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HARALD FERNANDO VICENTE DE BRITO

UTILIZAÇÃO DE CÉLULAS MONONUCLEARES DE MEDULA ÓSSEA PARA O TRATAMENTO DE SEQUELAS NEUROLÓGICAS DE CINOMOSE CANINA

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Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias, do Setor de Ciências Agrárias, da Universidade Federal do Paraná, como requisito parcial para a obtenção do título de Doutor em Ciências Veterinárias.
Orientadora: Professora Dra. Rosangela Locatelli Dittrich.
Coorientadora: Dra. Carmen Lúcia Kuniyoshi Rebelatto.

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UFPR

PARECER

A Comissão Examinadora da Defesa da Tese intitulada **"UTILIZAÇÃO DE CÉLULAS MONONUCLEARES DE MEDULA ÓSSEA PARA O TRATAMENTO DE SEQUELAS NEUROLÓGICAS DE CINOMOSE CANINA"** apresentada pelo Doutorando **HARALD FERNANDO VICENTE DE BRITO** declara ante os méritos demonstrados pelo Candidato, e de acordo com o Art. 79 da Resolução nº 65/09–CEPE/UFPR, que considerou o candidato APROVADO para receber o Título de Doutor em Ciências Veterinárias, na Área de Concentração em Ciências Veterinárias.

Curitiba, 31 de março de 2015

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RESUMO

A cinomose é uma doença infecciosa causada por um *Morbillivirus* pertencente a família *Paramyxoviridae*. Até 30% dos cães infectados com o vírus da cinomose apresentam complicações neurológicas e aproximadamente 10% morrem por leucoencefalite aguda. Os cães que recuperam podem apresentar sequelas permanentes. Até o momento, não existe nenhum tratamento específico para animais com sinais neurológicos da cinomose, assim, são necessárias novas intervenções terapêuticas. O transplante de células-tronco tem emergido como um novo e promissor modelo de terapia. Células mononucleares derivadas da medula óssea (CMNMO) podem ser isoladas e aplicadas como estratégia terapêutica em estudos clínicos e pré-clínicos. Porém, existem alguns desafios associados a essa abordagem terapêutica. Um deles é a falta de estudos sobre a caracterização das CMNMO dos cães. Heparina e citrato-fosfato-dextrose-adenina-1 (CPDA-1) são comumente utilizados para coletar medula óssea. Porém, o efeito destes anticoagulantes em termos de isolamento dessas células também não é conhecido. Outro ponto que precisa ser mais estudado é a via de administração para ação eficiente das células transplantadas. A via intravenosa (IV) tem sido utilizada porque é o método mais fácil e menos invasivo de transplante de células. A marcação e rastreamento das células permite avaliar se elas migram para o local da lesão. O PKH26 é uma molécula de marcação fluorescente, não citotóxica e estável por longo período. Devido a estas características, este corante parece ser ideal para o rastreamento das células transplantadas. A caracterização dos subtipos de CMNMO e uma melhor compreensão das funções destas células podem contribuir para o conhecimento de seu potencial e, assim, aumentar as possibilidades de sua utilização para ensaios clínicos em medicina veterinária. As CMNMO administradas por via intravenosa têm uma passagem pulmonar 30 vezes maior que as células-tronco mesenquimais (CTM), facilitando a migração dessas células para os tecidos lesados. Contudo, a migração dessas células para o sistema nervoso central (SNC), após administração intravenosa, em animais com sequelas de cinomose ainda não está bem estudada. O PKH é uma molécula de marcação fluorescente, que é incorporada na bicamada lipídica das membranas citoplasmáticas, que tem sido utilizado para a identificação da migração de células. Uma vez incorporado nas células *in vitro*, o PKH não é transferido para o meio ou para células não marcadas e não possui efeito tóxico ou imunogênico. CMNMO podem ser marcadas com PKH e utilizadas para transplante por via intravenosa para acompanhamento da migração dessas células. Os objetivos deste estudo foram comparar o rendimento de células mononucleares derivadas da medula óssea de cães, coletadas com heparina ou com CPDA-1, avaliar a migração das CMNMO após transplante alogênico em 10 animais com complicações neurológicas da cinomose, caracterizar fenotipicamente as CMNMO de cães e, avaliar a segurança e eficácia do transplante alogênico de CMNMO para o tratamento de sequelas neurológicas da cinomose em outros 23 cães. Para comparação do efeito dos anticoagulantes, a medula óssea de cinco cães foi coletada por punção na crista ilíaca, utilizando-se como soluções anticoagulantes CPDA-1 ou heparina. As células mononucleares foram isoladas em gradiente de densidade e caracterizadas por citometria de fluxo quanto à marcação para CD9 e CD44. Os resultados foram submetidos ao teste *t* pareado para comparação de médias. Nas amostras coletadas com CPDA-1 o rendimento médio variou entre 5,16 x 10^6 (±1,76 x 10^6) a 20,20 x 10^6 (±1,55 x 10^6) células

mononucleares/mL. Nas amostras coletadas com heparina o rendimento variou entre 4,56 x 10^6 (±0,69 x 10^6) a 24,30 x 10^6 (±2,12 x 10^6) células mononucleares/mL. Na citometria de fluxo, a média de células duplo-marcadas variou de 1,96% (±0,64%) a 5,01% (±0,73%) para CPDA-1 e de 2,23% (±0,70%) a 7,27% (±0,97%) para heparina. Não foram observadas diferenças estatísticas no rendimento ou na expressão de CD9 e CD44. Para avaliar a marcação das CMNMO com PKH26 e o recrutamento das células no sistema nervoso central, foram coletadas amostras de medula óssea de oito cães. As CMNMO foram isoladas e marcadas com PKH26 e administradas por via intravenosa nos cães com sequelas de cinomose. A migração celular foi avaliada no líquido cefalorraquidiano (LCR) dos pacientes em diferentes momentos. A marcação das CMNMO e o recrutamento de células no LCR foram avaliados quantitativamente por citometria de fluxo e qualitativamente em microscópio de fluorescência. A porcentagem de células marcadas variou nos diferentes tempos de coleta de LCR, observando-se maior percentual de CMNMO marcadas com PKH26 de 4:00-5:30 horas após a infusão, com posterior diminuição. Na análise qualitativa, foi demonstrado o recrutamento das CMNMO para o sistema nervoso central, pela presença de células marcadas com PKH26 no LCR. Para avaliação da segurança e eficácia do transplante de CMNMO em cães com sequelas de cinomose foi conduzido um ensaio clínico controlado por placebo, com distribuição aleatória em único-cego, com 46 cães alocados nos grupos tratamento e controle, com acompanhamento semanal por 35 dias. A medula óssea foi colhida de 23 doadores saudáveis e isolada em gradiente de densidade. As CMNMO de quatro dadores foram marcadas com anticorpos anti-CD8a, CD9, CD14, CD29, CD34, CD44, CD45 e CD90 para a caracterização fenotípica. Os cães do grupo tratamento receberam 1,0 x 10^8 CMNMO por via intravenosa. Em relação à segurança do transplante, nenhum efeito adverso grave relacionado ao procedimento foi relatado durante os 35 dias do estudo. A recuperação comportamental foi acompanhada de acordo com o escore de Olby com algumas modificações. Para o grupo tratamento, as medianas e amplitudes interquartis do escore funcional, com 0, 7, 14, 21, 28 e 35 dias após a administração foram 7 (4-13), 9 (5-14), 12 (6-15), 14 (6-15), 14 (6-16), e 14 (6-16), enquanto que no grupo placebo foram 6 (4-12), 7 (4-13), 7 (4-12), 7 (4-12), 6 (3-12), e 6 (3-12). Foram observadas diferenças estatisticamente significativas entre os grupos ($p < 0,05$), demonstrando a eficácia do transplante de CMNMO em cães com leucoencefalite causada por cinomose. Concluindo, esse estudo demonstrou que as CMNMO podem ser eficientemente marcadas com o corante PKH26 e que essas células podem ser monitoradas após o transplante IV em animais com complicações neurológicas de cinomose.

Palavras-chave: Terapia com células mononucleares; recuperação funcional; migração de células-tronco.

ABSTRACT

Canine distemper is an infectious disease caused by a *Morbillivirus* belonging to *Paramyxoviridae* family. Up to 30% of dogs infected with canine distemper virus showed neurological complications and approximately 10% die from acute leukoencephalitis. In addition, dogs that recover may have permanent sequels. There is no specific therapy for animals showing distemper neurological signs, and new therapeutic interventions are necessary, and cell transplantation has emerged as a promising new therapy model. Bone marrow mononuclear cells (BMMNC) can be easily isolated and broadly applied as a therapeutic strategy in a large number of preclinical and clinical studies. However, there are some challenges associated with this therapeutic approach. One of them is the lack of studies on the characterization of canine BMMNC. Heparin and citrate-phosphate-dextrose-adenine-1 (CPDA-1) are commonly used to harvest bone marrow. However, the comparison of effect of anticoagulants at harvest on terms of stem cell yield has not been studied. Another point that needs to be further studied is the route of administration for efficient cell delivery. Intravenous (IV) delivery has been used because it is easier and less invasive method of cell transplantation. The labelling and tracking of cells allow to evaluate if IV transplanted cells are attracted to the site of injury. The PKH26 is a fluorescent labeling molecule, noncytotoxic and is stable for long time period. Because of these characteristics, this dye seems to be ideal for cells tracking. The characterization of subsets of BMMNC and a better understanding of the functions of these cells may contribute to the knowledge of their potential and thus increase the possibilities to their use for clinical trials in veterinary medicine. The BMMMC administered intravenously have a lung passage 30 times larger than the mesenchymal stem cells (MSC), facilitating the migration of these cells to the injured tissues. However, the migration of these cells to the central nervous system (CNS), after intravenous administration, in animals with canine distemper sequels has not been studied. The PKH is a fluorescent labelling molecule, which is incorporated in the lipid bilayer of the cytoplasmic membrane, which has been used for the identification of cell migration. After incorporated into the cells *in vitro*, the PKH is not transferred to the medium or to unmarked cells, and has no toxic or immunogenic effect. BMMNC can be labeled with PKH and used for transplant intravenously, so this dye appears to be ideal for monitoring the migration of these cells. The aims of this study were the comparison of the yield of bone marrow-derived mononuclear cells harvested from dogs with two different anticoagulants, evaluate cell migration after allogeneic BMMNC transplantation in 10 animals with neurological complications of canine distemper, characterize phenotypically canine BMMNC and, evaluate the safety and efficacy of allogeneic BMMNC transplantation for treatment of neurological sequels of canine distemper in others 23 dogs. For comparison of the anticoagulants, the bone marrow from five dogs was harvest in heparin or CPDA-1, isolated in a density gradient, and stained for CD9 and CD44 for characterization by flow cytometry. The means were compared using Student's paired t-test. Samples harvested with CPDA-1 yielded an average of 5.16×10^6 ($\pm 1.76 \times 10^6$) to 20.20×10^6 ($\pm 1.55 \times 10^6$) mononuclear cells/mL, whereas the yield of samples harvested with heparin varied between 4.56×10^6 ($\pm 0.69 \times 10^6$) and 24.30×10^6 ($\pm 2.12 \times 10^6$) mononuclear cells/mL. By flow cytometry, the mean percentage of double-stained cells varied from 1.96% (±0.64%) to 5.01 % (±0.73%) for CPDA-1 and from 2.23% (±0.70%) to 7.27 % (±0.97%) for heparin. No significant statistical differences were observed on yield or CD9 and CD44 expression. Bone marrow was harvested from

eight donors and BMMNC were isolated, labeled with PKH26 dye and IV transplanted into the patients, to assess the labelling and tracking of cells. The cell migration was assessed in the cerebrospinal fluid (CSF) of patients at different periods of time and the percentage of labeled cells with PKH 26 dye was evaluated quantitatively by flow cytometry and qualitatively by fluorescence microscope. In the quantitative analysis by flow cytometer, it was observed a wide variation in the percentage of labeled cells in different CSF collection times. Also, there was an increase on the percentage of PKH26-labeled cells in the CSF, with highest percentage between 4 and 5 ½ hours after infusion with subsequent decrease. In the qualitative analysis, it was showed the presence of labeled BMMNC recruited by central nervous system on CSF. In order to evaluate the safety and efficacy of allogeneic BMMNC transplantation for treatment of neurological sequels of canine distemper, it was used a single blind randomized controlled trial in 46 dogs divided into treatment group and control group with weekly follow-up for 35 days. Bone marrow was harvested from 23 healthy donors and BMMNC were isolated by density gradient centrifugation. BMMNC from four donors were labeled with anti-CD8a, CD9, CD14, CD29, CD34, CD44, CD45, and CD90 for phenotypic characterization. Dogs from the treatment group received 1 x 10^8 BMMNC intravenously. Regarding the safety of cell transplantation, no serious adverse events related to the procedure were recorded during the 35 days of the study. Functional recovery was evaluated according to the Olby scoring system with some modifications. For the treatment group, the median and interquartile range of the functional score, with 0, 7, 14, 21, 28 and 35 days after injection were 7 (4-13), 9 (5-14), 12 (6-15), 14 (6-15), 14 (6-16), and 14 (6-16) respectively, whereas for the placebo group they were 6 (4-12), 7 (4-13), 7 (4-12), 7 (4-12), 6 (3-12), and 6 (3-12). The differences observed were statistically significant ($p < 0.05$), showing the effectiveness of BMMNC transplantation in dogs with distemper leukoencephalitis. In conclusion, this study demonstrates that BMMNC can be efficiently labeled with PKH26 dye and these cells can be monitored after IV transplantation in animals with neurological complications of canine distemper.

