UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

# AVALIAÇÃO DE TÉCNICAS DE ISOLAMENTO E COMPARAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS HUMANAS DE MEMBRANA AMNIÓTICA, MEMBRANA CORIÔNICA, CORDÃO UMBILICAL E DECÍDUA DA PLACENTA

ANELISE BERGMANN ARAÚJO

Porto Alegre
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Tese apresentada como requisito parcial para obtenção do título de Doutor em Medicina: Ciências Médicas, da Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Medicina: Ciências Médicas

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RESUMO

Base teórica: Células-tronco mesenquimais (CTMs) são células multipotentes, com capacidade de autorrenovação e diferenciação. Devido às suas características, têm sido estudadas para utilização na terapia celular de diferentes patologias. Podem ser isoladas da medula óssea, tecido adiposo, tecidos neonatais, polpa dentária, entre outros. A utilização de CTMs isoladas de tecidos neonatais apresenta vantagens sobre as fontes de tecidos adultos, como quantidade e facilidade de obtenção, sem envolvimentos éticos associados, ausência de traumas para o doador, células com menor possibilidade de incorporação de mutações, melhor diferenciação, menor imunogenicidade e melhor capacidade de *homing.* As características biológicas das CTMs, bem como o seu potencial terapêutico, podem variar dependendo da fonte e protocolo de isolamento utilizados. Objetivo: Comparar protocolos para isolamento de CTMs de quatro fontes humanas de um mesmo indivíduo: decídua da placenta (D-CTMs), membrana amniótica (A-CTMs), membrana coriônica (C-CTMs) e segmento do cordão umbilical (CU-CTMs), a fim de definir o melhor protocolo de isolamento para cada fonte tecidual. Além disso, comparar as CTMs isoladas destas fontes teciduais em relação à imunofenotipagem, capacidade de diferenciação, tamanho em suspensão e cultura, índice de polaridade e potencial de crescimento. Métodos: Foram isoladas D-CTMs, A-CTMs, C-CTMs e CU-CTMs de doadoras saudáveis utilizando quatro protocolos enzimáticos de isolamento. Os tecidos foram submetidos a quatro protocolos de isolamento. As CTMs isoladas foram submetidas a ensaio de diferenciação *in vitro*, imunofenotipagem por citometria de fluxo, ensaio de dobro populacional, comparação do tamanho em suspensão por citometria de fluxo, análise do comprimento e largura em cultura e cálculo do índice de polaridade. Todos estes parâmetros foram comparados entre as CTMs isoladas e foi realizada análise estatística, considerando significativo $p<0,05$. Resultados: Foi possível isolar e caracterizar as CTMs de todas as fontes estudadas. A- CTMs e CU-CTMs foram isoladas de todas as amostras utilizando protocolos com tripsina e colagenase; C-CTMs foram obtidas de todas as amostras com dois protocolos, sendo um utilizando colagenase isoladamente e outro associando colagenase e tripsina; D-CTMs foram isoladas em todas as amostras exclusivamente com o protocolo à base de colagenase. C-CTMs em suspensão apresentaram o tamanho menor, enquanto CU-CTMs apresentaram o maior comprimento e a menor largura quando em cultura. A-CTMs mostraram menor índice de polaridade e CU-CTMs foram as células mais alongadas, com o índice de polaridade mais elevado. C-CTMs, D-CTMs e CU-CTMs foram semelhantes em capacidade de crescimento até P8; C-CTMs apresentaram maior longevidade em cultura, enquanto A-CTMs apresentaram

proliferação insignificante. Conclusão: O protocolo utilizando colagenase foi considerado o ideal para obtenção de D-CTMs e C-CTMs, enquanto CU-CTMs puderam ser isoladas usando protocolos que utilizam associação de colagenase e tripsina. Não foi possível determinar um protocolo adequado para isolamento de CTMs de membrana amniótica. Tanto tecidos fetais, quanto o tecido materno podem ser utilizados como fonte de CTMs. Porém, considerando as vantagens de células imaturas, CTMs isoladas de cordão umbilical e membrana coriônica demonstraram ser uma opção mais apropriada para avançar em estudos visando a utilização em terapia celular.

Palavras-chave: células-tronco mesenquimais, membrana amniótica, membrana coriônica, cordão umbilical, placenta, anexos embrionários, fontes de células-tronco mesenquimais, terapia celular

ABSTRACT

Background: Mesenchymal stem cells (MSCs) are multipotent cells with differentiation and self renewal ability. From this, many studies involve the isolation, cultivation and application of MSCs for therapeutic use in a diversity of diseases. Studies using MSCs employed bone marrow and adipose tissue as principal sources. However, fetal adnexa appears as a good alternative for MSCs isolation, since it is usually discarded without use, obtained easily in large quantities, with non-invasive collection, without ethical implications associated, without trauma to the donor and presents lower adverse effects in clinical trials. Biological properties and therapeutic potential of MSCs depends on source and the isolation protocol used. Objective: The objective of this study was to compare the effectiveness of four enzyme-based protocols for MSC isolation from four human fetal adnexa (amniotic membrane – A-MSCs, chorionic membrane – C-MSCs, umbilical cord - UC-MSCs and placental decidua – D-MSCs) and to define the optimal protocol for isolation from each tissue. Moreover, to compare biological characteristics of MSCs isolated from these sources in order to verify immunophenotipe, differentiation ability, size, polarity index and growth kinetics. Methods: Tissues were collected from healthy mothers and four protocols were applied for isolation of MSCs. Immunophenotipe, differentiation ability, cell size, cell complexity, polarity index and grow kinetics of MSCs isolated from the four sources were analyzed. MSCs were compared considering all parameters. Differences were considered significant when $p<0.05$. Results: MSCs were successfully isolated from all four sources. The surface marker profile and differentiation ability were consistent with human MSCs. A-MSCs and UC-MSCs could be isolated from all samples using trypsin/collagenase-based protocols; C-MSCs could be isolated from all samples with collagenase- and trypsin/collagenase-based protocols; and D-MSCs were isolated from all samples exclusively with a collagenase-based protocol. MSCs in suspension were the smaller cells, while UC-MSCs presented the higher length and lower width. A-MSCs showed a lower polarity index and UC-MSCs were the more elongated cells, with the higher polarity index. C-MSCs, D-MSCs and UC-MSCs were similar in growth capacity until P8; C-MSCs presented better lifespan and insignificant proliferation was observed in A-MSCs. Conclusion: The collagenase-only protocol was best for C-MSCs and D-MSCs, while a combination of trypsin and collagenase was considered best for UC-MSCs. None of the tested protocols was adequate for isolation of A-MSCs; all yielded heterogeneous cultures with short lifespans and limited cell growth. Both fetal and maternal tissues serves as source of multipotent stem cells. However, chorionic membrane and umbilical cord were considered good options for future use in cell therapy because of its advantages of immature cells.

Key Words: mesenchymal stem cells, amniotic membrane, chorionic membrane, umbilical cord, placental decidua, fetal adnexa, MSCs sources, cell therapy

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## REVISÃO DA LITERATURA



## ARTIGO 1

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# LISTA DE SIGLAS E ABREVIATURAS

A-CTMs - células-tronco mesenquimais derivadas da membrana amniótica/A-MSCs

CD - *cluster of differentiation*

cm - centímetros

CTMs – células-tronco mesenquimais / MSCs (mesenchymal stem cells)

C-CTMs - células-tronco mesenquimais derivadas da membrana coriônica/C-MSCs

CU-CTMs - células-tronco mesenquimais derivadas do cordão umbilical/UC-MSCs

CXCR4 - chemokine receptor type 4 - receptor de quimiocinas presente na superfície de CTMs

D-CTMs - células-tronco mesenquimais derivadas da decídua da placenta/D-MSCs

DECH - doença do enxerto contra o hospedeiro / GVHD - graft versus host disease

FAK - focal adhesion kinase

g - gramas

hCG - gonatrofina coriônica humana

HLA - antígeno leucocitário humano

IL - interleucina

IP - índice de polaridade / PI - polarity index

IPs - induced pluripotent stem cells

LFA-3 - lymphocyte function-associated antigen

LPS - lipopolissacarídeo

µm - micrômetros

SCUP – banco de sangue de cordão umbilical e placentário

SDF-1 - stromal derived factor-1

TRAIL - tumor necrosis factor related apoptosis inducing ligand

Thy-1 - antígeno de superfície celular

ÍNDICE

# 1. INTRODUÇÃO

As células-tronco mesenquimais (CTMs) são células multipotentes (Horwitz et al., 2005), aderentes ao plástico de cultivo, inicialmente caracterizadas pela expressão positiva para CD 105, CD 73 e CD 90, expressão negativa para CD45, CD34, CD14 ou CD11b, CD79α ou CD19 e HLA-DR e pela capacidade de diferenciação em osteoblastos, condroblastos e adipócitos (Dominici et al., 2006). Estão distribuídas por diversos compartimentos do organismo (Meirelles et al., 2006; Hass et al., 2011; Liu et al., 2011; Yang et al., 2011; Hattori et al., 2015; González et al., 2015). Devido à sua capacidade de diferenciação e autorrenovação, são responsáveis pela reparação e manutenção de variados tecidos vivos (Caplan, 1991). Além disso, o efeito parácrino das CTMs contribui nos processos de uso terapêutico (Komiecik et al., 2013). Os achados sobre o comportamento imunomodulatório das CTMs aumentaram as perspectivas terapêuticas do uso dessas células (Talwadekar et al., 2015). Assim sendo, baseados nas características de auto-renovação, diferenciação e imunomodulação, diversos estudos envolvem o isolamento, cultivo e/ou aplicação de CTMs para uso terapêutico em diferentes patologias, como reparo de tecido cardíaco (Zhao et al., 2005, Simpson et al., 2012), doenças fetais pré e pós-natais (Shaw et al., 2011), fibrose pulmonar (Tzouvelekis et al., 2011), falência hepática (Parekkadan et al., 2007), doença do enxerto contra o hospedeiro pós transplante alogênico (Nauta e Fibbe, 2007; Wu et al., 2011); diabetes tipo 1 (Fiorina et al., 2009; Fiorina et al., 2011), colite (Parekkadan et al., 2011), lesão renal (Hattori et al., 2015), doenças auto-imunes (Tanaka 2015), entre outros tantos ensaios clínicos em andamento (https://clinicaltrials.gov/).

A utilização de CTMs humanas com finalidade terapêutica tem sido amplamente estudada a partir de isolamento de medula óssea (Tepliashin et al., 2005, Flemming et al., 2011), tecido adiposo (Tepliashin et al., 2005; He et al., 2011; Yang et al., 2011), sangue periférico e, mais recentemente, polpa dentária (Tomic et al., 2011), sangue de cordão umbilical e placentário (Bieback et al., 2004; Kawasaki-Oyama et al., 2008, Fan et al., 2009) e outros anexos embrionários, como geleia de Wharton, fluido amniótico, membrana amniótica, membrana coriônica e decídua da placenta (Soncini et al., 2007; Hass et al., 2011; Kanematsu et al., 2011; Shaw et al., 2011; Wu et al., 2011; Manochantr et al., 2013; Choudhery et al., 2013; Gonzalez et al., 2015). Estas fontes relacionadas ao embrião são origens alternativas de material biológico, usualmente descartadas sem utilização, obtidas com facilidade em grandes quantidades, com vantagens de coleta não invasiva, segura e sem grandes envolvimentos éticos associados ou traumas para o doador (Hass et al., 2011). Outra importante vantagem do isolamento de CTMs provenientes de tecidos perinatais é a obtenção de células jovens, com menor possibilidade de

incorporação de mutações (Bieback e Brinkmann, 2010), além de melhor diferenciação, menor imunogenicidade e melhor capacidade de *homing* (Martineli et al., 2016).

Para avaliação de células-tronco mesenquimais de qualidade adequada para posteriores estudos terapêuticos, é importante analisar a forma de isolamento e qual o melhor tecido para ser usado como fonte celular. Há uma grande diversidade de protocolos de isolamentos das CTMs nos diversos tecidos, com variáveis como o tipo e concentração de enzima e período de incubação. Porém, não há um consenso sobre a melhor forma de isolar as CTMs de cada tipo de fonte tecidual. Bortolotti et al. (2015) verificaram que a ação terapêutica das CTMs pode variar não só pela fonte de obtenção das células, mas, também, de acordo com o protocolo de isolamento. Em função da fonte de onde são isoladas, as CTMs apresentam grande variação em suas características de imunofenotipagem, diferenciação, proliferação, migração, entre outras. Assim sendo, o presente trabalho envolve o isolamento, caracterização, avaliação do potencial de crescimento, avaliação do potencial de diferenciação e avaliação do índice de polaridade de células-tronco mesenquimais provenientes de quatro fontes teciduais não invasivas para posterior uso em terapia celular. Salienta-se, também, que na maioria das amostras, foi possível a comparação de quatro fontes do mesmo contexto fisiológico, geneticamente idênticas ou haploidênticas, descartando, assim, as variáveis interpessoais.

# 2. REVISÃO DA LITERATURA

## 2.1 ESTRATÉGIAS PARA LOCALIZAR E SELECIONAR AS INFORMAÇÕES

Esta revisão da literatura está focada nos principais aspectos relacionados às características das células-tronco mesenquimais humanas e possibilidades de aplicação terapêutica. A busca de informações foi realizada nas bases de dados: SciELO e PubMed, além de consulta a livros, todos com período de publicação entre 1974 e 2016. Foram realizadas buscas utilizando principalmente os termos: mesenchymal stem cells, human mesenchymal stem cells, amniotic membrane, chorionic membrane, placenta, umbilical cord, isolation protocols, cellular therapy, cell size, mesenchymal stem cells sources, immunophenotype, sendo que foram utilizados 133 artigos para compor esta revisão bibliográfica.

