Eder Zucconi

**Isolamento e caracterização de células-tronco
caninas do cordão umbilical para uso potencial
em transplantes de cães distróficos**

São Paulo

2009

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da Universidade de São Paulo, para a
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tenho como exemplo de vida e por
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Epígrafe

“São fúteis e cheias de erros as ciências que não nasceram da experimentação, mãe de todo conhecimento”. (Leonardo da Vinci)

Nota do Autor

A proposta inicial deste estudo consistiu em isolar e caracterizar células-tronco provenientes do cordão umbilical canino que tenham capacidade de se diferenciar *in vitro* em músculo esquelético. Além disso, o potencial miogênico destas células foram avaliadas *in vivo* em cães “Golden Retriever Muscular Dystrophy” (GRMD), modelo animal relevante para a distrofia muscular de Duchenne.

Esta tese foi organizada no formato de capítulos, sendo o primeiro deles composto por uma revisão geral e objetivos deste estudo. Os capítulos seguintes consistem de artigos científicos publicados em revistas internacionais ou em processo de redação / publicação, os quais descrevem os resultados obtidos durante o período de doutoramento. Nos anexos encontram-se os trabalhos relacionados com o tema desta tese, também já publicados em revistas internacionais.

Inicialmente nosso estudo começou com as células-tronco hematopoiéticas provenientes do sangue do cordão umbilical canino. Frente as dificuldades encontradas no isolamento, cultivo e caracterização destas células, bem como os resultados descritos no Capítulo 2, decidimos continuar nosso estudo utilizando células-tronco mesenquimais (CTMs), também do cordão umbilical canino. Visto que o isolamento destas células ainda não estava descrito na literatura, começamos nosso estudo com as CTMs do cordão umbilical humano (Capítulo 3) e depois extrapolamos o protocolo de isolamento para o modelo canino (Capítulo 4). No Capítulo 5, avaliamos o potencial miogênico, *in vitro* e *in vivo*, das CTMs obtidas do cordão umbilical. Por fim, no Capítulo 6, relatamos um cão GRMD excepcional, Ringo, e discutimos novas condutas na avaliação dos testes pré-clínicos neste modelo animal e ferramentas futuras visando o tratamento das distrofias musculares.

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Resumo

A Distrofia Muscular de Duchenne (DMD) é a forma mais comum e grave de Distrofia Muscular Progressiva. Esta doença possui herança recessiva ligada ao X e é caracterizada pela ausência de distrofina na membrana das fibras musculares. A DMD afeta 1/3.000 meninos nascidos vivos, e até os 12 anos de idade os pacientes são confinados à cadeira de rodas. Os afetados raramente sobrevivem após a terceira década de vida. Atualmente nenhum tratamento efetivo ainda foi desenvolvido para esta doença. Deste modo, nosso trabalho tem como objetivo principal contribuir para o entendimento do potencial terapêutico das células-tronco adultas do cordão umbilical visando a regeneração muscular. Para tanto, utilizamos como modelo cães da raça Golden Retriever portadores de distrofia muscular (GRMD – Golden Retriever Muscular Dystrophy), uma vez que estes cães apresentam um quadro clínico muito semelhante com a patologia clínica humana. Demonstramos em nosso estudo que células hematopoiéticas provenientes do cordão umbilical canino não são capazes de restaurar a expressão de distrofina em níveis clinicamente relevantes em cães GRMDs. Frente a estes resultados, decidimos dar continuidade em nossos estudos com as células-tronco mesenquimais (CTMs) do cordão umbilical humano e canino as quais foram imunofenotipadas e caracterizadas, *in vitro*, quanto ao potencial de diferenciação. *In vivo*, demonstramos que as CTMs do cordão umbilical canino são capazes de chegar à musculatura de cães GRMDs, quando injetadas por via arterial, mas não de restaurar a expressão de distrofina em níveis clinicamente relevantes. Por fim, descrevemos um cão GRMD excepcional, Ringo, que apesar da completa ausência de distrofina nas fibras musculares, apresenta um fenótipo leve de distrofia muscular. Concluímos com nosso estudo que células-tronco do cordão umbilical parece não ser a fonte mais adequada para a regeneração muscular *in vivo*. Contudo é de extrema importância investigar novas estratégias visando melhorar o direcionamento destas células para o músculo esquelético.

Abstract

Duchenne Muscular Dystrophy (DMD) is the most frequent and severe form of muscular dystrophy. It is an X-linked recessive disorder caused by complete absence of dystrophin in muscle fibers. DMD affects 1/3.000 living boys, with loss of independent ambulation occurring at approximately 12 years old. Without any special care, affected boys rarely survive beyond the third decade of life. Currently there is no treatment available for these patients, thus, the main purpose of these study is to understand the therapeutic contribution of umbilical cord stem cells aiming muscular regeneration. To perform our investigation we chose Golden Retriever Muscular Dystrophy (GRMD) dogs, since it is a large animal model of DMD with clinical signs closely mimicking those observed in humans. Here we showed that hematopoietic cells from umbilical cord blood failed to restore dystrophin expression at clinically relevant levels in GRMD dogs. Due to these results, we continued our studies with human and canine mesenchymal stem cells (MSCs) from umbilical cord, which were immunophenotyped and had their multipotent plasticity demonstrated *in vitro*. Using GRMD dogs we also showed that human and canine MSCs from umbilical cord were able to engraft in muscle fibers but were not sufficient to restore dystrophin expression at clinically relevant levels *in vivo*. Finally, we described an exceptional GRMD dog, Ringo, that besides the complete absence of dystrophin in his muscle fibers is showing a very mild phenotype. In conclusion, umbilical cord stem cells transplantation may not be a promising source for skeletal muscle regeneration *in vivo*, but further studies must take place in order to improve stem cell delivery to skeletal muscle.

Capítulo 1

I – INTRODUÇÃO

1.1 Distrofias Musculares Progressivas

As Distrofias Musculares Progressivas (DMP) constituem um grupo de doenças hereditárias caracterizadas por degeneração e fraqueza muscular [1]. Já foram descritas mais de 30 formas de DMPs que diferem entre si quanto à idade de manifestação dos primeiros sinais e sintomas, velocidade de progressão, gravidade e localização preferencial das lesões, dentre outros sinais [2].

Dentre as características que unificam as DMPs destacam-se a variação no tamanho das fibras musculares, áreas de necrose, invasão de macrófagos, reposição do tecido muscular por gordura e tecido conjuntivo, e aumento nos níveis séricos da proteína creatina kinase (CK). Além disso, as DMPs podem ser subdivididas em seis grupos principais com base na distribuição da fraqueza muscular predominante (**FIG. 1**): Duchenne e Becker; Emery Dreifuss; distal; fácio-escápulo-umeral; oculofaríngea; e cinturas, que se caracteriza como o grupo mais heterogêneo [1].

1.2 Distrofia Muscular de Duchenne

O tipo mais grave e comum de DMP é a Distrofia Muscular de Duchenne (DMD), descrita em 1852 pelo médico inglês Edward Meryon que afeta 1 em cada 3.000 recém nascidos do sexo masculino.

A DMD manifesta-se nos primeiros 3 a 5 anos de vida. No estágio inicial da doença, observam-se níveis séricos aumentados da enzima creatina kinase (CK), pseudo-hipertrofia das panturrilhas, quedas freqüentes

e dificuldade em correr e subir escadas. Com a progressão da doença, ciclos repetitivos de degeneração superam a capacidade regenerativa do tecido muscular, causando sua substituição por tecido conjuntivo e adiposo. Por volta dos 10 a 12 anos de idade, a doença já progrediu de tal modo que os afetados não podem mais andar. A maioria dos afetados morre na segunda década de vida, excepcionalmente na terceira, por insuficiência respiratória ou cardíaca [3,4].

FIGURA 1 – Distribuição da fraqueza muscular predominante nos principais grupos das distrofias musculares progressivas. (A) Duchenne e Becker; (B) Emery Dreifuss; (C) cinturas; (D) fácio-escápulo-umeral; (E) distal; (F) oculofaríngea.

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1.3 Distrofina

Em 1985 o gene responsável pela DMD foi localizado no braço curto do cromossomo X (Xp21) [5-7]. Com 2,3 megabases (Mb), o gene DMD é o maior gene humano já caracterizado. É constituído por 79 exons, sendo transcrito em um mRNA de 14 kilobases (Kb). O produto protéico deste gene, denominado distrofina, possui 3685 aminoácidos e uma massa molecular de 427 kilodaltons (kDa) [8,9].

1.4 Complexo de Proteínas Associadas à Distrofina

A distrofina está localizada na face citoplasmática da membrana celular e interage com várias proteínas (**FIG. 2**), formando um complexo denominado DAPC – “Dystrophin Associated Protein Complex” [10-13]. Dentre as proteínas que fazem parte deste complexo, encontra-se a F-actina, uma proteína fundamental do citoesqueleto, a qual se encontra associada à extremidade amino-terminal da distrofina. À extremidade carboxi-terminal associam-se glicoproteínas transmembrana do complexo distroglicana-sarcoglicana que por sua vez interagem com proteínas extracelulares como a laminina. Portanto, uma das funções da distrofina seria coordenar a interação da matriz extracelular com o citoesqueleto, além de manter a integridade estrutural da célula muscular [3,14].

1.5 Mutações no gene DMD

Mutações que geram perda da função da distrofina são responsáveis pela degeneração muscular progressiva observada em pacientes com DMD. Destes, 65% possuem deleções, 5% possuem duplicações e os 30% restantes possuem mutações de ponto ou deleções/duplicações de baixa escala. Mutações que mantêm o quadro de leitura da distrofina, gerando uma proteína comprometida, mas funcional, são responsáveis pela Distrofia Muscular de Becker (DMB), alélica à DMD [15-17].

FIGURA 2 – Complexo de proteínas associadas à distrofina. Representação esquemática mostrando a interação da distrofina com proteínas citoplasmáticas, transmembrana e extracelulares no músculo esquelético.

ADAPTADO – Lydia Kibiuk

1.6 Modelos animais para DMD

Embora grandes avanços tenham sido obtidos no entendimento das bases moleculares e genéticas da DMD, nenhum tratamento efetivo para esta doença está disponível. O progresso nesta área depende de modelos animais fiéis à patologia clínica humana, uma vez que a preocupação ética limita a obtenção de dados experimentais em crianças afetadas por esta doença.

A descoberta do gene responsável pela DMD e de seu produto protéico, a distrofina, permitiu a identificação de modelos animais homólogos à DMD humana em camundongos, cachorros, gatos e peixe. Nos humanos e nos cães, o processo de degeneração e fibrose predominam, levando a uma perda progressiva da estrutura e função muscular. Já nos modelos felino e murino, observa-se pouca fibrose e o processo de regeneração supera a necrose do músculo, gerando um fenótipo brando ou ausente de distrofia muscular. Dessa forma, a utilização do modelo murino e felino tornam-se limitados, servindo apenas como modelo genético e bioquímico, mas não como modelo clínico-patológico da DMD humana [4,18].

Nos últimos anos, cães da raça Golden Retriever portadores de distrofia muscular (GRMD – “Golden Retriever Muscular Dystrophy”) têm se mostrados importantes para a realização de triagens experimentais, pois suas alterações musculares assemelham-se a patogênese humana mais fielmente que o modelo murino, além de possuírem uma massa muscular comparável a dos pacientes distróficos [4].

Os cães afetados possuem uma mutação de ponto (A>G) no sítio 3’ de “splicing” do intron 6 do gene da distrofina. Por consequência, durante o processamento do mRNA, ocorre a perda total do exon 7 ou a perda dos 5 nucleotídeos iniciais deste exon. Ambos estes variantes de “splicing” introduzem uma mudança no quadro de leitura e um códon de terminação prematuro, resultando em uma deficiência grave da proteína distrofina, com baixos níveis (~0,01%) de fibras musculares revertentes que expressam esta proteína [19-21].

1.7 Estratégias terapêuticas para DMD

Diferentes estratégias terapêuticas para a DMD já foram propostas ou testadas em modelos animais. Dentre elas, o transplante de mioblastos, apesar de restaurar a expressão de distrofina em camundongos mdx e pacientes com DMD, ainda apresenta limitações como baixa distribuição das células após injeção, rejeição imune e baixa sobrevivência celular [22-24]. Outra estratégia bastante utilizada é a terapia gênica. Durante muitos anos, o grande tamanho do mRNA da distrofina (14kb) foi o grande empecilho nesta área, pois dificultava a sua manipulação em vetores virais. Entretanto, a criação de miniaturas deste gene (micro e minidistrofinas) tornou possível sua introdução em vetores virais adenoassociados (AVV) e a realização de ensaios pré-clínicos em camundongos mdx [25-27]. Atualmente, os grandes obstáculos a serem resolvidos envolvem a dose necessária para transdução viral em todo o corpo humano sem causar toxicidade, sua baixa permanência e necessidade de reinjeção sem causar rejeição, e por último, a efetividade da terapia gênica em modelos animais de grande porte como os cães GRMD [28,29]. Concomitante aos estudos em modelos animais, já encontra-se em andamento estudos clínicos avaliando a segurança da administração de AVV em pacientes com DMD, administração de oligonucleotídeos antisense para indução de “exon skipping” e avaliação de drogas que ignoram o códon de parada em pacientes com mutações “nonsense” [30].

1.7.1 Uso de células-tronco

Mais recentemente, uma nova linha de pesquisa utilizando células-tronco vem sendo explorada [21,31-36]. Células-tronco são definidas pela sua capacidade de auto-renovação e diferenciação em múltiplos tipos celulares [37]. Estas células possuem origem tanto adulta como embrionária. Dentre as fontes adultas destacam-se o cordão umbilical, a medula óssea, polpa dentária, tecido adiposo, dentre outros [38-40]. Já as células-tronco embrionárias podem ser obtidas da massa celular interna de blastocistos, de um único blastômero sem interferir no potencial de desenvolvimento do embrião ou a partir da reprogramação de fibroblastos da pele, também

conhecidas com células iPS - “induced pluripotent stem cells” [41-44].

Dentre as células-tronco adultas, duas populações principais de células se destacam: células-tronco hematopoiéticas (CTHs) e células-tronco mesenquimais (CTMs). Tradicionalmente as CTHs são isoladas da medula óssea, porém, mais recentemente, o mesmo protocolo também está sendo realizado com o sangue do cordão umbilical. As CTHs por definição são capazes de originar toda a linhagem de células sanguíneas e as mesmas podem ser identificadas por uma série de marcadores de superfície celular como: CD34, CD38, CD45, CD133, dentre outros. Ao contrario das CTHs que crescem em suspensão *in vitro*, as CTMs são cultivadas aderidas nas placas de cultura. CTMs compreendem uma população de células progenitoras multipotentes capazes de diferenciar em vários tipos celulares de origem mesodérmica incluindo condrócitos, osteócitos e adipócitos [45,46]. Além disso, estas células podem ser caracterizadas com os marcadores de superfície celular CD29, CD90, SH2, SH3, SH4, dentre outros.

As células-tronco provenientes do cordão umbilical apresentam um forte atrativo para o uso em terapias celulares devido a presença da fração hematopoiética e mesenquimal. Ademais, o cordão umbilical é geralmente descartado após o parto e sua coleta é obtida por um método simples, seguro e não doloroso; não causando nenhum dano para a mãe ou para a saúde do recém-nascido. Sua fração hematopoiética vem sendo utilizada com sucesso, desde o final da década de 80, para tratar diversas doenças hematológicas. Já o potencial terapêutico da fração mesenquimal vem sendo explorada *in vitro* e *in vivo* devido à maior plasticidade destas células em relação à fração hematopoiética.

Apesar dos grandes esforços realizados para a utilização de células-tronco no tratamento de distrofias musculares, pouco progresso foi alcançado na identificação e caracterização confiável de células capazes de tolerar o ambiente pós-transplante e efetivamente regenerar o músculo em quantidades clinicamente relevantes. Desse modo, o entendimento da natureza heterogênea das células-tronco progenitoras musculares será de grande valia para o desenvolvimento de uma terapia celular eficiente para as

distrofias musculares. Dentre os estudos visando estes objetivos, Gang e colaboradores (2004) e Jazedje e colaboradores (2009) demonstraram o potencial de diferenciação em músculo esquelético *in vitro* das CTMs e CTHs, respectivamente, provenientes do cordão umbilical [47,48]. Dentre os estudos *in vivo*, Kong e colaboradores (2004) demonstraram o potencial miogênico de CTHs do cordão umbilical em camundongos distróficos [49]. No entanto, apenas três trabalhos foram descrito até hoje com células-troncos em cães GRMDs, nenhum deles avaliando o papel das células-tronco do cordão umbilical na regeneração muscular *in vivo*. O primeiro, relatado por Dell’Agnola e colaboradores (2004), demonstrou que CTHs da medula óssea não são capazes de restaurar a expressão de distrofina em níveis clinicamente relevantes em cães GRMDs [21]. Em seguida, Sampaolesi e colaboradores (2006) demonstraram que células-tronco isoladas dos vasos de biópsias musculares, denominadas mesoangioblastos, são capazes de restaurar a expressão de distrofina *in vivo* em cães GRMD após injeções consecutivas via artéria femoral [33]. Também utilizando este mesmo modelo, Kerkis e colaboradores (2008) compararam a eficiência entre injeções sistêmicas e locais utilizando CTMs de polpa de dente [34].

Desta forma, pretendemos extrapolar os resultados obtidos por estes pesquisadores e avaliar o potencial das células-tronco provenientes do cordão umbilical, testando sua capacidade de diferenciação em músculo esquelético e expressão de distrofina *in vitro*, e *in vivo* em cães GRMD.

II – OBJETIVOS

1. Isolar células-tronco do cordão umbilical canino;
2. Caracterizar por imunofenotipagem as células-tronco isoladas;
3. Avaliar o potencial de diferenciação destas células, *in vitro*, em diferentes linhagens celulares;
4. Verificar se células-tronco caninas do cordão umbilical são capazes de diferenciar em músculo esquelético, *in vitro*, e expressar distrofina;
5. Verificar se células-tronco caninas do cordão umbilical são capazes de restaurar a expressão de distrofina, *in vivo*, em cães GRMD.

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TRANSPLANTE DE CÉLULAS HEMATOPOIÉTICAS DO CORDÃO UMBILICAL EM CÃES GRMD

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Hematopoietic cell transplantation from umbilical cord blood fails to restore dystrophin expression in GRMD dogs

Eder Zucconi, Tatiana Jazedje, Natássia M. Vieira, Viviane Abreu Nunes, Mariz Vainzof, Mayana Zatz

Abstract

Duchenne muscular dystrophy (DMD) is the most frequent and severe form of muscular dystrophy. The possibility to replace the defective muscle through stem cell therapy has been of great interest. In order to evaluate whether hematopoietic cell transplantation (HCT) from umbilical cord blood (UCB) could regenerate skeletal muscle and restore dystrophin expression, 5 Golden Retriever Muscular Dystrophy (GRMD) dogs were given healthy dog leukocyte antigen (DLA)-identical cells, directly into muscle or systemically. Analysis of dystrophin expression in muscle biopsied after HCT did not show any remarkable difference as compared to pretransplantation biopsies. Taken together, our data indicate that HCT using UCB cells do not contribute to muscle regeneration in GRMD dogs at clinically relevant levels.