Key words: Bone marrow mononuclear cells therapy; functional recovery; cells tracking

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7-AAD	7-amino-actinomycin D
APC	allophycocyanin
BBB	blood brain barrier
BMMNC	bone marrow mononuclear cells
BrdU	bromodeoxyuridine
CDV	canine distemper virus
CFU-F	colony-forming-unit fibroblasts
CMFDA	chloromethylfluorescein diacetate
CMNMO	células mononucleares derivadas da medula óssea
CNS	central nervous system
CPDA-1	citrato-fosfato-dextrose-adenina-1 (*citrate-phosphate-dextrose-adenine-1*)
CSF	cerebrospinal fluid
CTM	células-tronco mesenquimais
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
IL	interleucina (*interleukin*)
IMDM	Iscove's modified Dulbecco's médium
IFN	interferon
IV	intravenosa (*intravenous*)
LCR	líquido cefalorraquidiano
MHC-II	histocompatibility complex class II
MNC	mononuclear cells
MSC	mesenchymal stem cells
NK	natural killer
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
SD	standard deviation
SNC	sistema nervoso central
TNF	fator de necrose tumoral (*tumor necrosis factor*)
TGF	fator de transformação do crescimento (*transforming growth factor*)

SUMÁRIO

1 INTRODUÇÃO

A utilização das células-tronco em terapia regenerativa tem aumentado durante a última década. As pesquisas focam principalmente o uso das células-tronco mesenquimais (CTM), também denominadas células estromais da medula ou células estromais mesenquimais multipotentes (HORWITZ et al., 2005). As CTM são células progenitoras multipotentes, com capacidade de auto renovação e estão presentes em vários tecidos adultos, como medula óssea, osso trabecular e tecido adiposo (ZUK et al., 2001).

As células mononucleares da medula óssea (CMNMO) compreendem todas as células da medula óssea com núcleo esférico e sem grânulos no citoplasma. Essas características fazem com que as CMNMO tenham densidade e tamanhos similares, e sejam diferentes das células progenitoras mielóides e eritróides. Essa diferença permite a separação física dessas células. As CMNMO adultas são as células progenitoras hematopoiéticas em diferentes estágios de maturação, como células linfóides, monócitos, macrófagos e várias células de linhagem não hematopoiética. Entre as células não hematopoiéticas identificadas na fração mononuclear da medula óssea do homem adulto normal, estão células estromais mesenquimais (SALEM e THIEMERMANN, 2010), células-tronco "*embryonic-like*", células progenitoras adultas multipotentes, hemangioblastos, células progenitoras endoteliais e células tronco "*tissue-committed*" (CUENDE et al., 2012).

Os resultados de pesquisas *in vivo* e *in vitro* demonstraram que as células da medula óssea estão fisiologicamente envolvidas na neovascularização e reparo tecidual. O transplante de medula óssea é utilizado em pacientes com leucemia, mas a partir do ano de 2000 as CMNMO começaram a ser usadas em novas terapias, como no tratamento de doenças isquêmicas (doenças cardíacas isquêmicas agudas e crônicas) e doença arterial periférica (IDEI et al., 2011; WILLIAMS et al., 2011).

As CMNMO são de fácil obtenção e não requerem expansão *in vitro* antes do uso na terapia regenerativa, porém, sendo uma combinação heterogênea de diferentes tipos de células progenitoras, nem todos os subtipos foram completamente caracterizados e, consequentemente, nem todas as funções são conhecidas (CUENDE et al., 2012; ALVAREZ-VIEJO et al., 2013). No entanto,

CMNMO caninas ainda não foram caracterizadas e, mesmo para CTM caninas existe pouco consenso sobre a caracterização fenotípica (SCREVEN et al., 2014).

Os efeitos benéficos das CMNMO recrutadas à lesão tecidual resultam de mecanismos parácrinos, como produção de citocinas e fatores de crescimento com diferentes funções, como a prevenção de apoptose, citoproteção de células nativas viáveis, efeitos anti-inflamatórios, redução de fibrose e recrutamento de células-tronco específicas, estimulando a angiogenese e regeneração tecidual mediadas por célula progenitoras residentes (KUBAL et al., 2006). A caracterização dos subtipos de CMNMO, os estudos de migração e a melhor compreensão das funções dessas células podem contribuir para o conhecimento do seu potencial e, consequentemente, incrementar as possibilidades de seu uso em ensaios clínicos na medicina veterinária.

As CMNMO administradas por via intravenosa têm uma passagem pulmonar 30 vezes maior que as CTM (FISCHER et al., 2009), facilitando a migração dessas células para os tecidos lesados. Contudo, a migração dessas células para o SNC, após administração intravenosa, em animais com sequelas de cinomose, por exemplo, ainda não está bem estudada.

A cinomose é uma doença infectocontagiosa causada por um Morbillivirus pertencente a família Paramyxoviridae (ALLDINGER et al., 2000; MORO et al., 2003; BEINEKE et al., 2008; BEINEKE et al., 2009). Dependendo da cepa viral e condições imunológicas do hospedeiro podem ocorrer variações nos sinais cínicos da doença (LEMPP et al., 2014). Cães infectados pelo vírus da cinomose podem apresentar afecção dos tratos respiratório e gastrintestinal (MORO et al., 2003; BEINEKE et al., 2008), sendo que até 30% deles têm também complicações neurológicas e aproximadamente 10% morrem por encefalite aguda (RUDD et al., 2006). Além disso, os cães que recuperam podem apresentar sequelas permanentes (BEINEKE et al., 2009). Manifestações neurológicas da infecção pelo vírus da cinomose, caracterizadas como leucoencefalite desmielinizante (ALLDINGER et al., 2000; BEINEKE et al., 2009), podem ocorrer em paralelo ou subsequentemente a outras afecções (LEMPP et al., 2014), mas geralmente têm início de uma a três semanas após a recuperação dos sinais clínicos sistêmicos (DEEM et al., 2000; GREENE e APPEL, 2006).

Os processos imunopatológicos e a degeneração axonal inicial que ocorrem na leucoencefalite de cães com cinomose são semelhantes a outras doenças

desmielinizantes, tais como esclerose múltipla (LEMPP et al., 2014). O vírus da cinomose provoca uma doença neurológica aguda e progressiva, tanto na substância branca, quanto na substância cinzenta (VON RÜDEN et al., 2012).

A invasão do vírus da cinomose no sistema nervoso central (CNS) ocorre provavelmente por via hematógena com afecção ependimária e da substância branca subependimária (BEINEKE et al., 2009). A patogênese da perda de mielina, na leucoencefalite desmielinizante causada pelo vírus da cinomose, é provavelmente um processo bifásico (FRISK et al., 1999; ALLDINGER et al., 2000), iniciando-se a infecção pela substância cinzenta, com subsequente disseminação do antígeno para sustância branca (LEMPP et al., 2014).

As lesões da leucoencefalite aguda relacinadas à cinomose são histologicamente caracterizadas por pequeno aumento do número de astrócitos e micróglia (ULRICH et al., 2014), em que 95% de todas as células infectadas são astrócitos (LEMPP et al., 2014). Alldinger et al. (2000) mostraram uma ligeira gliose e vacuolização como as únicas lesões visíveis na cinomose aguda, sem desmielinização. Este tipo de lesão pode ser observada entre 16 e 24 dias após infecção experimental (ULRICH et al., 2014). No entanto, leucoencefalite com desmielinização multifocal do SNC é a lesão mais comum associada à infecção pelo vírus da cinomose (BEINEKE et al., 2008; BEINEKE et al., 2009; LEMPP et al., 2014), observando-se esse tipo de lesão entre 29 e 63 dias após a infecção. Em cães com lesões neurológicas inflamatórias ou não-inflamatórias de cinomose observa-se sobre-regulação de interleucina (IL)-10. Nos animais com leucoencefalite subaguda ou crônica (lesões inflamatórias) ocorre também sobre-regulação de IL-6, fator de necrose tumoral (TNF)-α e fator de transformação do crescimento (TGF)-β (FRISK et al., 1999), portanto, a progressão das lesões parecem decorrer de uma alteração do equilíbrio necessário na expressão de citocinas no SNC, o que torna atrativo o emprego das células-tronco em ensaios clínicos para o tratamento de sequelas da cinomose, visto que o principal efeito das CMNMO recrutadas nos tecidos lesados é a ação parácrina, a qual inclui a expressão de citocinas e fatores de crescimento que possuem efeitos anti-inflamatórios e imunomodulatórios potentes, podendo modular o microambiente local e ativar progenitores endógenos (CUENDE et al., 2011; PITTENGER et al., 1999; JIANG et al., 2002).

Dentre as possibilidades de terapia celular, as CMNMO são atrativas pela facilidade em seu isolamento, não sendo necessária estrutura de laboratório

complexa para sua obtenção. Além disso, elas também podem ser recrutadas para os sítios de injuria (MA et al., 2014).

Heparina e citrato-fosfato-dextrose-adenina-1 (CPDA-1) são comumente utilizados para coletar medula óssea (BAUMERT et al., 2008). A heparina é um potente anticoagulante que tem efeito na presença de um componente chamado cofator da heparina (ROSENBERG, 1974), enquanto que o CPDA-1 é uma solução anticoagulante que contém adenina, usada para preservação do sangue por período prolongado (BEUTLER e WEST, 1979). Quando estes anticoagulantes foram comparados *in vitro*, em relação à viabilidade de linfócitos, o CPDA-1 resultou em melhor proteção dos linfócitos que a heparina (KLEIN et al., 1991). Porém, o efeito destes anticoagulantes em termos de isolamento de células-tronco não é conhecido.

O monitoramento das células após o transplante, permite confirmar se estas células migram para o local da lesão. O PKH é uma molécula de marcação fluorescente, que é incorporada na bicamada lipídica das membranas citoplasmáticas, que tem sido utilizado para a identificação da migração de uma variedade de células (JOHNSSON et al., 1997). Uma vez incorporado nas células *in vitro*, o PKH não é transferido para o meio ou para células não marcadas (SLEZAK e HORAN, 1989). O PKH não é tóxico nem imunogênico, e a marcação das células com este corante é estável por longo período (JOHNSSON et al., 1997). CMNMO podem ser marcadas com PKH e utilizadas para transplante por via intravenosa (KAMIYA et al., 2008). Portanto, a marcação com PKH parece ser ideal para monitoramento da migração de CMNMO transplantadas por via intravenosa (JOHNSSON et al., 1997).

Esta tese está dividida em três capítulos com os seguintes títulos:

1) “Efeito dos anticoagulantes no rendimento de células mononucleares da medula óssea de cães”;
2) “Recrutamento de células mononucleares derivadas da medula óssea para o sistema nervoso central após transplante alogênico em cães com sequelas de cinomose”;
3) “Avaliação da segurança e eficácia do transplante de células mononucleares derivadas da medula óssea para o tratamento de sequelas de cinomose”.

No primeiro capítulo os objetivos foram comparar o rendimento de células mononucleares derivadas da medula óssea de cães, coletadas com heparina ou com CPDA-1. No segundo capítulo os objetivos foram avaliar a marcação das BMMNC com o corante PKH26 e o recrutamento das células marcadas para o CNS, após o transplante alogênico em animais com complicações neurológicas da cinomose. No terceiro capítulo teve-se como objetivo avaliar a segurança e eficácia do transplante alogênico de CMNMO no tratamento de sequelas neurológicas de cães naturalmente infectados pelo vírus da cinomose.

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2 EFFECT OF ANTICOAGULANTS ON YIELD OF BONE MARROW-DERIVED MONONUCLEAR CELLS HARVESTED FROM DOGS

EFEITO DOS ANTICOAGULANTES NO RENDIMENTO DE CÉLULAS MONONUCLEARES DA MEDULA ÓSSEA DE CÃES

ABSTRACT

Cell therapy with bone marrow-derived mononuclear cells is an alternative to therapy with mesenchymal stem cell cultures. Heparin is an anticoagulant commonly used to harvest bone marrow, however, can affect the cell viability during extended contact. The aim of the present work was the comparison of the yield of bone marrow-derived mononuclear cells harvested from dogs with two different anticoagulants. Bone marrow was harvested from the iliac crest of five healthy dogs aged between 15 and 30 months, and the effect of two anticoagulant solutions, CPDA-1 (citrate phosphate dextrose adenine-1) and heparin, on the isolation of mononuclear cells was compared. Mononuclear cells were isolated in a density gradient and stained for CD9 and CD44 for characterization by flow cytometry. The means were compared using Student's paired *t*-test. Samples harvested with CPDA-1 yielded an average of 5.16×10^6 ($\pm 1.76 \times 10^6$) to 20.20×10^6 ($\pm 1.55 \times 10^6$) mononuclear cells/mL, whereas the yield of samples harvested with heparin varied between 4.56×10^6 ($\pm 0.69 \times 10^6$) and 24.30×10^6 ($\pm 2.12 \times 10^6$) mononuclear cells/mL. By flow cytometry, the mean percentage of double-stained cells varied from 1.96% (± 0.64%) to 5.01 % (± 0.73%) for CPDA-1 and from 2.23% (± 0.70%) to 7.27 % (± 0.97%) for heparin. No significant statistical differences were observed on yield or CD9 and CD44 expression. Further studies are recommended to assess efficacy of CPDA on mononuclear cell isolation.

