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Anticoagulants on yield of bone marrow-derived mononuclear cells harvested from dogs

Anticoagulantes no rendimento de células mononucleares da medula óssea de cães

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ABSTRACT

Cell therapy with bone marrow-derived mononuclear cells is an alternative to therapy with mesenchymal stem cell cultures. The aim of the present research 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. Means were compared using Student's paired t-test. Samples harvested with CPDA-1 yielded an average of $5.16x10^6$ ($\pm1.76x10^6$) to $20.20x10^6$ ($\pm1.55x10^6$) mononuclear cells/mL, whereas the yield of samples harvested with heparin varied between $4.56x10^6$ ($\pm0.69x10^6$) and $24.30x10^6$ ($\pm2.12x10^6$) mononuclear cells mL^{-1}. By flow cytometry, 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.

Key words: *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. O objetivo deste 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 $5,16 \times 10^6$ ($\pm1,76x10^6$) a $20,20x10^6$ ($\pm1,55x10^6$) células mononucleares mL^{-1}, enquanto que, nas amostras coletadas com heparina, o rendimento variou entre $4,56x10^6$ ($\pm0,69x10^6$) a $24,30x10^6$ ($\pm2,12x10^6$) 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. Recomendam-se estudos adicionais para avaliar melhor a eficácia do CPDA no isolamento de células mononucleares.

Palavras-chave: *célula-tronco mesenquimal, célula-tronco multipotente, imunofenotipagem.*

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 (MSCs) have potent immunomodulatory and anti-inflammatory effects, acting on tissue repair by means of local environment modulation, endogenous progenitor cell

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activation, direct cell-to-cell interaction and secretion of several factors (CHEN & TUAN, 2008).

MSCs 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., 2004) 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 *in vitro* anticoagulants on the viability of lynphocytes, KLEIN et al. (1991) showed that CPDA is better than heparin as regards the protection of lymphocytes. However, the comparison of anticoagulant effects 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 (JUNG et al., 2009; QUIMBY et al., 2011). Despite KAMISHINA et al. (2008) estimated the frequency of canine BMSCs to be 0.0042 (±0.0019%). These researchers observed a great variability among bone marrow samples.

Phenotypic characterization of canine bone marrow-derived MSCs 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 (JIANG at al., 2004).

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). Clones are also heterogeneous and 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, evaluated by gene sequencing, is an important criterion for evaluating the adequacy of such lineages in medical practice (SVERDLOV & MINEEV, 2013). However, this type of evaluation makes cell therapy highly expensive.

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

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 marrow bonewere withdrawn from each dog, one containing 2.0mL CPDA-1 (citrate phosphate dextrose adenine-1) anticoagulant solution and the other 2.0mL anticoagulant solution composed of 1.0mL heparin (5000 IU/mL) and 1.0mL 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 1.5mL Histopaque 1119 (Sigma®) and 1.5mL 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 30min. After centrifugation, the layer of mononuclear cells was collected with a serological pipette and washed twice with 5.0mL DMEM by centrifugation at 400*g* for 10min. After washing, cells were resuspended in PBS and evaluated in a hematology analyzer (BC2800Vet, Mindray).

For phenotypic assay of mononuclear cells the antibodies were used 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 $1.0x10^6$ cells from each sample were resuspended in 10µL PBS containing 1.0µL Anti-Human-CD9 (conjugated to R. Phyoerythrin) (AbDserotec; IgG concentration

1.0mg mL^{-1}) and 1.0µL Anti-Dog-CD44 (conjugated to FITC) antibodies (AbDserotec; IgG concentration 0.05mg mL^{-1}) and incubated for 40min. Cells were subsequently resuspended in 400µL PBS and analyzed by flow cytometry (FACSCalibur, BD).

Results for the number of obtained cells mL^{-1} 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.

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 1). Specifically, samples harvested with CPDA-1 yielded an average of $5.16x10^6$ ($\pm1.76x10^6$) to $20.20x10^6$ ($\pm1.55x10^6$) mononuclear cells/mL, whereas samples harvested with heparin yielded between $4.56x10^6$ ($\pm0.69x10^6$) and $24.30x10^6$ ($\pm2.12x10^6$) mononuclear cells mL^{-1}.

By flow cytometry, the mean percentage of CD9-stained cells varied 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) 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).

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 there is 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 has 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.

In the present research it 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.,

Table 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.

Table 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.

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 MSCs were 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) have 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 MSCs 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, CD9 and CD44 staining concomitantly, which characterizes mesenchymal cells, was 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., 2004; 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.

CONCLUSION

The results of the present study indicate promising possibilities to use of CPDA-1 to harvest bone marrow with the aim to isolate mononuclear cells. 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 Universidade Federal do Paraná, Southern Brazil (CEUA-SCA/UFPR number 030/2011).

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