Resumo

A distrofia muscular de Duchenne (DMD) é a forma mais comum e grave de distrofia muscular. A possibilidade de substituir o músculo defeituoso através da terapia com células-troncos tem sido alvo de grandes estudos. Desta forma, pretendemos avaliar se o transplante de células hematopoiéticas (TCH) provenientes do sangue do cordão umbilical é capaz de regenerar o músculo esquelético e restaurar a expressão de distrofina. Para tanto, cinco cães distróficos da raça Golden Retriever receberam células compatíveis diretamente no músculo ou por via sistêmica. A análise da expressão de distrofina nos músculos biopsiados após TCH não mostrou nenhuma diferença evidente quando comparado com as biópsias pré-transplante. Em conjunto, nossos dados sugerem que o TCH provenientes do sangue do cordão umbilical não contribui para a regeneração muscular em cães distróficos em níveis clinicamente relevantes.

Brief Article

Hematopoietic Cell Transplantation from Umbilical Cord Blood Fails to Restore Dystrophin Expression in GRMD Dogs

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Keywords

Hematopoietic Cell Transplantation, Umbilical Cord Blood, Muscular Dystrophy, Golden Retriever Muscular Dystrophy Dog

Running title

Hematopoietic Cell Transplantation into GRMD dogs

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Disclosures

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INTRODUCTION

Traditionally, bone marrow (BM) has been widely investigated as a source for hematopoietic cell transplantation (HCT) in many diseases, including muscular dystrophies. However, there is a great controversy regarding their clinical utility for muscle repair. Some groups reported success in skeletal muscle regeneration [1,2] while others did not confirm these findings [3,4].

More recently, umbilical cord blood (UCB) has been explored as an alternative source to BM, since it has the advantage to be obtained by a simple, safe and painless procedure at birth. We previously reported transdifferentiation and cell fusion of UCB hematopoietic stem cells into muscle *in vitro* [5]. Also, the myogenic differentiation of human UCB cells was reported after intravenous injection into the *sjl* dystrophic mice [6]. Here we evaluated the myogenic potential of UCB for HCT using the golden retriever muscular dystrophy (GRMD) dog, the best clinical and histopathological animal model for Duchenne muscular dystrophy (DMD). In humans, as observed in GRMD dogs, the disease is caused by a complete absence of dystrophin in muscle fibers, leading to progressive muscle degeneration and atrophy, fibrosis, contractures and raised serum creatine kinase levels [7,8].

Five GRMD dogs received canine UCB cells systemically or directly into muscle as described in Table 1. Donor-recipient pairs were chosen based on identity of highly polymorphic major histocompatibility complex (MHC) class I and class II microsatellite markers [9].

Dystrophin expression was compared in pre- and post-HCT biopsies using both NCL-DYS1 and NCL-DYS2 antibodies (Novocastra Laboratories) that recognizes the rod-domain and c-terminus domain of dystrophin protein, respectively. Immunohistochemistry analysis showed no remarkable increase of dystrophin positive fibers following umbilical cord blood HCT (Figure 1). Some isolated dystrophin positive fibers (Figure 1F and 1H) were seen but they were probably revertant fibers since their relative proportion was comparable to untreated muscle. Protein extracts from both sets of muscle

biopsies and necropsy samples were also investigated by western blot which confirmed a complete absence of dystrophin expression in all samples analyzed after HCT, using both NCL-DYS1 and NCL-DYS2 antibodies (data not shown).

Table 1. Summary of HCT treatment in GRMD dogs

Recipient (age at HCT, d)	Donor (genotype)	Cell type (number)	Via	Immuno- suppression	Status after HCT
Charles (117)	Lady (carrier)	UCBHC CD34+ (3×10^6 cells/kg)	Local (pectineus)	Tacrolimus	Death at 123 days
Peter (251)	Yasmine (carrier)	UCBHC (3×10^8 cells/kg)	Local (biceps femoralis and masseter)	Cyclosporin A	Dog alive but with cardiac dysfunction
Johnny (321)	Gaia (carrier)	UCBHC (3×10^8 cells/kg)	Local (biceps femoralis) + systemic (jugular vein)	-	Death at 403 days
Hercules (75)	BR3F6 (normal)	UCBHC (3×10^8 cells/kg)	Systemic (jugular vein)	-	Death at 1481 days
Zeus (75)	Xenna (carrier)	UCBHC (3×10^8 cells/kg)	Systemic (jugular vein)	-	Death at 261 days

HCT indicates hematopoietic cell transplantation; UCBHC, umbilical cord blood hematopoietic cell; and d, age in days at transplantation

In summary, we concluded that umbilical cord blood HCT failed to restore dystrophin expression in GRMD dogs in accordance to other groups who found similar results using bone marrow HCT in the same canine model [3,4]. Although reporting negative results may be disappointing, the publication of these investigations is extremely important to avoid private clinics to offer treatment to DMD boys based in apparently successful reports [10-12]. The identification of other stem cell sources for skeletal muscle regeneration and long-term expression of dystrophin at clinically relevant means remains of great interest. However, before going to translational medicine, a great amount of research has to take place.

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Figure 1. Dystrophin expression before and after umbilical cord blood HCT in GRMD dogs. Immunofluorescence staining was performed on muscle tissues sections using both NCL-DYS1 (A, E-H, K, L) or NCL-DYS2 (B-D, I, J) antibodies. A wild type dog was used as positive control (A, B). Pectineus from Charles before HCT (C) and at necropsy (D). Biceps femoralis from Peter before (E) and 6 months after HCT (F). Triceps braquialis from Johnny before (G) and 50 days after HCT (H). Biceps femoralis from Hercules before (I) and 6 months after HCT (J). Biceps femoralis from Zeus before (K) and 2 months after HCT (L). Original magnification X 20.

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Capítulo 3

ISOLAMENTO DE CÉLULAS-TRONCO MESENQUIMAIS HUMANAS DO CORDÃO UMBILICAL

Stem Cells 2008; 26:146-150

Multipotent stem cells from umbilical cord: cord is richer than blood!

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Abstract

The identification of mesenchymal stem cell (MSC) sources that are easily obtainable is of utmost importance. Several studies have shown that MSCs could be isolated from umbilical cord (UC) units. However, the presence of MSCs in umbilical cord blood (UCB) is controversial. A possible explanation for the low efficiency of MSCs from UCB is the use of different culture conditions by independent studies. Here, we compared the efficiency in obtaining MSCs from unrelated paired UCB and UC samples harvested from the same donors. Samples were processed simultaneously, under the same culture conditions. Although MSCs from blood were obtained from only 1 of the 10 samples, we were able to isolate large amounts of multipotent MSCs from all UC samples, which were able to originate different cell lineages. Since the routine procedure in UC banks has been to store the blood and discard other tissues, such as the cord and/or placenta, we believe our results are of immediate clinical value. Furthermore, the possibility of originating

different cell lines from the UC of neonates born with genetic defects may provide new cellular research models for understanding human malformations and genetic disorders, as well as the possibility of testing the effects of different therapeutic drugs.

Resumo

A identificação de fontes de células-tronco mesenquimais (CTMs) de fácil obtenção é de extrema importância. Diversos estudos tem apontado que CTMs podem ser isoladas a partir do tecido do cordão umbilical. No entanto, a presença de CTMs no sangue de cordão umbilical (SCU) é controversa. Uma possível explicação para a baixa eficiência no isolamento de CTMs do SCU é o uso de diferentes condições de cultura em estudos independentes. Portanto, comparamos a eficiência de isolamento de CTMs de amostras pareadas provenientes do tecido do cordão umbilical e do SCU obtidas do mesmo doador. As amostras foram processadas simultaneamente e sob as mesmas condições de cultivo. Embora a eficiência de isolamento das CTMs do sangue foi apenas de 1 das 10 amostras avaliadas, fomos capazes de isolar grandes quantidades de CTMs multipotentes de todas as amostras do tecido do cordão umbilical, as quais foram capazes de originar diferentes linhagens celulares. Uma vez que o procedimento de rotina nos bancos de cordão umbilical é armazenar os sangue e descartar outros tecidos como o cordão umbilical e a placenta, acreditamos que nossos resultados são de valor clínico imediato. Além disso, a possibilidade de originar diferentes linhagens a partir do tecido do cordão umbilical de neonatos com defeitos genéticos pode proporcionar novos modelos de pesquisa para entender malformações humanas e doenças genéticas, bem como proporcionar a possibilidade de testar o efeito de diferentes drogas terapêuticas.

Multipotent Stem Cells from Umbilical Cord: Cord Is Richer than Blood!

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Key Words. Human umbilical cord • Human umbilical cord blood • Mesenchymal stem cells • Umbilical cord banks

ABSTRACT

The identification of mesenchymal stem cell (MSC) sources that are easily obtainable is of utmost importance. Several studies have shown that MSCs could be isolated from umbilical cord (UC) units. However, the presence of MSCs in umbilical cord blood (UCB) is controversial. A possible explanation for the low efficiency of MSCs from UCB is the use of different culture conditions by independent studies. Here, we compared the efficiency in obtaining MSCs from unrelated paired UCB and UC samples harvested from the same donors. Samples were processed simultaneously, under the same culture conditions. Although MSCs from blood were obtained from only 1 of the 10

samples, we were able to isolate large amounts of multipotent MSCs from all UC samples, which were able to originate different cell lineages. Since the routine procedure in UC banks has been to store the blood and discard other tissues, such as the cord and/or placenta, we believe our results are of immediate clinical value. Furthermore, the possibility of originating different cell lines from the UC of neonates born with genetic defects may provide new cellular research models for understanding human malformations and genetic disorders, as well as the possibility of testing the effects of different therapeutic drugs. *STEM CELLS* 2008;26:146–150

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells (MSCs) are undifferentiated cells that are able to self-renew and that have a high proliferative capacity. These cells comprise a rare population of multipotent precursors that are capable of supporting hematopoiesis. Moreover, several reports suggest that MSCs were able to differentiate into various cell types, including chondrocytes, osteocytes, adipocytes, myocytes, and neurons [1–4]. MSCs can be isolated from different tissues, such as bone marrow (BM), adipose tissue, dental pulp, placenta, and umbilical cord blood (UCB), and from a variety of fetal tissues, such as spleen, lung, pancreas, kidneys, and amniotic fluid during midgestation [5–10]. Phenotypic and genetic evidence suggests that MSCs are an immature cell type, being a potentially useful model for developmental biology studies in normal and disease background, in addition to their therapeutic potential.

BM has been considered as one of the main sources of MSCs for both experimental and clinical applications, and most of the knowledge concerning MSCs comes from BM studies. However, MSCs from BM decrease significantly with age [11, 12], and their isolation is invasive and can cause infection, bleeding, and chronic pain. In past decades, human UCB has been regarded as an alternative source to BM cell transplantation and therapy because of its hematopoietic and mesenchymal components. Human UCB is obtained after full-term delivery of the newborn from a sample that would inevitably be discarded. The process is noninvasive, painless, and without harm for the mother or the infant.

Hematopoietic stem cells (HSCs) from UCB have already been proven to be useful in treating various hematological disorders [13–16]. However, the presence of MSCs in UCB is controversial. Some researchers succeeded in isolating these cells [8, 17–19], whereas others failed or obtained a low yield [20–22]. More recently, some groups have reported success in isolating and establishing MSCs cultures from umbilical cord (UC) vein and UC stroma, also called Wharton's jelly [3, 20, 23–25]. According to Tondreau et al., the discrepant results in isolating MSC from UCB might be explained by different methodologies used for obtaining and culture these cells [26]. To address this issue, we compared 10 samples of UCB and UC, obtained from the same donors, regarding the presence of MSCs, as well as the differentiation potential in culture. Our results show that UC are rich in MSCs that are able to differentiate into various cell lines in vitro, whereas MSCs from blood were obtained from only 1 of the 10 samples. Based on these findings, we strongly suggest storing UC, in addition to UCB, for future therapeutic applications and scientific investigation purposes.

MATERIALS AND METHODS

Harvesting of UCB and UC

Ten human UCB and UC matching units were collected after informed consent was obtained from the mother in accordance with the ethical committee of Institute of Bioscience of University of São Paulo and Jesus José e Maria Hospital. Informed consent was obtained from all subjects. All studies and laboratory procedures

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were carried out in the Human Genome Research Center, São Paulo, Brazil.

From each sample, UCB was harvested and conserved with 100 mM EDTA anticoagulant at 22°C. Sections of 8–10 cm of umbilical cords, routinely discarded, were internally washed with phosphate-buffered saline (PBS) containing 300 U/ml penicillin and 300 µg/ml streptomycin (Gibco, Grand Island, NY, <http://www.invitrogen.com>) and immediately immersed in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 300 U/ml penicillin, and 300 µg/ml streptomycin. All samples were processed within 12–15 hours after collection.

Isolation and Culture of Adherent Cells from UCB

After blood dilution of 3:1 with RPMI 1640 medium (Gibco), mononuclear cells (MNCs) were isolated by density gradient centrifugation at 400g for 30 minutes at room temperature using Ficoll-Paque Premium (GE Healthcare, Little Chalfont, U.K., <http://www.gehealthcare.com>). MNCs were washed twice with PBS and resuspended in proliferation medium consisting of DMEM-LG, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were plated at a density of 5×10^7 cells per ml in culture flasks (25 cm²) and maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 24 hours of incubation, nonadherent cells were removed, and culture medium was replaced every 3 days. Adherent cells were cultured until they reached 80%–90% confluence.

Isolation and Culture of Adherent Cells from UC

UCs were filled with 0.1% collagenase (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) in PBS and incubated at 37°C for 20 minutes. Each UC was washed with proliferation medium, and the detached cells were harvested after gentle massage of the UC. Cells were centrifuged at 300g for 10 minutes, resuspended in proliferation medium, and seeded in 25-cm² flasks at a density of 5×10^7 cells per ml. After 24 hours of incubation, nonadherent cells were removed and cultivated as described above.

Immunophenotyping

To analyze cell-surface expression of typical protein markers, adherent cells were incubated with the following anti-human primary antibodies: CD29-PECy5, CD34-PerCP, CD31-phycoerythrin (PE), CD45-fluorescein isothiocyanate (FITC), CD90-R-PE, CD117-PE, human leukocyte antigen (HLA)-ABC-FITC, HLA-DR-R-PE (Becton, Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>), and SH3 (kindly provided by Dr. Kerkis, Instituto Butantã, São Paulo). Unconjugated markers were reacted with anti-mouse PE secondary antibody (Guava Technologies, Hayward, CA, <http://www.guavatechnologies.com>). A total of 10,000 labeled cells were analyzed using a Guava EasyCyte flow cytometer running Guava ExpressPlus software (Guava Technologies).

Cell Differentiation Procedures

To evaluate MSC properties, adherent cells (third passage, at 80%–90% confluence) were subjected to adipogenic, chondrogenic, myogenic, and osteogenic differentiation *in vitro*, according to established protocols [1, 3]. Normal human dermal fibroblasts were used as a negative control in the differentiation studies.

Adipogenic Differentiation

Subconfluent cells were cultured in proliferation medium supplemented with 1 µM dexamethasone (Sigma-Aldrich), 500 µM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 60 µM indomethacin (Sigma-Aldrich), and 5 µg/ml insulin (Sigma-Aldrich). Adipogenic differentiation was confirmed on day 21 by intracellular accumulation of lipid-rich vacuoles stainable with oil red O (Sigma-Aldrich). For the oil red O stain, cells were fixed with 4% paraformaldehyde for 30 minutes, washed, and stained with a working solution of 0.16% oil red O for 20 minutes.

Chondrogenic Differentiation

A pellet culture system was used for chondrogenesis. Cells (2.5×10^3) were centrifuged in a 15-ml polypropylene tube at 500g for 5 minutes, and the pellet was resuspended in 10 ml of basal medium consisting of DMEM-LG supplemented with 100 nM dexamethasone, 50 µM ascorbic acid-2 phosphate (Sigma-Aldrich), 1 mM sodium pyruvate (Gibco), and 1% ITS-Premix (Becton Dickinson). Without disturbing the pellet, cells were resuspended in 0.5 ml of chondrogenic differentiation medium consisting of basal medium supplemented with 10 ng/ml transforming growth factor-β1 (R&D Systems Inc., Minneapolis, <http://www.rndsystems.com>). On day 1, tubes were flipped gently to acquire a single floating cell sphere. Medium was changed every 3–4 days, and cells were fixed on day 21 with 4% paraformaldehyde. Cryosections (10 µm thick) were stained with toluidine blue to demonstrate extracellular matrix mucopolysaccharides.

For chondrogenic differentiation in monolayer culture, adherent cells were cultured in chondrogenic differentiation medium for 21 days. Chondrogenesis was demonstrated by staining with toluidine blue.

Osteogenic Differentiation

To promote osteogenic differentiation, subconfluent cells were treated with proliferation medium supplemented with 50 µM ascorbate-2 phosphate, 10 mM β-glycerophosphate (Sigma-Aldrich) and 0.1 µM dexamethasone for 21 days. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate assessed by von Kossa stain. Briefly, cells were stained with 1% silver nitrate (Sigma-Aldrich) for 45 minutes under ultraviolet light, followed by 3% sodium thiosulfate (Sigma-Aldrich) for 5 minutes, and then counterstained with van Gieson stain.

Myogenic Differentiation

For myogenic differentiation, adherent cells from UC were cultured in proliferation medium supplemented with 0.1 µM dexamethasone (Sigma-Aldrich), 50 µM hydrocortisone (Sigma-Aldrich), and 5% horse serum (Gibco) for 30 days. After that, cells were fixed with 4% paraformaldehyde and blocked with a blocking solution containing 10% fetal bovine serum, 5% bovine serum albumin, and 0.1% Triton X-100 in PBS for 1 hour. Primary antibody was added at a concentration of 1:100 for Myosin (M7523; Sigma-Aldrich) and 1:20 for dystrophin (VP-D508; Vector Laboratories) and incubated at room temperature for 2 hours. After several washes, cells were incubated with secondary antibodies against mouse IgG tagged to FITC (green) or rabbit IgG tagged to cyanine 3 (red) for 2 hours at room temperature. Immunostaining controls were done in the same conditions but lacked the primary antibody. Slides were counterstained with 4,6-diamidino-2-phenylindole and mounted in Vectashield (Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>) solution. All images in the same set (samples and controls) were obtained using the same photographic parameters of exposition and speed. Images were captured using the Axiovision 3.0 image analysis system (Carl Zeiss, Jena, Germany, <http://www.zeiss.com>).

RESULTS

Isolation and Culture of Adherent Cells from UCB and UC

After plating MNCs from UCB, different cell types were observed. Most of them displayed an oval morphology (Fig. 1A), with few or no cytoplasmatic extensions. Some cells had an MSC-like phenotype (Fig. 1B), but most of them did not spread, migrate, or proliferate after 14 days in culture. Although we could isolate and expand MSCs from only one of the UCB units (data not shown), all the samples of UC generated primary adherent cultures, with cells displaying an MSC-like phenotype. After 4 days in culture, these cells grew in colonies, reaching confluence after 10–14 days. Most of the cells were spindle-

Figure 1. Morphology of adherent cells when isolated from umbilical cord blood. (A): Cells cultured for 4 days after initial plating displayed a round morphology. (B): Cells with an MSC-like phenotype (arrows) were also observed at day 4 but only in a few samples. However, these cells did not proliferate further than 14 days. Scale bars = 100 μ m.

shaped, resembling fibroblasts. Some clusters of cells with endothelial appearance, which spread weakly and practically did not proliferate, could also be observed. After the second passage, adherent cells were constituted by homogeneous cell layers with an MSC-like phenotype (Fig. 2A–2D). The number of MSC from UC decreased slightly after freezing and thawing, and the remaining viable cells were successfully expanded on consecutive days (data not shown).