Keywords: Mesenchymal stem cells; multipotent stem cell; immunophenotyping.

RESUMO

A terapia com células mononucleares de medula óssea é uma alternativa ao cultivo de células-tronco mesenquimais. A heparina é um anticoagulante comumente utilizado para a coleta de medula óssea, no entanto, com o contato prolongado ela pode afetar a viabilidade das células. O objetivo desse trabalho foi comparar o rendimento de células mononucleares derivadas da medula óssea de cães colhidas com dois anticoagulantes diferentes. Foram coletadas medulas ósseas de cinco cães hígidos, com idades variando entre 15 e 30 meses, por punção na crista ilíaca. Foi comparado o efeito da solução anticoagulante no isolamento das células mononucleares, utilizando-se CPDA-1 (citrato, fosfato, dextrose, adenina) ou heparina como soluções anticoagulantes. As células mononucleares foram isoladas em gradiente de densidade e caracterizadas fenotipicamente em citometria de fluxo. Os resultados foram submetidos ao Teste *t* pareado para comparação de médias. Nas amostras coletadas com CPDA-1 o rendimento médio variou entre 5,16 x 10^6 (± 1,76 x 10^6) a 20,20 x 10^6 (± 1,55 x 10^6) células mononucleares/mL, enquanto que nas amostras coletadas com heparina o rendimento variou entre 4,56 x 10^6 (± 0,69 x 10^6) a 24,30 x 10^6 (± 2,12 x 10^6) células mononucleares/mL. Na citometria de fluxo, a média de células duplo-marcadas variou de 1,96% (± 0,64%) a 5,01% (±0,73%) para CPDA-1 e de 2,23% (± 0,70%) a 7,27% (± 0,97%) para heparina. Não foram observadas diferenças estatísticas no rendimento ou na expressão de CD9 e CD44. Recomenda-se estudos adicionais para avaliar melhor a eficácia do CPDA no isolamento de células mononucleares.

Palavras-chave: Célula-tronco mesenquimal; célula-tronco multipotente, imunofenotipagem.

2.1 INTRODUCTION

In animals, stem cell therapy is being studied as a treatment option for inflammatory lesions and is generally performed by administration of autologous adipose-derived mesenchymal cells (BLACK et al., 2007). In addition to their structural contribution to tissue repair, mesenchymal stem cells (MSC) have potent immunomodulatory and anti-inflammatory effects, acting on tissue repair by means of local environment modulation, endogenous progenitor cell activation, direct cell-to-cell interaction and the secretion of several factors (CHEN e TUAN, 2008).

MSC can be isolated from bone marrow, adipose tissue or umbilical cord blood (KERN et al., 2006). Adult stem cells can be isolated from bone marrow and culture-expanded for therapeutic application (JIANG at al., 2002) or used in cell therapy without prior expansion (SOARES et al., 2004). However, stem cells isolated and expanded under different culture conditions differ in their properties and, most likely, in their therapeutic potential (PROCKOP, 2009).

Heparin and CPDA-1 are commonly used to harvest bone marrow (BAUMERT et al., 2008). Heparin is a potent anticoagulant that has effects only in the presence of a plasma component termed heparin-cofactor (ROSENBERG, 1974), while the CPDA-1 is an adenine-containing preservative solution used to anticoagulant blood over a wider range (BEUTLER & WEST, 1979). Comparing the effect of anticoagulants *in vitro* on the viability of lymphocytes, KLEIN et al. (1991) showed that CPDA is better than heparin regarding the protection of lymphocytes. However, the comparison of effect of anticoagulants at harvest on terms of stem cell yield has not been studied.

Adult bone marrow-derived stem cells can be obtained by density gradient centrifugation (SOARES et al., 2004). Therefore, the total bone marrow-derived mononuclear cell fraction offers a low-cost alternative to MSC culture (BRITO et al., 2010). Although there are some reports on the use of non-cultured cells for therapy (SOARES et al., 2004; BRITO et al., 2010), most studies use culture-expanded cells (QUIMBY et al., 2011; JUNG et al., 2009). Despite KAMISHINA et al. (2008) estimated the frequency of canine bone marrow-derived MSC to be 0.0042 (± 0.0019%). These researchers observed a great variability among bone marrow samples.

Phenotypic characterization of canine bone marrow-derived MSC expanded has been demonstrated (JUNG et al., 2008; SCREVEN et al., 2014). However, the phenotype of non-cultured canine bone marrow-derived multipotent cells is yet unknown and, there is a little consensus on the phenotypic characterization of canine stem cells (Screven et al., 2014).

According to GIMBLE et al. (2007), the phenotypic expression profile changes during culture as a function of time in passage and adherence to plastic. The cited authors further state that, despite differences in isolation and culture procedures, immunophenotyping is relatively consistent among laboratories.

Stem cell cultures are heterogeneous, comprising different subsets (clones). The clones are also heterogeneous and may contain mutations that accumulate during expansion and thus can acquire secondary mutations that may cause cancerous transformations after transplantation. For this reason, the mutation rate of a stem cell lineage is an important criterion for evaluating the adequacy of such lineages in medical practice (SVERDLOV & MINEEV, 2013). However, this type of evaluation renders cell therapy highly expensive.

The objective of the present study was to compare of the yield of bone marrow-derived mononuclear cells harvested from dogs with two different anticoagulants as well as evaluate the difference in the phenotype of the isolated cells for future use of non-expanded cells in cell therapy.

2.2 MATERIAL AND METHODS

Five healthy mixed-breed dogs aged between 15 and 30 months were used for the present study. Bone marrow was harvested from animals under general anesthesia by puncturing the iliac crest with disposable hypodermal needles (16 G) and disposable 10-mL syringes. Two 8-mL samples of bone marrow were withdrawn from each dog, one containing 2.0 mL CPDA-1 (citrate phosphate dextrose adenine-1) anticoagulant solution and the other 2.0 mL anticoagulant solution composed of 1.0 mL heparin (5000 IU/mL) and 1.0 mL phosphate-buffered saline (PBS). All samples were diluted with 10 mL Dulbecco's Modified Eagle Medium (DMEM, Sigma®). For density gradient isolation, six 15-mL Falcon tubes were prepared containing a bottom layer of 1.5 mL Histopaque 1119 (Sigma®) and 1.5 mL Histopaque 1077 (Sigma®) carefully layered on top. Samples were divided into six homogenous 3-mL aliquots and layered onto the Histopaque solutions by means of gravity sedimentation. Tubes were centrifuged at 950 *g* for 30 min. After centrifugation, the layer of mononuclear cells was collected with a serological pipette and washed twice with 5.0 mL DMEM by centrifugation at 400 *g* for 10 min. After washing, cells were resuspended in PBS and evaluated in a hematology analyzer (BC2800Vet, Mindray).

For phenotypic assay of mononuclear cells were used the antibodies according to a previous report for characterization of canine mesenchymal stem cells (JUNG et al., 2008). Homologous control was used as negative control. A total of 1.0×10^6 cells from each sample were resuspended in 10 µL PBS containing 1.0 µL Anti-Human-CD9 (conjugated to R. Phyoerythrin) (AbDserotec; IgG concentration 1.0 mg/mL) and 1.0 µL Anti-Dog-CD44 (conjugated to FITC) antibodies (AbDserotec; IgG concentration 0.05 mg/mL) and incubated for 40 min. Cells were subsequently resuspended in 400 µL PBS and analyzed by flow cytometry (FACSCalibur, BD).

Results for the number of obtained cells/mL of bone marrow and the percentage of stained cells in immunophenotyping were submitted to statistical analysis using Student's paired *t*-test for the comparison of means.

2.3 RESULTS

The mean yield of mononuclear cells was similar in all samples ($p > 0.05$) with regard to the anticoagulants used at harvest, *i.e.*, CPDA-1 or heparin (Table 2.1). Specifically, samples harvested with CPDA-1 yielded an average of 5.16×10^6 ($\pm 1.76 \times 10^6$) to 20.20×10^6 ($\pm 1.55 \times 10^6$) mononuclear cells/mL, whereas samples harvested with heparin yielded between 4.56×10^6 ($\pm 0.69 \times 10^6$) and 24.30×10^6 ($\pm 2.12 \times 10^6$) mononuclear cells/mL.

Table 2.1 Comparison of two anticoagulants (CPDA-1 and heparin) used for the harvest of canine bone marrow in terms of mean yield of cells and standard deviation (SD).

Animal	CPDA-1		Heparin	
	Mean	SD	Mean	SD
1	20.20×10^6	1.55×10^6	24.30×10^6	2.12×10^6
2	9.89×10^6	4.36×10^6	4.56×10^6	0.69×10^6
3	13.60×10^6	1.28×10^6	7.94×10^6	1.06×10^6
4	10.70×10^6	3.63×10^6	4.89×10^6	1.09×10^6
5	5.16×10^6	1.73×10^6	16.20×10^6	2.14×10^6

The observed differences were not statistically significant ($p > 0.05$).

CPDA-1: citrate phosphate dextrose adenine-1

By flow cytometry, the mean percentage of CD9-stained cells varying between 0.07 % (± 0.03 %) and 1.38 % (± 0.40 %) of the total cells in samples harvested with CPDA-1 and between 0.17 % (± 0.10 %) and 1.16 % (± 0.15 %) in samples harvested with heparin. For CD44-staining the variation was between 43.05 % (± 4.14 %) and 73.58 % (± 5.03 %) for samples harvested with CPDA-1 and between 43.87 % (± 2.39 %) and 69.67 % (± 7.64 %) for samples harvested with heparin. The mean percentage of double-stained cells (Table 2.2) varied between 1.96 % (± 0.64 %) and 5.01 % (± 0.73 %) for samples harvested with CPDA-1 and between 2.23 % (± 0.70 %) and 7.27 % (± 0.97 %) for samples harvested with heparin. However, the observed differences were not statistically significant ($p > 0.05$).

Table 2.2 Mean percentage and standard deviation (SD) in cells isolated from canine bone marrow double-stained (CD9 and CD44).

Animal	Double-stained cells with CPDA-1		Double-stained cells with Heparin	
	mean	SD	mean	SD
1	5.01	0.73	2.23	0.70
2	3.20	1.84	3.73	0.66
3	4.88	1.01	5.96	1.15
4	1.96	0.64	7.27	0.97
5	2.10	0.86	5.78	2.10

The observed differences were not statistically significant ($p > 0.05$).

CPDA-1: citrate phosphate dextrose adenine-1

2.4 DISCUSSION

In the present study, mononuclear cells were isolated according to a previous report (SOARES et al., 2004), and has been compared the yield of bone marrow-derived mononuclear cells harvested from dogs with two different anticoagulants as well as assessed the difference in the phenotype of the isolated cells.

Although KLEIN et al. (1991) emphasizes the importance of choosing the right anticoagulant when the viability of lymphocytes, in this study no differences were observed on the viability of cells obtained with CPDA or heparin.

In study focusing on the influence of different anticoagulants on chemotactic reactivity, the heparin was been considered the most suitable and safe anticoagulant for freshly isolated bone marrow hematopoietic stem and progenitor cells (BAUMERT et al., 2008). In this study, we have not found significant differences at harvest in terms of stem cell yield with both anticoagulants tested.

ABBIT & NASH (2001) compared the effect of anticoagulants in the characteristics of leucocyte adhesion and showed that whereas nearly all-adherent leucocytes were rolling with CDPA, a large proportion were stationary adherent with heparin. However, the authors found no significant differences in CD11b on neutrophils expression during storage.

This study was compared the concomitant expression of CD9 and CD44 by flow cytometry. Staining for CD9 was obtained in less than 2 % and for CD44 in approximately 70 % of the isolated cells regardless of the anticoagulant used for bone marrow harvest. The percentage of staining for CD44, a characteristic marker for bone marrow-derived mesenchymal cells (KOCHER et al. 2001), observed in the present study was equivalent to that reported by SCREVEN et al. (2014), who demonstrated staining for CD44 in 65.1 % of canine adipose-derived mesenchymal cells. However, the authors showed only 20-30% of the bone marrow-derived MSC was stained positive for CD44. The CD44, or H-CAM, is a multifunctional cell adhesion molecule (ROA et al., 2001) that was first identified as a lymphocyte-homing receptor and is highly expressed in antigen-activated T cells and in T cells with transendothelial migratory capacity. In peripheral blood, most $CD4^{+}$ cells have a $CD44^{low}$ phenotype (BRENNAN et al. 1999). H-CAM functions include cell-cell and cell-substrate adhesion (LEWINSOHN et al., 1990), as well as lymphocyte homing,

hematopoiesis, angiogenesis, cytokine release and hyaluronic acid metabolism and degradation (ROA et al., 2001).