## 2.2. CÉLULAS-TRONCO MESENQUIMAIS

Friedenstein e colaboradores (1974) descreveram o isolamento e cultivo *in vitro* deste grupo celular, quando observaram uma população celular da medula óssea semelhante a fibroblastos e com aderência ao plástico, sendo inicialmente denominadas Unidades Formadoras de Colônia semelhantes a Fibroblastos (UFC-F). A denominação células-tronco mesenquimais (mesenchymal stem cells - MSC) foi adotada posteriormente, conforme orientação da Sociedade Internacional de Terapia Celular (Horwitz et al., 2005). Células-tronco apresentam as características básicas de autorrenovação, ou seja, multiplicam-se gerando novas células idênticas, a fim de manter a população ativa no tecido onde se encontram; além da capacidade de diferenciação em tecidos especializados. A identificação da população isolada como CTMs depende de um conjunto de propriedades morfológicas, fenotípicas e funcionais (Dominici et al., 2006), as quais serão melhores descritas posteriormente.

No homem, estão distribuídas em diversos compartimentos do organismo, sendo que já foram isoladas em medula óssea (Friedenstein et al., 1974), tecido adiposo (Yang et al., 2011), tecidos neonatais (Hass et al., 2011; González et al., 2015), mucosa da laringe (Liu et al., 2011), dente (Tomic et al., 2011; Hattori et al., 2015; Ren et al., 2016), sangue menstrual (Patel e Silva, 2008), músculo esquelético e derme (Young et al., 2001), membrana sinovial (De Bari et al., 2001), cartilagem (Alsalameh et al., 2004), sangue periférico após mobilização medular com G-CSF (Kassis et al., 2006), entre outros. Meirelles et al (2006) defendem que, por sua localização perivascular, podem estar presentes em todos os tecidos vascularizados, como cérebro, pâncreas, timo, baço, rim, fígado, pulmão. Também há relatos de isolamento de CTMs de trofoblastos (Veryasov et al., 2014).

Muitos estudos em andamento e, com resultados promissores, utilizam CTMs isoladas de tecidos adultos, principalmente medula óssea e tecido adiposo. Porém, em função da facilidade de obtenção, insignificância dos componentes éticos associados e vantagens em características celulares, tem crescido a utilização de anexos embrionários como fonte de CTMs. CTMs provenientes de tecidos perinatais são células jovens, com menor possibilidade de incorporação de mutações (Bieback and Brinkmann, 2010), além de melhor diferenciação, menor imunogenicidade e melhor capacidade de *homing* (Martineli et al., 2016). Estas células possuem uma grande capacidade migratória tanto *in vitro* (Li et al., 2011) quanto *in vivo* (Kholodenko et al., 2012). A frequência de células no tecido e sua capacidade de diferenciação também estão relacionadas com a idade do doador (Baxter et al., 2004; Stolzing et al., 2008; Pappa et al., 2009). Pesquisas iniciais apontavam o uso do sangue de cordão umbilical e placentário para o isolamento alternativo das células-tronco mesenquimais. Porém, tendo sido identificada a dificuldade no isolamento de CTMs de sangue de cordão (Reinisch et al., 2007), que pode ser inferior a 50% (Malgieri et al., 2010), outros segmentos dos anexos embrionários têm sido explorados como alternativas para o isolamento de CTMs, como geleia de Wharton, fluido amniótico, membrana amniótica, membrana coriônica e decídua da placenta (Hass et al., 2011; Kanematsu et al., 2011; Shaw et al., 2011; Wu et al., 2011; Manochantr et al., 2013; Choudhery et al., 2013; González et al., 2015).

## 2.3. PLACENTA E TECIDOS FETAIS

O feto desenvolve-se submerso no líquido amniótico, em um espaço delimitado mais internamente pela membrana amniótica (âmnion) e, adjacente a esta, o córion liso, que, por sua vez, relaciona-se com a decídua placentária materna. A camada formada pelo córion e âmnion denomina-se de membrana cório-amniônica. O feto é ligado à placenta e membranas através do cordão umbilical (Figuras 1 e 2).

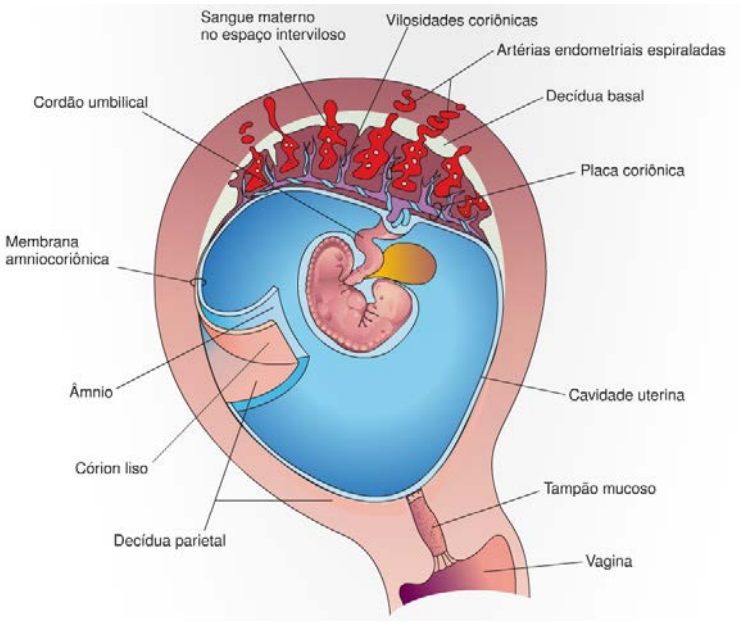


Figura 1: Esquema de um corte sagital de útero gravídico de 4 semanas (Fonte: Moore et al., 2012)

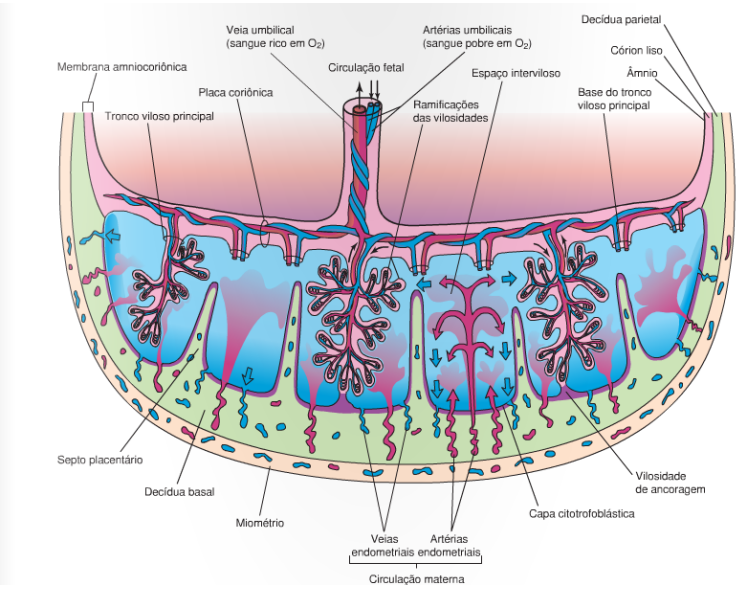


Figura 2: Esquema do corte transverso de uma placenta a termo (Fonte: Moore et al., 2012)

Âmnion: membrana mais interna, resistente e flexível, composta de cinco camadas: epitélio simples, membrana basal, camada de colágeno, camada rica em células de Hofbauer e fibroblastos e camada acelular esponjosa, de estruturas do celoma extra-embrionário. É desprovida de nervos, células musculares, vasos linfáticos e vasos sanguíneos e é adjacente ao cório (Fonseca et al., 2008). Células epiteliais e células mesenquimais, ambas com

características de células-tronco, podem ser obtidas por protocolos específicos aplicados à membrana amniótica (Kmieck et al., 2013).

Córion: composto por citotrofoblasto e células mesenquimais, divide-se em cório liso (avascular, adjacente ao âmnio) e córion frondoso/placa coriônica (porção mais vascularizada, diretamente conectada com o embrião, na região em torno da inserção do cordão umbilical). Duas populações celulares são obtidas desta membrana: células mesenquimais e células trofoblásticas (Kmieck et al., 2014)

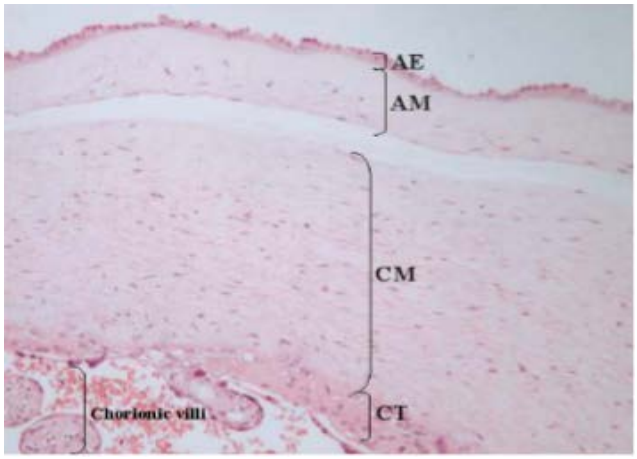


Figura 3 – Corte transversal de membranas fetais humanas (âmnion e córion). O âmnion é composto por uma camada epitelial de células cubóides e colunares, as quais se encontram sobre uma camada mesodérmica, constituída por uma camada compacta acelular superior e uma camada mais profunda contendo fibroblastos dispersos. A membrana coriônica consiste de uma camada mesodermal e uma camada de células trofoblásticas extravilosas. Abreviaturas: AE; epitélio amniótico; AM, mesoderme amniótica; CM, mesoderme coriônica; CT, trofoblasto coriônico. Fonte: Parolini et al. (2008)

Cordão umbilical: origina-se da vesícula vitelínica e liga o abdome fetal à placenta. Sendo recoberto pelo âmnio, é espiralado, apresenta aparência esbranquiçada e tamanho variado, de 30-100 cm comprimento (média 55 cm) e 0,8-2 cm diâmetro, contendo duas artérias e uma veia (Fonseca et al., 2008).

Placenta: órgão materno-fetal, específico da gestação, pesando 300-600g, altamente vascularizado, geralmente de formato redondo ou ovalado, desenvolvido a partir do endométrio materno sob ação hormonal. Apresenta a face fetal, em contato com a placa coriônica, e a face materna, ligada à parede uterina. É imprescindível para formação e manutenção do feto, pois tem inúmeras funções, dentre elas: trocas gasosas materno-fetais, transporte de nutrientes ao feto e excreção de metabólitos do feto e produção de enzimas e hormônios (hCG, tireotrofina coriônica humana, lactogênio placentário humano, corticotrofina coriônica humana, progesterona e estrogênios) (Fonseca et al., 2008; Moore et al., 2012).

Quando ocorre o parto, após 10-30 minutos do nascimento do recém-nascido, a placenta e as membranas fetais são expelidas pelo útero, processo chamado de dequitação (Fonseca et

al., 2008; Moore et al., 2012). A quantidade de tecido amniótico disponível em uma placenta é a mais escassa, seguido pelo córion e cordão umbilical, sendo a decídua da placenta o tecido mais abundante.

## 2.4. ISOLAMENTO DAS CÉLULAS-TRONCO MESENQUIMAIS

Atualmente, a maioria dos estudos, *in vitro* ou *in vivo*, envolvendo células-tronco mesenquimais utiliza células obtidas de medula óssea, tecido adiposo ou tecidos perinatais. O isolamento de CTMs dos diversos tecidos tem sido realizado através de protocolos variados (Parolini et al., 2008). Bortolotti et al. (2015) verificaram que a ação terapêutica das CTMs depende não só da fonte celular, mas também do protocolo de isolamento. Originalmente, o isolamento de CTMs da medula óssea foi realizado pela capacidade de aderência das células ao plástico quando colocadas em placas de cultivo (Friedenstein et al., 1974). A concentração das CTMs pré cultivo é realizada com o auxílio de solução com gradiente de densidade específica para formação de uma camada contendo as células mononucleares (Rebelatto et al., al., 2011). CTMs de tecido adiposo são obtidas preferencialmente utilizando digestão com colagenase tipo I (Blande et al., 2009; Rebelatto et al., 2008; Vishnubalaji et al., 2012) e tipo IV (Choudery et al., 2013). Também as CTMs de origem dentária podem ser isoladas através da digestão enzimática do tecido com colagenase (Ren et al., 2016) ou associação de enzimas contendo colagenase (Hattori et al., 2015).

O isolamento de CTMs de sangue de cordão umbilical, assim como da medula óssea, também é realizado com o auxílio de solução de gradiente de centrifugação com densidade específica (Bieback et al., 2004; Rebelatto et al., 2008; Avanzini et al., 2009; Zhang e al., 2011). Já para isolamento de CTMs de tecidos perinatais como membrana amniótica, membrana coriônica, decídua da placenta e cordão umbilical, muitos protocolos utilizam a digestão enzimática com colagenase dos tipos I (Barlow et al., 2008; Kanematsu et al., 2011; Kim et al., 2011; Seo et al., 2013), II (Lu et al., 2013; Zhu et al., 2013) ou IV (Lee et al., 2012), podendo estas serem usadas isoladamente (Kang et al., 2012) ou associadas a outras enzimas. Também, alguns estudos utilizam apenas a tripsina para o tratamento tecidual no isolamento de CTMs de anexos embrionários (Yen et al., 2005; Castrechini et al., 2010; Manochantr et al., 2013; Martini et al., 2013). Os protocolos utilizando digestão enzimática combinada incluem o uso de colagenase I/DNase I/dispase (Kanematsu et al., 2011), colagenase I/DNase I (Barlow et al., 2008; Brooke et al., 2008; Kim et al., 2011), colagenase II/dispase II (Portmanz-Lanz et al., 2006), colagenase I/tripsina (Koo et al., 2012; Seo et al., 2013), colagenase II/tripsina (Lu et al., 2013), colagenases I e II/tripsina (González et al., 2015). Estas substâncias são usadas em

diferentes concentrações e o tempo de digestão enzimática pode variar de 10 minutos (Yen et al., 2005; Castrechini et al., 2010) até mais de 18 horas (Fu et al., 2006). Também é descrito o isolamento não enzimático de CTMs, apenas com adesão do tecido da placa em presença de meio de cultivo (Bosch et al., 2012; Zhu et al., 2013; González et al., 2015; Mahmood et al., 2015). Ainda, alguns estudos incluem no protocolo de isolamento o uso de gradientes de densidade para separação de camada mononuclear após a digestão enzimática do tecido (Barlow et al., 2008; Brooke et al., 2008; Lu et al., 2013; Zhu et al., 2013).