Immunophenotypic Analyses

All adherent cells derived from UC did not express hematopoietic lineage markers (CD34, CD45, and CD117) and endothelial markers (CD31), as assessed by flow cytometry. In addition, the majority of cells expressed high levels of adhesion markers (CD29 and CD90) and MSC markers (SH3). The isolated cells from UC were also positive for HLA-class I (HLA-ABC) but negative for HLA-class II (HLA-DR) (Fig. 3). In comparison with our fibroblast control, no obvious difference in the expression of these surface antigens could be observed (data not shown). Thus, the MSC property of isolated cells was further confirmed with cell differentiation studies.

Multilineage Differentiation Potential

The plasticity of adherent cells obtained from cord blood (CB) and UC was assessed 3 weeks after mesodermal induction. Osteogenic, adipogenic, and chondrogenic differentiation was demonstrated by the calcium deposits, lipid vacuoles, and mucopolysaccharide-rich extracellular matrix, respectively. No evident differences in MSCs from CB and UC differentiation potential were detected. Furthermore, an osteogenic, adipogenic, or chondrogenic phenotype was not observed in induced fibroblasts (negative controls; Fig. 4).

In addition, the potential of adherent cells from UC to differentiate into skeletal muscle cells was investigated. The myogenic differentiation was demonstrated by the expression of myogenic markers (myosin and dystrophin). As shown in Figure 5, myosin and dystrophin were specifically expressed in the differentiated cells rather than in untreated cells (noninduced controls). Together, these results confirmed the mesenchymal nature of the isolated cells and their multipotency.

DISCUSSION

In this study we compared, for the first time, the efficiency in obtaining MSCs from match-paired UCB and UC samples harvested from the same donors, which were processed simultaneously and under the same culture conditions. Although MSCs from blood were obtained from only 1 of the 10 samples, we were able to generate primary MSCs cultures from all cord

Figure 2. Adherent cells in primary cultures of UC. (A–D): The morphology and growth of cells with an MSC-like phenotype after 4, 7, 10, and 14 days of culturing, respectively. Some residual clusters of cells with endothelial appearance (arrow) could also be identified. Scale bars = 200 μ m.

samples with a 100% yield. MSCs from UC are isolated by a fast and simple procedure using short enzymatic digestion (which provides a large number of cells without risk to the donor) and can easily be expanded in vitro, stored cryogenically, and thawed. MSCs have been reported to be isolated from UC by others, using different protocols [3, 23, 24, 27–30]. Likewise, difficulties in obtaining MSCs from UCB have been reported previously [8, 17]. Based on our experience, the efficiency in isolating MSC from blood in approximately 100 umbilical cord units stands around 10% (unpublished data).

Crucial parameters for the isolation of MSCs from UCB, such as time between collection and processing, the volume of samples, and the amount of MNC have been already described. Even so, the MSCs yield is never greater than 60% [19]. Recently, MSCs have successfully been derived from CD133+ hematopoietic stem cells originated from UCB [26]. However, this method would not allow the simultaneous isolation of hematopoietic and mesenchymal progenitors.

In mobilized peripheral blood, controversial results have been reported about the presence or absence of MSCs [22, 31–33]. da Silva Meirelles et al. reported that MSCs could not be detected in circulating blood in adult rats and suggested that all tissues have MSCs reservoirs localized in the perivascular niche [33]. According to these authors, it might be possible that during harvesting procedures only a few cells would detach from this perivascular niche as a result of needle syringe friction in blood vessel wall or the pressure applied in the UC during blood collection. On the other hand, a full success in obtaining MSCs from UC by our group and others might be explained by enzymatic dissociation processes within the UC perivascular niche. It is questionable whether the results observed in rats can be extrapolated to human samples and whether both MSC populations described here share the same genetic repertoire.

It has been suggested that the removal of myeloid and osteoclast-like cells seems to favor the isolation of MSCs from UCB [19]. Therefore, cocultivation with other hematopoietic cell types could inhibit the proliferation of the few MSCs present in the sample. Although some adherent primary cultures of UC contained cells with an endothelial phenotype, in our conditions, such contaminants did not interfere with MSCs growth. Others have also suggested that MSCs occurred at a low

Figure 3. Immunophenotyping of adherent cells isolated from umbilical cord. Values represent the mean percentage of all assessed cells positively stained by the indicated antigens and analyzed by flow cytometry. Graphs show forward scatter versus fluorescence intensity. Abbreviations: HLA-ABC, human leukocyte antigen-ABC; HLA-DR, human leukocyte antigen-DR; SH3, Src homology 3.

Figure 4. Differentiation potential of adherent cells isolated from CB and UC. (A): Osteogenic differentiation from adherent cells from CB and UC was demonstrated by calcium deposition shown by von Kossa stain. Scale bar = 50 μ m. (B): Adipogenesis was detected by the formation of intracytoplasmic lipid droplets stained with oil red O. Scale bar = 200 μ m. (C): Cell spheres from CB and UC were stained with toluidine blue to confirm chondrogenic differentiation. Mucopolysaccharide-rich extracellular matrix is shown in pinkish metachromatic areas. (D): Chondrogenic differentiation in monolayer culture of MSC from UC was demonstrated by stained with toluidine blue. Scale bars = 50 μ m. (A, B, D): Osteogenic, adipogenic, or chondrogenic phenotype was not observed in induced fibroblasts (negative controls). Scale bars = 200 μ m. More details are given in Materials and Methods. Abbreviations: CB, cord blood; UC, umbilical cord.

frequency in UCB and that their survival could be affected by different culture conditions [26]. However, our results show that the low yield of MSCs in UCB is likely not due to different culture methodologies.

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Figure 5. Myogenic differentiation potential of adherent cells isolated from umbilical cord. (A): Myogenic differentiation was assessed by immunocytochemistry. Induced cells were strongly labeled with anti-human myosin monoclonal antibody (Sigma-Aldrich) in red and, with anti-human dystrophin (dys1) monoclonal antibody (Vector Laboratories) in green. Counterstaining with Dapi (in blue) was used to identify all nuclei. (B): Noninduced controls. (C): Negative controls (without the first antibody). Scale bar = 50 μ m. Abbreviation: Dapi, 4,6-diamidino-2-phenylindole.

An important issue in cellular therapy studies is the availability of alternative stem cell sources and the efficacy of isolation techniques to yield a reasonable amount of viable cells that could be successfully expanded. Despite the advantages of HSC from UCB in hematopoietic reconstitution [13–15], results from the present study demonstrated that UC, and not UCB, is the best choice for isolating MSCs for future applications. Until very recently, BM has been considered the main source of MSCs. Panepucci et al. demonstrated that MSCs derived from UC and BM are highly similar at the transcriptional level, reinforcing the usefulness of UC from neonates [34].

In short, based on the present results and other studies, we believe that UC is the easiest obtainable biological source of MSCs. Storing UCB in private or public banks has been recently the subject of many ethical dilemmas. Here we show that regardless of being public or private, these banks are discarding a precious source of MSCs. In addition, since human UC contain a significant amount of MSCs, we suggest donors to split samples, storing in both public and private banks. Storing both UCB and UC would allow maximum recovery of HSCs and MSCs for possible therapeutic applications in the future. Furthermore, the possibility of originating different UC-derived cell lines from babies born with human malformations and genetic disorders may provide new research models for understanding pathological mechanisms responsible for these conditions. In addition, storing both UCB and UC would also create the possibility of testing the effects of therapeutic drugs in these different cell lineages.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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ISOLAMENTO DE CÉLULAS-TRONCO MESENQUIMAIS CANINAS DO CORDÃO UMBILICAL

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Mesenchymal stem cells derived from canine umbilical cord vein – a novel source for cell therapy studies

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Abstract

The canine model provides a large animal system to evaluate many treatment modalities using stem cells. However, only bone marrow (BM) protocols have been widely used in dogs for preclinical approaches. BM donation consists of an invasive procedure and the number and differentiation potential of its mesenchymal stem cells (MSCs) decline with age. More recently, umbilical cord was introduced as an alternative source to BM since it is obtained from a sample that is routinely discarded. Here, we describe the isolation of MSCs from canine umbilical cord vein (cUCV). These cells can be obtained from every cord received and grow successfully in culture. Their multipotent plasticity was demonstrated by their capacity to differentiate in adipocytic, chondrocytic and osteocytic lineages. Furthermore, our results open possibilities to use cUCV cells in preclinical trials for many well characterized canine model conditions homologues to human diseases.

Resumo

O modelo canino possibilita avaliar várias modalidades terapêuticas utilizando células-tronco em um sistema animal de grande porte. No entanto, apenas protocolos com células-tronco de medula óssea tem sido amplamente utilizados em abordagens pré-clínicas no modelo canino. A doação de medula óssea consiste em um procedimento invasivo, e o número e o potencial de diferenciação das células-tronco mesenquimais (CTMs) diminuem com a idade do doador. Mais recentemente, o cordão umbilical foi introduzido como um fonte alternativa à medula óssea uma vez que este é obtido de um material que é rotineiramente descartado. Sendo assim, descrevemos o isolamento de CTMs da veia do cordão umbilical canino (VCUc). Estas células podem ser obtidas de todo cordão coletado e expandida em cultura com sucesso. A plasticidade destas células foi demonstrada pela sua capacidade de diferenciar em adipócitos, condrócitos e osteócitos. Além disso, nossos resultados abrem possibilidades para utilização das células VCUc em ensaios pré-clínicos de várias condições ou doenças muito bem estabelecidas no modelo canino e homólogas em seres humanos.

Mesenchymal Stem Cells Derived from Canine Umbilical Cord Vein—A Novel Source for Cell Therapy Studies

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The canine model provides a large animal system to evaluate many treatment modalities using stem cells (SCs). However, only bone marrow (BM) protocols have been widely used in dogs for preclinical approaches. BM donation consists of an invasive procedure and the number and differentiation potential of its mesenchymal stem cells (MSCs) decline with age. More recently, umbilical cord was introduced as an alternative source to BM since it is obtained from a sample that is routinely discarded. Here, we describe the isolation of MSCs from canine umbilical cord vein (cUCV). These cells can be obtained from every cord received and grow successfully in culture. Their multipotent plasticity was demonstrated by their capacity to differentiate in adipocytic, chondrocytic, and osteocytic lineages. Furthermore, our results open possibilities to use cUCV cells in preclinical trials for many well-characterized canine model conditions homologs to human diseases.

Introduction

RESEARCHES AIMING REGENERATIVE MEDICINE have developed fast in recent years and are causing great expectation among scientists and the general public. Current approaches are being developed to repair and restore the function of damaged or disease tissues. Animal models are of immense importance in this field and most of the knowledge and advances achieved came from preclinical studies.

Historically, the dog has been an useful model for studying mechanisms and testing new therapies of several human pathologies including prostate cancer, cardiovascular diseases, bone regeneration, diabetes, leukemia, spinal cord injury, as well as solid organ transplantation [1–7]. Dogs are also a source of many well-characterized genetic diseases homologs to human, such as X-linked severe combined immunodeficiency (XSCID) and Duchenne muscular dystrophy [8–10]. In addition, they are an ideal large animal model to study various treatment modalities such as gene therapy and stem cell (SC) transplantation.

Studies carried out over the last decades suggest that SCs could be of great value for the development of new therapies. SCs are defined by their ability to self-renewal and to differentiate into multiples cell types and by their capacity of in vivo reconstitution of a given tissue [11]. They can

be derived from the inner cell mass of an early embryo as well as numerous postnatal sources, including bone marrow (BM), umbilical cord (UC), placental tissue, and dental pulp [12–16]. Much of the knowledge concerning canine SCs comes from the numerous studies of hematopoietic and mesenchymal components from BM [17–21]. In recent reports, adult and embryonic canine SC lines were established from novel sources such as the blood vessels from muscle biopsies, termed mesoangioblasts, and from embryo blastocysts, respectively [22–25]. Moreover, dog somatic cell nuclear transfer demonstrated by Lee and colleagues will provide new possibilities to evaluate the concept of therapeutic cloning in this animal model [26].

Given that many advances achieved in the dog are directly transferable to humans since it constitutes a large animal model, it could be of great value for the development of new therapies. Isolating SCs in the dog model will provide the opportunity to evaluate the efficacy and safety of gene therapy and cell transplantation. Here, for the first time, we isolated canine mesenchymal stem cells (MSCs) from UC. These cells, termed canine umbilical cord vein (cUCV), are simple to harvest in large amount without risk to the donor, may be expanded in vitro, cryogenic stored, and thawed. We also demonstrated that cUCV express mesenchymal markers

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and were able to differentiate into, at least, adipocytes, chondrocytes, and osteocytes.

Materials and Methods

Isolation and culture of canine adherent cells from UCV

All experimental protocols were approved by the ethical committee of animal use of Institute of Biosciences, University of Sao Paulo. For this study, Golden Retriever newborn dogs were obtained from Peter White Kennel, Sao Paulo and the Brazilian Colony of Golden Retriever Muscular Dystrophy, Faculty of Veterinary Medicine, and Zootechny of University of Sao Paulo. Full-term UCVs were collected immediately after birth and washed with phosphate-buffered saline (PBS) containing 300 U/mL penicillin and 300 µg/mL streptomycin (Gibco) and placed in Dulbecco's modified Eagle's medium low glucose (DMEM-LG; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco), 300 U/mL penicillin, and 300 µg/mL streptomycin. All samples were processed within 10–12 h after collection.

In the laboratory, the UCVs were filled with 0.1% collagenase (Sigma, St. Louis, MO) in PBS and incubated at 37°C for 15 min. Then, the UCVs were washed internally with proliferation medium consisting of DMEM-LG supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin. Detached cells were harvested after gentle massage of the UC and centrifuged at 300g for 10 min. Cells were resuspended in proliferation medium, seeded in 25 cm² flasks, and maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h incubation, nonadherent cells were removed and culture medium was replaced every 3 days. Adherent cUCV cells were cultured until reaching 90% confluence and passaged using TrypLE (Invitrogen, Carlsbad, CA).

Immunocytochemistry

Canine umbilical cord vein (cUCV) cells at passage 3 were seeded onto chamber slides and allowed to grow until reaching 70%–80% confluence. For antibody staining, growth medium was removed and the cells were fixed with 4% paraformaldehyde for 15 min. Blocking was performed using 1% bovine serum albumin (BSA, Sigma, St. Louis, MO) in PBS-Tween (PBST) for 30 min. The cells were then incubated with the following primary antibodies (15 µg/mL): anti-canine CD29 (Veterinary Medical Research & Development, Inc., Pullman, WA) or anti-canine CD90 (Becton Dickinson, Franklin Lakes, NJ) for 1 h at room temperature. The secondary antibody anti-mouse IgG-Cy3 conjugated (Chemicon), diluted 1:100, was incubated in the same conditions described earlier. Negative control samples were incubated with PBS instead of primary antibody or only with secondary antibody. Cells were further counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and analyzed on Axiovert 200 microscope (Carl Zeiss) using appropriate filters. Images were captured using Axiovision 3.1 software (Carl Zeiss).

Immunophenotyping

To analyze cell-surface expression of typical protein markers, cUCV adherent cells, at passage 3, were incubated

with the following primary antibodies: CD14, CD45 (all from Veterinary Medical Research & Development); CD34-PE, CD44-FITC, CD117-PeCy5 (all from Becton Dickinson). Unconjugated markers were reacted with anti-mouse phycoerythrin (PE) secondary antibody (Guava Technologies, Hayward, CA). A total of 10,000 labeled cells were acquired using EasyCyte flow cytometry (Guava Technologies). Negative control samples were incubated with PBS instead of primary antibody or only with anti-mouse-PE secondary antibody. All plots generated were analyzed in Guava ExpressPlus software (Guava Technologies).

Expansion characteristics

cUCV cells at passage 2 were monitored for 28 days to determinate their cumulative population doubling level (cpdl). Cell plating density was set at 1×10^5 cells in a 35-mm dish and cultivated 4 days in proliferation medium until near confluence. Each passage, in a total of seven, was performed using TrypLE and recovered cells were replated at initial density as passage 2 cells and thereafter. Cell number was determined by three independent direct counts in Neubauer chamber. The population doubling (pd) at each subculture was calculated by using the following equation: $pd = \ln(N_f/N_i)/\ln 2$, where N_i and N_f are initial and final cell numbers, respectively, and $\ln$ is the natural log. The pds of continuous subculture were added to obtain the cpdl.

Karyotype analysis

For evaluation of any abnormal karyotype at latter passages, chromosomes preparations were performed in cUCV cells cultures. Briefly, metaphase cells were arrested with 0.1 µg/mL colchicine (Sigma) for 20 min. Then, cUCV cells were detached from cultures flasks using TrypLE (Gibco), resuspended in a hypotonic solution (0.075 M KCl), and incubated for 20 min at 37°C. Cells were pelleted at 1,000 rpm for 10 min and fixed by washing three times in methanol:acetic glacial acid (3:1). Chromosome spreads were obtained by pipetting suspension drops onto clean glass slides and air-dried. The best metaphases were captured with Axioplan 2 microscope (Zeiss) and analyzed using Ikaros 3 software (Zeiss).

Differentiation studies

cUCV cells were subjected to differentiation protocols to evaluate their MSC property. For this purpose, adipogenic, chondrogenic, and osteogenic inductions were assessed in the third passage cultures. Noninduced control samples were maintained in proliferation medium during all the differentiation study.

Adipogenesis. Subconfluent cells (90%) were cultured in DMEM high glucose medium (Gibco, Grand Island, NY) supplemented with 5% rabbit serum (Gibco), 5 µM rosiglitazone (GSK), 1 µM dexamethasone (Sigma, St. Louis, MO), and 5 µg/mL insulin (Sigma). Adipogenic differentiation was confirmed on day 21 by intracellular accumulation of lipid-rich vacuoles stainable with Oil Red O (Sigma). For the Oil Red O stain, cells were fixed with 4% paraformaldehyde for 30 min, washed, and stained with a working solution of 0.16% Oil Red O for 20 min.

Chondrogenesis. For chondrogenic differentiation, cells were cultured as monolayer or cell sphere. For the last one,

2.5 × 10⁵ cells were centrifuged in a 15-mL polypropylene tube at 500g for 5 min and the pellet resuspended in 10 mL of basal medium consisting of DMEM-LG supplemented with 100 nM dexamethasone, 50 µM ascorbic acid-2 phosphate (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Gibco), and 1% ITS-Premix (Becton Dickinson, Franklin Lakes, NJ). Without disturbing the pellet, the cells were resuspended in 0.5 mL of chondrogenic differentiation medium consisting of basal medium supplemented with 10 ng/mL transforming growth factor beta 1 (TGF-β1) (R&D Systems, Minneapolis, MN). On day 1, the tubes were flipped gently to acquire a single floating cell sphere. To perform monolayer cell culture, subconfluent cells (90%) were also cultured in chondrogenic differentiation medium. In both cases, the medium was changed every 3–4 days, and then cells were fixed on day 21 with 4% paraformaldehyde. Cryosections (10 µm thick) of cell spheres or monolayer cultures were stained with Toluidine Blue to demonstrate extracellular matrix mucopolysaccharides. Staining solution was prepared by adding 1% of Toluidine Blue dissolved in distilled water containing 1% of sodium borate, followed by filtering. One drop of this solution was added to each sample for 1 min and then washed with distilled water and left to air-dry.