The CD9 antigen is a transmembrane protein of the tetraspanin superfamily (KLEIN-SOYER et al., 2000). In the hematopoietic system, CD9 is expressed in young B cells, platelets, eosinophils, basophils and activated T lymphocytes (BOUCHEIX et al., 1991; ANTON et al., 1995). CD9 is not expressed by hematopoietic progenitors or non-activated lymphocytes (BOUCHEIX et al., 1991). In B cells and platelets, CD9 regulates cell activation and aggregation, possibly by association with an integrin. In other cells, CD9 regulates cell motility (ANTON et al., 1995). CD9 has also been reported in cells of the central and peripheral nervous system (MEISTER at al., 2007). MARTIN et al. (2002) obtained approximately 92 % CD9-stained cells in second- and third-passage cultures of bone marrow-derived cells harvested from cats.

Although KERN et al. (2006) have reported strong expression of CD90 in adult human bone marrow-derived stem cells, studies with bone marrow and adipose-derived canine mesenchymal stem cells (TAKEMITSU et al., 2012) and with canine umbilical cord blood-derived mesenchymal stem cells (SEO et al., 2009) have showed low expression of this marker. TAKEMITSU et al. (2012) and SEO et al. (2009) also reported low expression of CD73, however this marker is not commercially available.

Concomitant CD9 and CD44 staining has been reported for MSC phenotyping in dogs (JUNG et al., 2009) and cats (MARTIN et al., 2002). However, there are no studies showing the phenotypic characterization of non-cultured cells harvested from dogs. According to GIMBLE et al. (2007) immunophenotyping is relatively consistent among laboratories despite the differences in isolation and culture procedures.

The reported frequency of MSC in canine bone marrow was estimated to be 0.0042 (± 0.0019%) of mononuclear cells as demonstrated in studies on colony-forming-unit fibroblasts (CFU-F) (KAMISHINA et al., 2008). Interestingly, concomitant CD9 and CD44 staining, which characterize mesenchymal cells, were obtained for 1.96 % to 7.27 % of mononuclear cells in the present study. Considering that phenotyping is generally performed on cultured cells after the first passage (JIANG at al., 2002; JUNG et al., 2009; MARTIN et al., 2002; SCREVEN et al., 2014), the differences in double-staining and the frequency reported in studies on CFU-F may be due to the loss of non-adherent mesenchymal cells.

2.5 CONCLUSIONS

The results of this study indicate promising possibilities to use CPDA 1 for bone marrow harvest with the aim to isolate mononuclear cells. This use should be considered when it is not possible to isolate and use the cells immediately after collection. Further studies are needed to evaluate the influence of CPDA-1 on expanded cells and in the expression on non-expanded cells of several additional surface markers.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study protocol was approved by the Animal Use Ethics Committee of the Agricultural Sciences Department of the Federal University of the State of Paraná, Southern Brazil (CEUA-SCA/UFPR number 030/2011).

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3 BONE MARROW MONONUCLEAR CELL RECRUITMENT TO THE CENTRAL NERVOUS SYSTEM AFTER ALLOGENEIC CELL TRANSPLANTATION IN DOGS WITH DISTEMPER SEQUELS

ABSTRACT

Canine distemper is an infectious disease that causes demyelinating encephalitis in dogs. Neurological signs of distemper are variable and progressive, and some of them may be permanent. There is no specific therapy for dogs that show distemper clinical signs and the use of new alternative treatment must be considered. Stem cell therapy has been studied as an option for treating several injuries, including brain injury. Bone marrow mononuclear cells (BMMNC) can be easily isolated and widely used as a therapeutic strategy in a large number of preclinical and clinical studies. However, one challenge associated with this therapeutic approach is the route of administration for efficient cell delivery. Intravenous (IV) delivery has been used because it is the easiest and least invasive method of cell transplantation. Cell labelling and tracking allow to evaluate if IV transplanted cells are migrating towards sites of injury. The PKH26 is a fluorescent labelling molecule, noncytotoxic and is stable for long time period. Because of these characteristics, this dye seems to be ideal for cells tracking. The aim of this study was to evaluate cell migration after allogeneic BMMNC transplantation in 10 animals with neurological complications of canine distemper. Bone marrow was harvested from eight donors and BMMNC were isolated, labeled with PKH26 dye and IV transplanted into the patients. The cell migration was assessed in the cerebrospinal fluid (CSF) of patients at different periods of time and the percentage of labeled cells with PKH 26 dye was evaluated quantitatively by flow cytometry and qualitatively by fluorescence microscopy. The mean volume of harvested bone marrow was 28.38 mL (±6.46 mL) and the average of mononuclear cells obtained was 8.84×10^6 ($\pm 2.56 \times 10^6$) cells/mL. Flow cytometry analyses revealed that the average of PKH labeled BMMNC was 93.0% (±9.9%), and the average cell viability was 87.6% (±12.8%). Labelling with PKH26 had no negative effect on cell viability. In the quantitative analysis by flow cytometer, it was observed a wide variation in the percentage of labeled cells in different CSF collection times. Also, there was an increase on the percentage of PKH26-labeled cells in the CSF, with the highest percentage between 4 and 5 ½ hours after IV infusion with subsequent decrease. In the qualitative analysis, it was observed the presence of labeled BMMNC recruited by central nervous system on CSF. In conclusion, this study demonstrated that BMMNC can be efficiently labeled with PKH26 dye and these cells can be monitored after IV transplantation in animals showing neurological complications of canine distemper.

Key words: Bone marrow mononuclear cells; cells tracking; intravenous transplantation.

3.1 INTRODUCTION

Canine distemper is an infectious disease, caused by Morbillivirus of the Paramyxoviridae family, which affects the central nervous system (CNS) of the dogs, causing demyelinating encephalitis (ALLDINGER et al., 2000; BEINEKE et al., 2009). Neurologic manifestations of canine distemper usually begin 1-3 weeks after recovery from systemic illness (DEEM et al., 2000; GREENE and APPEL, 2006). Acute or chronic neurologic signs are typically progressive (GREENE and APPEL, 2006) and depend on viral distribution into the CNS (TIPOLD et al., 1992; DEEM et al., 2000). Neurological complications are varied and progressive and include myoclonus, nystagmus, ataxia, postural reaction deficits and paraparesis or tetraparesis (TIPOLD et al., 1992; DEEM et al., 2000; GREENE and APPEL, 2006; BEINEKE et al., 2009), cervical rigidity, seizures and cerebellar and vestibular signs (DEEM et al., 2000; GREENE and APPEL, 2006). Myoclonus is considered untreatable and irreversible (TIPOLD et al., 1992; GREENE and APPEL, 2006). Neuroinvasion of canine distemper virus (CDV) occurs predominantly via the hematogenous route with ependymal and subependymal white matter infection, indicating path of infection along the cerebrospinal fluid (BEINEKE et al., 2009).

There is no specific therapy for animals with distemper clinical signs (DEEM et al., 2000; GREENE and APPEL, 2006). However, nonspecific and supportive treatments are beneficial to reduce mortality (GREENE and APPEL, 2006). Careful attention to nutrition and hydration is an important quality-of-life issue. Stem cell therapy has been studied as a treatment option for inflammatory and degenerative lesions (BLACK et al., 2007).

Bone marrow has been used extensively as a source of stem cells (LINON et al., 2014; GUO et al., 2015). Bone marrow mononuclear cell (BMMNC) is a term used to denominate all cells present in bone marrow whose nuclei are unilobulated or rounded without granules in the cytoplasm (CUENDE et al., 2012). BMMNC contain a subset of cells denominated mesenchymal stem cells, as well as hematopoietic stem cells (KAMIYA et al., 2008; CUENDE et al., 2012), besides very small embryonic-like stem cells, multipotent adult progenitor cells, hemangioblasts, endothelial progenitor cells, and tissue-committed stem cells (CUENDE et al., 2012). BMMNC can be easily obtained by density gradient and do not require *in vitro*

expansion before their use for therapy (SOARES et al., 2004; MATHIEU et al., 2009; ALVAREZ-VIEJO et al., 2013).

However, the route of administration for efficient cell delivery is a challenge in the use of BMMNC for regenerative medicine. Intravenous (IV) stem cell delivery has been used in both experimental and clinical trials because it is the easiest and least invasive method of cell transplantation. However, recent data suggest that the majority of administered stem cells are initially trapped in the lungs. Pulmonary passage is a major obstacle for systemic intravenous stem cell administration with smaller number of stem cells crossing over to the arterial system and the majority of cells being trapped inside the lungs (ROCHEFORT et al., 2005; KANG et al., 2006). Both cell size and receptor-mediated adhesion and/or stem cell type appear to be crucial variables for pulmonary stem cells passage. BMMNC have advantages over other types of stem cells like mesenchymal stem cells (MSC), due to their smaller size that could explain improved cell passage through the pulmonary microvasculature (FISCHER et al., 2009). The number of cells reaching the arterial circulation after IV cell transplantation still represents only a small fraction of the originally administered amount. Therefore, an efficient IV stem cell therapy requires a sufficient number of stem cells reaching the target organ, to increase the number of stem cells reaching the arterial circulation. Some studies show that BMMNC injected intra-arterially and intravenously migrated into the CNS showing a significant neuroprotective effect after transient focal cerebral ischemia in rats (KAMIYA et al., 2008; KAMIYA et al., 2014). In dogs, stem cells were used by intra-arterial route for the treatment of cerebral ischemia, with evidence of migration of labeled cells to the site of injury (CHUNG et al., 2009). However, studies that showed the migration of cells into the CSF after intravenous delivery in dogs were not found in our review of the literature.

One method for evaluating whether IV transplanted cells were attracted to the site of injury is the labelling and tracking of cells. Different dyes may be used for cell labelling and monitoring after transplantation, such as CM-DiI, a fluorescent dye, non cytotoxic membrane marker and long half-life (CAI et al., 2014); 5-chloromethylfluorescein diacetate (CMFDA), a fluorescent dye with low cytotoxicity which does not affect viability and proliferation of the cells remaining three to six generations (SARTORE et al., 2005); 5- and 6-([(4-chloromethyl)benzoyl]-amino) tetramethylrhodamine a fluorescent intracellular dye (VAAGS et al., 2011);

bromodeoxyuridine (BrdU) a nuclear marker (COUSINS et al., 2014; NAGYOVA et al., 2014) and the PKH-26.

The PKH is a fluorescent labelling molecule, which is incorporated into the lipid bilayer of cytoplasmic membranes and has been used for labelling a variety of cells (JOHNSSON et al., 1997). Once incorporated into the cells *in vitro*, the PKH neither leaks out to the medium nor transfers to nonlabeled cells (SLEZAK and HORAN, 1989). The labelling with PKH is stable for long time period and the PKH dye is not toxic or immunogenic, therefore, this dye seems to be ideal for cells tracking (JOHNSSON et al., 1997).

The nonvital dye 7-amino-actinomycin D (7AAD) has the ability to diffuse into the cell due to increased plasma membrane permeability and can be used conjugated to FITC or PE to discriminate dead cells from viable cells by flow cytometry (SCHMID et al., 1992; SCHUURHUIS et al., 2001).

The aim of this study was to evaluate the recruitment of BMMNC into the CNS after IV allogeneic transplantation in dogs with neurological complications caused by canine distemper.

3.2 MATERIALS AND METHODS

This study was approved by the Animal Use Ethics Committee of the Agricultural Sciences Department of the Federal University of the State of Paraná, Southern Brazil (CEUA-SCA/UFPR number 030/2011).

3.2.1 ISOLATION OF BONE MARROW MONONUCLEAR CELLS

Eight healthy mixed-breed dogs aged between 12 and 48 months were used as bone marrow donors. Bone marrow was harvested under general anaesthesia, by puncturing the iliac crest with disposable hypodermal needles (16 G) and disposable 10-mL syringes containing 1.0 mL heparin (5,000 IU/mL) anticoagulant solution. After collection, the bone marrow was mixed into culture medium, Iscove's modified Dulbecco's medium (IMDM) (Gibco), in the ratio 1:3. Bone marrow was carefully loaded onto Histopaque (1.077 g/mL) (Sigma Chemical) to isolate bone marrow mononuclear cells (MNC). MNC were isolated by density gradient centrifugation (400g, 30 minutes, room temperature) and washed twice with IMDM (BÖYUM, 1967).

3.2.2 BONE MARROW MONONUCLEAR CELLS LABELING WITH PKH26

BMMNC were stained with PKH26 (Sigma) according to the manufacturer's instructions to evaluate cells migration. Briefly, 2 x 10^8 cells were placed in a conical tube (TTP), and the cells were washed with serum-free medium by centrifuging at 400 *g* for 10 minutes. The supernatant was discarded and the diluent C was added to the cell pellet and the cells were resuspended. The dye solution (PKH 4x10-6 M + diluent C) was added to the cell pellet and incubated for 10 minutes with periodic agitation. Dye activity was stopped by adding 1 mL of protein solution (PBS + fetal bovine serum (FBS: Gibco)) and incubated for 5 minutes at 37°C. The cells were washed twice at 400 *g* for 10 minutes at room temperature with PBS containing medium to remove the excess of dye (HAAS et al., 2000; LEE et al., 2004; HEMMRICH et al., 2005). The cells were resuspended in culture medium and underwent counting in a Neubauer chamber.