Outras variáveis podem imprimir pequenas diferenças entre a metodologia de isolamento, dentre estas, utilização ou não do processo de filtração para separação dos fragmentos não digeridos, lavagem ou não do material durante o processo de isolamento, diferentes soluções de lavagem (tampão fosfato salino-PBS, solução balanceada salina de Hank-HBSS, soro fisiológico), centrifugação para utilização apenas do *pellet* no cultivo celular e tempo de troca do meio/tempo de adesão das CTMs.

## 2.5. CARACTERIZAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS

Conforme descrito anteriormente, dentre os critérios para classificação de CTMs (Dominici et al., 2006), as células isoladas, quando submetidas a estímulos apropriados, devem apresentar habilidade de diferenciação em linhagens mesodermais: adipócitos, condrócitos e osteócitos. Após indução *in vitro* para adipogênese, condrogênse e osteogênese, as células apresentam vacúolos lipídicos citoplasmáticos, depósitos citoplasmáticos de cálcio e formação de cartilagem, com matriz extracelular rica em glicosaminoglicanos, respectivamente. Esta característica deve-se ao fato destas células serem "estromais", ou seja, derivadas do folheto embrionário intermediário (mesoderma), responsável pela formação dos tecidos adiposo, cartilaginoso e ósseo. Porém, muitos estudos relatam a capacidade das CTMs originarem outros tipos celulares (Hu et al., 2009), incluindo células de linhagem neuronal, hepatócitos, células pancreáticas, células epiteliais e renais (Squillaro et al., 2016). Já foi descrita a capacidade de células mesenquimais de âmnion diferenciarem-se em células das três camadas germinativas: neural (ectoderme), muscular esquelética, cardiomiocítica e endotelial (mesoderme) e pancreática (endoderme), além da diferenciação na triade básica necessária para critérios de células-tronco mesenquimais (Kmiecik et al., 2013). CTMs do córion também apresentam capacidade de diferenciação em miócitos e células semelhantes às neuronais (Komiecik et al., 2013). CTMs isoladas da geleia de Wharton do cordão umbilical mostraram potencial de diferenciação em linhagens de adipócitos, osteócitos, células endócrinas, neurais (Montanucci et

al., 2011). O amplo potencial de diferenciação das CTMs em diversas linhagens celulares incrementa as perspectivas de sua aplicabilidade em terapia celular.

Vários antígenos de superfície são utilizados para a identificação de CTMs isoladas de diversos tecidos, embora não existam marcadores específicos para este tipo celular. Para padronização dos estudos em CTMs foi proposto um conjunto de marcadores mínimos utilizados como critério para classificação de CTMs (Dominici et al., 2006). As células devem apresentar expressão positiva para CD105 (endoglina), CD73 (ecto-5'nucleotidase) e CD90 (molécula de adesão Thy-1), expressão negativa para CD45 (marcador de células hematopoéticas), CD34 (marcador de células-tronco hematopoéticas), CD14 (receptor LPS, marcador de monócitos) ou CD11b (integrina expressa principalmente em granulócitos, macrófagos e células NK), CD79α (IgA, expressa, principalmente, em linfócitos B maduros) ou CD19 (marcador de linfócitos B) e HLA-DR (pertence ao complexo de histocompatibilidade classe II), embora a proposta é que, no decorrer dos progressos nos estudos de CTMs, estes parâmetros devam ser revisados. Destes, CD105 geralmente apresenta uma maior variação de resultados entre os marcadores positivos para CTMs (Bieback et al., 2004; Portman-Lanz et al., 2006; Soncini et al., 2007; Kanematsua et al., 2011; Kim et al., 2011; Veryasov et al., 2014). Entretanto, a expressão das proteínas de superfície de pode variar de acordo com a fonte de células (Rebelatto et al., 2008) e com o número de passagens das células (Ren et al., 2016).

Muitos outros marcadores intracelulares ou de superfície celular vem sendo investigados para complementar a caracterização das CTMs, bem como a relação destes com alguma propriedade específica das células. Moléculas do sistema HLA classe I (Tse et al., 2003), além de CD29 e CD44 (Bieback et al., 2004; Barlow et al., 2008; Rebelatto et al., 2008; Blande et al., 2009; Kanematsu et al., 2011; Bortolotti et al., 2015; Paladino et al., 2015), antígenos relacionados à adesão celular, também são expressos significativamente na superfície de CTMs, bem como os antígenos CD13 e CD166 (Soncini et al, 2007; Wetzig et al., 2013), mesmo que estes sejam expressos também por outros grupos celulares. CD54, molécula de adesão intracelular, e CD49d, importantes na migração pelas barreiras endoteliais até o sítio de inflamação, também auxiliam na caracterização das CTMs por apresentarem-se com alta expressão na superfície das células (Tse et al., 2003; Karlsson et al., 2011).

A presença de HLA-G na superfície ou intracelular tem sido pesquisada devido à sua relação com as propriedades imunomodulátórias das CTMs (Nasef et al., 2007, Kim et al., 2011; Ding et al., 2016). Em CTMs de âmnion, há relatos de 17% da expressão HLA-G na superfície (Wang et al., 2014), enquanto que em CTMs de placa coriônica e cordão umbilical, aproximadamente 10% de HLA-G na superfície celular foi descrito (Kim e al., 2011). Em outro

estudo, 90,8% HLA-G intracelular foram relatados em CTMs de cordão umbilical por citometria de fluxo e, também, a positividade extracelular foi demonstrada utilizando outros ensaios (Ding et al., 2016). Os marcadores CD10, CD49a e Stro-1, aparecem em torno de 10% das células-tronco mesenquimais de medula óssea (Weitzig et al., 2013). Alguns estudos relacionam a expressão de CD56 com maior potencial clonogênico (Batulla et al., 2009), bem como a expressão de CD146 com a capacidade de diferenciação osteogênica (Kaltz et al., 2010). O percentual de positividade de CD56 varia em diferentes relatos, visto que em certos estudos apresenta baixo percentual de positividade em CTMs de cordão umbilical (Ding et al., 2016), enquanto pode ser descrito com positividade superior a 50% também em CTMs de cordão umbilical, além de CTMs de membrana coriônica (González et al., 2015). A presença de CD271 na superfície de CTMs está associada à maior capacidade de proliferação, migração (Latifi-Pupovci et al., 2015), um maior potencial condrogênico (Alvarez et al., 2015), maior potencial osteogênico e clonogênico (Soncini et al., 2007). CD58 (molécula de adesão LFA-3) também pode ser expressa em quantidade baixa nas CTMs (Tse et al., 2003).

A ausência de antígenos HLA da classe II na superfície, bem como dos marcadores CD40 (relacionado à ativação do sistema imune), CD80 e CD86 (coetimuladores de linfócitos T) (Tse et al., 2003; Abumaree et al., 2013), contribuem fortemente para que as células-tronco mesenquimais sejam consideradas pouco imunogênicas em transplantes alogênicos. CTMs isoladas de placa coriônica também não expressam as moléculas CD83 e B7H2 (Abumaree et al., 2013).

Marcadores de outros tipos celulares, os quais podem estar contaminando as culturas de CTMs, além dos marcadores que já foram citados nos critérios mínimos, também são pesquisados a fim de verificar a pureza das células em cultivo, tais como: CD31, marcadores de células epiteliais; CD3 e CD19, marcadores de linfócitos. Geralmente, são descritas quantidades insignificantes de marcadores de células hematopoiéticas (CD34, CD45, CD3 e CD14). Porém, alguns estudos demonstram contaminação das culturas com células positivas para estes marcadores, que pode ser superior a 20% de CD34 e CD14 para as CTMs de placa coriônica (Indumathi et al., 2013), 16,7% de CD45 para CTMs da decídua da placenta (Kanematsu et al., 2011) e 17% de CD45 para CTMs de membrana coriônica (Koike te al., 2014). CD133 não foi encontrado na superfície de CTMs de medula óssea, âmnion e córion (Soncini et al., 2007).

A idade do doador de CTMs pode influenciar nas características das células isoladas. Baxter et al., (2004) e Stolzing et al. (2008) verificaram diferenças na expressão dos marcadores de superfície e no potencial de diferenciação de CTMs isoladas de medula óssea em relação à

idade do doador. Zhou et al. (2008) testaram a capacidade de diferenciação em osteoblastos e também verificaram diminuição em relação a CTMs de doadores mais velhos.

## 2.6. CRESCIMENTO *IN VITRO*

Em função da sua capacidade de autorrenovação, as CTMs realizam divisão mitótica, multiplicando-se em curto espaço de tempo. Quando em cultura, claramente pode ser observado o fenômeno de expansão celular, visto que uma vez visualizada uma célula isolada, após um período em condições de cultura, pode ser observada uma colônia de células formada a partir da célula inicial; após plaqueamento de um número celular, um número superior ao inicial poder ser recuperado.

Por definição, as CTMs não têm um número limite de divisões mitóticas que podem realizar (Caplan, 1991). Entretanto, Banfi et al. (2000) afirmam que a há diminuição do potencial da taxa de proliferação *in vitro* desde as primeiras passagens, havendo perda gradual das propriedades progenitoras iniciais. Apesar dos dados contraditórios acerca do número de passagens em que as células mesenquimais param a proliferação, alguns autores referiram que CTMs podem ser cultivadas até P9-P15 (Komiecik et al., 2013). A capacidade de proliferação de CTMs de cordão umbilical até mais de P25 foi relatada (Ding et al., 2016). Blande et al. (2009) mantiveram crescimento constante de CTMs de tecido adiposo até P10. Bruder et al. (1997) mantiveram o crescimento de CTMs da medula óssea até uma média de 38 passagens em experimento de dobro populacional, quando se tornaram maiores e mais achatadas, provavelmente associado ao fenômeno de quiescência. Izadpanah et al. (2008) observaram declínio na eficiência proliferativa de medula óssea e tecido adiposo depois de P10, mas as células mantiveram crescimento até P15 e P30, respectivamente. Já Baxter et al. (2004) observaram o término de crescimento de CTMs de medula óssea em P10, com, em média, 197 dias em cultura. CTMs de membrana coriônica mantiveram-se em cultura até P15 (Soncini et al., 2007). CTMs de membrana amniótica apresentam muita variação de crescimento entre os estudos. Soncini et al. (2007), mantiveram culturas até P15, enquanto Portmann-Lanz et al. (2006) reportaram morte das células entre P4-P5. Mesmo com alguns dados encontrados na literatura, a maioria dos estudos finaliza as análises antes das CTMs encerrarem seu crescimento, dificultando a definição do limite de passagens em que as células podem realizar a expansão em número. No entanto, é bem descrito que existem diferenças nas taxas de proliferação de CTMs de diferentes fontes (Barlow et al., 2008; Zhang et al., 2011; Choudery et al., 2013; Indumathi et al., 2014; González et al., 2015; Ren et al., 2016).

A duração do período de crescimento das CTMs é influenciada pela idade do doador. Doadores de medula óssea para isolamento de CTMs foram divididos em doadores jovens (18-29 anos) e doadores idosos (68-81 anos). CTMs de doadores idosos apresentaram decréscimo no limite de vida celular em cultivo em quase metade (dobro populacional CTMs idosos 24 *versus* 41 de CTMs jovens) (Stenderup et al., 2003). Estes dados são confirmados por outros estudos envolvendo CTMs de medula óssea, onde também a menor idade do doador está relacionada com células com maior potencial de crescimento e que se expandem por maiores períodos (Baxter et al, 2004; Stolzing et al., 2008; Zhou et al., 2008). Também esta tendência pode ser comprovada pela superioridade do crescimento de CTMs de tecidos neonatais, quando comparados a CTMs de tecidos adultos. CTMs de sangue de cordão apresentam maior potencial de proliferação e expansão se comparadas a CTMs de tecido adiposo e meia vida das células da medula óssea (Zhang et al., 2011), bem como, também as CTMs obtidas da membrana amniótica e fluido amniótico apresentam maior crescimento do que CTMs isoladas de medula óssea (Hu et al., 2009; Shaw et al., 2011).

Embora seja conhecida a capacidade de CTMs manterem-se em cultura por muito tempo *in vitro*, estudos mostram que as CTMs apresentam instabilidade genética e podem apresentar alterações moleculares ao longo das passagens, mesmo, muitas vezes, não perdendo as propriedades de diferenciação, crescimento e imunomodulação (Binatto et al., 2008). O número de cromossomos das CTMs de medula óssea e tecido adiposo permaneceu normal em estudo que analisou células em passagens iniciais e tardias (até P20 e P30, respectivamente), assim como também não foram observados rearranjos cromossômicos. Entretanto, os dados sugerem uma descompensação do ciclo celular de CTMs em longo tempo de expansão *in vitro* (Izadpanah et al., 2008).

## 2.7. COMPARAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS DE DIFERENTES TECIDOS

Estudos têm demonstrado que as propriedades das CTMs variam de acordo com a fonte (Kim et al., 2011, Zhang et al., 2011, Indumathi et al., 2013, Veryasov et al., 2014; González et al., 2015; Ren et al., 2016) e o método de isolamento utilizado (Bortoliti et al., 2015). Muitos estudos comparam as características celulares de CTMs isoladas de tecidos adultos e perinatais, mas ainda existem divergências sobre a superioridade de alguma fonte tecidual.