Osteogenesis. To promote osteogenic differentiation, subconfluent (90%) cUCV cells were treated with proliferation medium supplemented with 50 µM ascorbate-2 phosphate, 10 mM β-glycerophosphate (Sigma), and 0.1 µM dexamethasone for 21 days. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate assessed by von Kossa stain and Alizarin Red. Briefly, for von Kossa stain, the cells were stained with 1% silver nitrate (Sigma) for 45 min under ultraviolet light, followed by 3% sodium thiosulfate (Sigma) for 5 min, and then counterstained with van Gieson. To perform Alizarin Red stain, ethanol (50%) fixed cells were incubated for 30 min at room temperature (RT) in

a solution containing 1% Alizarin Red and 1% ammonium hydroxide. Cells were rinsed twice with distilled water and allowed to dry completely.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs were isolated from cells using Trizol (Invitrogen) under conditions recommended by the manufacturer. Extracted RNA was solved in diethylpyrocarbonate-treated water (DEPC water) and cDNAs were synthesized from 0.2 µg total RNA using Superscript III reverse transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed using specific primers to amplify the target sites listed in Table 1. Twenty-five microliters of PCR were prepared with 1 µL cDNA, 10 pmol of each primer, 1 unit of Taq polymerase (GE Healthcare), 1× PCR buffer (GE Healthcare), and final concentration of 160 µM dNTPs. Cycle conditions were as follows: 94°C for 4 min; 30–35 cycles at 94°C for 1 min; optimal annealing temperature (Table 1) for 1 min; 72°C for 1 min; followed by 72°C for 5 min. The PCR products were separated on 6% polyacrylamide gel by electrophoresis, stained with ethidium bromide, and visualized under UV light. Digital images were captured with ImageQuant (GE Healthcare).

Results

Isolation and morphological characteristics of cUCV cells

The results reported in this work were representative of 30 cUCV cell lineages established in our laboratory. We were able to generate primary adherent cell culture from all UC samples with a 100% yield. These cells were obtained

TABLE 1. PRIMERS USED IN RT-PCR

Markers	Gene	Primer sequence (5'–3')	Amplicon size	Ann. Temp. (°C)	Reference
Adipocytes	<i>FABP4</i>	Forward ATCAGTGTAACGGGGATGTG Reverse GACTTTTCTGTCATCCGCAGTA	117	60	[42]
	<i>LEPTIN</i>	Forward CTATCTGTCCTGTGTTGAAGCTG Reverse GTGTGTGAAATGTCATTGATCCTG	102	60	[42]
	<i>LPL</i>	Forward ACACATTCACAAGAGGGTCAC Reverse CTCTGCAATCACACGGATG	132	60	
Condrocytes	<i>COL2A</i>	Forward GAAACTCTGCCACCCTGAATG Reverse GCTCCACCAGTTCTTCTTGG	156	64	[42]
	<i>SOX9</i>	Forward GCTCGCAGTACGACTACACTGAC Reverse GTTCATGTAGGTGAAGGTGGAG	101	60	[42]
	<i>AGGRECAN</i>	Forward ATCAACAGTGCTTACCAAGACA Reverse ATAACCTCACAGCGATAGATCC	122	58	[42]
Osteocytes	<i>OSTEOPONTIN</i>	Forward CATATGATGGCCGAGGTGATAG Reverse CAAGTGATGTGAAGTCCTCCTC	114	60	
	<i>COL1A1</i>	Forward GTAGACACCACCCTCAAGAGC Reverse CCAGTCGGAGTGGCACAT	118	62	
	<i>BSP</i>	Forward TTGCTCAGCATTTTGGGAAT Reverse AACGTGGCCGATACTTAAAGAC	295	60	
Housekeeping	<i>GAPDH</i>	Forward CCATCTTCCAGGAGCGAGAT Reverse TTCTCCATGGTGGTGAAGAC	97	60

FIG. 1. Adherent cells isolated from cUCV. (A, B) Morphology of cells after 3 days in culture. (A) Cells with a wide cytoplasm (arrow) dispersed among fibroblast-like cells. (B) Cluster of cells with endothelial appearance (arrow). (C) cUCV cells after 6 days in culture. (D) Cells at passage 5. Scale bars = 500 μ m.

from cUCV by rapid digestion of the umbilical vein wall. Then, the UCVs were washed internally with proliferation medium and the collected cells were plated in plastic culture flask to isolate the adherent cell population.

Freshly cUCV adherent cells showed heterogeneity during the first days in culture. Most of cells displayed a fibroblast-like appearance with a spindle-shaped morphology, while some cells appeared with a wide cytoplasm (Fig. 1A). During the first week of cultivation, it could also be observed some clusters of cells with endothelial appearance, which spread weakly and practically did not migrate or proliferate (Fig. 1B). After the third week, the cells occupied the whole plastic surface and were constituted by a homogeneous layer of fibroblast-like cells (Fig. 1C). This morphology

became more frequent and was maintained in subsequent passages (Fig. 1D).

Expansion characteristics

When cultivated in proliferation medium, cUCV cells presented a cpdl of 15 in 28 days. These cells could be cultured for at least seven passages without changing their morphology or proliferation capacity (Fig. 2A). Furthermore, cUCV could be frozen and thawed without losing their viability and expansion characteristics (data not shown). At both early and late passages, cells maintained a diploid karyotype of 78 chromosomes (Fig. 2B).

Immunophenotypic characterization of cUCV cells

To characterize the adherent cells population derived from cUCV, expression of typical surface proteins were examined by immunocytochemistry and flow cytometry. Passage 3 cUCV cells were positively labeled with CD29 (β 1 integrin) and CD90 (Thy1) adhesion molecules (Fig. 3A and B). No signal was observed in the negative control sample incubated only with the secondary antibody (Fig. 3C). By flow cytometry, all adherent cells derived from cUCV did not express hematopoietic lineage markers (CD14, CD34, CD45, and CD117) but express the adhesion molecule CD44 (Supplementary Fig. 1). (supplementary figure is available online at <http://www.liebertpub.com>) As these surface markers are not sufficient for the identification or definition of MSC, cUCV cells were subjected to differentiation studies for further confirmation of their MSC property.

Multilineage differentiation potential

The plasticity of adherent cells obtained from cUCV was investigated under conditions favorable to adipogenic, chondrogenic, and osteogenic inductions. Under the influence of adipogenic differentiation medium, cUCV cells achieved an adipocytic phenotype by the end of the third week. The presence of multisized intracytoplasmic lipid droplets was confirmed by Oil Red O staining only in induced cultures

FIG. 2. Proliferation characteristics of cUCV. (A) Mean values of cumulative population doublings from cUCV lineage cultured for seven passages in proliferation medium. (B) Karyotype of cUCV lineage after seven passages, showing an euploid number of chromosomes.

FIG. 3. Immunophenotyping of cUCV cells. The majority of cells labeled positively, in red, for (A) CD29 and (B) CD90. Negative control was performed after staining with anti-mouse Cy3 secondary antibody alone (C). Counterstaining with DAPI (blue) was used to identify all nuclei present. Scale bars = 100 μ m.

(Fig. 4B), but not in noninduced cultures (Fig. 4A). Expression of *FABP4* and *LPL* was seen only in adipo-induced cells (Fig. 5A). On the other hand, basal level of *LEPTIN* mRNA was observed in noninduced control cells, and the expression level was increased following adipogenic induction. To verify the chondrogenic ability, cUCV cells were subjected to monolayer (Fig. 4C and D) or cell sphere (Fig. 4E and F) culture systems. Induced (Fig. 4D–F) and noninduced (Fig. 4C) cultures were stained with Toluidine Blue to confirm chondrogenic differentiation. As demonstrated in Figure 4E, cUCV cells originated a single floating spherical cell mass. After staining, these sections showed irregular groups

of cells embedded in a mucopolysaccharide extracellular matrix surrounded by a thin capsule (Fig. 4F). Chondrogenic treatment resulted in specific expression of *COL2A*, *SOX9*, and *AGGRECAN*, all of which were undetected or had basal expression in noninduced cells (Fig. 5B). Finally, cUCV cells exposed to osteogenic medium for 3 weeks showed direct evidences of calcium mineralization seen by Alizarin Red (Fig. 4H) and von Kossa staining (Fig. 4J). Noninduced control cultures did not show spontaneous differentiation for osteogenic lineages (Fig. 4G and I), respectively. Expression of *OSTEOPONTIN*, *COL1A1*, and *BSP* was observed only in induced cells, with no basal expression in control cells

FIG. 4. Differentiation potential of cUCV cells. (A,B) Adipogenic differentiation. (A) Noninduced cUCV cells stained with Oil Red O. (B) Induced cUCV cells. Adipogenesis was detected by the formation of intracytoplasmic lipid droplets stained with Oil Red O. (C–F) Chondrogenic differentiation. Noninduced monolayer culture (C), induced monolayer culture (D), and 3D cell sphere-induced culture (E) stained with Toluidine Blue to confirm chondrogenic differentiation. (F) Higher magnification of (E) showing mucopolysaccharide-rich extracellular matrix in pinkish metachromatic areas. A clear capsule surrounding the entire sphere was also evident (arrow). (G–J) Osteogenic differentiation. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate assessed by Alizarin Red (G, H) and von Kossa stain (I, J). Noninduced cells are shown in (G, I) and induced cells in (H, J). Scale bars = 100 μ m (B, F); Scale bars = 200 μ m (A, C, D); Scale bars = 500 μ m (E, G–J).

FIG. 5. mRNA expression of specific differentiation markers. (A) Adipogenic markers; *FABP4*, *LEPTIN*, and *LPL*. (B) Chondrogenic markers; *COL2A*, *SOX9*, and *AGGRECAN* (*AGC*). (C) Osteogenic markers; *OSTEOPONTIN* (*OPN*), *COL1A1*, *BONE SIALOPROTEIN* (*BSP*). The expression of *GAPDH* was used as reference for evaluating the quality of mRNA. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

(Fig. 5C). In short, these results confirmed the mesenchymal nature of the adherent cells isolated from cUCV and their multipotent property.

Discussion

Umbilical cord stem cells (UCSCs) have been used as an alternative source to BM for both experimental and clinical applications [14,27–34]. UCSCs are obtained after full-term delivery of the newborn, from a sample that is routinely discarded. In the case of dogs, after each puppy delivery, UC was collected and then the mother could ingest the amniotic sac and placenta, replenishing the nutrients and energy lost during the partition. The process is noninvasive, as opposed to the obtention of other sources of adult SCs, and does not cause any harm to the mother or the newborn.

Mesenchymal stem cells from human UC have been isolated from umbilical vein, UC matrix, perivascular region, and cord blood [14,32–36]. The last one is a controversial source of MSCs as described by some researchers [14,37,38]. Recently, our group compared the efficiency of MSCs isolation from UC tissue and UC blood from the same patient and under the same conditions. We demonstrated that the UC tissue and not the blood is the most abundant source of MSCs [39]. On the other hand, cord blood is rich in hematopoietic SCs that have already been proven useful to treat various hematological disorders [27–30].

The results obtained here suggest that cUCV contains a substantial amount of MSCs that could be isolated by a fast

and simple procedure using a rapid enzymatic digestion. Using this methodology, we were able to isolate adherent cells from all 30 UC obtained, yielding a 100% of success. Other groups had already reported a harvesting efficiency of 100% using human UC. Such results were described by Sarugaser et al. [32] who examined the perivascular cell population of human UC; Weiss et al. [34] who isolated cells from human UC matrix; and Secco et al. who studied the human UC tissue [39].

During the first day of cultivation some cells with endothelial appearance were present in our cultures but they spread weakly and practically did not proliferate. This result is in accordance with Romanov et al. [14] who isolated MSCs from human umbilical vein. Their methodology was used in this study, with few modifications, to isolate and establish our cUCV lineages. After 3 weeks of cultivation, attached cells from cUCV had a fairly uniform fibroblast-like morphology commonly described in MSCs populations from a variety of sources.

Adherent cells were obtained from cUCV and could be expanded in vitro, up to numbers that would be sufficient for a preclinical assay, without any numeric chromosome alteration. The proliferation potential of cUCV cells (cpdl = 15 in 28 days) was very similar to canine adipose tissue-derived MSC reported by Neupane et al. [40] (~15 cpdl in 30 days) although quite inferior to human UC blood MSC reported by Bieback et al. [36] and Kern et al. [41] (both cpdl = 20 in seven passages).

As reported by Kern et al. [41] and Wagner et al. [42] as well as by many other groups, MSCs isolated from different tissues express a well-defined set of cell-surface markers that include CD29, CD44, CD90, SH2 (CD105), and SH3 (CD73). To characterize the adherent cells obtained from cUCV, we performed immunocytochemistry to investigate the expression of CD29 and CD90. Both adhesion markers were positively labeled in passage 3 cUCV cells. By flow cytometry we could also demonstrate that these cells do not express any hematopoietic marker but they express CD44 as shown in dog mesoangioblasts described by Sampaolesi et al. [22]. However, as reported previously by Wagner et al. [42] no obvious difference in the expression of these surface antigens could be observed between MSCs and fibroblasts. Their results show that this panel of surface markers is not itself sufficient to define or identify MSCs. In order to evaluate the MSC property of cUCV cells, we subjected them to differentiation studies.

SCs are defined by their ability to self-renewal and their capacity to generate committed cells in vitro and in vivo [11]. MSCs can be induced to differentiate along the adipogenic, chondrogenic, and osteogenic lineages using specific culture medium [41,42]. In all six of our 30 lineages obtained from cUCV, we demonstrated their multipotency and plasticity. Here, adipogenic, chondrogenic, and osteogenic differentiation were confirmed by the appearance of lipid vacuoles, mucopolysaccharide-rich extracellular matrix, and calcium deposits, respectively. Furthermore, the differentiation studies were confirmed by expression of specific mesoderm lineage markers.

Results from the present study demonstrate that adherent cells isolated from cUCV could be defined as multipotent MSC with ability to give rise to at least three mesodermal lineages. This is the first report of UCMSC isolated from dogs. In addition to canine bone marrow mesenchymal stem cell

(BMMSC), other sources had previously been described in this model, such as embryonic stem cells, mesoangioblasts, and adipose-derived MSC [17–25,40].

Traditionally, rodent models of human diseases have been widely used in preclinical studies since they are easy to be manipulated. However, large animal models, such as the dog, are more faithful to reproduce human pathologies. Dogs represent a source of many well-characterized genetic diseases homologs to human making them ideal for in vivo approaches, such as heterologous stem cells transplantation or autologous genetically corrected stem cells transplantation [8–10,22]. Furthermore, stem cells lineages established from dogs with genetic disease will represent a valuable tool for in vitro and in vivo screening of therapeutic drugs. Also, cell therapy for many others pathologies such as bone fracture, medullar lesion, and myocardial infarction could be tested in this model.

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Author Disclosure Statement

The authors indicate no potential conflicts of interest.

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AQ1: Please approve the insertion of manufacturer location
AQ2: Please provide manufacturer location
AQ3: Please provide manufacturer location
AQ4: Please provide manufacturer location

SUPPLEMENTAL FIG. 1. Flow cytometry of adherent cells isolated from cUCV. Values represent the mean percentage of all assessed cells positively stained by the indicated antigens. Graphs show relative number cells (counts) versus fluorescence intensity. Unmarked cells served as negative control for analysis.

AVALIAÇÃO DO POTENCIAL MIOGÊNICO DAS CÉLULAS-TRONCO DO CORDÃO UMBILICAL

I – MATERIAL E MÉTODOS

1.1 ESTUDOS *IN VITRO*

1.1.1 Ensaio de diferenciação miogênica

CTMs provenientes do cordão umbilical canino – “canine umbilical cord vein” (cUCV), passagem 3, foram expandidas em meio de proliferação até atingirem a confluência de 90%. Em seguida, estas células foram transferidas para dois tipos diferentes de meios miogênicos, denominados miogênico 1 e miogênico 2. O meio miogênico 1, descrito por Zuk e colaboradores (2001), foi preparado com meio de proliferação suplementado com 50µM hidrocortisona (Sigma); 0,1µM dexametasona (Sigma) e 5% de soro de cavalo (Sigma) [1]. Já o meio miogênico 2 foi preparado com Dulbecco’s Modified Eagle Medium “low glucose” – DMEM-LG suplementado com 3% de soro de cavalo. Os meios de cultura foram trocados a cada 4 dias, durante um período de 29 dias. O grupo controle negativo consistiu de células cUCV mantidas durante todo o período experimental em meio de proliferação.

1.1.2 Isolamento de RNA e transcrição reversa por reação em cadeia da polimerase (RT-PCR)

O RNA total das células não tratadas (controle) e tratadas com meio de diferenciação foi extraído utilizando Trizol (Gibco) de acordo com recomendações do fabricante. 250ng de cada RNA total foi reversamente transcrito utilizando a SuperScript III (Invitrogen), conforme instruções do fornecedor. Após a síntese de cDNA, a reação em cadeia da polimerase (PCR) foi realizada utilizando primers listados no **QUADRO 1**, específicos para a diferenciação miogênica. A reação de PCR foi preparada com 1µl cDNA, 10 pmol de cada primer, 1U de Taq polimerase (GE Healthcare), 1X tampão de PCR (GE Healthcare) e 160µM de dNTPs, totalizando um volume final de 25µl. As condições da reação foram: 94°C por 4 minutos; 35 ciclos de 94°C por 1 minuto; 60°C por 1 minuto; 72°C por 1 minuto seguidos por 72°C por 5 minutos. Todos os primers foram determinados por sequências depositadas em bancos de dados. Como controle endógeno e de eficiência da reação, foi avaliado a expressão do gliceraldeído-3-fosfato desidrogenase (GAPDH). Os produtos de PCR foram analisados em gel de poliacrilamida 6%, corados com brometo de etídeo e visualizados sob luz ultravioleta. Imagens digitais foram capturadas com o sistema ImageQuant300 (GE Healthcare).

QUADRO 1**Primers utilizados na RT-PCR.**

Gene	Sequencia do primer (5'-3')	Fragmento (pb)	Acesso no GenBank
MyoD	Forward GACGGCATGATGGACTACAG	118	XM_849663.1
	Reverse ACACCGCAGCACTCTTCC		
Myf5	Forward TTTGACAGCATCTACTGTCCTGA	104	XM_847069.1
	Reverse TTGGTGATTGATCCACTATGC		
Miogenina	Forward CAGTCCCTCAACCAGGAG	102	XM_547345.2
	Reverse CTGCAGGAGGCACTGTGG		
MRF4	Forward GACCCCTTCAGCTACAGACC	110	XM_849210.1
	Reverse TCCACGATGGAAGACAGG		
Distrofina	Forward AAACACAGTGGTAGCCCACAAGAT	116	NM_001003343.1
	Reverse TGGTGACAGCCTGTGAAATC		
GAPDH	Forward CCATCTTCCAGGAGCGAGAT	97	AB038240.1
	Reverse TTCTCCATGGTGGTGAAGAC		

1.2 ESTUDOS *IN VIVO*

1.2.1 Animais experimentais

Neste estudo, utilizamos animais provenientes da colônia brasileira de cães GRMD da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (USP). Todos os protocolos experimentais foram aprovados pelo comitê de ética em experimentação animal do Instituto de Biociências da USP.