3.2.3 PRE-TRANSPLANTATION ASSESSMENT OF CELL VIABILITY AND CELL LABELING BY FLOW CYTOMETRY

Pre-transplantation samples were assessed by flow cytometry to perform a quantitative analysis of the percentage of BMMNC stained with PKH-26 and assess cells viability. A total of 1.0 x 10^6 cells were washed in 500 μL PBS by centrifugation at 400 *g* for 10 min. After being washed the cells were resuspended with 5.0 μL of 7-amino-actinomycin D (7-AAD) (BD Pharmigen) and incubated at room temperature for 20 min. The cells were washed again in 500 μL PBS by centrifugation at 400 *g* for 10 minutes and 1% paraformaldehyde-fixed (300 μL). Non-labeled BMMNC were used as negative control. Quantitative analyses were performed using a FACSCalibur flow cytometer (Becton Dickinson) and FlowJo software (Flowjo).

3.2.4 CELLS TRANSPLANTATION AND CEREBROSPINAL FLUID COLLECTION

Ten mixed-breed dogs, which were identified with numbers from 1 to 10, weighing 5 to 35 kg and showing neurological signs of subacute or chronic distemper encephalitis, were intravenously transplanted with BMMNC. The number of transplanted cells was 1.0 x 10^8 PKH-26 labeled cells per animal in 4.0-mL IMDM.

The canine distemper was confirmed by detection of CDV antigen using a commercial immunochromatographic test (Alere), or Real-time PCR (carried out in commercial laboratory facilities: IDEXX Laboratories Inc.), and detection of CDV antibody in the CSF using commercial immunochromatographic test (Alere). The progression of clinical signs was considered in all cases.

After PKH26-labeled BMMNC transplantation, approximately 1 mL of CSF was obtained by suboccipital puncture from each animal, under general anaesthesia with propofol (Cristalia) and meperidine (Dolosal: Cristalia), at different times. Only in dogs 1 and 6 the CSF was collected twice, with an interval of three hours between collections in the dog 1 and 17 ½ hours in the dog 6. All samples were divided into 0.5 mL aliquots. One aliquot was used to determine the percentage and viability of cells labeled with PKH26 by flow cytometry. 7-AAD-dye was used to assess the cells viability as described above. Pre-transplantation samples of PKH26-labeled BMMNC were used as a positive control.

Another aliquot of CSF was concentrated by centrifugation at 200 *g* for 8 min and fixed with 50 μL of methanol/acetic acid (2:1) to assess labelling by immunofluorescence microscopy. Slides of CSF were prepared by cytospinning (200 *g* for 5 min) and the cells nuclei were counterstained with DAPI antifade (4,6-diamidino-2-phenylindole) (Cytocell, Cambridge, UK). Coverslips were mounted on glass slides and images were acquired using a fluorescence microscope (Leica DM4000). The positive control for qualitative analysis was PKH26 labeled BMMNC fixed with 100 μL of methanol/acetic acid (2:1) prepared on slides by cytospinning (200 *g* for 5 min) with the cells nuclei counterstained with DAPI antifade and the coverslips mounted onto the glass slides.

3.3 RESULTS

The mean volume of bone marrow harvested per dog was 28.38 mL (±6.46 mL). The average mononuclear cells yield obtained was 8.84 x 10^6 (±2.56x10^6) cells/mL.

Regarding the tracking after transplantation of cells stained with PKH26 dye in pre-transplantation samples, an average PKH26-labeled BMMNC of 92.9% (±9.9%) was observed by flow cytometry. The average cell viability, assessed by 7AAD staining, was 87.7% (±13.6%) as shown in Figure 3.1. Labelling with PKH26 had no significant negative effect on cell viability.

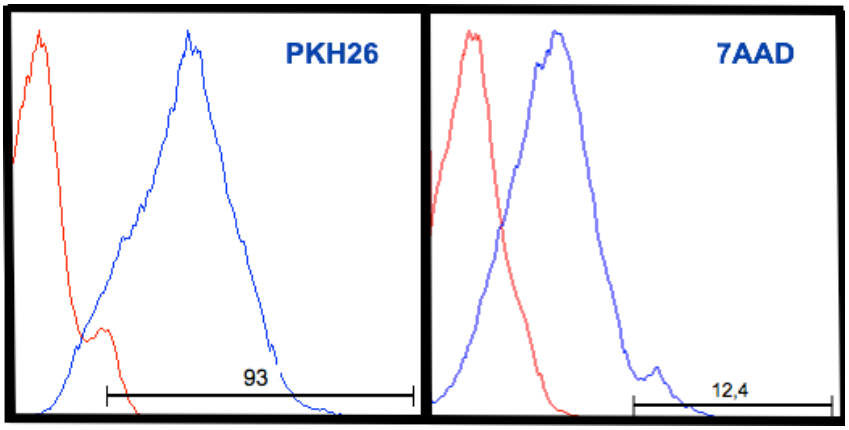

Figure 3.1 Percentage of PKH26-labeled BMMNC, and cell viability assessed by 7AAD staining demonstrated by flow cytometry, in the pre-transplant sample (donor for dog 7).

Pre-transplantation samples of cells labeled with PKH26 dye were also analyzed by fluorescence microscopy and were used as positive control (Figure 3.2A). In the qualitative analysis, it was showed the presence of labeled BMMNC recruited by central nervous system on CSF of transplanted dogs (Figure 3.2B).

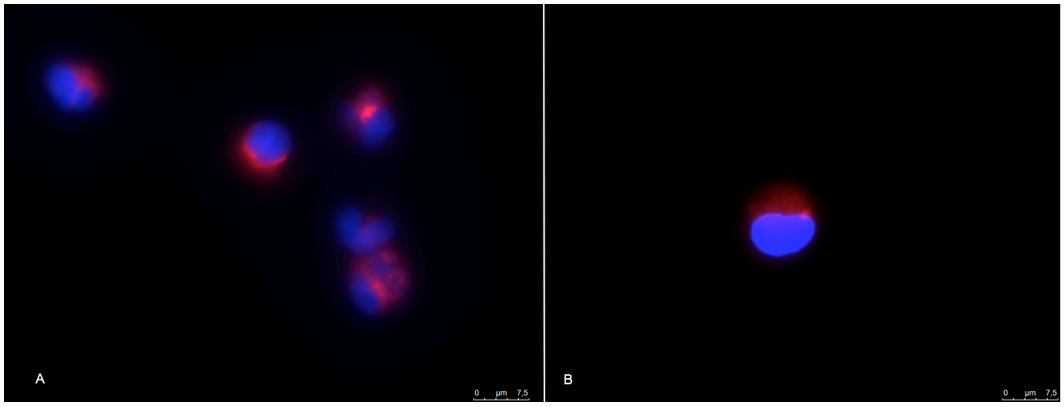

Figure 3.2 PKH26 (Sigma) labeled bone marrow mononuclear cells (BMMNC) before administration (positive control) (A). Representative picture of PKH26-labeled BMMNC present in the CSF, 5:30h after intravenous BMMNC transplantation in dog (7) with distemper. Nuclei of cells counterstained with DAPI antifade (4,6-diamidino-2-phenylindole) (Cytocell) (B).

In the quantitative analysis by flow cytometer, a wide variation in the percentage of labeled cells was observed at different CSF collection times. Also, there was an increase in the percentage of PKH26-labeled cells in the CSF, with the highest percentage between 4 and 5 ½ hours after infusion of PKH26-labeled BMMNC, with subsequent decrease (Table 3.1).

Table 3.1 Quantitative analysis and cell viability of PKH26 labeled cells in the cerebrospinal fluid (CSF) of distemper dogs, after intravenous bone marrow mononuclear cells (BMMNC) transplantation, evaluated by flow cytometry. The collection of CSF was performed at different time points after transplantation, represented in hours.

Dogs	Weight (kg)	Pre-transplant samples		Time after transplantation (h)	Post-transplant CSF samples	
		Labeled cells (%)	Cell viability (%)		Labeled cells (%)	Total of labeled cells / mL
1	22	69.5	80.50	1:00	0.89	254
2	34	99.1	98.15	2:30	2.85	5,692
3	7.0	97.1	-------	3:00	11.30	1,718
4	9.6	98.8	60.50	3:30	4.96	1,674
1	22	69.5	80.50	4:00	24.10	166
5	35	97.7	94.95	4:00	19.60	10,574
6	30	97.0	98.07	4:30	52.00	4,874
7	5.0	93.0	87.60	5:30	65.20	14,172
8	14	90.8	94.45	5:30	74.40	3,636
9	5.8	90.8	94.45	6:00	7.86	3,130
10	9.2	97.7	94.95	16:00	9.45	1,350
6	30	97.0	98.07	23:00	1.72	1,792

3.4 DISCUSSION

This study investigated the tracking of BMMNC to CNS after intravenous administration in animals showing neurological complications of canine distemper.

The protocol used in this study allowed a large number of BMMNC to be efficiently labeled with PKH26, which enabled cell traceability even after IV transplantation. Labeling cells using dyes allows this traceability after transplantation. In this study, the PKH26 staining method, which is a lipophilic dye, offering half-life greater than 100 days, was used (UENG et al., 2007). The fluorescence emitted from the cells is not transferred to neighboring cells but to the daughter cells during cell division (NIU et al., 2009). Other studies using canine BMMNC marked with PKH26 also demonstrated that this dye is effective in tracking transplanted cells (LINON et al., 2014; WU et al., 2015). These results are consistent with those observed by others authors reporting intense and uniform labeling in lymphocytes of rats and human hematopoietic cells (JOHNSSON et al., 1997; OH et al., 1999). The average cell viability showed in this study is also consistent with those reported by other studies showing that this dye has not significant toxic side effect on the cell viability (SLEZAK and HORAN, 1989; JOHNSSON et al., 1997; OH et al., 1999; RIECK, 2003; WU et al., 2015).

The presence of PKH26-labeled cells in CSF showed that BMMNC migrated to CNS after IV administration. Although the blood brain barrier (BBB) provides a protective obstacle into the CNS, BMMNC may cross the BBB by diapedesis through the endothelial cells, or via modifications of tight junctions (ABBOTT et al., 2010). Furthermore, canine distemper virus infection induces a disruption of the BBB, favouring the invasion of cells into CNS (LEMPP et al., 2014), which corroborates the results obtained in this study. Others authors have also demonstrated the migration of PKH26 labeled cells injected IV (BEAVIS and PENNLINE, 1994; JOHNSSON et al., 1997; KAMIYA et al., 2014).

Canine distemper is a systemic disease, so IV BMMNC transplantation was a choice for this study. Direct cells injection into the lesion site is usually more efficient (DIMMELER et al., 2008), but to some organs, such as the brain, systemic administration may be preferable. In addition, strategies for local stem cell delivery increase risks and side effects such as bleeding and tissue injury with direct tissue

injection or occlusion and embolization associated with direct arterial administration (OTT et al., 2005; SHERMAN et al., 2006). Intravenous transplantation of cells would be ideal, given broad biodistribution and easy access (FISCHER et al., 2009). Also, it can be widely applied as a therapeutic strategy in a large number of preclinical and clinical studies (MOAZZAMI et al., 2014; KANAMARU et al., 2015).

However, there is a concern about the number of cells remaining in the area of injury with a systemic IV approach. In this study, the variability in percentage of labeled cells observed in the CSF after transplantation was great, depending on the post-transplant evaluation time, with the highest percentage between 4 and 5 ½ hours after intravenous infusion and subsequent reduction. This can be clearly observed in the results of the dog 6, wherein the total and the percentage of cells in the CSF in the first collection (4 ½ hours after injection) were significantly higher than in the second collection (23 hours after injection). This reduction may indicate that most of the cells were implanted in the lesion site. Kamiya et al. (2008) demonstrated PKH26-positive cells in the brain of the rats with focal ischemia 24 hours after intravenous and intra-arterial BMMNC transplantation. The results of this study also indicate that the migration of BMMNC to the CNS does not seem to be related to the amount of administered cells or the size of the patient. However, in dog 1 the total cells in CSF was significantly lower than the other dogs. This is probably related to concomitant infection because this dog was also suffering from cystitis, and it is likely that the cells were attracted to the site of inflammation (Zhou et al., 2015). Nevertheless, the percentage of labeled cells present in CSF of this dog was greater in the second collection (4 hours after injection) than in the first (1 hour after injection). No other dog presented concomitant infection.

Although the migration of stem cells to CNS has been investigated in rats after intra-arterial and intravenous transplantation of BMMNC (Kamiya et al., 2008) or intravenous transplantation of neural stem cell (Cheng et al., 2015) or when the cells are infused into the CSF (Ide et al., 2010), this migration has not been well-researched in dogs, although stem cells have already been used in dogs for the treatment of spinal cord injury, administered intrathecally or percutaneously near the lesion (Jung et al., 2009; Lee et al., 2011; RYU et al., 2012) or for the treatment of cerebral ischemia, administered intra-arterially near the injury (Chung et al., 2009; Lu et al., 2013). However, there are no published data on BMMNC migration to CNS in dogs with distemper for comparison with the results of this study.

Fischer et al. (2009) investigated the effect of pulmonary first-pass on migration of mesenchymal stromal cells, multipotent adult progenitor cells, BMMNC, and neural stem cells in rats. The authors demonstrated also that can be seen a clear signal in the lung that the majority of infused cells to be trapped inside the pulmonary system, although BMMNC passage was 30-fold increased as compared to mesenchymal stromal cells. The cell size and receptor-mediated adhesion and/or stem cell type appear to be crucial variables for pulmonary stem cells passage.