### 2.7.1. Comparação CTMs adultas

CTMs de medula óssea e tecido adiposo apresentam expressão similar de marcadores de superfície (Vishnubalaji et al., 2012). Células mesenquimais isoladas do tecido adiposo apresentam maior poder imunossupressor em comparação às células mesenquimais de medula

óssea quanto à inibição da diferenciação de monócitos em células dendríticas, expressão de moléculas de superfície coestimulatórias, secreção de quimiocinas e estímulo na secreção de IL-10 (Ivanova-Todorova et al., 2011). Por outro lado, Puissant et al. (2005) encontraram similaridade nas propriedades imunossupressoras de células-tronco adultas de tecido adiposo e CTMs de medula óssea. Também foi observado que o potencial osteogênico de CTMs de medula óssea é superior ao obtido por CTMs de tecido adiposo (Vishnubalaji et al., 2012).

2.7.2. Comparação CTMs tecidos neonatais x CTMs adultas

CTMs sangue cordão umbilical e placentário (SCUP): As CTMs de sangue de cordão apresentam maior potencial de proliferação (Zhang et al., 2011; Jin et al., 2013), expansão e diferenciação em condrócitos, se comparadas a CTMs de tecido adiposo e medula óssea (Zhang et al., 2011). Avanzini et al. (2009) relataram semelhança em potencial de proliferação, diferenciação e imunofenótipo entre CTMs de SCUP e medula óssea. Também Zhang et al. (2011) consideraram a expressão de antígenos de superfície bastante semelhantes em CTMs isoladas de SCUP e medula óssea, com a vantagem de as CTMs isoladas do SCUP apresentarem menor expressão de HLA-ABC, podendo diminuir a reação do hospedeiro. Zhang et al. (2011) não encontraram diferenças do potencial imunomodulador comparando CTMs de sangue de cordão, tecido adiposo e medula óssea. Porém, Avanzini et al. (2009) e Jin et al. (2013) encontraram maior capacidade de imunossupressão em CTMs de SCUP do que CTMs de medula óssea. O sucesso no isolamento das CTMs é bastante superior nas amostras de medula óssea do que SCUP (Avanzini et al., 2009).

CTMs cordão umbilical: CTMs isoladas da geleia de Wharton do cordão umbilical apresentaram superior potencial proliferativo e imunossupressor do que células mesenquimais da medula óssea (Wu et al., 2011). CTMs de cordão umbilical e placenta foram similares na expressão de marcadores de superfície e capacidade de diferenciação em gordura, osso e cartilagem, mas CTMs de cordão umbilical mostraram maior potencial proliferativo (Choudery et al., 2013). Considerando como fontes: cordão umbilical, sangue menstrual e polpa dentária, o potencial de crescimento foi maior em CTMs de cordão umbilical, enquanto CTMs de polpa dentária se sobrepuseram na diferenciação osteogênica e menor apoptose e senescência (Ren et al., 2016).

CTMs placenta: CTMs isoladas da placenta humana apresentam similaridade às isoladas de medula óssea em características de proliferação, morfologia, tamanho e marcadores de superfície em diferentes condições de cultura (tipos de meio de cultivo, diferentes suplementos, tipos de frasco de cultivo e tempo de troca de meio). Entretanto as células oriundas da placenta apresentaram maior e mais rápido crescimento além de uma menor capacidade de

diferenciação adipogênica quando comparadas às células isoladas de medula óssea (Barlow et al, 2008). Em outro estudo, CTMs isoladas da placenta também apresentaram características semelhantes às de medula óssea e foram utilizadas com segurança em transplante alogênico com sangue de cordão (Brooke et al., 2008). Ainda, células isoladas da decídua de placenta humana "a termo" apresentaram boa diferenciação em condrócitos, moderada diferenciação em adipócitos e pouca evidência de osteogênese, quando comparadas a células mesenquimais isoladas de medula óssea. (Kanematsua et al., 2011).

CTMs membrana amniótica: CTMS de membrana amniótica apresentaram expressão de marcadores de superfície similares a CTMs de medula óssea (Soncini et al., 2007). As CTMs obtidas da membrana amniótica apresentam maior crescimento do que CTMs isoladas de medula óssea (Hu et al., 2009). Também as CTMs obtidas da membrana amniótica apresentam maior expressão de OCT-4, marcador de células pluripotentes, do que CTMs isoladas de medula óssea, além de serem livres de contaminação com células não fibroblásticas (Hu et al., 2009).

CTMs membrana e placa coriônica: CTMS de membrana coriônica apresentaram expressão de marcadores de superfície similares a CTMs de medula óssea (Soncini et al., 2007). CTMs isoladas de placa coriônica apresentaram vantagem nos efeitos imunomoduladores, quando comparadas às CTMs isolada de medula óssea e tecido adiposo (Lee et al., 2012).

CTMs fluido amniótico: Também as CTMs isoladas de fluido amniótico resultaram em células de alto potencial proliferativo quando comparadas às CTMs derivadas de medula óssea (Shaw et al., 2011).

### 2.7.3. Comparação CTMs de tecidos neonatais

O isolamento e cultivo de CTMs é menos eficiente no sangue de cordão umbilical, quando comparado ao segmento da parede do cordão e de outros anexos embrionários, devido à baixa densidade de células mesenquimais neste material (Bieback e Brinkmann, 2010). Marcadores de superfície foram semelhantes em culturas de CTMs de membrana amniótica, placenta e cordão umbilical. Por outro lado, CTMs do cordão umbilical se destacaram na capacidade de diferenciação adipogênica e CTMs de placenta e âmnion na diferenciação osteogênica (Veryasov et al., 2014).

Comparando CTMs da decídua da placenta, membrana amniótica e membrana coriônica, Indumathi et al. (2013) verificaram superior capacidade proliferativa nas CTMs da decídua da placenta, mas superior capacidade migratória e de *homing* nas CTMs provenientes da membrana amniótica.

Segundo Kim e colaboradores (2014) CTMs de cordão podem ser obtidas em maior quantidade, apresentam maior potencial de proliferação, melhor potencial de diferenciação

hepatogênica, mas menor potencial de diferenciação adipogênica do que CTMs isoladas da placa coriônica. A análise dos marcadores para pluripotência mostrou-se semelhante entre os dois grupos celulares. Já em comparação entre decídua da placenta, cordão umbilical e córion, CTMs da placenta e cordão umbilical mostraram maior capacidade proliferativa, CTMs de cordão e córion foram superiores na capacidade de diferenciação e CTMs de córion foram mais capazes de angiogênese (González et al., 2015).

No estudo de Talwakedar e colaboradores (2015), a proliferação de linfócitos T teve maior redução na co-cultura de CTMs obtidas da placenta do que com CTMs obtidas de cordão umbilical. Em outro estudo comparando imunossupressão de CTMs de placenta, cordão umbilical e membranas fetais, as CTMs de membranas fetais mostraram-se mais eficientes na capacidade imunossupressora (Karlsson et al., 2011). Também comparando placenta, cordão umbilical e membrana coriônica, CTMs da membrana fetal mostraram melhor capacidade de inibição das células T (González et al., 2015).

De acordo com os dados descritos, fica evidente a diferença entre os resultados de estudos comparativos de CTMs de tecidos neonatais. Inclusive, resultados indicativos da melhor fonte de CTMs são controversos. Indumathi et al. (2013) sugeriram que CTMs de âmnion foram superiores a CTMs de córion e decídua da placenta, enquanto Soncini et al. (2007) observaram que A-CTMs e C-CTMs são similares. Já Gonzalez et al. (2015) considerou que a membrana coriônica foi a melhor fonte de CTMs, quando comparada com decídua da placenta e cordão umbilical. Kim et al. (2011) consideraram semelhantes CTMs da placa coriônica e cordão umbilical para uso em tratamentos terapêuticos.

## 2.8. APLICAÇÃO TERAPÊUTICA

De acordo com descrição prévia, as células-tronco mesenquimais apresentam características básicas de autorrenovação e diferenciação. Além disso, a capacidade imunomoduladora de células mesenquimais vem sendo apresentada em diversos estudos como: capacidade de diminuição da diferenciação de monócitos em células dendríticas (Ivanova-Todorova et al., 2009), supressão da proliferação de células mononucleares (Flemming et al., 2011; Karlsson et al., 2011; Tomic et al., 2011; Wu et al., 2011; Gonzalez et al., 2015; Talwakedar et al., 2015), expressão de molécula de superfície coestimulatórias e alteração na secreção de quimiocinas (Flemming et al., 2011; Tomic et al., 2011; Ivanova-Todorova et al., 2011). Baseados nas características de autorrenovação, diferenciação e imunomodulação, diversos estudos envolvem o isolamento, cultivo e/ou aplicação de CTMs para uso terapêutico em diferentes patologias, como reparo de tecido cardíaco (Zhao et al., 2005, Simpson et al.,

2012), doenças fetais pré e pós natais (Shaw et al., 2011), fibrose pulmonar (Tzouvelekis et al., 2011), falência hepática (Parekkadan et al., 2007), doença do enxerto contra o hospedeiro (DECH) pós transplante alogênico (Nauta e Fibbe, 2007; Wu et al., 2011); diabetes tipo 1 (Fiorina et al., 2009; Fiorina et al., 2011), colite (Parekkadan et al., 2011), lesão renal (Hattori et al., 2015), entre outros.

Doenças hematológicas e DECH: em razão das propriedades imunomodulatórias e capacidade de produção de citocinas que podem apoiar na reconstituição medular, as CTMs têm sido estudadas em pacientes pós-transplante alogênico de células-tronco hematopoéticas, para redução de DECH em pacientes refratários a corticosteroides e auxílio na hematopoese. A maioria dos ensaios está em fase I/II ou II, com poucos ou nenhuns efeitos adversos (Le Blanc et al., 2004; Le Blanc et al., 2006; Nauta e Fibbe, 2007; Le Blanc et al., 2008; Muller et al., 2008; Chao et al., 2011; Wu et al., 2011; Xiao et al., 2013; Squillaro et al., 2015, Zhao et al., 2015).

Doenças cardiovasculares: os estudos de CTMs com doenças cardiovasculares estão ocorrendo (Zhao et al., 2005, Simpson et al., 2012) em número representativo (14,8%) e exibem resultados promissores em infarto do miocárdio, doença arterial coronariana e cardiomiopatia isquêmica. Evidências pré-clínicas apontavam para a hipótese do aumento da reconstituição do tecido cardiovascular injuriado. Apesar dos benefícios do enxerto de CTMs pré-diferenciadas, os efeitos destas evidenciam ser menores e pouco duradouros, comparados às CTMs indiferenciadas (Squillaro et al., 2015).

Doenças neurológicas: estudos pré-clínicos mostraram que CTMs parecem suprimir a neuroinflamação, diminuir as lesões locais e reduzir os sintomas do déficit das funções neuronais. A maioria dos estudos, de fases I/II e II, foca no tratamento da esclerose múltipla, esclerose lateral amiotrófica e lesão da medula espinhal, sendo o uso autólogo seguro e capaz de promover melhora neurológica (Squillaro et al., 2015).

Doenças ósseas e cartilaginosas: a capacidade intrínseca de diferenciação das CTMs em osteócitos e condrócitos aponta para uma forte possibilidade de uso terapêutico em doenças de ossos e cartilagens. Neste sentido, há muitos ensaios clínicos com o uso de CTMs registrados no Instituto Nacional de Saúde dos EUA. Embora precoces, os estudos para uso em osteoartrite e osteogênese imperfeita são promissores, mas necessitam maiores investigações (Squillaro et al., 2015).

Lesões hepáticas, pulmonares e renais: observações clínicas demonstram a eficácia de CTMs quando infundidas para melhora de casos de lesão dos tecidos renal (Hattori et al., 2015), hepático (Parekkadan et al., 2007) e pulmonar (Tzouvelekis et al., 2011). Foi observado um

aumento dos estudos clínicos para o tratamento destas patologias a partir de 2012 (Squillaro et al., 2015).

Doenças inflamatórias e autoimunes: as descobertas sobre o comportamento imunológico das CTMs aumentaram as perspectivas terapêuticas do uso dessas células, especialmente na doença do enxerto contra o hospedeiro (Nauta & Fibbe, 2007; Wu et al., 2011; Chao et al., 2011). Porém, também estão sendo estudadas para outras doenças autoimunes como: artrite reumatóide (Tanaka, 2015), esclerose sistêmica (Cras et al, 2015) e lúpus eritematoso sistêmico (Cras et al, 2015, Klinker et al, 2015), doença inflamatória intestinal (Klinker et al, 2015), doença de Crohn (Ciccocioppo et al., 2015) e diabetes (Katuchova et al, 2015; Xie et al, 2016).

Doenças oncológicas: Embora haja contradição e a preocupação da utilização de células-tronco em função da possibilidade de formação de massas tumorais, as CTMs tem sido estudadas para terapia celular em doenças oncológicas devido a um possível potencial antitumor, associado à sua natureza imune, habilidade de migração para o sítio do tumor e capacidade de diferenciação em várias linhagens celulares. Entre as citocinas liberadas pelas CTMs no sítio de ação, a TRAIL (tumor necrosis factor related apoptosis inducing ligand) é conhecida pela propriedade pró-apoptótica de células tumorais (Ramdasi et al., 2015). Além disso, como já descrito, CTMs podem ser auxiliares em tratamentos oncológicos que utilizem material biológico alogênico.

Transplante de órgãos: o conhecimento dos resultados promissores para uso de CTMs em DECH sinaliza para o uso em terapia no transplante de órgãos sólidos. A maioria dos estudos clínicos avalia o uso de CTMs após transplantes hepáticos e renais (Squillaro et al., 2015).

No período de 2012 a 2015, o número de ensaios clínicos usando CTMs mais que dobrou, sendo a maioria de fase I/II, seguido por fase I, fase II, fase III e fase II/III. Em análise dos estudos em andamento e/ou concluídos até 2015, Squillaro e colaboradores (2015) concluíram que a infusão de CTMs parece bem tolerada e que a eficácia da terapia com CTMs está inicialmente restrita a DECH e doenças hematológicas, ósseas e cartilaginosas, baseada nos benefícios proporcionados pelas características de imunomodulação e potencial regenerativo. Focando para aplicação terapêutica, alguns pontos críticos necessitam atenção, como: heterogeneidade dos doadores, falta de padronização do isolamento e expansão *ex vivo*, criopreservação, pureza das populações de células-tronco, determinação de dose, entre outros (Squillaro et al., 2015).