1.2.2 Genotipagem

A triagem dos cães normais, afetados e portadores foi realizada segundo Bartlett e colaboradores [2]. A purificação do DNA genômico foi realizada a partir de linfócitos do sangue periférico utilizando o kit comercial GFXTM Genomic (GE Healthcare), conforme recomendações do fabricante. A região contendo a mutação de ponto foi amplificada por PCR utilizando os pares de primers: GF2 (5' – CTT AAG GAA TGA TGG GCA TGG G – 3') e GR2 (5' – ATG CAT AGT TTC TCT ATC ATG C – 3') localizados, respectivamente, no intron 6 e exon 7 do gene da distrofina. Os amplicons gerados (310 pb) foram incubados por 2 horas a 37°C com a enzima de restrição *Sau96 I* (GE Healthcare), capaz de reconhecer o sítio mutado. Os produtos da digestão enzimática foram analisados em gel de poliacrilamida 6% e visualizados após coloração com brometo de etídeo. A documentação das imagens foi realizada no aparelho ImageQuant300 (GE Healthcare).

1.2.3 Tipagem de histocompatibilidade (DLA - Dog Leukocyte Antigen)

Para determinar qual o doador mais adequado para um transplante *in vivo* de células-tronco, foi realizada análise de histocompatibilidade utilizando três marcadores de microssatélites polimórficos localizados no cromossomo 12 e marcados com fluoróforos descritos no **QUADRO 2**. O marcador C. 2200 encontra-se justaposto ao MHC de classe I canino, o C.

2202, justaposto ao MHC de classe II canino, e o C. 07003, justaposto aos outros dois marcadores anteriores [3,4]. Estes microssatélites foram amplificados por PCR conforme condições descritas por Wagner e colaboradores [3]. Os amplicons gerados, para cada amostra, foram diluídos 1:10 e reunidos em volume final de 20µl. Por último, as amostras foram analisadas no MegaBACE 1000 (GE Healthcare), utilizando o kit MegaBACE ET550-R Size Standard (GE Healthcare). A determinação dos alelos foi realizada utilizando o programa Genetic Profiler versão 2.2 (GE Healthcare).

QUADRO 2

Primers utilizados na tipagem de Histocompatibilidade

Marcador	Sequencia do primer (5'- 3')	Fluoróforos
C. 2200	Forward CATGATCCTGGAGTCCCG	Fam
	Reverse GAAAGCTGCTTCAGTGGACC	
C. 2202	Forward GTTGAGTGGTTGCCTTTAGC	Hex
	Reverse CAGGATCTTCATATGTCACC	
C. 07003	Forward GAGCCTGCTTCTGCCTCTCC	Hex
	Reverse GTATTAATGGATGGATTGCA	

1.2.4 Injeção intra-arterial de CTMs do cordão umbilical em cães GRMD

Aproximadamente 1×10^7 CTMs provenientes do cordão umbilical foram injetadas no terço proximal ou medial da artéria femoral de cães GRMD com ajuda de um cateter intravenoso 22 gauge (BD). O isolamento e cultivo das CTMs do cordão umbilical humano e canino foram realizados conforme descrito nos capítulos 3 e 4, respectivamente. As células cultivadas para a injeção foram recuperadas utilizando TripLE e ressuspensas em um volume final de 5-10 mLs de “Hank’s Balanced Salt Solution” (HBSS) 1x. Para determinar o número total de células injetadas em cada animal, foi realizada a contagem em câmara de Neubauer conforme especificações do fabricante. As injeções foram realizadas nos cães sedados com 0,05mg/Kg de acepromazina e 2 mg/Kg de cloridrato de peptidina por via intramuscular. Durante todo o procedimento os principais sinais vitais, como os batimentos cardíacos e a respiração, foram monitorados. No total, sete injeções consecutivas foram realizadas em intervalos de aproximadamente 30 dias.

Para a realização das injeções três e sete, as células foram marcadas com o corante Dil (Invitrogen), momentos antes da injeção, seguindo as especificações do fornecedor. A confirmação da marcação foi realizada por citometria de fluxo onde 10.000 eventos foram adquiridos utilizando o citômetro EasyCyte (Guava Technologies). Como controle negativo foram utilizadas amostras não marcadas com o corante Dil. Todos os gráficos gerados foram analisados no software Guava Express Pro, versão 4.2 (Guava Technologies).

1.2.5 Ensaio de viabilidade celular

A viabilidade celular foi determinada utilizando o corante iodeto de propídio (PI – “propidium iodide”) a 2µg/mL. Para tanto, amostras obtidas da seringa, logo após o processo de injeção, foram incubadas por 1 minuto com PI e analisadas no citômetro de fluxo EasyCyte. Controles negativos foram realizados utilizando as mesmas amostras, porém não marcadas com este corante. A análise dos dados gerados foi realizada no software Guava Express Pro, versão 4.2. Ao todo foram analisados 10.000 eventos para cada experimento.

1.2.6 Biópsias musculares

Dois dias após a terceira injeção (144 dias de idade) e três dias após a sétima injeção (312 dias de idade), foram realizadas as biópsias designadas 3 meses (B3M) e 8 meses (B8M) pós-injeção, respectivamente. Para realização das biópsias, os cães tratados com CTMs provenientes do cordão umbilical foram sedados com 0,05mg/Kg de acepromazina e 2 mg/Kg de cloridrato de peptidina por via intramuscular. Em seguida os cães foram submetidos a anestesia local com lidocaína (1MI) na região da pele, tecido subcutâneo e fáscia muscular. Durante todo o procedimento os sinais vitais foram monitorados. Fragmentos provenientes do bíceps femoral e tibial

cranial foram coletados e preservados para análises de DNA, RNA e proteínas (Western Blot - WB e imunofluorescência).

Os fragmentos destinados à análise de DNA, RNA e WB foram preservados a 4°C em gaze umedecida com soro fisiológico e estocadas posteriormente em nitrogênio líquido. Os fragmentos destinados a imunofluorescência foram também preservados a 4°C em gaze umedecida com soro fisiológico até a preparação das rolhas, onde estes foram envoltos em Tissue-Tek (Sakura) e talco visando a manutenção da estrutura do tecido e das proteínas musculares, sem artefatos, durante o congelamento em nitrogênio líquido.

1.2.7 Análise de DNA

Amostras de DNA foram extraídas das células-tronco mesenquimais do cordão umbilical canino e humano, utilizados nos transplantes, e dos tecidos musculares biopsiados pós-injeções. No primeiro caso, utilizamos o kit comercial GFX™ Genomic (GE Healthcare) conforme especificações do fornecedor. Já no caso das biópsias, pequenos fragmentos de músculos foram incubados durante a noite com tampão de lise (100mM Tris-HCl pH 8,5; 5mM EDTA; 0,2% SDS; 200mM NaCl) contendo 20ng/μl de proteinase k. Após este passo, as amostras foram centrifugadas a 3.000rpm por 10 minutos e aos sobrenadantes foram adicionados álcool isopropílico na proporção de 1:1. Em seguida, as amostras foram centrifugadas por 15 minutos a 12.000rpm e os pellets formados foram ressuspensos em tampão TE (10mM Tris-HCl; 1mM EDTA; pH 8,0). A dosagem de DNA destas amostras foi realizada no espectrofotômetro NanoDrop ND-1000 (NanoDrop).

Para avaliar a presença do cromossomo Y nas amostras, utilizamos como marcador o gene *SRY* ("Sex-determining Region Y"). Para amplificar esta região do cromossomo Y utilizamos os primers descritos por Olivier e colaboradores [5], conforme detalhado no **QUADRO 3**. As condições da PCR, denominadas programa "Touchdown", foram: 94°C por 1 minuto; 14 ciclos de 94°C por 30 segundos, 62°C por 40 segundos e 72°C por

2 minutos, diminuindo 0,5°C por ciclo; 23 ciclos de 94°C por 30 segundos, 62°C por 40 segundos e 72°C por 2 minutos, diminuindo 0,5°C por ciclo; 72°C por 10 minutos; 10°C ∞. Como controle positivo da reação de PCR, utilizamos os mesmos primers empregados na genotipagem de cães GRMD [2], descritos no **QUADRO 3**. Os amplicons gerados foram analisados em gel de poliacrilamida 6% e visualizados após coloração com brometo de etídeo. A documentação das imagens foi realizada no aparelho ImageQuant300 (GE Healthcare).

QUADRO 3

Primers utilizados nas análises de DNA

Gene	Especificidade	Sequencia do primer (5'-3')	Fragmento (pb)
SRY	Humano/Canino	Forward CTCGCGATCAAAGGCGCAAGAT	104
	Humano/Canino	Reverse TCGGCTTCTGTAAAGCATTTTC	
DYS	Canino	Forward CTTAAGGAATGATGGGCATGGG	310
	Canino	Reverse ATGCATAGTTTCTCTATCATGC	

1.2.8 Análise de RNA

O RNA total das células-tronco mesenquimais do cordão umbilical canino e humano, utilizados nos transplantes, e dos tecidos musculares biopsiados pós-injeções foi extraído utilizando Trizol (Invitrogen) de acordo com recomendações do fabricante. A RT-PCR foi realizada com 250ng de cada RNA total, utilizando o kit da enzima SuperScript III (Invitrogen), de acordo com as especificações do fornecedor. Após a síntese de cDNA, a PCR foi realizada com os primers listados no **QUADRO 4**. Como controle endógeno e de eficiência da reação, foi avaliado a expressão do gliceraldeído-3-fosfato desidrogenase (*GAPDH*). Os 25µl da PCR foram preparados com 1µl de cDNA; 20pmol de cada primer; 0,1 unidades de Taq (GE Healthcare); 0,2mM dNTPs e 1x tampão PCR (Gibco). As condições de ciclagem foram: 94°C por 5 minutos; 30 ciclos de 94°C por 30 segundos, 60°C por 30 segundos e 72°C por 1 minuto; 72°C por 7 minutos; 4°C∞. Os amplicons gerados foram analisados em gel de poliacrilamida 6% e corados com brometo de etídeo. A documentação das imagens foi realizada no aparelho ImageQuant300 (GE Healthcare).

QUADRO 4**Primers utilizados na RT-PCR**

Gene	Especificidade	Sequencia do primer (5'-3')	Fragmento (pb)
DYS	Canino	Forward AAACACAGTGGTAGCCCACAAGAT	116
	Canino	Reverse TGGTGACAGCCTGTGAAATC	
DYS	Humano	Forward GAATAAGTCAGTGACCCAGAAGAC	116
	Humano	Reverse TGGTGACAGCCTGTGAAATC	
GAPDH	Humano/Canino	Forward CCATCTTCCAGGAGCGAGAT	97
	Humano/Canino	Reverse TTCTCCATGGTGGTGAAGAC	

1.2.9 Análise de proteína**Western blot**

Extratos de proteína foram preparados a partir de fragmentos musculares coletados nas biopsias pós-injeção, B3M e B8M. Para tanto, os mesmos foram tratados com tampão de lise (10mM Tris-HCl pH 8.0; 150mM NaCl; 5mM EDTA; 1% Triton X-100 e 60mM octilglicosídeo) e incubados por 2 minutos a 100°C. Após esta etapa, as amostras foram centrifugadas a 14.500rpm por 15 minutos para remover o debris insolúveis, sendo o sobrenadante estocado a -20 °C até o uso. Para a realização do WB, as amostras de proteína foram tratadas com o tampão contendo: 62,5mM Tris-HCl pH 6,7; 2% SDS; 10% glicerol; 50mM 2-mercaptoetanol e 0,1% de azul de bromofenol; e incubadas por 5 minutos a 100 °C. As proteínas foram separadas em gel de SDS-PAGE 6% e transferidas para membrana de nitrocelulose Hybond-C (GE Healthcare). A confirmação da transferência das proteínas do gel para a membrana de nitrocelulose e a visualização da quantidade de proteínas aplicadas foram analisadas após coloração com Ponceau (Sigma). Após esta etapa, as membranas foram bloqueadas por 1h em temperatura ambiente com tampão TBS-T (50mM Tris-HCl pH7,0; 50mM NaCl; 0,05% Tween-20) contendo 5% de leite desnatado. Logo em seguida, as membranas foram incubadas "overnight" a 4 °C com os anticorpos primários NCL-Dys1 1:100 (Vector) ou Mandys106/2C6 1:100 (cortesia do Dr. Glenn E. Morris – "Center for Inherited Neuromuscular Diseases", Reino Unido). Após três lavagens com TBS-T por 5 minutos, as membranas foram incubada com o anticorpo secundário "goat anti-mouse" HRP (Santa Cruz)

1:2000 por 1 hora. As proteínas associadas aos anticorpos foram detectadas por quimioluminescência utilizando o kit ECL (GE Healthcare) de acordo com instruções do fabricante.

Imunofluorescência (IF)

Cortes seriados de 6µm dos fragmentos de biopsias musculares coletados pós-injeção, B3M e B8M, foram corados com hematoxilina-eosina (H&E) ou preparados para estudos de IF. Neste ultimo caso, os cortes histológicos foram fixados por 15 minutos em 4% de paraformaldeído diluído em PBS 1x. Em seguida, os cortes foram tratados duas vezes por 10 minutos em solução de lavagem (100mM glicina diluído em PBS 1x). O bloqueio dos sítios inespecíficos foi realizado tratando os cortes por 1 hora em solução de bloqueio (5% soro de albumina bovina; 10% soro fetal bovino; PBS 1x; 0,1% Triton X-100). Logo após esta etapa, os cortes foram incubados em câmara úmida, durante a noite, em solução de bloqueio contendo os seguintes anticorpos primários: (1) NCL-Dys1 1:20 (Novocastra Laboratories); (2) NCL-Dys2 1:20 (Novocastra Laboratories); (3) Mandys106/2C6 1:100 e (4) anti-núcleo humano MAB1281 1:20 (Chemicon). Subseqüentemente, os cortes foram tratados duas vezes por 10 minutos com solução de lavagem e incubados por 1 hora com solução de bloqueio contendo o anticorpo secundário IgG-Cy3 1:100 (Chemicon). Por fim, os cortes foram tratados 2 vezes por 10 minutos em solução de lavagem e, quando necessário, realizamos a marcação do núcleo com 0,1 µg/ml Dapi (Sigma) por 1 minuto. As lâminas foram analisadas no microscópio Axiovert200 (Carl Zeiss) sob o filtro adequado. As imagens foram documentadas utilizando o software Axiovision 3.1 (Carl Zeiss) com o mesmo tempo de exposição.

II – RESULTADOS

2.1 ESTUDOS *IN VITRO*

2.1.1 Diferenciação miogênica

Para investigar se as células aderentes isoladas do cordão umbilical canino tem o potencial de diferenciar em músculo esquelético *in vitro* e expressar distrofina, células cUCV foram tratadas com meio miogênico 1 ou meio miogênico 2 por 4 semanas. Em diferentes intervalos de tempo, analisamos a expressão de RNA mensageiros (mRNA) dos marcadores miogênicos, por RT-PCR, nas células induzidas e não induzidas com os meios de diferenciação (**FIG. 1**). Como controle positivo utilizamos músculo esquelético canino, o qual expressa tantos os marcadores precoces (MyoD e Myf5) como os tardios (miogenina, MRF4 e distrofina) da via miogênica. Nas células não tratadas com meio miogênico não observamos a expressão de nenhum desses marcadores, conforme o esperado. No entanto, após 15 dias de diferenciação *in vitro*, fomos capazes de observar a expressão de distrofina nas células cUCV induzidas com os meios miogênicos 1 ou 2. Esta expressão se manteve aos 29 dias de indução, também para ambos os meios avaliados.

2.2 ESTUDOS *IN VIVO*

2.2.1 Genotipagem

No dia 25 de Março de 2007 nasceu uma ninhada na colônia brasileira de cães GRMD proveniente do cruzamento natural entre o macho afetado Ringo (**ver ANEXO II**) e a fêmea portadora Lady. Como a distrofia Muscular de Duchenne possui herança recessiva ligada ao X e letal, este cruzamento permitiu gerar fêmeas afetadas, além de aumentar as chances de geração de cães afetados. Conforme demonstrado na **FIG. 2**, foram gerados nesta ninhada 1 fêmea portadora, 2 fêmeas afetadas, 2 machos

afetados e 2 machos normais, sendo que o animal L3M4 veio a óbito nas primeiras semanas de vida.

FIGURA 1 – Diferenciação miogênica das células cUCV. Os níveis de mRNA dos marcadores miogênicos MyoD, Myf5, MRF4, miogenina (Myog) e distrofina (Dys) foram avaliados por RT-PCR durante o processo de diferenciação muscular. Músculo esquelético canino e células cUCV não induzidas com meio miogênico serviram como controle experimental positivo e negativo, respectivamente. A expressão de *GAPDH* foi utilizada como referência para avaliar a qualidade dos mRNAs.

FIGURA 2 - Genotipagem dos cães GRMD. O DNA genômico foi extraído de linfócitos do sangue periférico dos cães recém-nascidos e amplificado por PCR. Os amplicons gerados foram digeridos com a enzima de restrição *Sau96I*, capaz de reconhecer o sítio mutado no gene da distrofina, submetido à eletroforese e visualizado após coloração com brometo de etídeo. As bandas dos animais normais e afetados estão indicadas pelas setas.

2.2.2 Tipagem de Histocompatibilidade (DLA – Dog Leukocyte Antigen)

Visando determinar os pares compatíveis para a realização dos transplantes de CTMs provenientes do cordão umbilical, analisamos a segregação de 3 marcadores polimórficos de microssatélites: C.2200, C.2202 e C.07003, descritos por Wagner e colaboradores [3] e disponível no site da Universidade Estadual de Michigan – Michigan State University (MSU) [4].

Conforme demonstrado na TAB. 1, dos 3 cães afetados vivos e disponíveis para o transplante apenas 2, Dani (L3F1) e Caju (L3M6),

possuem pares normais compatíveis. Deste modo, decidimos utilizar estes pares nos transplantes de CTMs de cordão umbilical e, no caso da Rosa (L3F2), por não ter par compatível, optamos por avaliar o potencial miogênico *in vivo* de uma das linhagens de CTMs humana, do sexo masculino, obtidas do cordão umbilical, conforme previamente descrito no **CAPÍTULO 3**. Este último caso facilitará o rastreamento das células injetadas, uma vez que marcadores específicos para a espécie humana poderão ser avaliados nas biópsias musculares caninas. Além disso, o fato excepcional da geração de 2 fêmeas afetadas nesta ninhada somado à compatibilidade de uma delas com um macho normal (L3M5) permitirá rastrear as células injetadas utilizando marcadores do cromossomo Y.

TABELA 1**Tipagem de histocompatibilidade (DLA)**

Código	Nome	Genótipo	2200		2202		7003	
			Alelo 1	Alelo 2	Alelo 1	Alelo 2	Alelo 1	Alelo 2
M1M4	Ringo	Afetado	38	60	21	32	32	33
BR2F4	Lady	Portadora	22	26	34	34	35	35
L3F1	Dani	Afetada	26	60	32	34	33	35
L3F2	Rosa	Afetada	26	38	21	34	32	35
L3F3		Portadora	26	60	32	34	33	35
L3M4		Afetado	22	60	32	34	33	35
L3M5		Normal	26	60	32	34	33	35
L3M6	Caju	Afetado	22	38	21	34	32	35
L3M7		Normal	22	38	21	34	32	35

NOTA – Os números indicados nos alelos representam o número relativo de repetições de microssatélites para cada marcador testado. Em azul e verde encontram-se assinalados os pares histocompatíveis.