Furthermore, an efficient intravenous stem cell therapy requires a sufficient number of stem cells reaching the arterial circulation. In order to increase the possibility that IV BMMNC transplanted reached the CNS, a large number of cells was transplanted in this study (1×10^8). However, there is no consensus on the numbers of cells needed for transplantation. In rats, Fischer et al. (2009) used 2×10^6 BMMNC intravenously to evaluate the effect of the pulmonary passage in the stem cell delivery, while, Kamiya et al. (2013) used up to 3×10^7 BMMNC to treatment focal cerebral ischemia. Nonetheless, in a study in dogs, Chung et al. (2009) used 1×10^6 human umbilical cord blood-derived mesenchymal stem cells and LU et al. (2013) used 3×10^6 bone marrow-derived mesenchymal stem cells, intraarterially, in cerebral ischemia and showed a decrease in the infarction volume.

3.5 CONCLUSIONS

This study demonstrated that BMMNC can be efficiently labeled with PKH26 dye and these cells can be monitored after IV transplantation in animals with neurological complications of canine distemper.

A large number of labeled cells observed in the CSF after transplantation indicates that delivery of cells and number of transplanted cells were suitable for this study.

Due to the high percentage of labeled cells observed in this study 5 ½ hours after transplantation, this time is indicated for assessment of cell migration into the CSF. However, further studies with a larger number of receivers must be performed in order to better evaluate the migration of cells into the CNS after intravenous transplantation of BMMNC.

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4 EVALUATION OF SAFETY AND EFFICACY OF BONE MARROW-DERIVED MONONUCLEAR CELLS TRANSPLANTATION FOR THE TREATMENT OF CANINE DISTEMPER SEQUELS

ABSTRACT

Canine distemper is regarded as one of the most important viral disease of dogs, which may cause multifocal demyelinating leukoencephalitis. Neurological complications are progressive and can be permanent. Currently, there is no specific therapy for animals with distemper clinical signs, so new therapeutic interventions are clearly needed and cell transplantation has emerged as a new therapy option. Bone marrow mononuclear cells (BMMNC) have been used to treat various diseases. These cells are chemo-attracted to sites of injury and may produce cytokines and trophic factors that act in tissue regeneration. Nevertheless, a challenge associated with this procedure is the lack of studies on the characterization of canine BMMNC. The objectives of this study were to evaluate safety and efficacy of allogeneic BMMNC transplantation for the treatment of neurological sequels of canine distemper and to contribute with BMMNC characterization data. The design of the study was a single blind randomized controlled trial in 46 dogs divided into treatment group and control group with weekly follow-up for 35 days. Bone marrow was harvested from 23 healthy donors and BMMNC were isolated by density gradient centrifugation. BMMNC from four donors were labeled with anti-CD8a, CD9, CD14, CD29, CD34, CD44, CD45, and CD90 for phenotypic characterization. Dogs from the treatment group received 1 x 10^8 BMMNC intravenously. Regarding the safety of cell transplantation, no serious adverse events related to the procedure were recorded during the 35 days of the study. Functional recovery was evaluated according to the Olby scoring system with some modifications. For the treatment group, the median and interquartile range of the functional score, with 0, 7, 14, 21, 28 and 35 days after injection were 7 (4-13), 9 (5-14), 12 (6-15), 14 (6-15), 14 (6-16), and 14 (6-16) respectively, whereas for the placebo group they were 6 (4-12), 7 (4-13), 7 (4-12), 7 (4-12), 6 (3-12), and 6 (3-12). The differences observed were statistically significant ($p < 0.05$), showing the effectiveness of BMMNC transplantation in dogs with distemper leukoencephalitis.

Key words: Bone marrow mononuclear cells therapy, functional recovery, remyelination.

4.1 INTRODUCTION

Canine distemper virus (CDV) is a member of the genus *Morbillivirus* belonging to the *Paramyxoviridae* family (ALLDINGER et al., 2000; MORO et al., 2003; BEINEKE et al., 2008; BEINEKE et al., 2009) that causes gastrointestinal, respiratory and neurological clinical signs in dogs (MORO et al., 2003; BEINEKE et al., 2008).

Canine distemper is regarded as one of the most important viral disease of dogs (DEL PUERTO et al., 2010). Up to 30% of dogs exhibit neurological complications caused by CDV infection, around 10% die from acute encephalitis (RUDD et al., 2006) and the dogs that recover may show lifelong residual signs (BEINEKE et al., 2009). Neurological complications often occur in parallel or subsequently to other organ affections (LEMPP et al., 2014). Canine distemper virus causes acute and progressive neurological disease in both gray and white matter (VON RÜDEN et al., 2012). The invasion of CDV in central nervous system (CNS) is more likely to occur by hematogenous route with ependymal and subependymal white matter infection (BEINEKE et al., 2009). The pathogenesis of myelin loss caused by canine distemper demyelinating leukoencephalitis, is probably a biphasic process (FRISK et al., 1999; ALLDINGER et al., 2000). Canine distemper leukoencephalitis usually starts with grey matter infection followed by antigen spread into the white matter (LEMPP et al., 2014). Acute leukoencephalitis lesions are histologically characterized by slightly increased numbers of astrocytes and microglia (ULRICH et al., 2014), wherein 95% of all infected cells are astrocytes (LEMPP et al., 2014). Alldinger et al. (2000) showed a slight gliosis and vacuolation as the only visible lesion in acute distemper, without demyelination. Immunopathological processes and early axonal degeneration of canine distemper leukoencephalitis are similar to other demyelinating diseases, such as multiple sclerosis (LEMPP et al., 2014).

Leukoencephalitis with multifocal demyelination is the most common CNS disorder associated with CDV infection (BEINEKE et al., 2008; BEINEKE et al., 2009; LEMPP et al., 2014). Frisk et al. (1999) demonstrated the presence of interleukin (IL)-10 mRNA in dogs' cerebrospinal fluid (CSF) with non-inflammatory lesions as well as inflammatory lesions. The authors also showed an up-regulation of IL-6,

tumor necrosis factor (TNF) and TGF (transforming growth factor) in dogs with subacute or chronic leukoencephalitis.

There is no specific therapy for animals showing distemper clinical signs (DEEM et al., 2000; GREENE and APPEL, 2006). However, nonspecific and supportive treatments are beneficial to reduce mortality (GREENE and APPEL, 2006). Careful attention to nutrition and hydration is an important quality-of-life issue.

New therapeutic interventions are clearly needed, and cell transplantation has emerged as a new therapy option. Stem cell therapy has been studied as therapeutic alternative for several diseases, showing good results mainly in the treatment of inflammatory and ischemic diseases (SOARES et al., 2004; MATHIEU et al., 2009) and even neuronal damage (OHTA et al., 2004; JUNG et al., 2009; BRITO et al., 2010).

Bone marrow mononuclear cells (BMMNC) contain a subset of hematopoietic progenitor cells and several cells of non-hematopoietic lineage (KAMIYA et al., 2008; GIRALDI-GUIMARÃES et al., 2009). Clinical studies have focused on the use of the entire BMMNC fraction, assuming that functional effects depend on a correct balance among multiple cell types and stem cell precursors (MATHIEU et al., 2009). In addition, the cells and precursors of the BMMNC fraction produce large amounts of cytokines and trophic factors (BATTISTELLA et al., 2011). Also, BMMNC have are chemo-attracted to sites of injury, and may produce cytokines and trophic factors (GIRALDI-GUIMARÃES et al., 2009). These cells have been used to treat various diseases and their therapeutic effect is possibly due to an increased angiogenesis (TAGUCHI et al., 2004). Bone marrow mononuclear cells may be easily obtained by a low-cost method and isolated in a short time just before transplantation, minimizing the risks of contamination. Besides, the isolation of BMMNC does not require a laboratory with a more complex structure, which makes it a more accessible procedure in clinical trials with animals. Another advantage that has been exploited in BMMNC regards rapidity that such cells can be used, due to the fact that they do not need to be cultured (KAMIYA et al., 2008; GIRALDI-GUIMARÃES et al., 2009; KAMIYA et al., 2014). Furthermore, it was demonstrated that, when administered intravenously, BMMNC resulted in a 30-fold pulmonary passage increase when compared to other types of stem cells, such as mesenchymal stem cell (MSC) (FISCHER et al., 2009).

Despite the fact that BMMNC have been used as regenerative strategies for tissue injuries, there are many challenges associated with this therapeutic approach. One of them is the characterization of canine BMMNC that is poorly defined compared to human cells. The limited knowledge of phenotype of the canine BMMNC is partly due to the lack of species-specific monoclonal antibodies for many of the cellular markers used for the characterization of human cells. As a result, non-validated cross-reacting antibodies from other species, such as human and mouse are commonly used (SCREVEN et al., 2014). These limitations hamper the characterization of canine BMMNC, which is of great importance for the use of these cells in research and clinical trials. On the other hand, some canine monoclonal antibodies are commercially available and some cross-reacting antibodies have been compared to those of the target species (COBBOLD and METCALFE, 1994; ALLDINGER et al., 1999; SCHUBERTH et al., 2007), but there are no studies in the literature on the characterization of BMMNC. Therefore, the use of specific or validated antibodies for canine cells characterization may contribute to the definition of a panel of cellular markers to identify canine BMMNC. The markers used in this study to characterize canine bone marrow mononuclear cells are presented in Table 4.1.

Table 4.1 Description of markers used to characterize canine bone marrow mononuclear cells.

CD marker	Population of labelled cells	Comments	References
CD8a	NK cells and thymocytes	CD8 blinds to MHC class I	Moore et al. (1992) Lin et al. (2010)
CD9	Young B cells, activated lymphocytes, platelets, eosinophils, basophils, monocytes and MSC	Not expressed in hematopoietic progenitors cells	Boucheix et al. (1991) Anton et al. (1995) Jung et al. (2009)
CD14	Macrophage, monocyte and endothelial progenitor cells	Low expression in MSC	Lai et al. (1998) Rehman et al. (2003) Dvorakova et al. (2008)
CD29	MSC, endothelial cells, monocytes, lymphocytes, NK cells, eosinophil, basophil and platelets	Not expressed in neutrophils	De Schauwer et al. (2011) Takemitsu et al. (2012) Wong et al. (1995)
CD34	Hematopoietic stem cells and endothelial progenitor cells	Highly variable expression	Bara et al. (2015) Henrich et al. (2015)
CD44	MSC and others cells with transendothelial migratory capacity	Not expressed in platelets	Lewinsohn et al. (1990) Patel et al. (1997) Screven et al. (2014)
CD45	Hematopoietic cells (mature and immature) and endothelial progenitor cells	Not expressed in red blood cells and platelets	Brodersen et al. (1998) Henrich et al. (2015)
CD90	MSC, T lymphocytes and monocytes	Weak expression in granulocytes	Cobbold and Metcalfe (1994) Lai et al. (1998) Screven et al. (2014)

NK: natural killer; MSC: mesenchymal stem cells.

The aims of this study were evaluate the safety and efficacy of allogeneic BMMNC transplantation for the treatment of neurological sequels of canine distemper and contribute to characterization of canine BMMNC.

4.2 MATERIALS AND METHODS

This work was performed according to protocol 030/2011, approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Federal University of Paraná.

4.2.1 BONE MARROW DONORS

Twenty tree healthy mixed-breed dogs aged between 18 and 36 months were used as bone marrow donors. The routine pretreatment evaluations included a complete medical history, physical examination and hematology and biochemical profiles (serum proteinogram, urea, creatinine, alkaline phosphatase and alanine aminotransferase) to assess the health status of donors.

4.2.2 BONE MARROW HARVESTING AND BMMNC ISOLATION

Animals received general anesthesia with propofol (Cristalia) and meperidine (Dolosal: Cristalia) for bone marrow aspiration, which was performed by puncturing the iliac crest with disposable hypodermal needles (16 G) and disposable 10 mL syringes containing 1.0 mL heparin (5,000 IU/mL) anticoagulant solution. All bone marrow was diluted in Dulbecco's modified Eagle medium (DMEM: Gibco) at a ratio 1:3, and BMMNC were isolated by Ficoll-Hypaque (Sigma Chemical) density gradient (density 1,077 g/cm^3) as previously reported (BÖYUM, 1967). After isolation, the BMMNC were washed with medium to remove excess Ficoll-Hypaque and finally resuspended in medium. Cell counting and cell viability were determined using Trypan blue (Sigma Chemical) exclusion test in a Neubauer chamber.

4.2.3 EXPERIMENTAL GROUPS

This study was a single blind randomised controlled trial with follow-up 35 days after injection. Forty six male and female mixed-breed dogs with neurological signs of canine distemper were randomly assigned to two groups for cell transplantation: Group 1: treatment group with 23 animals that received 1 x 10^8

BMMNC resuspended in 4.0 mL of DMEM, intravenously; Group 2: placebo group with 23 animals that received 4.0 mL of DMEM intravenously. The exact ages of the dogs were unknown, but they were estimated to be between six months and six years old.