De acordo com dados do Instituto Nacional de Saúde dos Estados Unidos (US National Institute of Health), em julho de 2016, aproximadamente 600 ensaios clínicos com o uso de CTMs estão cadastrados, em diferentes fases de execução (www.clinicaltrials.gov), sendo 12 no Brasil.

Ainda corroborando para o incremento do uso de CTMs em terapia celular, o uso destas é preferencial a IPs ou células embrionárias, devido à grande disponibilidade de células e pouca manipulação celular antes do uso, além de apresentar poucos efeitos adversos em ensaios clínicos (Marquez-Curtis et al., 2015).

## 2.9. ADMINISTRAÇÃO DE CTMs: *HOMING,* MIGRAÇÃO CELULAR E SEGURANÇA

A utilização de CTMs na terapia celular depende da migração das células até a inflamação ou local de injúria (*homing*) (Sohni e Verfaillie, 2013). Então, a capacidade de migração das CTMs é essencial para o processo de *homing* e pode aumentar terapeuticamente a competência das células. São evidentes os benefícios da administração local das CTMs (Kim et al., 2013), porém, a administração intravenosa é mais prática e minimamente invasiva, sendo uma opção com maior aceitabilidade para administração de doses seriadas e em locais mais inacessíveis. Apesar da possibilidade de retenção pulmonar das células infundidas por via intravenosa (Schrepfer et al., 2007), vários estudos demonstram que CTMs migram por vários tecidos, já tendo sido encontradas no cérebro (Jackson et al., 2010; Kholodenko et al., 2012), medula espinhal (Kim et al., 2013), coração (Zhang et al., 2008), cólon (Liang et al., 2011), rim (Devine et al., 2003; Zhuo et al., 2013), tecidos gastrointestinais, fígado, pulmão, timo e pele (Devine et al., 2003) após administração intravenosa.

A migração celular é um processo complexo que depende da polarização celular, extensão da membrana, formação e maturação da adesão celular, contractilidade do corpo celular e desprendimento da célula (Lauffenburger and Horwitz, 1996). Considerando que as células devem adquirir uma morfologia polarizada para migrar (Lauffenbrger e Horwitz, 1996), a análise da polaridade celular pode ser um parâmetro sugestivo de capacidade de motilidade das CTMs. A célula mais alongada é geralmente associada a um melhor desempenho migratório (Torrente et al., 2014; Schneider et al., 2015) O índice de polaridade das CTMs pode ser determinado pela divisão do comprimento do eixo maior (paralela à protrusão da membrana) pelo comprimento do eixo perpendicular, que intersecta o centro do núcleo da célula (Figura 2). O índice de polaridade celular pode ser alterado em presença de drogas e condições especiais de lesão (Torrente et al., 2014; Schneider et al., 2015).

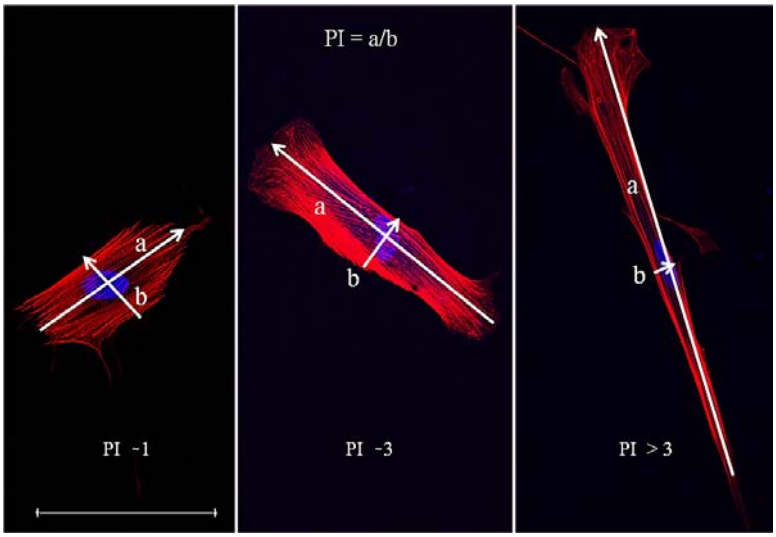


Figura 4 - Análise do índice de polaridade - IP / *polarity index - PI* (Fonte: Schneider et al., 2015)

A mobilidade celular é, então, afetada por características do citoesqueleto, o qual é composto por filamentos de actina, microtúbulos e filamentos intermediários. A interferência da organização das fibras de actina nos diferentes estágios de migração celular já está descrita (Tojkander et al., 2012; Vallenius, 2013).

Além disso, o processo migratório é dependente da relação entre as quimiocinas liberadas por células do sítio inflamatório (quimioatratores) e os receptores presentes na membrana das CTMs. Um dos receptores mais estudados é o CXCR4, responsável pela ligação ao quimioatrator SDF-1 (stromal derived factor-1) (Sohni e Verfaillie, 2013; Marquez-Curtis e Janowska-Wieczorek, 2013). Diferentes condições fisiológicas ou farmacológicas também podem afetar o movimento celular (Geissler et al., 2012; Lamers et al., 2011; Schneider et al., 2015). A velocidade de deslocamento e trajetória migratória das CTMs são essenciais para o processo de *homing*.

A capacidade migratória das CTMs pode variar dependendo do tecido de onde foram isoladas as células (Li et al., 2011). Porém, ainda são escassos na literatura os dados de comparação das condições migratórias das CTMS isoladas dos diferentes tecidos da placenta e outros anexos.

Também o tamanho das células pode interferir no processo de migração celular e na segurança da infusão intravenosa na administração terapêutica. As CTMs infundidas podem ficar retidas sem alcançar o sítio de ação e serem causa de obstrução de vasos. Estudo publicado recentemente descreve esta relação entre o maior tamanho das CTMs e a taxa de retenção no coração após injeção intracoronariana. Os autores verificaram que a injeção das CTMs reduziu o

fluxo coronariano, sugestivo de embolia coronariana, o que foi comprovado pelos achados histológicos (Campbell et al., 2016). Outros estudos já demonstraram a relação do tamanho das CTMs como causa de obstrução vascular (Janowski et al., 2013, Ge et al., 2014), gerando os efeitos lesivos relacionados ao quadro obstrutivo. Janowski et al. (2013) encontraram 25μm como diâmetro médio das CTMs e verificaram que a infusão destas, comparadas a outros tipos celulares de menor diâmetro, causaram um decréscimo no fluxo sanguíneo cerebral. Ge et al. (2014) dividiram as CTMs em três grupos de tamanhos distintos e verificaram relação direta do aumento do tamanho celular com o aumento severidade da obstrução vascular e incidência de acidente vascular cerebral após injeção das CTMs via carótida nos modelos experimentais. Neste sentido, os estudos sugerem que as células sejam selecionadas em relação ao tamanho para melhorar a segurança do transplante intravascular de CTMs. Complementando, Janowski et al. (2013) afirmam que o tamanho celular, associado à velocidade de injeção das células e dose celular, são determinantes para a segurança do transplante de CTMs pela carótida.

Assim, a avaliação dos caracteres que podem interferir no processo migratório pode ser útil na determinação de células com maior capacidade de sucesso na terapia celular. Além disso, a avaliação de caracteres que podem estar relacionados à segurança na administração terapêutica, também é importante para a escolha da melhor CTM para uso terapêutico.

## 3. JUSTIFICATIVA

Considerando a grande variedade de protocolos para isolamento de CTMs de anexos embrionários e que não foram encontrados estudos comparando a eficiência de protocolos de isolamento nestes tecidos, é importante determinar qual seria a melhor forma de isolar as CTMs de cada tipo de fonte tecidual, a fim de otimizar os processos laboratoriais com o uso de um protocolo de menor custo e menor demanda de tempo, para obtenção de células de boa qualidade para uso *in vitro* e, posteriormente, *in vivo*. Além disso, a caracterização e comparação dos potenciais de crescimento, tamanho das células em suspensão e cultura e índice de polaridade de CTMs obtidas de cada tecido são importantes como indicadores de uma boa fonte celular. Assim sendo, o presente trabalho envolve o isolamento, caracterização, avaliação do potencial de crescimento, avaliação do potencial de diferenciação, avaliação do tamanho e complexidade e avaliação do índice de polaridade de células-tronco mesenquimais provenientes de quatro fontes teciduais não invasivas para posterior uso em terapia celular. Salienta-se, também, que na maioria das amostras, foi possível a comparação de quatro fontes do mesmo contexto fisiológico, geneticamente idênticas ou haploidênticas, descartando, assim, as variáveis interpessoais.

# 4. OBJETIVOS

## 4.1. OBJETIVO PRIMÁRIO

- Avaliar diferentes protocolos enzimáticos para obtenção de células-tronco mesenquimais a partir de membrana amniótica, membrana coriônica, cordão umbilical e decidua da placenta e comparar as células obtidas quanto a potencial de crescimento, diferenciação, imunofenotipagem e morfologia.

## 4.2. OBJETIVOS SECUNDÁRIOS

- Determinar o protocolo mais eficiente para obtenção de CTMs de cada tipo de tecido analisado.
- Determinar o melhor tecido para obtenção de CTMs.
- Avaliar a capacidade de diferenciação em adipócitos, condrócitos e osteócitos das CTMs isoladas pelos diferentes protocolos nas diferentes fontes.
- Caracterizar fenotipicamente as CTMs isoladas pelos diferentes protocolos nas diferentes fontes.
- Avaliar o potencial proliferativo das CTMs isoladas pelos diferentes protocolos nas diferentes fontes.
- Comparar tamanho e complexidade das CTMs isoladas das diferentes fontes.
- Verificar o índice de polaridade das CTMs isoladas das diferentes fontes.

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## 6. ARTIGO 1

# Isolation of human mesenchymal stem cells from amniotic membrane, chorionic membrane, placental decidua and umbilical cord: comparison of four enzymatic protocols

## Protocols for MSC isolation from fetal adnexa

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**Abstract**

*Background:* Mesenchymal stem cells (MSCs) have been isolated from different tissues using different protocols, and there are no standard protocols for each source. The objective of this study was to compare the effectiveness of four enzymatic protocols for isolation of MSCs from four different human fetal adnexal sources. *Methods:* Four enzymatic Protocols were applied for isolation of MSCs from amniotic membrane (A-MSC), chorionic membrane (C-MSC), umbilical cord (UC-MSC) and placental decidua (D-MSC). General characteristics of MSCs isolated from each protocol were analyzed. *Results:* MSCs were successfully isolated from all four sources. A-MSCs and UC-MSCs could be isolated and characterized from all samples using trypsin/collagenase-based protocols; C-MSCs could be isolated from all samples with collagenase- and trypsin/collagenase-based protocols; and D-MSCs were isolated from all samples exclusively with a collagenase-based protocol. *Discussion:* Of the four protocols tested, none could be used as standard for isolation of MSCs from all tissues of interest. The protocol that used trypsin alone was least efficient. None of the tested protocols was adequate for isolation of A-MSCs; all yielded heterogeneous cultures with short lifespans and limited cell growth. The collagenase-only protocol was best for C-MSCs and D-MSCs, while a combination of trypsin and collagenase was considered best for UC-MSCs.

**Key Words:** amniotic membrane, cell therapy, chorionic membrane, fetal adnexa, fetal membranes, human placenta, isolation protocols, mesenchymal stem cells, placental decidua, umbilical cord.

**Abbreviations:**

A-MSC: amniotic membrane-derived mesenchymal stem cells
C-MSC: chorionic membrane-derived mesenchymal stem cells
DMEM: Dulbecco's modified Eagle's medium
D-MSC: placental decidua-derived mesenchymal stem cells
FBS: fetal bovine serum
MSC: mesenchymal stem cells
PBS: phosphate buffered saline
PD: population doubling
UC-MSC: umbilical cord-derived mesenchymal stem cells

**Introduction**

Mesenchymal stem cells (MSCs) are multipotent cells [1] characterized by adherence to plastic, positive expression of CD 105, CD 73 and CD 90, absence of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR expression, and ability to differentiate into osteoblasts, chondroblasts and adipocytes [2]. MSC can be derived from different adult tissues, such as fat, lung, kidney and bone marrow [3], as well as from fetal adnexa, such as Wharton's jelly, amniotic fluid, amniotic membrane, chorionic membrane, placental decidua and umbilical cord [4-9]. Because of the age-related decrease in frequency and differentiating capacity of MSC, fetal tissues are considered a valid alternative source of stem cells [10]. Moreover, fetal stem cells have several advantages over their adult counterparts, including greater differentiation, lower immunogenicity, homing and engraftment potency [11].

Isolation of MSCs from different tissues has been accomplished using a variety of protocols [12]. However, Bortolotti et al (2015) affirm that the therapeutic potential of MSCs depends not only on their source, but also on the isolation protocol [13]. For isolation of MSCs from fetal adnexa, many isolation protocols use enzymatic digestion with type I [4,14-16], type II [17-18] or type IV collagenase [19]. In some studies, isolated trypsin has been used for tissue disaggregation and MSC isolation from embryonic appendages [8,20-22]. Combined enzymatic digestion protocols include the use of type I collagenase/DNase I/dispase [4], collagenase I/DNase I [14-15,23], collagenase II/dispase II [24], collagenase I/trypsin [16,25], collagenase II/trypsin [17], and collagenases I and II/trypsin [9]. These enzymes are used at different concentrations and digestion time can range from 10 minutes [20-21] to more than 18 hours [26]. Non-enzymatic isolation protocols, which involve plating tissue in the presence of culture medium, have also been described [7,9,18,27-29]. Some studies have also included

density gradients into the protocol isolation in order to separate the mononuclear layer after enzymatic digestion [14,17-18,23]. Other variables can account for small differences across isolation methods, including the use of filtration to separate undigested fragments, the method of washing during the isolation procedure, use of different washing solutions (phosphate buffered saline [PBS], Hank's balanced salt solution [HBSS], saline), centrifugation for pellet cell culture, erythrocyte lysis and time to first medium change.