2.2.3 Injeção intra-arterial de CTMs do cordão umbilical em cães GRMD

O transplante *in vivo* de CTMs do cordão umbilical foi realizado nos cães GRMDs Dani (L3F1), Rosa (L3F2) e Caju (L3M6), **FIG. 3A**. L3F1 e L3M6 receberam células cUCV normais provenientes dos pares compatíveis L3M5 e L3M7, respectivamente. Já L3F2 recebeu CTMs humanas provenientes do cordão umbilical (hUCMSC-15). A idade de início dos transplantes foi aos 51 dias, após os cães receberem a primeira dose de

vacinação. Ao todo foram realizados 7 transplantes utilizando como via de injeção a artéria femoral (**FIG. 3B**). O membro de injeção preferencial foi o direito. No entanto, algumas injeções tiveram que ser realizadas no membro oposto devido a dificuldades no acesso da artéria femoral. O intervalo médio entre as injeções foi de 30 dias, sendo que em dois momentos, injeções 3 e 4, tivemos que adiá-las devido a problemas na expansão *in vitro* das células e a uma infecção por giardíase nos cães transplantados. O número total de células utilizadas, por injeção, girou em torno de 1×10^7 células. O resumo destas informações e detalhes de cada injeção encontram-se expostos na **TAB. 2**.

Para verificar a viabilidade das células injetadas nos cães, utilizamos o corante fluorescente iodeto de propídio que é permeável à membrana celular apenas quando esta já está se fragmentando, um indicativo de morte celular. As leituras foram realizadas por citometria de fluxo, em amostras que sobraram na seringa utilizadas nas injeções, e que foram levadas ao laboratório logo após o transplante. O resultado obtido na **FIG. 4** representa a viabilidade da terceira injeção. Nesta, obtivemos uma viabilidade celular de 71,2% para a linhagem cUCV L3M5; 96,4% para a cUCV L3M7 e 92,4% para a linhagem hUCMSC-15. Com exceção desta leitura, as outras injeções obtiveram viabilidade celular superior à 90% para todas as amostras avaliadas.

FIGURA 3 – Injeção de CTMs caninas e humanas provenientes do cordão umbilical em cães GRMDs e biópsias musculares. (A) Os cães L3F1, L3F2 e L3M6, com 267 dias de vida e minutos antes à sedação, para a realização da sexta injeção de CTMs. **(B)** Injeção de CTMs via artéria femoral. A seta indica o retorno de sangue venoso na seringa, parâmetro utilizado para confirmar o acesso à artéria femoral. **(C)** Biópsia muscular. Após sedação e anestesia local os cães foram submetidos à biópsia nos músculos bíceps femoral e tibial cranial. As fotos demonstram a segunda biópsia muscular, realizada 4 dias após a sétima injeção, onde os cães apresentavam 312 dias de vida.

TABELA 2

Cronograma das injeções de CTMs nos cães GRMDs

	RECEPTOR	L3F1 - DANI - 3L	L3F2- ROSA - 3L7	L3M6 - Caju - 3L6
	DOADOR	L3M5 cUCV	hUCMS-15	L3M7 cUCV
	COMPATIVEL	SIM	NÃO	SIM
	BIOPSIA TO	NÃO	NÃO	NÃO
1A INJEÇÃO	DATA	15.05.2007	15.05.2007	15.05.2007
	IDADE (DIAS)	51	51	51
	PESO (KG)	1.900	2.100	0.800
	NO. CÉLULAS	1,41E+107	9,03E+106	1,93E+107
	PASSAGEM	6	7	6
	VOL. INFUNDIDO (ML)	1.5	1.5	1.5
	MEMBRO INJETADO	Esquerdo	Esquerdo	Direito
	VIA	ARTERIA FEMORAL	ARTERIA FEMORAL	ARTERIA FEMORAL
	OBSERVAÇÃO			
2A INJEÇÃO	DATA	18.06.2007	15.06.2007	21.06.2007
	IDADE (DIAS)	85	82	88
	PESO (KG)	9.500	8.500	8.000
	NO. CÉLULAS	1,99E+07	1,84E+07	6,88E+06
	PASSAGEM	7	6	7
	VOL. INFUNDIDO (ML)	10	15	10
	MEMBRO INJETADO	Direito	Direito	Direito
	VIA	ARTERIA FEMORAL	ARTERIA FEMORAL	ARTERIA FEMORAL
	OBSERVAÇÃO	L3F1 RECEBEU CELS. HUMANAS (LIN15) E L3F2 RECEBEU CELS. L3M5		
3A INJEÇÃO	DATA	14.08.2007	14.08.2007	14.08.2007
	IDADE (DIAS)	142	142	142
	PESO (KG)	14.300	15.000	13.000
	NO. CÉLULAS	1,84E+07	3,75E+07	6,08E+06
	PASSAGEM	6	7	7
	VOL. INFUNDIDO (ML)	10	10	5
	MEMBRO INJETADO	Direito	Esquerdo	Esquerdo
	VIA	ARTERIA FEMORAL	ARTERIA FEMORAL	ARTERIA FEMORAL
	OBSERVAÇÃO	3 CAES RECEBERAM CELULAS MARCADAS COM Dil. BIOPSIA 2 DIAS APÓS INIEÇÃO		
4A INJEÇÃO	DATA	11.10.2007 (3)	11.10.2007 (1)	11.10.2007 (2)
	IDADE (DIAS)	200	200	200
	PESO (KG)	18.000	18.900	18.700
	NO. CÉLULAS	1,29E+106	7,38E+106	5,46E+105
	PASSAGEM	5	9	6
	VOL. INFUNDIDO (ML)	5	5	5
	MEMBRO INJETADO	Direito	Esquerdo	Esquerdo
	VIA	ARTERIA FEMORAL	ARTERIA FEMORAL	ARTERIA FEMORAL
	OBSERVAÇÃO			
5A INJEÇÃO	DATA	21.11.2007 (3)	21.11.2007 (2)	21.11.2007 (1)
	IDADE (DIAS)	241	241	241
	PESO (KG)	20.100	19.000	19.400
	NO. CÉLULAS	1,58E+107	1,19E+107	3,22E+106
	PASSAGEM	6	8	7
	VOL. INFUNDIDO (ML)	10	10	10
	MEMBRO INJETADO	Esquerdo	Direito	Direito
	VIA	ARTERIA FEMORAL	ARTERIA FEMORAL	ARTERIA FEMORAL
	OBSERVAÇÃO			
6A INJEÇÃO	DATA	17.12.2007	17.12.2007	17.12.2007
	IDADE (DIAS)	267	267	267
	PESO (KG)	20.000	19.000	19.580
	NO. CÉLULAS	5.3E+106	4.9E+106	1.9E+106
	PASSAGEM	5	7	6
	VOL. INFUNDIDO (ML)	5	5	5
	MEMBRO INJETADO	Direito	Direito	Direito
	VIA	ARTERIA FEMORAL	ARTERIA FEMORAL	ARTERIA FEMORAL
	OBSERVAÇÃO			
7A INJEÇÃO	DATA	28.01.2008 (1)	28.01.2008 (2)	28.01.2008 (3)
	IDADE (DIAS)	309	309	309
	PESO (KG)	22	21	22
	NO. CÉLULAS	8.9E+106	1.5E+107	6.6E+106
	PASSAGEM	6	8	7
	VOL. INFUNDIDO (ML)	10	10	10
	MEMBRO INJETADO	DIREITO	DIREITO	DIREITO
	VIA	ARTERIA FEMORAL	ARTERIA FEMORAL	ARTERIA FEMORAL
	OBSERVAÇÃO	3 CAES RECEBERAM CELULAS MARCADAS COM Dil. BIOPSIA 3 DIAS APÓS INIEÇÃO

FIGURA 4 – Ensaio de viabilidade das CTMs utilizadas na segunda injeção dos cães GRMDs. Os gráficos representam o número relativo de células *versus* à intensidade da fluorescência. Em verde encontram-se as populações de células não marcadas com PI e em vermelho as populações marcadas com este corante. Os valores indicados nos gráficos demonstram a porcentagem de células mortas, ou seja, marcadas com PI, para cada uma das linhagens analisadas: L3M5 cUCV, L3M7 cUCV e hUCMSC-15.

2.2.4 Biópsias musculares

Dois dias após a segunda injeção e três dias após a sétima injeção, os cães transplantados foram submetidos à biópsia muscular (**FIG. 3C**). Visando o rastreamento das células injetadas nos músculos biopsiados, as células cUCV e hUCMSC-15 foram marcadas, previamente ao transplante, com o corante fluorescente Dil. Este corante possui coloração vermelho-alaranjado, é lipofílico e possui alta afinidade com a membrana celular. Além disso, o Dil permite rastrear as células por até 3-4 gerações de divisão celular. A **FIG. 5A** apresenta as linhagens utilizadas nos transplantes *in vivo* e marcadas com o corante Dil. Por citometria de fluxo foi possível quantificar a marcação das células utilizadas nas injeções 2 e 7, as quais apresentarem uma marcação positiva superior a 90% e superior a 85%, para este corante, respectivamente (**FIG. 5B-C**).

FIGURA 5 – Marcação das linhagens de CTMs, provenientes do cordão umbilical, com o corante fluorescente lipofílico Dil. Visando o rastreamento das células injetadas, as linhagens cUCV L3M5, cUCV L3M7 e hUCMSC-15 foram submetidas a marcação com o corante Dil. **(A)** Células previamente marcadas com o corante Dil e utilizadas nos transplantes *in vivo*. Aumento 100X. **(B-C)** Quantificação das células marcadas com o corante Dil. Os gráficos representam o número relativo de células *versus* à intensidade da fluorescência. Os valores indicados demonstram a porcentagem de células previamente marcadas com Dil e utilizadas na injeção 2 **(B)** e injeção 7 **(C)**.

2.2.5 Análise de DNA

Dentre os animais utilizados neste experimento, duas fêmeas (L3F1 e L3F2), receberam CTMs caninas e humanas, respectivamente, ambas do sexo masculino. Para verificar se as CTMs do cordão umbilical injetadas na artéria femoral são capazes de atravessar a barreira endotelial dos vasos sanguíneos e atingirem os músculos distróficos, analisamos se as biópsias pós-transplante das duas fêmeas injetadas apresentavam o gene *SRY*, um marcador do cromossomo Y, por análise de DNA.

Conforme mostrado na **FIG. 6A**, foi possível detectar a presença das CTMs do cordão umbilical canino nas biópsias B3M e B8M realizadas no bíceps femoral. Já as CTMs humanas do cordão umbilical foram detectadas apenas na biópsia B8M, também realizada no bíceps femoral. Os primers utilizados também permitiram confirmar que as CTMs caninas do cordão umbilical injetadas eram do sexo masculino (amostras 1 e 2), bem como as CTMs humanas utilizadas nos transplantes (amostra 11). Além disso, pode-se observar que o mesmo primer é específico para linhagens masculinas pois os mesmos não amplificaram amostras femininas caninas (amostra 5) e humanas (amostra 10). Finalmente, para descartar a possibilidade de falso-negativo na amostra 8, realizamos um PCR com primers que amplificam a distrofina canina para confirmar a presença de DNA nesta amostra (**FIG. 6B**).

FIGURA 6 – Análise de DNA. Rastreamento das CTMs masculinas provenientes do cordão umbilical após injeção em fêmeas GRMDs. **(A)** PCR para o marcador do cromossomo Y, *SRY* (“Sex-determining Region Y). **(B)** PCR controle utilizando o gene da distrofina (*DYS*). Amostras 1 e 2: CTMs caninas provenientes do cordão umbilical; Amostras 3 e 4: biópsias do músculo bíceps femoral provenientes do cão L3M6 – Caju; Amostras 6 e 7: biópsias do músculo bíceps femoral provenientes da cadela L3F1 – Dani; Amostras 8 e 9: biópsias do músculo bíceps femoral provenientes da cadela L3F2 – Rosa; Amostra 5: DNA de fêmea canina; Amostra 10: DNA humano sexo feminino; Amostra 11: CTM humana proveniente do cordão umbilical. B3M: Biópsia 3 meses pós-injeção; B8M: Biópsia 8 meses pós-injeção.

2.2.6 Análise de RNA

Para avaliar se as CTMs humanas injetadas em cães GRMDs foram capazes de expressar distrofina humana neste modelo animal, analisamos a expressão de mRNA, por RT-PCR, nos músculos biopsiados pós-injeção (**FIG. 7**). Conforme esperado, ao utilizar pares de primers específicos para a espécie canina (**FIG. 7A**), verificamos a presença de mRNAs da distrofina apenas em amostras dos músculos caninos biopsiados (amostras 2 a 8) e não nas amostras de músculo humano (amostra 1) ou nas amostras das células-tronco utilizadas nas injeções (amostras 9 a 11).

Ao utilizarmos pares de primers específicos para espécie humana (**FIG. 6B**), constatamos a presença de mRNA da distrofina apenas na amostra provenientes de músculo humano (amostra 1), como esperado, mas não nas biópsias provenientes das fêmeas que receberam as CTMs humanas de cordão umbilical (amostras 6 a 8).

Como controle endógeno e de eficiência da reação, foi avaliado a expressão do *GAPDH*, conforme mostrado na **FIG. 7C**.

FIGURA 7 – Análise de RNA. Avaliação da presença de mRNAs da distrofina humana em cães GRMDs transplantados com CTMs humanas provenientes do cordão umbilical. **(A)** RT-PCR utilizando primers específicos para a distrofina canina. **(B)** RT-PCR utilizando primers específicos para a distrofina humana. **(C)** Controle endógeno, RT-PCR utilizando primers para

GAPDH. Amostra 1: biópsia muscular humana; Amostras 2 a 8: Biópsias musculares caninas; Amostras 9 a 11: CTMs provenientes do cordão umbilical e utilizadas nas injeções. B3M: Biópsia 3 meses pós-injeção; B8M: Biópsia 8 meses pós-injeção.

2.2.7 Análise de proteínas

Estudos de proteínas expressas nos músculos biopsiados pós-transplante foram realizados pela técnica de western blot (WB) e imunofluorescência (IF). A **FIG. 8** mostra os resultados obtidos pela técnica de WB, onde foram analisados os anticorpos NCL-Dys1 (**FIG. 8A**), que reconhece tanto a distrofina humana como a canina, e Mandys106/2C6 (**FIG. 8B**), específico apenas para a distrofina humana. Conforme demonstrado na **FIG. 8A**, podemos notar a banda de 427kDa da distrofina na amostra contendo extrato de músculo normal canino (amostra 2), mas nenhuma expressão significativa de distrofina nos músculos biopsiados pós-transplante. Já a **FIG. 8B** mostra a especificidade do anticorpo Mandys106/2C6 pela a distrofina humana (amostra 3) mas não pela canina (amostra 2). Além disso, também não foi possível notar nenhuma expressão significativa de distrofina humana nas biopsias provenientes dos cães que receberam CTMs humanas do cordão umbilical (amostras 9 a 11).

O cachorro experimental L3M6 (Caju) veio a óbito aos 414 dias de idade, três meses após o recebimento da última injeção. Visando verificar se as CTMs caninas provenientes do cordão umbilical, injetadas na artéria femoral, foram capazes de restaurar a expressão de distrofina em diferentes grupos musculares, coletamos amostras do bíceps femoral, bíceps braquial, tríceps, quadríceps, tibial cranial, diafragma, sartório e gastrocnêmio, durante a realização da necropsia. Estes músculos foram processados para análise de WB e os resultados encontram-se evidenciados na **FIG. 9**. Conforme pode-se observar, em nenhum dos músculos do cão L3M6 foi possível encontrar expressão evidente de distrofina.

FIGURA 8 – Western blot pós-injeção de CTMs. Análise da expressão de distrofina em biopsias de cães GRMDs submetidos a múltiplas injeções de CTMs provenientes do cordão umbilical. **(A)** Reação com o anticorpo NCL-Dys1 e **(B)** reação com o anticorpo Mandys106/2C6. A banda de miosina permite comparar a quantidade de proteínas aplicadas para cada amostra.

FIGURA 9 – Western blot necrópsia L3M6. Análise da expressão de distrofina utilizando o anticorpo NCL-Dys1, em diferentes grupos musculares, 3 meses após a última injeção de CTMs caninas provenientes do cordão umbilical. A banda de miosina permite comparar a quantidade de proteínas aplicadas para cada amostra.

Análises da expressão de distrofina também foram realizadas pela técnica de IF em cortes seriados dos músculos biopsiados pós-transplante (**FIG. 10**). Neste caso, nenhuma expressão evidente de distrofina foi encontrada nos cortes analisados tanto com o anticorpo NCL-Dys1 (**FIG. 10B**), que reconhece a extremidade amino-terminal da proteína distrofina, como o anticorpo NCL-Dys2 (**FIG. 10C**), que reconhece a extremidade carboxi-terminal da distrofina. Através da coloração de H&E (**FIG. 10A**), observamos que todos os músculos dos cães que receberam CTMs apresentavam características típicas de músculos distróficos: alteração no calibre das fibras musculares, degeneração, fibrose, substituição por tecido conjuntivo e processos inflamatórios.

FIGURA 10 – Análise da estrutura muscular e expressão de distrofina.

Cortes seriados obtidos das biópsias musculares pós-injeção de células-tronco foram analisadas pela **(A)** coloração de H&E, **(B)** reação como o anticorpo NCL-Dys1 e **(C)** reação com o anticorpo NCL-Dys2. Os campos obtidos por IF foram adquiridos sob o mesmo tempo de exposição. Aumento 200x.

Também pela técnica de IF, avaliamos a expressão de distrofina humana nas biópsias musculares da cadela L3F2, que recebeu injeções seriadas de CTMs humanas do cordão umbilical. Conforme demonstrado na **FIG. 11**, podemos constatar que o anticorpo Mandys106/2C6 é específico para a distrofina humana, uma vez que o mesmo não reage em cortes de músculo canino (**FIG. 11B**), mas com os de corte muscular humano (**FIG. 11A**). Além disso, nenhum dos cortes musculares provenientes da cadela L3F2 apresentaram expressão de distrofina evidente (**FIG. 11C-E**).

FIGURA 11 – Análise da expressão de distrofina humana. Cortes seriados de biópsias musculares foram analisados, por IF com o anticorpo Mandys106/2C6, após múltiplas injeções de CTMs humanas de cordão umbilical na cadela L3F2. Os campos obtidos por IF foram adquiridos sob o mesmo tempo de exposição. Aumento 200x.

Por fim, para confirmar o enxerto de células humanas no músculo canino, realizamos IF com o anticorpo MAB1281 que reconhece núcleo humano (**FIG. 12A**), mas não núcleo canino (**FIG. 12D**). Além disso, neste experimento contra-coramos as lâminas com Dapi que marca os núcleos humanos e caninos em azul (**FIG. 12B,E**). Assim, após a sobreposição das imagens temos os núcleos humanos em roxo (**FIG. 12C**) e os núcleos caninos em azul (**FIG. 12F**). Dentre os múltiplos campos analisados fomos capazes de distinguir núcleos humanos na cadela L3F2 (**FIG. 12G-H**), a qual recebeu injeções seriadas de CTMs humanas do cordão umbilical. Estes dados também foram confirmados por análise de DNA (**FIG. 6**).

FIGURA 12 – Imunofluorescência para núcleo humano. Cortes musculares provenientes de **(A-C)** músculo normal humano; **(D-F)** músculo normal canino e **(G-I)** biópsia 8 meses pós-transplante da cadela L3F2. **(A, D, G)** imunofluorescência para núcleo humano (vermelho); **(B, E, H)** coloração Dapi (azul) e **(C, F, I)** sobreposição de imagens. Nos quadros em vermelho encontram-se evidenciados os núcleos humanos. Estes são mostrados em detalhe abaixo dos campos **G, H, I**. Os campos obtidos por IF foram adquiridos sob o mesmo tempo de exposição. Aumento 200x.