The inclusion criteria were presence of canine distemper with neurological sequels that affect ambulation, such as proprioceptive deficit and/or ataxia, paresis, or paralysis, onset of neurological signs for more than 10 days and less than three months, no progression of clinical signs 10 days before injection (LEMPP et al., 2014), and without concomitant disease. According to these criteria, only dogs showing subacute or chronic lesions were included in this study.

Canine distemper was confirmed by the detection of CDV antigen using a commercial immunochromatographic test (Alere), or Real-time polymerase chain reaction (carried out in commercial laboratory facilities: IDEXX Laboratories Inc.), and detection of CDV antibody in the CSF using a commercial immunochromatographic test (Alere). The progression of clinical signs was considered in all cases.

4.2.4 PHENOTYPIC CHARACTERIZATION OF BMMNC

For phenotypic characterization, samples of BMMNC of four donors were incubated with commercial monoclonal antibodies to analyze canine cell-surface expression of typical marker proteins: CD45 and CD44 conjugated with fluorescein isothiocyanate (FITC), CD90, CD29, CD34 and CD9 conjugated with phycoerythrin (PE); CD14 conjugated with allophycocyanin (APC) (BD Pharmingen, CA, USA) and CD8a conjugated with peridinin chlorophyll protein (PerCP) (BD Pharmingen, CA, USA). Cells of four donors were washed with PBS, and incubated in the dark for 30 minutes at room temperature with the respective antibody. Cells were then washed with phosphate-buffered saline (PBS: Gbico) and resuspended in 500 µL of 1% formaldehyde solution. Mouse isotype IgG1 antibodies were employed as controls (BD Pharmingen). Approximately twenty thousand labeled cells were passed through a FACS Calibur flow cytometer (Becton Dickinson) and were analyzed by FlowJo software (Flowjo).

4.2.5 BMMNC TRANSPLANTATION

1 x 10^8 BMMNC were resuspended in 4.0 mL DMEM for transplantation in the animals of treatment group. The dogs belonging to the treatment group received intravenous transplantation of resuspended BMMNC through the cannulated cephalic vein, while the animals of placebo group underwent the same procedure, and were injected 4.0 mL of DMEM.

4.2.6 EVALUATION OF SAFETY AND EFFECTIVENESS POST-TRANSPLANT

The presence of adverse effects such as gastrointestinal disorders, cardiopulmonary complications, pruritus or skin lesions and behavior changes was observed during the 35 days of the study to assess the safety of cell transplantation.

The efficacy analysis was based on behavioral assessment that was performed before injection and at 7, 14, 21, 28, and 35 days after injection to assess the functional recovery of ambulation. Functional recovery was evaluated according to Olby scores (OLBY et al., 2001) with some modifications (Appendix I). Tree points were added for normal response to assess proprioceptive response in the forelimb; two points were added when the response was reduced with ventral support; one point were added when the response was decreased weight-bearing, and zero points for lack of proprioceptive response.

4.2.7 STATISTICAL ANALYSIS

Continuous variables were presented as median ± standard error, and it was showed also the interquartile range for each group. Categorical variables were presented as frequency and percent. Scores regarding functional recovery were analyzed by nonparametric Fisher test, between groups and between times, and values of $p < 0.05$ were considered statistically significant. Analysis was performed with the SPSS V.14 software package.

4.3 RESULTS

4.3.1 CHARACTERIZATION OF CANINE BMMNC

The bone marrow harvest volume varied from 18 to 36 mL. The average number of BMMNC cells obtained per mL was 1.14 x 10^7 (±0.32 x 10^7), and the average cells viability was 91.7% (± 5.6%).

BMMNC cell-surface antigen expression was evaluated by flow cytometry in four samples (Figure 4.1). The average cell viability was 83.36% (±6.84%). The results were expressed as the intensity of expression of these markers in cells. With few exceptions, all four sources displayed similar immunophenotypes for the markers analyzed (Table 4.2).

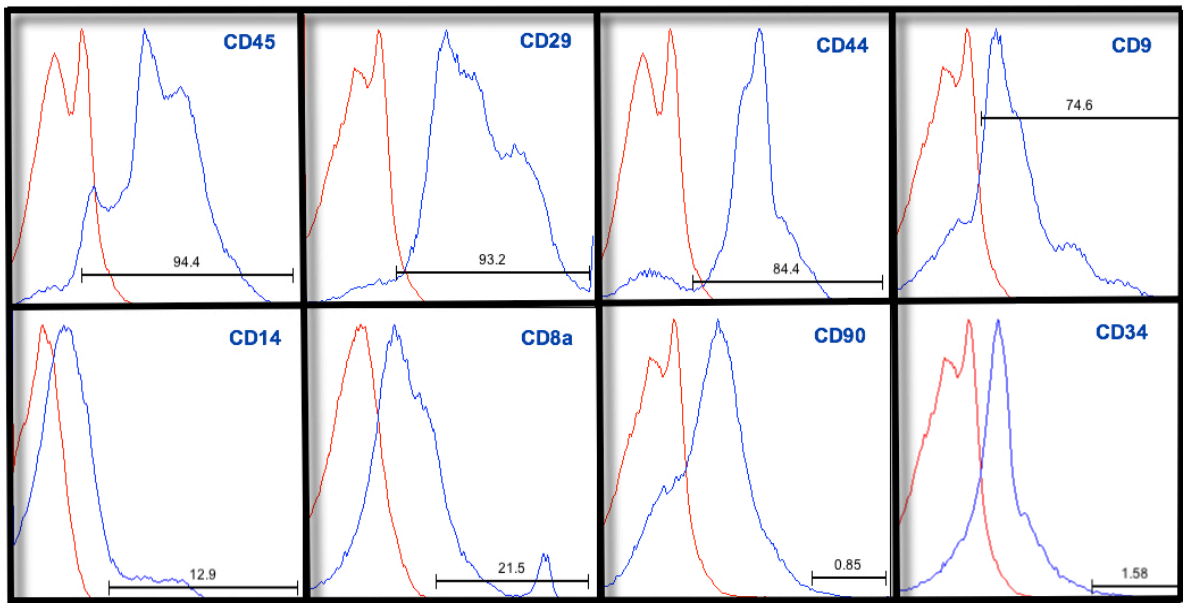

Figure 4.1 Immune phenotype by flow cytometry. The bone marrow mononuclear cells were labeled with antibodies against the indicated antigens, and analyzed by flow cytometry. Representative histograms of sample 4 are displayed. On the y-axis is the % of Max (the cell count in each bin divided by the cell count in the bin that contains the largest number of cells) and the x-axis is the fluorescence intensity in a log (10^0 – 10^4) scale. Isotype control is shown as a thick red line histogram

Table 4.2 Expression of surface proteins of mononuclear cells derived from four samples of canine bone marrow analyzed by flow cytometry.

Canine	Cell surface molecule labelling (%)									
BMMNC	CD8a	CD9	CD14	CD29	CD34	CD44	CD45	CD90	7AAD	Anexina
1	18.50	69.60	38.90	96.50	1.04	92.70	96.70	0.40	23.70	3.01
2	16.90	88.80	35.60	97.30	1.47	91.20	98.50	1.41	21.00	5.05
3	18.00	84.90	8.92	97.40	0.50	90.10	87.70	1.83	12.70	10.60
4	21.50	74.60	12.90	93.20	1.58	84.40	94.40	0.85	9.16	0.61

4.3.2 ALLOGENEIC TRANSPLANTATION OF BMMNC

Regarding the safety of cell transplantation, no adverse events related to the procedure were observed in the 23 patients analyzed in the BMMNC group, during the 35 days of the study, although the study population was in poor health condition.

In the clinical evaluation, the functional score of each dog was evaluated weekly. The median and interquartile ranges of the functional scores are presented in Table 4.3. In the treatment group, it was observed that animals with intermediate to high scores tended to recover, increasing median of the group's score, whereas those with low scores tended to stabilize. In the placebo group, it was observed a stabilization of the scores from the first evaluation; however, some animals also recovered, reaching the maximum score. Nonetheless, the observed differences between treatment group and placebo group were statistically significant ($p < 0.05$). Differences between times statistically significant ($p < 0.05$) were observed only in the treatment group (Table 4.3). Overall comparison of the group scores is shown in Figure 4.2.

Table 4.3 Median and interquartile range of the functional scores performed to evaluate the functional recovery of dogs with distemper sequels. The animals of the treatment group received 1 x 10^8 BMMNC resuspended with 4-mL DMEM intravenously, whereas the animals of the placebo group received 4-mL DMEM intravenously.

Groups	Days after injection					
	0	7	14	21	28	35
Treatment	7 (4-13) **Aa**	9 (5-14) **Aa**	12 (6-15) **Ab**	14 (6-15) **Ab**	14 (6-16) **Ab**	14 (6-16) **Aa**
Placebo	6 (4-12) **Aa**	7 (4-13) **Aa**	7 (4-12) **Ba**	7 (4-12) **Ba**	6 (3-12) **Ba**	6 (3-12) **Ba**

Different letters between lines indicate significant difference in Fisher test ($p < 0.05$).

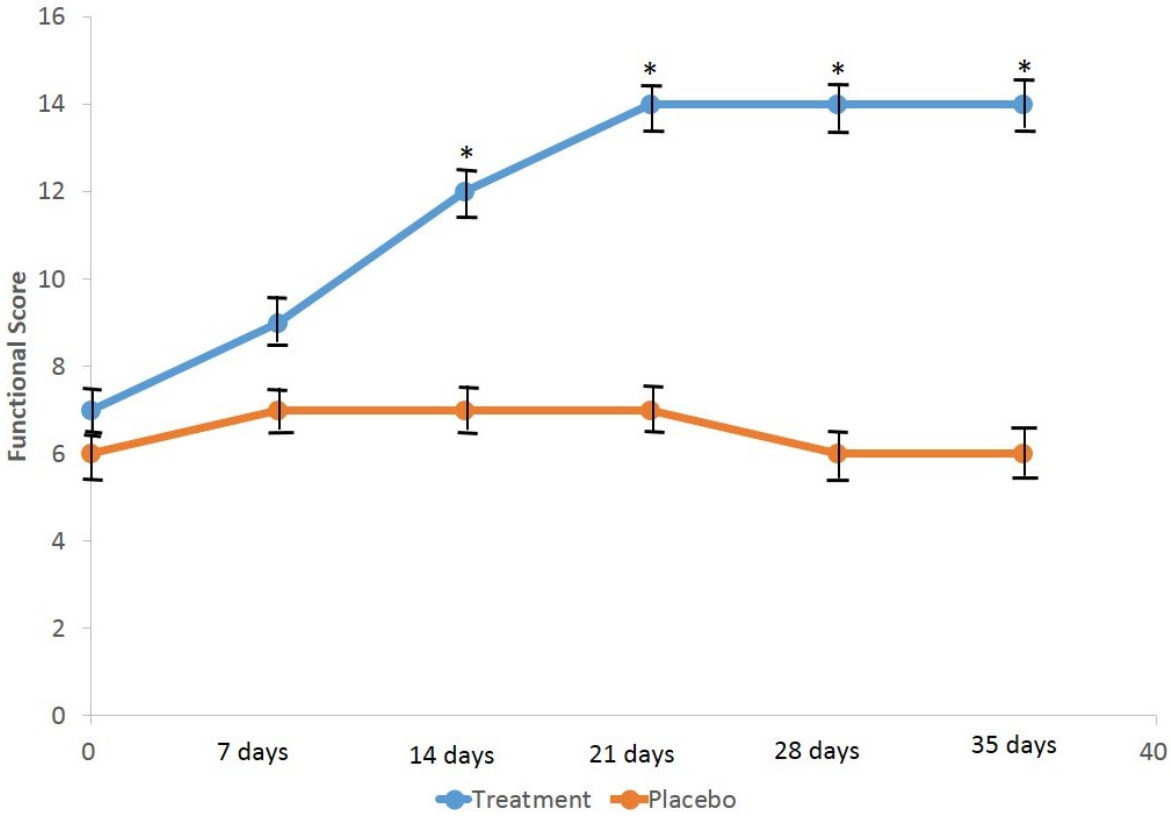

Figure 4.2 Median and standard error of functional scores follow-up at 35 days after BMMNC injection (treatment group) or DMEM injection (placebo group). The scores in the treatment group were significantly higher from 14 days after cells administration (* $p < 0.05$).

4.4 DISCUSSION

Bone marrow mononuclear cells comprise a pool of different cells types that can act significantly when used for the treatment of many diseases. Several evidences can be obtained from animal and human models (LARIONOV et al., 2011; MOAZZAMI et al., 2014). Moreover, the bone marrow was obtained from young animals, aged between 18 and 36 months, because there is an age-related decline in overall BMMNC “fitness” which could interfere in cell-based therapies (STOLZING et al., 2007).

The number of BMMNC isolated from canine bone marrow in this study (1.14 x 10^7 BMMNC / mL ± 0.32 x 10^7) was consistent with those reported by others authors (KAMISHINA et al., 2008; SPENCER et al., 2012). The characterization of these cells is well established in humans (CAPIOD et al., 2009; BARA et al., 2015), but in the veterinary field the lack of species-specific monoclonal antibodies for many of the cellular markers makes it more difficult to characterize BMMNC. Therefore, the precise nature of the cellular product obtained from bone marrow used in therapeutic strategies remains unclear. In this study, it was evaluated the expression of some cell surface molecules, some of which are commonly used for mesenchymal stem cells characterization as CD8a, CD9, CD29, CD44 and CD90 (JUNG et al., 2009; KANG and PARK, 2014; QUINTANILHA et al., 2014; SCREVEN et al., 2014; TAKEMITSU et al., 2012) as well as CD14, CD34 and CD45, which are negative markers for mesenchymal cells. The results of the expression of CD9, CD29, CD34 and CD44 were consistent with the results obtained by other authors for characterization of mesenchymal stem cells harvested from dogs. However, the results of CD90 expression were significantly lower then those previously reported (ALVES et al., 2014; SCREVEN et al., 2014).