Considering the diversity of protocols for MSC isolation from fetal adnexa, it seems important to determine the best technique to isolate MSCs from each tissue type and establish a robust, practical, low-cost protocol that yields high-quality MSCs for in vitro or in vivo application. Within this context, the objective of this study was to compare the effectiveness of four enzyme-based protocols for MSC isolation from four human fetal adnexa (amniotic membrane, chorionic membrane, umbilical cord and placental decidua) and to define the optimal protocol for isolation from each tissue.

**Methods**

After obtaining written informed consent, tissues were collected from six healthy donors to the Umbilical Cord Blood Bank of Hospital de Clínicas de Porto Alegre (HCPA), Brazil. This study was approved by the HCPA Research Ethics Committee (26563613.1.0000.5327).

The maternal inclusion criteria, in accordance with Brazilian public umbilical cord blood bank legislation [30], were: maternal age > 18 years, gestational age ≥ 35 weeks, rupture of membranes for no longer than 18 h, minimum of two antenatal visits during pregnancy, no abnormalities during labor and no infection or other disease during pregnancy that might interfere with placental vitality. Mothers were also

questioned for family history and high-risk behavior for infectious diseases during pregnancy, and samples were collected for screening serologies.

After placental expulsion and collection of umbilical cord blood for public blood bank storage, segments of umbilical cord and placenta including decidua, chorionic membrane and amniotic membrane were collected. These segments were placed in sterile saline-filled vials, transported to the laboratory and processed immediately. The time elapsed from collection to processing was always less than 1 hour. Amniotic and chorionic membranes were separated from the placental decidua with forceps and a scalpel and the umbilical cord vessels were removed. Each tissue specimen was washed thoroughly with saline solution and fragmented into small pieces. Then, 1.5-2.5g of each tissue was set aside for use in each isolation protocol (Supplementary Figure S1).

The MSC isolation protocols tested were as follows (Figure 1):

*Protocol I – Collagenase solution 1 mg/mL*

In this protocol, standardized in our laboratory, tissues were digested with type I collagenase 1 mg/mL (Gibco), incubated for 2 hours at 37°C with agitation every 10 minutes. After enzymatic inactivation with Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), filtration was carried out using a 100-µm filter (BD Falcon), followed by centrifugation at 500*g*/6 min/20°C. Cells were cultured in a 6-well plate in DMEM 20% FBS, 1% penicillin-streptomycin antibiotic solution (Gibco) and 2 mM L-glutamine (Sigma-Aldrich), at 37°C, in a humidified 5% $CO_2$ atmosphere.

The duration of the procedure was approximately 5 hours.

*Protocol II – Trypsin–EDTA 0.05% and collagenase solution 2 mg/mL*

In this protocol, adapted from Seo et al. (2013) [16], tissues were incubated with 0.05% trypsin-EDTA solution (Sigma-Aldrich) for 1 h at 37°C, with agitation every 10 minutes. After enzymatic inactivation, specimens were washed three times with saline

and a new round of enzymatic digestion performed with type I collagenase 2 mg/mL for 3 h at 37°C, with agitation every 10 minutes. After inactivation, samples were washed with phosphate buffered saline (PBS)(Laborclin, Brazil) and centrifuged at 500*g* for 6 min at 20°C. Cells were cultured in a 6-well plate in DMEM supplemented with 20% FBS, 1% penicillin-streptomycin antibiotic solution, and 2 mM L-glutamine, at 37°C, in a humidified 5% $CO_2$ atmosphere.

The duration of the procedure was approximately 8 hours.

*Protocol III – Trypsin–EDTA 0.25%*

In this protocol, adapted from Martini et al. (2013) [22], tissues were incubated with 0.25% trypsin-EDTA solution for 20 min at 37°C with agitation every 10 minutes. After inactivation, samples were cultured in a 6-well plate in DMEM supplemented with 20% FBS, 1% penicillin-streptomycin antibiotic solution and 2 mM L-glutamine, at 37°C, in a humidified 5% $CO_2$ atmosphere.

The duration of the procedure was approximately 3 hours.

*Protocol IV – Collagenase solution 2 mg/mL and trypsin–EDTA 0.25%*

In this protocol, adapted from Wang et al. (2004) [31], tissues were washed with DMEM 10% FBS and digested with collagenase type I 2 mg/mL, incubated for 16 hours at 37°C. After enzymatic inactivation, a second round of digestion with trypsin-EDTA was carried out for 30 min at 37°C. After inactivation, samples were cultured in a 6-well plate in DMEM supplemented with 20% FBS, 1% penicillin-streptomycin antibiotic solution and 2 mM L-glutamine, at 37°C, in humidified 5% $CO_2$ atmosphere.

The duration of the procedure was approximately 24 hours (2 hours on day 1, 16 hours of incubation overnight and 2 hours on day 2).

Protocol IV was used only for amniotic membrane and umbilical cord tissue, because the other protocols did not yield robust MSC isolation.

For all protocols, culture medium was changed after 3-4 days initially and twice a week thereafter until 80-90% confluence or overlapping colonies with risk of

detachment were observed. These samples were trypsinized (0.05% trypsin–EDTA solution). Primary culture was maintained for 1 month, after which time samples without colonies or signs of cell growth were discarded.

*Cell characterization*

Immunophenotype analysis was performed by flow cytometry (BD Stemflow hMSC Analysis Kit, BD Biosciences, USA). CD73, CD90, CD105, CD44, CD45, CD34, CD11b, CD19 and HLA-DR were assessed, as well as CD29-APC conjugated (BD Pharmingen, BD Biosciences, USA). Staining was performed according to manufacturer instructions and 10,000 gated events per sample were collected for each analysis. Data were acquired using a FACSCanto II flow cytometer (BD Biosciences) and analysis was performed using FlowJo software (FlowJo LLC, USA).

Differentiation induction was carried out in osteocytes (10-14 days), adipocytes (28 days) and chondrocytes (28 days) using the STEMPRO® Differentiation Kit (Gibco). Medium was changed twice a week. Alizarin Red staining was used to confirm calcium mineralization in osteogenic induction, Oil Red staining was used to confirm adipogenic differentiation and Alcian Blue staining was used to confirm chondrogenic potential.

*Growth kinetics*

Cell growth kinetics were evaluated by counting the total number of cells in a 6-well plate after an initial density of $5x10^4$ cells/well, in duplicates, from P1 until P15, once a week for 103 days or until there was no appreciable cell growth for 2 weeks. Population doubling was calculated using the formula: PD = (log N(t)-log N(to))/log 2, where N(t) was the number of cells per well when trypsinized after 1 week and N(to) was the number of cells initially plated. Cell counts were performed in a Neubauer chamber under Trypan Blue staining 9Sigma-Aldrich).

*Criteria*

Cultures that met the following criteria were considered *bona fide* MSCs: adhesive capacity; expression of typical surface markers (CD90, CD105, CD73, CD 29, CD 44); no expression of CD34, CD45, CD19, CD 11b and HLA-DR; and adipogenic, osteogenic and chondrogenic differentiation capability.

The criteria used to determine the best isolation protocol were: isolation of MSCs in homogeneous culture; immunophenotypic characterization of the isolated cells as MSCs; ability of isolated MSCs to differentiate into adipocytes, chondrocytes and osteocytes; superior in vitro proliferation capacity; least isolation time and lowest cost.

*Statistical Analysis*

Growth kinetics data were analyzed using Generalized Estimating Equations (GEE) and multiple comparisons with Bonferroni correction. Statistical analyses were performed using PASW, version 18.0. Differences were considered significant at $p<0.05$.

**Results**

Samples were collected from six mothers to acquire the data presented in this study. To minimize interpersonal variability, tissues from the same donor were used whenever the size and quality of material received by the laboratory allowed.

MSCs were successfully isolated from amniotic membrane (A-MSC), chorionic membrane (C-MSC), placental decidua (D-MSC) and umbilical cord (UC-MSC) using at least one of the tested protocols (Table 1).

Differences in the mean number of days for first passage were observed between cells isolated from the same tissue using different protocols: for amniotic membrane,

AII-MSC<AIV-MSC (8-9<15 days); for chorionic membrane, CI-MSC<CII-MSC (15<25 days); and for umbilical cord, UCIV-MSC<UCII-MSC (16 days<21-22 days).

As shown in Table 1, A-MSCs could be isolated from all samples using protocols II and IV and, occasionally, using protocols I and III; C-MSCs could be isolated with protocols I and II from all samples; D-MSCs were isolated in all samples exclusively when protocol I was used; and UC-MSCs were obtained with protocols II and IV from all samples.

All A-MSC trypsin isolation protocols produced a heterogeneous population, composed of two predominant cellular types: circular or polygonal-like cells and fibroblast-like cells. MSCs isolated from other tissues resulted in homogenous populations already in the first passage.

*Characterization of MSCs*

Regardless of differences in origin and isolation protocol, all MSCs cultures exhibited a characteristic marker profile. MSCs were positive for CD73, CD90, CD105, CD 44 and CD29 and negative for CD45, CD34, CD11b, CD19 and HLA-DR.

All MSCs isolated were able to differentiate into adipogenic, osteogenic and chondrogenic lineages.

*Growth kinetics*

A-MSCs cultures isolated using protocols I, II and IV presented short lifespans (usually until P3-P5) and low growth rates (PDmax=1.6). A-MSCs isolated by protocol III exhibited a very different growth pattern, with PD values that reached 3.9 and up until P14, but this pattern could not be reproduced because AIII-MSC isolation was successful in only one sample. For C-MSCs and UC-MSCs, no statistically differences

in cell proliferation were observed across the different protocols. D-MSCs could only be isolated using protocol I.

Figure 2 illustrates population doublings for MSCs isolated with different protocols.

**Discussion**

The first objective of this study was to define an optimal protocol for MSC isolation from four sources: amniotic membrane, chorionic membrane, placental decidua and umbilical cord. There are no standard protocols for MSC isolation from theses sources. Bortolotti et al. (2015) provide a rationale for the need for standardization of a gold-standard procedure for MSC isolation [13]. Currently, several protocols – usually based on tissue digestion – are used for MSC isolation. Three options with different enzyme combinations were selected initially, and a fourth protocol was used when protocols I, II and III failed to yield robust MSC isolation. Comparison of four sources from the same physiological matrix was intended to minimize interpersonal variability.

We isolated MSCs successfully from all tested tissues, and the isolated cells exhibited the desired MSC characteristics of adherence, immunophenotype and differentiation capacity. The isolation efficiency of extraembryonic tissues was 100% using at least one of the tested protocols, which corroborates previous studies involving one or more of these sources [7,32]. However, as in a study by González et al. (2015), isolation of A-MSCs was difficult [9].

A-MSCs also exhibited the greatest variability in behavior across the tested protocols. In all A-MSCs obtained using trypsin (protocols II, III and IV), the resulting culture was heterogeneous, with a combination of circular/polygonal-like and fibroblast-like cells. Numerous cells were observed in primary culture, but without longevity. We

believe that the second population observed in A-MSC isolations was composed of epithelial stem cells, as the amniotic membrane consists of epithelial and stromal cells [33-34]. Other authors who used enzymatic digestion with trypsin and collagenase to isolate A-MSCs also found heterogeneous cultures [33,35]. In this study, the trypsin-only protocol resulted in heterogeneous culture in one sample, similar to a culture obtained by combined enzymatic digestion, but with better growth. A-MSCs isolated using collagenase alone resulted in less heterogeneity, but lower growth as well. Heterogeneous primary culture has also been observed in cells originated by amniotic membrane isolation without enzymatic digestion [28]. Despite contradictory data about the number of passages at which epithelial and mesenchymal stem cells stop proliferating, some authors have reported completion of epithelial cell growth at P2-P6, while MSCs can be cultured until P9-P15 [34]. Thus, none of the protocols tested herein were successful for A-MSCs isolation, because we obtained a heterogeneous cultures with limited growth and short lifespans. The difficulty of isolating and maintaining A-MSCs cultures has also been reported by Gonzalez et al (2015) [9]. However, some studies consider that A-MSCs present powerful reproductivity [33,36].

C-MSCs were successfully isolated using two protocols (I and II). MSCs obtained by both protocols were capable of differentiating into mesodermal lineages, had characteristic immunophenotypic features and exhibited similar growth capacity. Protocol I was considered best for isolation of MSCs from the chorionic membrane, as CI-MSCs cultures exhibited a shorted mean time to first passage than CII-MSCs and protocol I was faster and less expensive. As in this study, C-MSCs have been isolated using enzymatic digestion with collagenase [18-19,37] or trypsin with collagenase [9,25] in previous experiments. C-MSCs can also be isolated with a combination of collagenase, trypsin, dispase and DNase [15]. Kim et al. (2011) suggested that MSCs

obtained by chorionic plate by digestion with collagenase after trypsin are inevitably contaminated with amniotic epithelial cells [15]. However, we used this combination in chorionic membrane tissue and did not observe such contamination.

UC-MSCs were also successfully isolated using two protocols (II and IV). No statistically superiority in cell growth was observed with either protocol. Thus, incubation time with collagenase and the order of enzymes (the main differences between protocols II and IV), do not seem to affect UC-MSCs isolation if an intermediate washing is not performed. A homogeneous fibroblast population were obtained with both protocols, as reported in previous studies [7,15]. Conversely, heterogeneous populations have been observed in UC-MSC isolation using collagenase-based and non-enzymatic isolation protocols [32]. Kim et al (2011) note that prolonged exposure to enzymes may result in contaminated cultures [15], but in the present study, this was not observed in UCIV-MSCs cultures, which were incubated with collagenase overnight. Several protocols have been used for successful isolation of UC-MSCs via non-enzymatic digestion [9,18,27,29].