III – DISCUSSÃO

As células aderentes provenientes do cordão umbilical canino foram capazes de se diferenciar em músculo esquelético *in vitro* e expressar distrofina quando submetidos a dois protocolos distintos de diferenciação miogênica. Para tanto, avaliamos a expressão de mRNAs específicos da via de diferenciação muscular. Dentre eles, Myf5 and MyoD são marcadores precoces da diferenciação muscular e são requisitados para a determinação de linhagens de músculo esquelético. Já a miogenina e MRF4 são marcadores tardios da diferenciação muscular e são responsáveis por regular a fusão celular e a diferenciação terminal [6]. Apesar de não observar a expressão de todos estes marcadores citados nas células cUCV submetidas à miogênese, outros grupos relataram a expressão dos mesmos quando investigaram o potencial de diferenciação muscular das CTMs provenientes do sangue de cordão umbilical humano [6]. A não reprodução destes dados em nossos experimentos podem ser explicadas por diferenças no cultivo celular ou por se tratar da investigação de espécies diferentes (humana x canina). Conforme descrito no **CAPÍTULO 3**, também fomos capazes de confirmar o potencial miogênico das CTMs provenientes do cordão umbilical humano.

Em relação aos estudos *in vivo*, os dados analisados até o momento permitiram demonstrar que as CTMs caninas e humanas provenientes do cordão umbilical, quando injetadas na artéria femoral de cães GRMDs, são capazes de atingir o músculo distrófico. Estes dados puderam ser confirmados por análise de DNA e por IF. No entanto, em nenhuma das biopsias analisadas pós-transplante, B3M e B8M, não observamos a restauração da expressão de distrofina tanto por IF ou WB.

Até a presente data, três grupos independentes avaliaram o potencial das células-tronco na regeneração muscular utilizando cães GRMDs. No primeiro estudo, sem muito sucesso, Dell'Agnola e colaboradores (2004) descreveram que injeções de células hematopoiéticas

provenientes da medula óssea não são capazes de restaurar a expressão de distrofina em cães GRMDs [7]. Neste estudo, 7 cães receberam as células hematopoiéticas de irmãos compatíveis sob o regime de imunossupressão. Todos os cães transplantados tiveram enxerto completo ou quase completo das células hematopoiéticas nos compartimentos mielóide e linfóide do sangue periférico e medula óssea. No entanto, o número de fibras positivas para a distrofina não aumentou após o transplante de células-tronco hematopoiéticas.

No segundo estudo, Sampaolesi e colaboradores (2006) demonstraram que células-tronco mesenquimais caninas isoladas das veias de biópsias musculares, denominadas mesoangioblastos, possuem capacidade de restaurar a expressão de distrofina *in vivo* [8]. Estes autores realizaram 5 injeções seriadas, com intervalo mensal, na artéria femoral de cães GRMDs. Ao todo 10 cães foram utilizados no estudo, divididos em 3 grupos principais: a) 4 cães recebendo aos 4 meses de idade transplante autólogo de mesoangioblastos transduzidos com vetor lentiviral expressando microdistrofina humana; b) 4 cães recebendo aos 2 meses de idade transplante heterólogo sob regime de imunossupressão e c) 2 cães aos 4 meses de idade recebendo transplante heterólogo sob regime de imunossupressão. Ao final do experimento os autores concluíram que o transplante autólogo associado a terapia gênica é menos efetivo que os transplantes heterólogos.

As células cUCV são obtidas das paredes das veias sangüíneas assim como os mesoangioblastos. Devido a esta origem de obtenção comum, decidimos utilizar um protocolo de injeção semelhante ao realizado por Sampaolesi e colaboradores (2006), os quais avaliaram a eficiência dos mesoangioblastos, obtidos de pequenos vasos de biópsias musculares, na regeneração muscular *in vivo* em cães GRMDs [8]. Estes mesmos mesoangioblastos, segundo relatos do mesmo grupo, expressam proteínas as quais são utilizadas pelos linfócitos para ultrapassar a parede dos vasos e atingirem o tecido intersticial [9]. Diferente de Sampaolesi e colaboradores (2006) nossos transplantes se iniciaram aos 51 dias contra 75-159 dias realizado por este grupo, pois acreditamos que quanto menor o estágio de

degeneração muscular, ou seja, quanto menor a idade, maior a chances de sucesso no transplante celular [8]. Assim como Sampaolesi e colaboradores (2006) nosso grupo realizou transplantes seriados de CTMs via artéria femoral, ambos com intervalo de um mês [8]. Contudo, nos nossos experimentos realizamos um total de 7 injeções contra 5 realizadas pelo grupo italiano. Outro ponto a ser ressaltado em relação ao nosso protocolo experimental foi a ausência de realização de imunossupressão. Conforme relatado por Grounds & Davies (2007), o efeito da ciclosporina e outras drogas anti-inflamatórias reduzem a gravidade da distrofia muscular [10]. Devido a este fato, os resultados apresentados por Sampaolesi e colaboradores (2006) foram amplamente criticados devido à associação de ciclosporina, rapamicina ou rapamicina + IL10 nos transplantes heterólogos de mesoangioblastos em cães GRMDs [8].

O terceiro e mais recente trabalho publicado, compara a eficiência de injeções locais e sistêmicas em cães GRMDs transplantados com CTMs humanas de polpa de dente [11]. Os estudos começaram em cães com idade entre 28 a 40 dias sem tratamento com imunossupressores. De acordo com estes autores, as CTMs humanas de polpa de dente apresentaram enxerto significativo nos músculos de cães GRMDs, embora a expressão de distrofina humana observada foi modesta e limitada a poucas fibras musculares.

Em contraste com os resultados obtidos com mesoangioblastos, Kerkis e colaboradores (2008) e os resultados aqui apresentados não mostraram um aumento significativo na expressão de distrofina em cães GRMDs após injeção de CTMs [11]. Segundo Kerkis e colaboradores (2008) apenas um cachorro, denominado Chokito, apresentou expressão de distrofina humana, visto por imunofluorescência mas não por western blot [11]. Já nos nossos dados, nenhum dos cães avaliados apresentou restauração da distrofina. No entanto, tanto os dados obtidos com CTMs de polpa de dente como de cordão umbilical mostraram que estas células são capazes de migrarem para o tecido muscular esquelético, na ausência de imunossupressão, quando injetadas na artéria femoral de cães GRMDs.

IV – CONCLUSÕES

1. As células cUCV foram capazes de se diferenciar em músculo esquelético *in vitro* e expressar distrofina quando submetidos a dois protocolos distintos de diferenciação miogênica;
2. CTMs humanas e caninas provenientes do cordão umbilical foram rastreadas, pela presença do cromossomo Y, em biopsias musculares de fêmeas GRMDs que receberam transplante destas células pela via arterial;
3. Nenhuma expressão de distrofina humana foi observada por RT-PCR, imunofluorescência ou western blot na cadela L3F2 que recebeu a linhagem de CTMs humanas proveniente do cordão umbilical;
4. Nenhuma expressão de distrofina foi observada por imunofluorescência ou western blot nos cães GRMDs que receberam CTMs caninas do cordão umbilical;
5. Núcleos humanos foram detectados na cadela L3F2, por imunofluorescência, na biópsia de 8 meses pós-transplante;
6. Nenhuma evidência de expressão de distrofina, avaliada por western blot, foi encontrada nos diferentes músculos coletados na necropsia do cão L3M6.

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Capítulo 6

RINGO: UM CÃO GRMD EXCEPCIONAL

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Ringo: Discordance between the molecular and clinical manifestation in a Golden Retriever Muscular Dystrophy dog

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Abstract

The best clinical and histopathological animal model for DMD is the Golden Retriever Muscular Dystrophy (GRMD) dog, which presents a variable, but always severe and progressive muscle weakness. Among 77 GRMD dogs born in our colony in Brazil, we have identified a very mildly affected dog, Ringo, born July 2003. Among his descendants, at least one male, Suflair, is also showing a mild course. In an attempt to better characterize these two dogs, we studied the pattern of muscle proteins expression in Ringo and Suflair, as compared to severely affected and normal control dogs. Dystrophin was absent in both and utrophin was overexpressed in a pattern similar to the observed in severely affected dogs. Understanding the mechanism that is protecting Ringo and Suflair of the deleterious effect of the dystrophin gene mutation is of utmost interest. In addition it points out that the clinical impact of therapeutic trials should be interpreted with caution.

Resumo

O melhor modelo animal clínico e histopatológico para a distrofia muscular de Duchenne (DMD) é o cão distrófico da raça Golden Retriever, o qual apresenta fraqueza muscular variável mas sempre grave e progressiva. Dentre os 77 cães distróficos nascidos em nossa colônia no Brasil, identificamos um cachorro com fenótipo leve, chamado Ringo, nascido em Julho de 2003. Dentre os seus descendentes, pelo menos um macho, Suflair, também está apresentando um quadro leve. Com o intuito de melhor caracterizar estes dois cães, estudamos o padrão de expressão de proteínas musculares no Ringo e Suflair e comparamos com cães afetados com quadro grave e cães normais. Nossos resultados mostraram ausência de distrofina no Ringo e Suflair e super-expressão de utrofina, similar ao observado em cães com quadro grave. Desta forma, será de grande interesse entender o mecanismo que está protegendo Ringo e Suflair do efeito deletério da mutação no gene da distrofina. Além disso, nossos resultados enfatizam que os testes pré-clínicos neste modelo animal devem ser interpretados com cuidado.

Ringo: Discordance between the molecular and clinical manifestation in a Golden Retriever Muscular Dystrophy dog

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Keywords

Golden Retriever Muscular Dystrophy; Duchenne Muscular Dystrophy; dystrophin; mild phenotype

1. Introduction

Duchenne Muscular Dystrophy (DMD), a recessive lethal X-linked disease, is the most frequent and severe form of muscular dystrophy. It is caused by a complete absence of dystrophin in skeletal muscle due to mutations in the dystrophin gene [1]. DMD is manifested in young boys and the clinical course is severe and progressive. Affected individuals usually are confined to a wheelchair by 10-12 years of age and without special care and assisted ventilation death owing to respiratory or cardiac failure occurs usually before the third decade [2]. The Golden Retriever Muscular Dystrophy (GRMD) dogs represent an excellent model for human DMD. Affected animals have a frameshift point mutation within the splice acceptor site in intron 6 of the dystrophin gene, which results in the absence of the muscle protein. GRMD dogs and DMD patients have many phenotypic and biochemical similarities, including early progressive muscle degeneration and atrophy, fibrosis, contractures and raised serum creatine kinase levels [3]. However, differently from humans, GRMD may have difficulties in swallowing and loss of ambulation is uncommon. Death may occur within the first weeks but is most frequent around 1 or 2 years of age as a result of failure of respiratory or cardiac muscles.

In opposition to human DMD, there is a great clinical variability among GRMD dogs. Perinatal death is frequent but some dogs may have a longer survival and be less severely affected. Since we have established the Brazilian GRMD colony, which was initiated in April 2000 with the female carrier Beth, donated by Dr. Joe Kornegay (University of North Carolina), 77 affected dogs were born. Among them, we identified Ringo (Fig. 1a), born in July 2003 presenting a mild phenotype clearly distinguishable from other affected dogs, despite complete muscle dystrophin deficiency [4].

Ringo is able to run, to jump and stay in his rear paws more easily than the other affected dogs. Furthermore, Ringo is, to our knowledge, the only affected GRMD dog that opens doors (Fig. 1c) and able to breed naturally, originating an offspring of 26 affected dogs (18 males and 8 females), 12 female carriers and 11 normal males (Fig. 2). Interestingly, one of his male descendant named Suflair (Fig. 1b), currently aged 3 years, is showing a

milder course, although less than his father, which rules out the possibility of an unknown modifier X-linked gene responsible for this milder phenotype.

Understanding the mechanism that is protecting these two dogs of the deleterious effect of the dystrophin gene mutation is of utmost interest aiming future therapies. On the other hand, the great phenotypic variability observed among GRMD dogs turns very difficult to analyze the clinical success of therapeutic trials since the existence of animals like Ringo and Suflair could lead to misleading or inconclusive results in preclinical studies using this dog model.

Fig. 1. Ringo (a) and Suflair (b). Both dogs are able to run, jump, and stand in their rear paws. Ringo also learned how to open doors (c).

Fig. 2. Ringo's pedigree. Dogs were genotyped based on a restriction endonuclease site polymorphism generated by the mutation in the dystrophin gene. (a) All dogs in our kennel carry the same DNA mutation transmitted by the female carrier, Beth, founder of our dog colony. (b) Among the 221 dogs generated until now, we identified one dog, Ringo (arrow), who presents a very mild course. He was able to breed naturally and had until now 49 descendants. One of his male offspring, Sufclair is showing a relatively milder phenotype.

2. Materials and methods

2.1. Animals

All animals were housed and cared for in the Faculty of Veterinary Medicine and Zootecny of the University of São Paulo, and genotyped at birth as previously described [5]. Studies were done after approval by the ethical committee of animal use of the Institute of Biosciences, University of São Paulo.

2.2. Biopsy sampling and histology

Samples of biceps femoralis from normal and GRMD dogs were crioprotected and frozen in liquid nitrogen. Biopsy 7- μ m sections were used for routine histology and immunohistochemistry. Hematoxylin and eosin stain was carried out for morphology studies.

Quantification of collagen was performed in muscle section stained with picrosirius red (for connective fibrous tissue). Slides were examined with an Axioscope (Carl Zeiss). Using polarizing filters, three randomly selected viewing fields were digitized and the collagen cross-sectional area was determined using the software KS400 (Carl Zeiss).

2.3. Morphometry

H&E slides were used to perform morphometric analysis in Axiovert 200 microscope (Carl Zeiss). At least 300 fibers per animal were evaluated, in randomly selected fields, using Axiovision 3.1 software (Carl Zeiss). Photomicrographs were taken using 20X objective. For each animal investigated, measurements were made using the minimal Feret's diameter. Variability coefficients were calculated by dividing the standard deviation by the mean fiber diameter and multiplying this by 1000 [6].

2.4. Protein analysis

For protein analysis, immunohistochemical and western blot were performed according to methodologies previously described [7]. The following primary antibodies were used: anti-dystrophin NCL-DYS1 and NCL-DYS2 (Novocastra Laboratories); anti b-SG, g-SG and d-SG (Novocastra Laboratories); anti-utrophin MANCHO3 (a kind gift from Dr. Glenn Morris and Dr. Nguyen thi Man, from the North East Wales Institute, Wrexham, UK).

For immunohistochemistry, anti-mouse IgG-Cy3 conjugated secondary antibody (Chemicon) was used. Slides were examined in Axiovert 200 microscope (Carl Zeiss) and images were captured using Axiovision 3.1 software (Carl Zeiss).

For western blot, horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology) was used to detect immunoreactive bands with ECL plus chemiluminescence kit (GE Healthcare) or through colorimetric procedures using alkaline phosphatase (AP)-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology). Blots were scanned on Storm image system (GE Healthcare) and band densities were measured using ImageJ software (<http://rsb.info.nih.gov/ij/>).

3. Results

3.1. Histological analysis

Histopathological analysis from biceps femoralis muscles showed typical features of dystrophic process such as atrophy, splitting, necrosis, central nuclei, rounded fibers, hyaline fibers regeneration in the two unrelated and severely affected dogs (Fig. 3d and e), as well as in the two GRMD dogs with mild phenotype (Fig. 3b and c). A normal dog was analyzed as control (Fig. 3a). Sections stained with Picrosirius Red also showed high quantity of perimysium and endomysium connective fibrous tissue in severely (Fig. 3i and j) and mildly affected dogs (Fig. 3g and h) as compared with normal control

dog (Fig. 3f). This difference was quantified by measuring the red positive area as showed in Fig. 3k.

Fig. 3. Histological analysis. (a-e) H&E staining, (f-j) picrosirius red staining, (k) quantification of collagen content determined by picrosirius red staining. Biceps femoralis biopsies were investigated in normal dog, Silver (a, f); mildly affected dogs, Ringo (b, g) and Sufclair (c, h); severely affected dogs, Thor (d, i) and Lucky (e, j). Bars represent mean $\pm$ SEM. Magnification 200X.

3.2 Morphometry

Variation in fiber size was also present in dystrophic dogs. Quantitative measurements using fiber minimal diameter were assessed in two normal dogs (Fig. 4a and b), Ringo (Fig. 4c), Sufclair (Fig. 4d) and two severely affected dogs (Fig. 4e and f). Besides the mild phenotype, Ringo and Sufclair presented a fiber distribution much more similar to severely affected dogs than normal dogs. Furthermore, all affected dogs had the peak of fiber diameter shifted to the left (Fig. 4) and the mean fiber diameter was

decreased (Table 1), in relation to the control dogs. The variability coefficients of all affected dogs, despite of their phenotype, were greater than the generally accepted normal value of up to 250 [6] (Table 1).

Fig. 4. Bar histograms representing the fiber diameter distribution of muscle biopsies. The muscle fibers were grouped in size classes of 10 μm and the number of fibers in each class was plotted. (a) GRN-7 and (b) Silver normal dogs; (c) Ringo and (d) Sufclair, mildly affected dogs; (e) Thor and (f) Lucky, severely affected dogs.

Table1. Morphometric studies of GRMD dogs

Dog	Mean fiber diameter (μm)	Standard deviation	Variability coefficient
GRN-7	52,34	10,77	205,94
Silver	52,35	13,79	263,40
Ringo	36,63	15,37	419,61
Sufclair	31,70	10,17	320,94
Thor	40,75	15,71	385,66
Lucky	36,88	12,44	337,45

3.3 Dystrophin and sarcoglycans expression analysis

Immunohistochemistry showed a complete dystrophin deficiency in both severe (Fig. 5c and d) and mildly (Fig. 5e-h) affected GRMD dogs using rod-domain DYS1 (Fig. 5a, c, e and g) and C-terminal DYS2 (Fig. 5b, d, f and h) anti-dystrophin antibodies. The expression of sarcoglycan proteins b-SG, g-

SG and d-SG was greatly reduced in all affected dogs examined (data not shown), with no differences in the pattern of distribution or quantity.

Fig. 5. Dystrophin expression analysis. (a, c, e, g and i) immunoreaction with anti-dystrophin rod domain DYS1 antibody. (b, d, f, h and j) immunoreaction with anti-dystrophin c-terminus domain DYS2 antibody. Muscle biopsies were taken from biceps femoralis of: normal dog (a, b) GRN-1 and normal human muscle (FEM-3); severely affected dogs (c, d) Lucky and Thor; mildly affected dogs (e, f) Ringo and (g, h) Suflair. Images were acquired with the same exposure time and magnification of 200x. Myosin content in the Ponceau S pre-stained blot was used to assess the amount of loaded proteins.

For western blot, tissue lysates from Ringo, Suflair and two severely affected dogs (Lucky and Thor) were compared with human and dog normal muscles. The dystrophin band of 427 kDa was completely absent in Ringo, Suflair and the severely affected dogs, but was present in normal amount in the control samples, when analyzed with both DYS1 (Fig. 5i) and DYS2 (Fig. 5j) antibodies.

3.4 Utrophin expression analysis

In order to verify if the mild course could be due to differences in utrophin expression, utrophin distribution on muscle fibers was analyzed using the specific MANCHO3 antibody. This protein was present at neuromuscular junction in the normal dog but was highly concentrated in the sarcolemma of muscle fibers from both severely and mildly affected dogs, as usually observed in DMD patients as well (data not shown).