In relation to markers with strongly positive result, it was observed that the expression of CD29 ranged between 93.20 and 97.40% in this study, consistent with the results obtained by Takemitsu et al. (2012) and Alves et al. (2014) that showed high expression of CD29 (98.41% ±0.53% and 96.00% ±3.00% respectivelly) in canine bone marrow MSC. Nonetheless, Screven et al. (2014) showed that CD29+ cells ranged between 2.06 and 2.96% also in canine bone marrow MSC. It is noteworthy that CD29 is a migration and adhesion molecule expressed in MSC,

endothelial cells, lymphocytes, monocytes, eosinophil, basophil, platelets and NK, but is not expressed in neutrophils (DE SCHAUWER et al., 2011; TAKEMITSU et al., 2012; WONG, 1995).

The expression of CD45 obtained in this study ranged from 87.70 to 98.50%. CD45 is a pan leukocyte marker and is also expressed in endothelial progenitors cells and hematopoietic stem cells, but is not expressed in platelets and MSC-precursor; therefore, this marker is used as a negative marker for MSC (BRODERSEN et al., 1998; HENRICH et al., 2015). Takemitsu et al. (2012) and Alves et al. (2014) obtained expression of CD45 in 0.24% (±0.07%) and 1.45% (±0.60%) of canine bone marrow MSC, respectively. Although Henrich et al. (2015) have used CD45 expression to characterize human BMMNC, these authors showed only concomitant expression of this marker with CD34 or with CD34 and CD133.

The result of CD9 expression obtained in this study (69.60 to 88.80%) is consistent with those reported by Jung et al. (2009), who used this marker to characterize canine bone marrow MSC. CD9 is expressed in young B cells, platelets, eosinophils, basophils and activated T lymphocytes (BOUCHEIX et al., 1991; ANTON et al., 1995), but is not expressed by hematopoietic progenitors or non-activated lymphocytes (BOUCHEIX et al., 1991).

In this study, a strong expression of CD44 (84.40 to 92.70%) was also observed in canine BBMNC. This result is consistent with those reported by Takemitsu et al. (2012) that showed expression of CD44 in 98.90% (0.25%) of canine bone marrow MSC. However, Screven et al. (2014) obtained CD44+ cells that ranged between 19.90 and 34.20% in canine bone marrow MSC. CD44 is a multifunctional cell adhesion molecule expressed in MSC and others cells with transendothelial migratory capacity as antigen-activated T cells. CD44 antigen functions include cell-cell and cell-substrate adhesion, lymphocyte homing, hematopoiesis, angiogenesis, cytokine release and hyaluronic acid metabolism and degradation (LEWINSOHN et al., 1990; ROA et al., 2001). Nonetheless, CD44 is not expressed in platelets (PATEL et al., 1997).

In this study, a moderate expression of CD8a (16.90 to 21.50%) and CD14 (12.90 to 38.90%) was also observed. CD8a is a surface glycoprotein expressed in NK cells and thymocytes that facilitates specific antigen recognition. In addition, CD8a antibody binds to MHC class I and plays a role in mature T cell activation

(Moore et al., 1992; Lin et al., 2010). Screven et al. (2014) showed great variation on expression of MHC class I (4.07 to 12.80%) in canine bone marrow-derived MSC.

Regarding the markers with weak expression in this study, CD34+ cells and CD90+ cells ranged from 0.50 to 1.58% and from 0.40 to 1.83%, respectively. The result of CD34 expression is consistent with the result obtained by Henrich et al. (2015) that showed expression of this marker around 1-2% in human BMMNC. Nonetheless, Screven et al. (2014) obtained expression of CD34 between 1.11 and 2.65% in canine bone marrow-derived MSC. CD34 is expressed in hematopoietic stem cells and endothelial progenitor cells, but is considered as a negative marker for MSC (Bara et al., 2015; Henrich et al., 2015). Our results for CD90 expression are significantly lower than those obtained by Screven et al. (2014), that showed CD90+ cells ranging from 17.1 to 27.8% in canine bone marrow-derived MSC. However, this marker is expressed in MSC, T lymphocytes and monocytes, with weak expression in granulocytes (Cobbold and Metcalfe, 1994; Lai et al., 1998; Screven et al., 2014). Henrich et al. (2015) have not used this marker to characterize human BMMNC. Therefore, the results of this study are consistent with those reported by Henrich et al. (2015) that BMMNC are a heterogenous mixture of diverse cell types. Nevertheless, these results should be confirmed by other researchers, given the limited number of studies in the literature about canine BMMNC characterization.

The absence of adverse events directly related to the procedure indicates that it is feasible, provided that the protocols implemented in this study are followed, as they aim at the patient's protection and safety. Other studies also indicate that intravenous infusion of BMMNC is safe (SAVITZ et al., 2011; PRASAD et al., 2014).

During this study, functional recovery was evaluated weekly according to the Olby scores (OLBY et al., 2001) with some modifications. The Olby scoring system is frequently used to evaluate functional recovery of dogs with spinal cord injury (OLBY et al., 2004; WEBB et al., 2004; JUNG et al., 2009; RYU et al., 2009). In this study, some modifications were necessary because this scoring system was developed to evaluate spinal cord injuries in dogs. Nonetheless, in canine distemper leukoencephalitis, the CDV spreads via CSF, and may infect ependymal lining cells of the ventricles, glial cells and neurons (LEMPP et al., 2014). Therefore, in CDV infections the dogs can show complications that affect ambulation visualized in the forelimb.

Dogs receiving BMMNC transplantation showed recovery of functional score after 14 days compared to the placebo group. Nonetheless, some animals of the placebo group also recovered neurological functions, and one of them reached maximum score. Remyelination is a possible phenomenon in demyelinating disease, even if it does not lead to complete recovery. The spontaneous healing process is probably due to an upregulation of anti-inflammatory cytokines with low production of pro-inflammatory cytokines (BEINEKE et al., 2008; LEMPP et al., 2014). However, the observed differences statistically significant in the medians of functional score between treatment group and placebo group, with similar amplitude variation, suggest that the treatment had influenced the recovery. The influence of treatment can also be observed by the statistically significant differences between moments.

There is evidence that one of the beneficial effects of bone marrow cells recruited by injured tissues results from a paracrine mechanism. This includes the production of significant amounts of cytokines and growth factors with different actions at the tissue level, including prevention of apoptosis, cytoprotection of native viable cells, anti-inflammatory effects, reduction of fibrosis, and recruitment of specific stem cells, leading to a robust stimulation of angiogenesis and tissue regenerative mechanisms directly mediated by resident progenitor cells (KUBAL et al., 2006; TAKAHASHI et al., 2006).

It has been well reported that the cytokine expression has an important role in induction, progression and possible remission of pathologic changes in the brain and spinal cord (FRISK et al., 1999). In distemper leukoencephalitis, the resident activated cells as well as attracted leukocytes can produce pro- and anti-inflammatory cytokines (LEMPP et al., 2014). Beineke et al. (2008) demonstrated, in subacute and chronic distemper leukoencephalitis, strong up-regulation of interleukin (IL)-6, IL-8, IL-12, and tumor necrosis factor (TNF)-α in the cerebra of distemper dogs, with mild to moderate up-regulation of the major histocompatibility complex class II (MHC-II) antigen, while IL-1, IL-2, and interferon (IFN)-γ were not expressed. However, Frisk et al. (1999) showed an up-regulation of IL-10 and IL-6 followed by TNF-α and transforming growth factor (TGF)-β1, showing expression of IL-12 and IFN-γ in only one dog. IL-12 is a pro-inflammatory cytokine secreted by neurodegenerative activated macrophages, whereas, neuroregenerative activated macrophages may produce anti-inflammatory cytokine, including the expression of

TGF-β and IL-10, which are beneficial effects to CNS recovery (LEMPP et al., 2014). Nevertheless, the immunomodulatory properties of cells present in the bone marrow could induce behavioral recovery after transplantation. Furthermore, BMMNC therapy can significantly decreases inflammation and fibrosis (SOARES et al., 2004). Therefore, the clinical improvement in the group that received BMMNC transplantation could be related to the immunomodulation effect and the creation of favorable environment for remyelination.

The animals received a single injection of BMMNC for the design of this study. However, after 21 day it was observed the stabilization of the functional scores. This suggests that multiple doses could be inserted at this point.

The above-mentioned results open up new perspectives in the treatment of canine distemper through the regenerating capacity of stem cells. Our data suggest that intravenously transplantation of BMMNC in patients with canine distemper sequels is feasible, potentially safe and effective, bringing symptomatic relief and improving the quality of life.

On the other hand, further studies focused on elucidating the mechanisms leading to improvement after therapy are clearly needed. While the accurate identification of subpopulations involved in the repair process is an important issue, it is essential to evaluate cautiously whether other methods for purifying these subpopulations, indeed more expensive than the simple purification of the mononuclear fraction used today in clinical trials, will bring more benefit to patients.

Considering the role of cytokines in the pathophysiology of this disease, we suggest that further studies may be conducted to monitor the expression of cytokines before and after BMMNC transplantation in distemper leukoencephalitis.

4.5 CONCLUSIONS

This study shows the potential of BMMNC use in dogs suffering from distemper leukoencephalitis. A significant difference was observed between the groups that received the cells and the placebo group, demonstrating the effectiveness of these cells for the treatment of the patients.

The maximum treatment effect, with only one BMMNC transplant, was observed between 14 and 21 days.

Regarding the safety of BMMNC transplantation, no adverse events related to treatment were recorded during this study.

Characterization of cell surface molecule may contribute to defining a panel of cellular markers to identify canine BMMNC.

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5 CONSIDERAÇÕES FINAIS

Os resultados deste estudo indicam possibilidades promissoras para utilização do transplante alogênico de células mononucleares derivadas da medula óssea de cães para o tratamento de sequelas de cinomose.

No primeiro capítulo desta tese foi demonstrado que o CPDA-1 pode ser considerado como o anticoagulante para a colheita de medula óssea, principalmente quando as células forem isoladas e utilizadas imediatamente após a colheita. No entanto, ainda são necessários estudos adicionais para avaliar a influência do CPDA-1 em células cultivadas.

No segundo capítulo foi demonstrado que as células mononucleares de medula óssea (CMNMO) podem ser eficientemente marcadas com o corante PKH26 e que essas células podem ser monitoradas após transplante intravenoso em animais com complicações neurológicas da cinomose, sendo demonstrada a presença de células transplantadas no líquido cefalorraquidiano (LCR) quantitativamente por citometria de fluxo e qualitativamente em microscopia de fluorescência. Contudo, uma limitação do estudo foi o número relativamente pequeno de receptores disponíveis para receber células marcadas com PKH. Por esse motivo, não foi possível avaliar determinados momentos após o transplante, nem aumentar o número de repetições.

No terceiro capítulo foi demonstrado o potencial terapêutico das CMNMO em cães com sequelas de cinomose, sendo avaliada eficácia e segurança do transplante. Foi demonstrada diferença estatisticamente significativa entre o grupo que recebeu células e o grupo que recebeu placebo, com efeito máximo observado entre 14 e 21 dias após o transplante. Além disso, não foram registrados eventos adversos relacionados ao tratamento durante este estudo.

APPENDIX I

Parameters to evaluate of the functional score (Scale of 0 to 17). Adapted from Olby et al. (2003).

Body posture and attitude:
0 – The dog cannot support its weight.
1 – Weight bearing only with assistance.
2 – Weight-bearing without assistance (less than 10 seconds). But, the dog cannot get up without help.
3 – Weight-bearing without assistance (more than 10 seconds). But, the dog cannot get up without help.
4 – Weight-bearing without assistance.

Proprioceptive response of the pelvic limbs:
0 – Lack of proprioceptive response.
1 – Proprioceptive response decreased with the dog supported weight bearing.
2 – Proprioceptive response reduced with ventral support.
3 – Normal proprioceptive response.

Proprioceptive response of the forelimbs:
0 – Lack of proprioceptive response.
1 – Proprioceptive response decreased with the dog supported weight bearing.
2 – Proprioceptive response reduced with ventral support.
3 – Normal proprioceptive response.

Voluntary movements of the pelvic limbs and ambulation:
0 – No pelvic limb or tail movement.
1 – No pelvic limb movement but voluntary tail movement.
2 – Voluntary movement of pelvic limbs and tail, but no ability to walk.
3 – Voluntary movement of pelvic limbs and tail, but ataxic gait.
4 – Normal gait.

Deep pain sensation:
0 – No deep pain sensation.
1 – Large decrease of deep pain sensation (Dog only retracts the clamped limb).
2 – Decrease of deep pain sensation (Dog only retracts the clamped limb).
3 – Normal deep pain sensation.