Amnion and umbilical cord behaved similarly across all tested protocols, with the best results obtained in protocols II and IV, both of which combine collagenase and trypsin digestion, but in reverse order. According to Fonseca et al. (2008), the fact that the amniotic membrane covers the umbilical cord [38] may contribute to this similarity in results; on the other hand, we did not observe heterogeneous populations in UC-MSCs as we did in cultures of A-MSCs isolated using the same protocols.

Placental decidua was the source with the poorest response to the tested protocols. D-MSCs were obtained just using enzymatic digestion with collagenase (protocol I) (DI-MSC). This protocol was considered a good option for D-MSCs because there was successful isolation in all tested samples and DI-MSCs presented

MSCs characteristics with longer and satisfactory growth. In contrast, D-MSCs have been isolated with trypsin-only [21] and trypsin/collagenase protocols [9], but this may be due to protocol heterogeneity in factors such as enzyme concentration and incubation time. D-MSCs can also be obtained using a combination of collagenase, dispase and DNase [4].

None of the four tested protocols could be defined as standard for all tissues analyzed. Protocol I was only effective for D-MSCs and C-MSCs; protocol II was successful for isolation of A-MSCs, C-MSCs, and UC-MSCs, but not D-MSCs; protocol III did not yield satisfactory isolation of MSCs from any of the tissues studied; and protocol IV was only effective for isolation of UC-MSCs and A-MSCs. From these results, we can conclude that the least effective protocol was that based on trypsin alone (protocol III).

**Conclusion**

In this study, MSCs were successfully isolated from all tested tissues (amniotic membrane, chorionic membrane, umbilical cord, and placental decidua). We were unable to determine an optimal protocol for isolation of A-MSCs. C-MSC isolation was possible using two different protocols, but the collagenase-only protocol was considered best for this tissue type. UC-MSCs could be obtained using two protocols, which used enzymatic digestion with trypsin and collagenase, and both were considered good options. D-MSCs could only be isolated with a collagenase-only protocol, which was considered effective for this purpose.

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**Disclosure of Interest:**

The authors declare no conflicts of interest.

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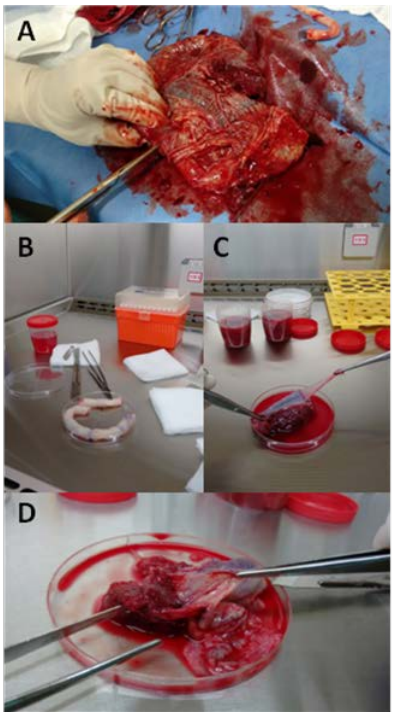
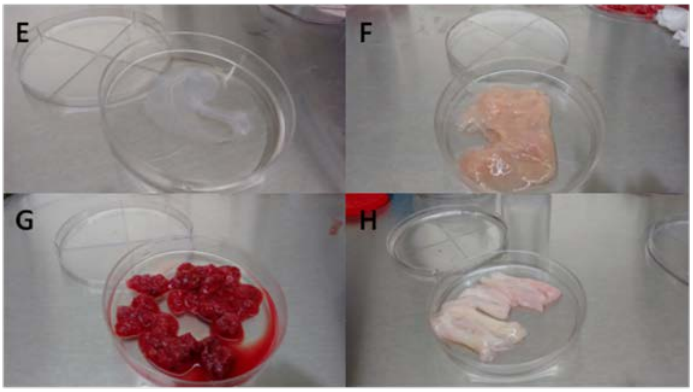
Supplementary Figure S1. Representative images of collected tissues. After cord blood collection, segments of umbilical cord and placenta were collected (A). The umbilical cord (B), amniotic membrane (C), chorionic membrane and placental decidua (D) were separated. Tissues were thoroughly washed with saline before fragmentation, and the umbilical cord vessels were removed. Amniotic membrane (E), chorionic membrane (F), placental decidua (G) and umbilical cord (H).

Figure 1. Schematic representation of the tested MSC isolation protocols.

Table 1. Protocols used for MSC isolation from amniotic membrane, chorionic membrane, placental decidua and umbilical cord.

Figure 2. Proliferation potential of A-MSCs (A), C-MSCs (B), D-MSCs (C) and UC-MSCs (D) obtained with the tested protocols. All samples were assayed in duplicate; figures present mean values. *Experiment stopped due to contamination.

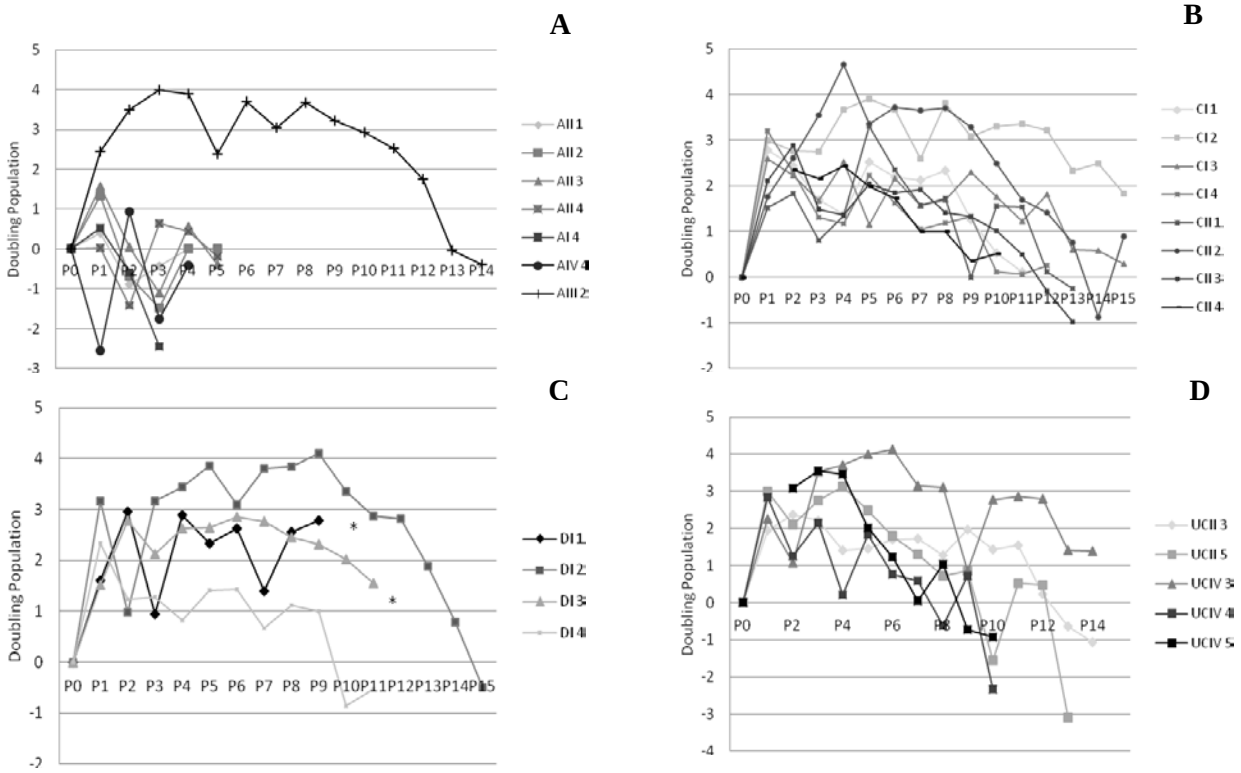



| PROTOCOL I | PROTOCOL II | PROTOCOL III | PROTOCOL IV |
|---|---|---|---|
| TISSUE | TISSUE | TISSUE | TISSUE |
| ⇩ | ⇩ | ⇩ | ⇩ |
| FRAGMENTATION | FRAGMENTATION | FRAGMENTATION | FRAGMENTATION |
| ⇩ | ⇩ | ⇩ | ⇩ |
| COLLAGENASE I 1mg/mL 2h/37°C | TRYPSIN-EDTA 0.05% 1h/37°C | TRYPSIN-EDTA 0.25% 20 min/37°C | COLLAGENASE I 2mg/mL16h/37°C |
| ⇩ | ⇩ | ⇩ | ⇩ |
| FILTRATION 100µm | COLLAGENASE I 2 g/mL 3h/37°C | | TRYPSIN-EDTA 0.25% 30 min/37°C |
| ⇩ | ⇩ | ⇩ | ⇩ |
| CULTURE 6-WELL PLATE DMEM 20%FBS/L-GLU 3-4 DAYS | CULTURE 6-WELL PLATE DMEM 20%FBS/L-GLU 3-4 DAYS | CULTURE 6-WELL PLATE DMEM 20%FBS/L-GLU 3-4 DAYS | CULTURE 6-WELL PLATE DMEM 20%FBS/L-GLU 3-4 DAYS |

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| AI | * | N | N | * | - | - |
| AII | Y | Y | Y | Y | - | - |
| AIII | * | Y | N | * | - | - |
| AIV | - | - | - | Y | - | Y |
| CI | Y | Y | Y | Y | - | - |
| CII | Y | Y | Y | Y | - | - |
| CIII | * | N | N | N | - | - |
| DI | Y | Y | Y | Y | - | - |
| DII | N | N | N | N | - | - |
| DIII | N | N | N | N | - | - |
| UCI | - | - | N | N | N | - |
| UCII | - | - | Y | Y | Y | - |
| UCIII | - | - | N | N | N | - |
| UCIV | - | - | Y | Y | Y | - |

Y, successful isolation of MSCs; N, no isolation of MSCs; *characteristic cells were isolated, but growth was insufficient for characterization assays. A, amniotic membrane; C, chorionic membrane; D, placental decidua; UC, umbilical cord. I, II, III and IV are protocol identifiers; 1-5 are tissues donors.



## 7. ARTIGO 2

# Comparison of mesenchymal stem cells from four fetal adnexa sources: amniotic membrane, chorionic membrane, placental decidua and umbilical cord



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**Abstract**

*Background:* Mesenchymal stem cells (MSCs) are being investigated as a potential alternative for cellular therapy. This study was designed to compare the biological characteristics of MSCs isolated from amniotic membrane (A-MSCs), chorionic membrane (C-MSCs), placental decidua (D-MSCs) and umbilical cord (UC-MSCs) to ascertain whether any one of these sources is superior to the others for cellular therapy purposes. *Methods:* MSCs were isolated from amniotic membrane, chorionic membrane, umbilical cord and placental decidua. Immunophenotype, differentiation ability, cell size, cell complexity, polarity index and growth kinetics of MSCs isolated from these four sources were analyzed. *Results:* MSCs were successfully isolated from all four sources. Surface marker profile and differentiation ability were consistent with human MSCs. C-MSCs in suspension were the smallest cells, while UC-MSCs presented the greatest length and least width. A-MSCs had the lowest polarity index and UC-MSCs, as more elongated cells, the highest. C-MSCs, D-MSCs and UC-MSCs exhibited similar growth capacity until P8; C-MSCs presented better lifespan, while insignificant proliferation was observed in A-MSCs. *Discussion:* Fetal and maternal tissues can serve as sources of multipotent stem cells. Some characteristics of MSCs obtained from four fetal adnexa were compared and differences were observed. Amniotic membrane was the least useful source of MSCs, while chorionic membrane and umbilical cord were considered good options for future use in cell therapy because its known advantages of immature cells.

**Key Words:** amniotic membrane, cell therapy, chorionic membrane, fetal adnexa, fetal membranes, human placenta, MSCs sources, mesenchymal stem cells, placental decidua, umbilical cord.

**Abbreviations:**

A-MSC: amniotic membrane-derived mesenchymal stem cells
C-MSC: chorionic membrane-derived mesenchymal stem cells
DMEM: Dulbecco's modified Eagle's medium
D-MSC: placental decidua-derived mesenchymal stem cells
FBS: fetal bovine serum
MSC: mesenchymal stem cells
PBS: phosphate buffered saline
PD: population doubling
UC-MSC: umbilical cord-derived mesenchymal stem cells

## Introduction

Mesenchymal stem cells (MSCs) are being investigated as a potential alternative for cellular therapy. Much research has focused on the isolation, cultivation, and application of MSCs for therapeutic use, based on their characteristics of self-renewal, differentiation and immunomodulation [1-8]. Most studies using MSCs have employed bone marrow and adipose tissue as principal cell sources [9-11]. However, fetal adnexa appear to be a good alternative for MSC isolation: these tissues are usually discarded without use, can be obtained easily, non-invasively, and atraumatically in large quantities, have no ethical implications [12] and are associated with fewer adverse effects in clinical trials [13]. Another important advantage of fetal adnexa is that they provide immature cells, which have a lower risk of mutations [14] and exhibit superior cellular activity, including greater differentiation, homing and engraftment potency, and lower immunogenicity [15]. MSCs from umbilical cord blood are difficult to isolate [16], with a yield of <50% [17-20], and other fetal adnexa are being studied, such as Wharton's jelly, amniotic fluid, amniotic membrane, chorionic membrane and placental decidua [5,7,12,21-25].

Comparative studies of different fetal adnexa sources have found discrepancies in the suitability of these tissues for use in cellular therapy [25-27]. Within this context, the present study was designed to compare the biological characteristics of MSCs isolated from amniotic membrane (A-MSCs), chorionic membrane (C-MSCs), placental decidua (D-MSCs) and umbilical cord (UC-MSCs) to ascertain whether any one of these sources is superior to the others for cellular therapy purposes. To the best of our knowledge, this is the first study comparing the characteristics of MSCs isolated from these four tissues.