Utrophin expression was also investigated by western blot in Ringo, Suflair and compared to one normal and 3 severely affected dogs. The expression was upregulated in all affected dogs, independently of the severity or their clinical course (Fig. 6a). No striking difference in utrophin expression was observed in Ringo or Suflair as compared to other affected dogs (Fig. 6b).

Fig. 6. Utrophin expression analysis. (a) Western blot using utrophin MANCHO3 antibody was investigated in a normal dog (GRN-7), three severely affected dogs (Lola, Trakinas and Amandita) and two mildly affected dogs (Ringo and Suflair). (b) Relative quantification of utrophin expression in mildly and severe affected dogs compared with normal control dog. Data was normalized to myosin content in the Ponceau S pre-stained blot, used to assess the amount of loaded proteins.

4. Discussion

A normal phenotype despite the complete absence of muscle dystrophin has been reported in a 6-year-old boy [8] but apparently this was never observed in a GRMD dog. Although the phenotype is highly variable, even among dogs from the same litter, most of the GRMD dogs in our kennel are severely affected. Parameters such as dog motility, posture, contractures, hopping, standing up, barrier crossing and food prehension are used to evaluate the phenotype variability among our dogs.

GRMD dogs as well as DMD affected patients have gene mutations which lead to dystrophin deficiency. Although, scattered revertant dystrophin-positive fibers have been described in both [9,10], the quantity of revertant fibers could never be correlated with a better clinical course [11,12].

Here, all examined dogs, either severely or mildly affected, showed the

same pattern of dystrophin deficiency. The expression of sarcoglycan proteins was greatly reduced in all affected dogs examined, with no differences in the pattern of distribution or quantity. Therefore, the milder clinical course cannot be explained by a mechanism that could restore dystrophin in these dogs.

It has been suggested that up-regulation of utrophin might mitigate the clinical phenotype in the absence of dystrophin [13,14]. However, utrophin analysis in Ringo and his son Suflair showed a pattern that did not differ from severely affected dogs and therefore could not provide an explanation for their milder course.

Pedigree analysis showed that among Ringo's descendants there is a great phenotypic variability: 9 dogs died in the first 2 weeks, 5 dogs died before 1 year and 3 dogs after 1 year of age. However, at least one from the 9 alive descendants, Suflair, old enough to be clinically characterized is also showing a relatively milder course (although more severe than Ringo). This father to son transmission rules out the possibility of an X-linked modifier gene responsible for this mild phenotype. Therefore it is not known if this unusual phenotype is due to Mendelian inheritance with low penetrance, multifactorial inheritance, a different genetic background, several modifier genes with a small effect or other epigenetic factors.

On the other hand, although all GRMD dogs carry the same gene mutation, the great phenotypic variability observed among them turns very difficult to analyze the clinical success of therapeutic trials. A recent study demonstrated that repeatedly injections of mesoangioblasts in GRMD dogs reduced the severity of the disease [15]. However, a careful evaluation of each dog before and after any treatment is necessary to avoid misleading interpretations when examining the results of clinical trials [16-18].

The present report indicates that in the canine model it is possible to have a milder phenotype despite the complete absence of dystrophin. Understanding what protects this animal or other very rare patients [8] from the deleterious effects of the dystrophin mutation is of utmost interest and might open new avenues for treating these disorders.

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Capítulo 7

I - DISCUSSÃO GERAL

Até o presente momento, nenhuma terapia eficaz está disponível para os pacientes com distrofia muscular de Duchenne (DMD). Desta forma, a avaliação do uso terapêutico de células-tronco provenientes de várias fontes é alvo de estudo em diversos centros de pesquisas.

Os resultados aqui relatados tem como objetivo principal contribuir para o entendimento do potencial terapêutico das células-tronco adultas do cordão umbilical visando a regeneração muscular. Neste contexto, nosso estudo teve um enfoque *in vitro*, no qual isolamos e estabelecemos linhagens de células-tronco, que foram caracterizadas e expandidas em cultura para a realização de estudos *in vivo* em um modelo animal de grande relevância para a DMD.

Na primeira etapa do nosso estudo (**CAPÍTULO 2**), avaliamos o potencial terapêutico do transplante de células hematopoiéticas (TCH) provenientes do sangue do cordão umbilical canino em cães da raça Golden Retriever portadores de distrofia muscular (GRMD – “Golden Retriever Muscular Dystrophy”). Em nenhum dos cinco cães avaliados não encontramos expressão de distrofina nas amostras de músculos biopsiados ou necropsiados em níveis clinicamente relevantes após o TCH. Estes dados vão de acordo com estudos previamente descritos na literatura, os quais também relatam resultados similares ao transplantar células hematopoiéticas provenientes da medula óssea em cães GRMDs [1,2]. Além disso, chamamos a atenção para a publicação de resultados negativos para que pesquisadores da mesma área não precisem repetir estes experimentos além de alertar os pacientes que buscam informações por terapias, protegendo os mesmos de danos financeiros ou psicológicos.

Devido ao insucesso dos TCH do sangue do cordão umbilical em cães GRMDs, partimos para o estudo das células tronco mesenquimais (CTMs) também provenientes do cordão umbilical. Neste sentido, comparamos a eficiência do isolamento desta população no sangue e no próprio tecido do cordão umbilical humano de amostras pareadas provenientes do mesmo paciente, as quais foram processadas e cultivadas nas mesmas condições (**CAPÍTULO 3, ANEXO I**). Nossos resultados mostraram que não o sangue e sim o tecido do cordão umbilical é rico em CTMs. Uma vez que a rotina nos bancos de cordão umbilical é guardar o sangue e descartar outras fontes como o tecido do cordão umbilical, alertamos a população e a comunidade científica de que os bancos estão descartando uma fonte preciosa de CTMs. Apesar das células-tronco hematopoiéticas terem grande utilidade no tratamento de doenças hematológicas [3-6], as CTMs possuem teoricamente uma plasticidade bem maior devido a sua origem no mesoderma, podendo originar osso, músculo, cartilagem e gordura, abrindo um leque maior de utilização na medicina regenerativa.

No **CAPÍTULO 4** extrapolamos a metodologia de isolamento de CTMs do cordão umbilical humano para o modelo canino. Assim como relatado para humanos, obtivemos um sucesso de 100% no isolamento de CTMs do cordão umbilical canino. Estas linhagens, denominadas cUCV – “canine umbilical cord vein” foram avaliadas quando a sua plasticidade, sendo capazes de se diferenciar em adipócitos, condrócitos e osteócitos *in vitro*.

Com o intuito de avaliar o potencial miogênico das CTMs provenientes do cordão umbilical realizamos estudos *in vitro* e *in vivo*. Demonstramos que tanto as CTMs humanas (**CAPÍTULO 3**) e caninas (**CAPÍTULO 5**) provenientes do cordão umbilical são capazes de se diferenciar em músculo esquelético e expressar distrofina, *in vitro*. Nos estudos *in vivo*, realizamos 7 injeções consecutivas de CTMS masculinas do cordão umbilical em 3 cães GRMDs (**CAPÍTULO 5**). Análises preliminares permitiram rastrear as CTMs humanas e caninas através da presença do cromossomo Y no músculo das fêmeas transplantadas. No entanto, não observamos expressão de distrofina nas amostras de biopsias ou necropsias musculares. Além disso, nossos resultados levantaram diversos questionamentos ainda não respondidos,

dentre eles: Por quanto tempo as células permanecem no tecido muscular? Elas são rejeitadas após certo tempo? Estas células são capazes de se diferenciar em músculo e expressar distrofina *in vivo* a longo prazo? Ou as mesmas teriam apenas um efeito parácrino benéfico? Visando dar continuidade a este estudo, decidimos avaliar o potencial miogênico das CTMs do cordão umbilical canino e humano em camundongos *sjl*, modelo animal para a distrofia muscular de cinturas tipo 2B [7]. Esta abordagem será baseada em um estudo publicado recentemente pelo nosso grupo que descreve o transplante de CTMs humanas de gordura de lipoaspiração nestes camundongos [8]. Estes autores demonstraram que estas células são capazes de fundir com o músculo murino e expressar proteínas musculares humanas, melhorando a performance muscular do camundongo *sjl*. Assim, pretendemos utilizar a mesma metodologia visando comparar a eficiência das CTMs do cordão umbilical na regeneração muscular com os dados previamente obtidos em nosso grupo (em andamento).

Por último, descrevemos um cão GRMD excepcional (**CAPÍTULO 6, ANEXO 2**). Dentre os 77 cães GRMDs gerados em nosso canil até hoje, um deles vêm nos chamando muita atenção. Ringo, apesar da completa ausência de distrofina nas fibras musculares, apresenta um fenótipo leve de distrofia muscular quando comparado com outros cães afetados. O mesmo vem sendo observado para o seu filho, Suflair, o qual também apresenta um quadro leve comparável ao seu progenitor. Embora todos os cães GRMDs carregam a mesma mutação, a variabilidade fenotípica entre eles é enorme, o que torna difícil avaliar o sucesso de abordagens terapêuticas. Contudo, chamamos atenção para que cada cão GRMD seja avaliado cuidadosamente antes e após a abordagem terapêutica para evitar conclusões precipitadas nos ensaios pré-clínicos com este modelo animal. Além disso, o entendimento do mecanismo que está protegendo Ringo e Suflair do efeito deletério da mutação no gene da distrofina abrirá novas frentes de pesquisa para o tratamento da DMD.

II – CONCLUSÕES

- 1.** Células-tronco hematopoiéticas provenientes do cordão umbilical canino não são capazes de restaurar a expressão de distrofina em níveis clinicamente relevantes em cães GRMDs;
- 2.** O tecido do cordão umbilical é a fonte mais rica e de fácil obtenção de células-tronco mesenquimais, comparando-se com o sangue do cordão umbilical;
- 3.** Células-tronco mesenquimais caninas multipotentes podem ser isoladas de maneira simples e rápida de todas as amostras de cordão umbilical obtidas;
- 4.** Células-tronco mesenquimais caninas e humanas do cordão umbilical são capazes de se diferenciar em músculo esquelético e expressar distrofina *in vitro*;
- 4.** Células-tronco mesenquimais caninas e humanas do cordão umbilical são capazes de chegar à musculatura de cães GRMDs, quando injetadas por via arterial, mas não de restaurar a expressão de distrofina em níveis clinicamente relevantes;
- 5.** Cães GRMDs, apesar da completa ausência de distrofina nas fibras musculares, podem apresentar variabilidade fenotípica enorme, desde um quadro leve até extremamente grave.

III – REFERENCIAS BIBLIOGRÁFICAS

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Anexo I

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Mesenchymal stem cells from umbilical cord: do not discard the cord!

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Annotation

Mesenchymal stem cells from umbilical cord: Do not discard the cord!

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On 10th February 2007, English entrepreneur Sir Richard Branson, best known for his Virgin brand of over 360 companies, announced the establishment of a cord blood bank, the Virgin Health Bank, in London. What makes this bank different is a dual public–private approach. A fifth of the cord blood would be stored for private use for the child or a family member and the rest would be donated to the public part of the bank which will be accessible to anyone in the world who needs it, at no cost. Additionally, Branson has pledged to donate his 50% of proceeds from Virgin Health Bank to researchers investigating the potential of cord blood stem cells [1].

The idea for the dual bank emerged when he was visited by a senior director of the National Blood Center, asking for his support in a charitable role, because children were dying through lack of umbilical cord blood. Initially Sir Richard offered 3 million pounds to the National Health Service to help them increase their storage capacity for umbilical cord blood, but the center was not comfortable with accepting funds from private sources. So Sir Richard decided to set up a company to do the job.

This is an initiative that should be applauded but we want to draw attention to a very important and urgent aspect. The routine procedure in umbilical cord banks has been to store the blood and discard other tissues, such as the cord and/or placenta, which is a much better source of mesenchymal stem cells than blood [2]. Umbilical cord

blood is rich in haematopoietic stem cells [3–6]. However the successful isolation of mesenchymal stem cells, which are undifferentiated cells able to self-renew, with high proliferative capacity, from the cord blood is controversial [7–13]. These cells comprise a rare population of multipotent precursors that in addition to supporting haematopoiesis are able to differentiate into various cell types such as chondrocytes, osteocytes, adipocytes and myocytes [14–17].

Bone marrow has been considered as one of the main sources of mesenchymal stem cells for both experimental and clinical applications and most of the knowledge concerning these cells came from bone marrow studies. However, obtaining them is painful and yields a low number of cells [18,19]. More recently, human umbilical cord blood taken after full term delivery of the newborn, from a sample that would be inevitably discarded has been regarded as an alternative source for transplantation and therapy because of its haematopoietic and mesenchymal components. However, while some researchers succeeded in the isolation of mesenchymal stem cells from umbilical cord blood [7–10] others failed or obtained low yield [11–13]. More recently, some groups have reported success in isolating and establishing mesenchymal stem cells cultures from umbilical cord vein and UC stroma, also called Wharton's jelly [11,16,20,21]. According to Tondreau et al. [22], these discrepant results might be explained by different isolation and culture methodologies.

In order to address this question we compared, for the first time, the efficiency in obtaining mesenchymal stem cells from 10 match-paired umbilical cord units harvested from the same donors, where the blood and the cord were

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processed simultaneously and under the same culture conditions [2]. Mesenchymal stem cells from blood were obtained from only one among the 10 cord samples collected but we were able to generate large amounts of primary mesenchymal stem cells cultures from all cord samples, with a 100% yield, which showed that the low yield of these progenitor cells in umbilical cord blood is not due to different culture methodologies. These results are in accordance with our previous experience where we observed that the efficiency in isolating mesenchymal stem cells from approximately 100 umbilical cord blood units stands around 10% (unpublished data). These cells isolated by a fast and simple procedure using short enzymatic digestion, may be expanded *in vitro*, cryogenic stored and thawed [2]. Despite the advantages of umbilical cord blood in hematopoietic reconstitution [3–5], results from our study demonstrated that the umbilical cord and not the blood is the best choice for isolating mesenchymal stem cells for future applications.

Storing umbilical cord blood in private or public banks has been recently the subject of many ethical dilemmas. A great obstacle to the altruistic proposal of Sir Richard Branson to split umbilical cord blood samples in both private and public banks is that the number of cells from one umbilical cord blood unit is usually not sufficient for an adult weighing more than 50 kg, which is a main limiting factor. In short, the important take home message is that regardless of being public or private, these banks are discarding the umbilical cord, a precious source of mesenchymal stem cells. Therefore, we suggest storing both the blood and the umbilical cord, which would allow maximum recovery of hematopoietic and mesenchymal stem cells for possible therapeutic applications in the future. Moreover, the possibility of originating different cell lines obtained from umbilical cord of neonates with malformations or genetic disorders, in particular myogenic lineages from patients with mutations responsible for neuromuscular disorders, may open new avenues for research of vast interest in addition to speed up screening process for therapeutic drugs.

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Anexo II

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Ringo, a golden retriever muscular dystrophy (GRMD) dog with absent dystrophin but normal strength.

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Clinical Casebook

Ringo, a Golden Retriever Muscular Dystrophy (GRMD) dog with absent dystrophin but normal strength

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The closest model to human Duchenne Muscular Dystrophy (DMD) is the Golden Retriever Muscular Dystrophy (GRMD) dog, which carries a point mutation in the splice acceptor site in intron 6 of the orthologous X-linked dystrophin gene, leading to the absence of protein in the muscles. These dogs present clinical signs within the first weeks of life involving the limbs as well as masticatory muscles. Diaphragmatic and intercostal muscles impairment leads to progressive respiratory failure. Death occurs from bronchopneumonia and cardiac arrest, usually before 2 years of age.

Here, we report the case of Ringo, an exceptional GRMD dog showing an unusually mild course. Currently, at age 4 years and 10 months he is able to run, jump and open doors while standing on his rear paws. He was also able to breed naturally, which apparently has never been reported before.

Ringo is descendant of Beth, a GRMD female carrier donated by Dr. Joe Kornegay (University of North Carolina, USA), and all affected descendants carry the same original mutation. The diagnosis in all dogs was established right after birth through DNA genotyping and elevated serum creatine kinase (CK). At birth, Ringo's serum CK level was increased 10-fold as compared to his three normal sibs. One affected brother had a comparable serum CK while the other had a 20-fold increase. At 15 days serum CK in the affected dogs was 4- to 5-fold higher than in normal siblings. Pedigree analysis (Fig. 1) revealed that Ringo had two affected brothers from the same litter. One of them died at 2 weeks of age. The other one is still alive but with a

much more severe phenotype, comparable to the usual clinical phenotype. Ringo was able to copulate naturally with four carrier females, which resulted in a total of 38 descendants: 12 affected males, 7 affected females, 11 carriers and 8 normal dogs. As the result of the first mating, with Xenina, 9 puppies were born: 1 affected female, 3 normal males and 5 carrier females. As the result of the second mating, with the female Hope, 12 puppies were born: 7 affected males, 1 affected female, 3 carrier females and one normal male. Four males showed a severe course and died of acute respiratory failure in the first days of life. However, interestingly, one male, Suflair, is also showing a mild course. He was born in April 2006 and at age 2 years and 1 month is able to run and jump, presents a normal posture tone and no contractures. The third mating was in 2007 with Lady, a carrier female from the kennel that was found to be accidentally pregnant. There were two possible fathers: Ringo and Suflair. Paternity testing (with microsatellite markers) confirmed that Ringo was the culpable father, which generated 7 additional offspring(s): 2 affected females, 2 affected males, 2 normal males and 1 carrier female. The fourth mating, with Kira, generated 10 puppies born in September of 2007: 4 affected males, 2 affected females, 2 carrier females and 2 normal males. However, 4 normal dogs died of a viral infection.

Ringo's performance has apparently never been observed in GRMD dogs. Although their phenotype may be highly variable, even among dogs from the same litter, most of the GRMD dogs are severely affected and die before 24 months of age. In the Brazilian colony, we observed that 24% died within the first 2 weeks, 17% died before 6 months, 24% before 12 months, 11% before 18 months and 24% after 24 months of age.

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Fig. 1. Ringo's Pedigree. The numbers below each symbol represent how many animals in each category: ■ affected male, ● affected female, ⊙ carrier female, ○ non-carrier female and □ normal male.

Interestingly, histopathological and immunohistochemistry analysis from Ringo's biceps biopsy showed typical features of a dystrophic process with variability in fiber size, splitting, degeneration and connective tissue as well as complete dystrophin deficiency, comparable to severely affected GRMD dogs (with both rod- and C-terminal anti-dystrophin antibodies). Sarcoglycans were partially deficient, and utrophin was overexpressed, in a pattern similar to the observed in severely affected dogs.

How much dystrophin is required in order to mitigate the clinical phenotype has always been an open question. Ringo's phenotype, which is not explained by higher amounts of dystrophin or up-regulation of utrophin, shows that GRMD dogs may have a mild course despite the complete absence of dystrophin, which opens a new avenue of investigations. On the other hand this observa-

tion reinforces the importance of taking into account clinical variability in the assessment of results of therapeutic trials that are currently underway. What is protecting Ringo and Suffair from the mitigating effect of the dystrophin mutation? The observed father to son transmission rules out X-linked inheritance and therefore this unusual phenotype could be due to modifier genes, to multifactorial inheritance or to other epigenetic factors. Complete absence of dystrophin expression associated with a mild clinical course has been recently reported in humans (Neuromuscul Disord 2006;16:865–6). “*Treasure your exceptions*”, says Victor Dubowitz. Maybe Ringo and his descendants could enhance our comprehension on unknown mechanisms that might protect skeletal muscle to degenerate despite the absence of dystrophin.

Biografia

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