: is an animal's life worth less than the life of a human being? What if the worst happens- tell the costumer to buy another pet?

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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CONCLUSÃO GERAL

O uso de células-tronco mesenquimais do tecido adiposo, pela via intra-arterial, em cães com leucoencefalite desmielinizante provocada pelo vírus da cinomose, mostrou ser um procedimento sem efeitos adversos no período de 12 meses após a aplicação, com moderada eficácia no quadro neurológico.

Todos os cães apresentaram melhora na qualidade de vida durante o ano de acompanhamento após as aplicações, o que demonstra relevância clínica com grande importância para pesquisa em pacientes humanos com esclerose múltipla, devido às semelhanças na fisiopatologia da doença. Entretanto, assim como os artigos supramencionados em revisão, que abordaram o uso de células-tronco derivadas do tecido adiposo, em doenças de cães e gatos com ocorrência espontânea, a fundamentação científica ainda é pouco clara, e dessa forma, expomos a preocupação médica de que a ausência de um posicionamento regulatório possa afetar negativamente o desenvolvimento legítimo de terapias celulares, além de expor os pacientes a riscos de danos físicos e exploração financeira, mediante o comércio de terapias ainda experimentais.