## Methods

### *Cell isolation*

MSCs were isolated from full-term human placentas provided by healthy donors to the Umbilical Cord Blood Bank of Hospital de Clínicas de Porto Alegre, Brazil. The maternal inclusion criteria, in accordance with Brazilian public umbilical cord blood bank legislation [28], were: maternal age > 18 years, gestational age ≥ 35 weeks, rupture of membranes for no longer than 18 h, minimum of two antenatal visits during pregnancy, no abnormalities during labor and no infection or other disease during

pregnancy that might interfere with placental vitality. Family history and high-risk behavior for infectious diseases during pregnancy were also evaluated. Maternal samples were collected for serological screening. Written informed consent was obtained from all donors, and this study was approved by the institutional Research Ethics Committee (26563613.1.0000.5327).

After umbilical cord blood collection, segments of umbilical cord and placenta containing fetal and maternal tissues were separated aseptically and immediately transported to the laboratory in sterile saline for processing. Time from collection to processing was always <1 hour. Forceps and a scalpel were used to separate fetal and maternal placental tissues and devascularize the umbilical cord. Tissues were washed thoroughly with saline solution and fragmented into small pieces. Then, 1.5-2.5 g of each tissue was set aside for isolation protocols.

Chorionic membrane (n=4) and placental decidua (n=4) were digested with type I collagenase 1 mg/mL (Gibco), incubated for 2 hours at 37°C with agitation every 10 minutes. After enzymatic inactivation with Dulbecco's modified Eagle's medium (DMEM)(Gibco) 10% fetal bovine serum (FBS)(Gibco), filtration was carried out using a 100-µm filter (BD Falcon), followed by centrifugation at 500g/6 min/20°C. Cells were cultured in a 6-well plate in DMEM supplemented with 20% FBS, 1% penicillin-streptomycin antibiotic solution (Gibco) and 2 mM L-glutamine (Sigma-Aldrich), at 37°C, in a humidified 5% $CO_2$ atmosphere.

A-MSCs were isolated by digestion with 0.05% trypsin-EDTA solution (Sigma-Aldrich) in amniotic membrane (n=4) for 1 h at 37°C, with agitation every 10 minutes. After enzymatic inactivation, specimens were washed three times with saline and a new round of enzymatic digestion performed with type I collagenase 2 mg/mL for 3 h at 37°C, with agitation every 10 minutes. After inactivation, samples were washed with phosphate buffered saline (PBS) (Laborclin, Brazil) and centrifuged at 500g for 6 min at 20°C. Cells were cultured in a 6-well plate in DMEM supplemented with 20% FBS, 1% penicillin-streptomycin antibiotic solution and 2 mM L-glutamine, at 37°C, in a humidified 5% $CO_2$ atmosphere.

For UC-MSC isolation, umbilical cords (n=3) were washed with DMEM 10% FBS and digested with type I collagenase 2 mg/mL for 16 hours at 37°C. After enzymatic inactivation, another enzymatic digestion with 0.25% trypsin-EDTA was performed. After inactivation, samples were cultured in a 6-well plate in DMEM

supplemented with 20% FBS, 1% penicillin-streptomycin antibiotic solution and 2 mM L-glutamine, at 37°C, in a humidified 5% $CO_2$ atmosphere.

Culture medium was changed after 3-4 days initially and twice a week thereafter, until 80-90% confluence or overlapping colonies were observed. At this point, cells were trypsinized.

*Characterization assays*

To determine whether the isolated cells were indeed MSCs, immunophenotypic characterization and *in vitro* differentiation into mesodermal lineages were performed (P3-P5).

Immunophenotypic identification for CD105, CD73, CD90, CD44, CD45, CD34, CD11b, CD19 and HLA-DR was performed (BD Stemflow hMSC Analysis Kit, BD Biosciences, USA). Expression of the surface markers CD29-APC conjugated (BD Pharmingen, BD Biosciences, USA), CD14 (BD Pharmingen, BD Biosciences, USA), CD3 (Exbio, Czech Republic), HLA-G (Exbio, Czech Republic) and CD56 (BD Pharmingen, BD Biosciences, USA) was also assessed. Related isotype antibodies were used as control. Analysis was performed using FACSCanto II (BD Biosciences) and FlowJo software (FlowJo LLC, USA).

Capacity to differentiate into chondrocytes (28 days), adipocytes (28 days) and osteocytes (14 days) was evaluated using the STEMPRO® Differentiation Kit (Gibco) and staining with Alcian Blue, Oil Red and Alizarin Red, respectively.

*Growth kinetics*

A 5 x $10^4$ cellular suspension was cultured in a 6-well plate and passaged once a week using 0.25% trypsin-EDTA, at which time cells were counted under Trypan Blue staining (Sigma-Aldrich) and cultured again. All cells were cultured until P15 or until a decrease in growth was observed in 2 weeks. Population doubling (PD) was calculated using the formula: PD = (log N(t)-log N(to))/log 2, where N(t) was the number of cells per well at the time of trypsinization after 1 week and N(to) was the number of cells initially plated.

*Cell morphology: size, complexity and cell polarity comparison*

Comparisons of cell size and complexity in suspension were performed using flow cytometry. The MFI (median fluorescence intensity) of cell size and cell complexity of MSCs from each source were used for statistical analysis. Evaluation of length, width and cell polarity of MSCs in culture was performed in ImageJ software, using photos acquired after 1 day of culture in a cover slip coated with fibronectin. Four cultures of each cell type (A-MSC, C-MSC, D-MSC and UC-MSC) were analyzed. The polarity index was calculated as the length of the major axis (parallel to the membrane protrusion) divided by the length of the perpendicular axis that intersects the center of the cell nucleus. All experiments were performed using P3-P5 MSCs.

*Statistical analysis*

The parameters of interest were compared using ANOVA, generalized estimating equations (GEE) and multiple comparisons with Bonferroni correction, Tukey, Kruskal Wallis and Kaplan Meier. Analysis was performed in PASW Statistics 18.0. Results were considered significant if $p<0.05$.

**Results**

MSCs from amniotic membrane, chorionic membrane, placental decidua and umbilical cord were successfully isolated in all samples analyzed (isolation rate=100%). The mean time to first passage was 8.5 days for A-MSCs, 15 days for C-MSCs, 16 days for UC-MSCs and 17 days for D-MSCs.

*Characterization assays*

The results of cell-surface antigen expression analysis by flow cytometry are presented in Table 1. The profile surface marker was consistent with human MSCs. No contamination with hematopoietic cells was observed. A-MSCs showed a more heterogeneous population, because levels of some common MSC markers were not as high as in other MSCs sources. The greatest variation among the analyzed groups was observed for markers CD105 and CD56. Levels of CD56 and HLA-G, used as additional markers for characterization, were low or scarce in all tested MSCs.

All MSCs isolated from fetal adnexa demonstrated adipogenic, osteogenic and chondrogenic differentiation capacities (Supplementary Figure S1), typical of

mesodermal cells, but with variable efficiencies. MSCs from all sources presented relatively low adipogenic differentiation ability, while osteogenic differentiation was superior in MSCs from all tested sources.

**Cell morphology**

On microscopic examination, there was a homogeneous population of fibroblast-like cells in first-passage of C-MSCs, D-MSCs and UC-MSCs. A-MSCs cultures were most heterogeneous, with a fibroblast-like population and a circular/polygonal population, as shown in Supplementary Figure S2. The heterogeneity of A-MSCs cultures was proven statistically by comparing the standard deviation of groups on morphology analysis ($p<0.01$).

Size comparison of MSCs in suspension showed that C-MSCs were the smaller cells (mean MFI 111295) ($p<0.01$). In contrast, D-MSCs were largest (mean MFI 119207) ($p<0.01$), while A-MSCs and UC-MSCs were similar (MFI mean 114020 and 114122, respectively). When cell complexity was analyzed, UC-MSCs had the lower cell complexity, followed by A-MSCs, D-MSCs and C-MSCs ($p<0.01$) (mean MFI 93607, 99186, 112579 and 120583, respectively).

Analysis of morphological characteristics of MSCs in culture (length and width) also revealed significant differences between groups (Figure 1). UC-MSCs were longer (mean/median 173.8/161µm) than A-MSCs (mean/median 158.4/140µm) ($p<0.05$) and D-MSCs (mean/median 155.6/140µm) ($p<0.01$). Width was smallest in UC-MSCs (mean/median 36/31.8µm) ($p<0.01$) and largest in A-MSCs (mean/median 64/50µm) ($p<0.01$). C-MSCs (mean/median 43.1/41.2µm) and D-MSCs (mean/median 43.6/40µm) presented similar width. In this context, UC-MSCs showed a higher polarity index (mean/median 5.8/5.0), as the most elongated cells ($p<0.01$), while A-MSCs had the lowest polarity index (mean/median 3.8/2.2) ($p<0.05$), with a more rounded cell shape. There was no significant difference in polarity index between C-MSC (mean/median 4.9/3.7) and D-MSCs (mean/median 4.6/3.7). As shown in Figure 1, it was possible o observe that A-MSCs were the most heterogeneous population.

**Growth kinetics**

The proliferation assay (Figure 2A) showed satisfactory growth of C-MSCs, D-MSCs and UC-MSCs, but an insignificant proliferation of A-MSCs, which had lower

PD values and a short lifespan (until P3-P5), with a significant difference from P1 ($p<0.001$ compared to C-MSCs and $p<0.05$ compared to D-MSCs and UC-MSCs). C-MSCs, D-MSC and UC-MSCs maintained similar population doubling until P8, when UC-MSCs showed decreased growth as compared with C-MSCs and D-MSCs ($p<0.01$). C-MSCs and D-MSCs presented a similar growth rate until P9. UC-MSC cultures had a superior mean PD value (3.1 in P3). C-MSCs maintained proliferation until P15, with mean PD=1.06. A survival analysis was conducted for evaluation of MSC lifespan (Figure 2B). A-MSCs cells presented the lowest lifespan ($p<0.01$). Considering C-MSC, D-MSC and UC-MSC survival, statistical analysis suggested that differences were only marginally significant ($p=0.07$); however, results indicated biological differences among the analyzed groups, with C-MSCs exhibiting a longer lifespan.

**Discussion**

A-MSCs, C-MSCs, D-MSCs and UC-MSCs represents alternative sources of multipotent cells, with many advantages over adult tissues [7,29-30]. As the therapeutic potential of MSCs depends on the cell source [31], this study intended to compare four types of fetal adnexa as a source of human MSCs. The advantage of comparison of these sources is the remission of donor-associated variability: fetal adnexa offer the opportunity to compare haploidentical or genetically identical MSCs exposed to the same environmental influence.

In this study, several characteristics of MSCs obtained from four fetal adnexa were compared and some differences were observed. Initially, visual examination of cells of amniotic membrane origin revealed population heterogeneity, a finding reported in some previous studies [21,27,32], but contradicting another [26]. This heterogeneous culture was confirmed by immunophenotypic and morphological analysis. We believe that the second population presented in MSCs cultures of all donors was composed of epithelial stem cells, since amniotic membrane is composed by epithelial and stromal cells [21,33]. Controversially, MSCs from chorionic membrane, placental decidua and umbilical cord exhibited homogeneous populations. However, in other studies, MSCs from these sources were found to yield heterogeneous cultures [27,34].

In accordance with previous reports using MSCs from fetal adnexa, all MSCs obtained in this study were capable of differentiating into mesodermal lineages [21,25,27]. As in our study, variations in magnitude of differentiation have been observed elsewhere, with adipogenic differentiation being especially weak [35-37].

Contradicting our findings, Karlsson et al (2011) observed that MSCs from all placental sources failed to differentiate into bone [37].

Immunophenotypic analysis characterized the isolated cells as MSCs (positive for CD90, CD73, CD105, CD29 and CD44; negative for CD34, CD45, CD14, CD3 and HLA-DR), in accordance with prior studies [23,25,36]. However, the surface-protein expression profile may vary depending on cell source [17]. C-MSCs, D-MSCs and UC-MSCs showed similarity in most markers analyzed in this study, while flow cytometry analysis corroborated the heterogeneity of A-MSCs cultures. Such heterogeneity has been reported elsewhere [21,39]. The degree of CD105 positivity varied in this study, which is consistent with previous investigations of MSCs from fetal adnexa [21-22,27,35,40-41]. C-MSCs, UC-MSCs and D-MSCs expressed high levels of CD29 and CD44, superior to those found in A-MSC cultures. This marker is related to cell adhesion. MSCs isolated in our study exhibited insignificant levels of hematopoietic cell markers (CD34, CD45, CD3 and CD14) when compared with MSCs isolated in other studies, which have been reported to exceed 20% for CD34 and CD14 in MSCs from chorion plate [26], 16.7% for CD45 in D-MSCs [22] and 17% for CD45 in A-MSCs [32]. Additionally, cells were tested for HLA-G because of its association with immune-inhibitory properties [42]. Both maternal and fetal sources exhibited low levels of HLA-G. In A-MSCs, 17% positivity for HLA-G surface has been reported [43], while in chorion plate-derived MSCs and UC-MSCs, approximately 10% of cells were positive for HLA-G in a previous investigation [41]. On intracellular analysis using flow cytometry, 90.8% of UC-MSCs were positive for HLA-G, and extracellular positivity has been demonstrated using other assays [42]. CD56 analysis was performed for supplemental characterization, as some studies have reported that CD56+ cells are proliferative and exhibit elevated clonogenic potential [44]. In our study, positivity for this marker was low across all MSC types. This is consistent with a previous study of UC-MSCs [42], but stands in contrast to another study involving MSCs from the same sources, wherein CD56 positivity was >50% in C-MSCs and UC-MSCs [25].

MSC size may interfere with the safety of transplantation, because large cells are a potential cause of vascular obstructions [45,46] and could cause acute toxicity [36]. Therefore, considering the present analysis, C-MSCs can be considered the safest cell for intravascular infusion, while D-MSCs might cause adverse effects because of their larger size. Previous comparisons between MSCs from adult tissues and fetal adnexa did not reveal any difference in cell size when comparing MSCs from adipose tissue,

umbilical cord blood and bone marrow [17] and MSCs from bone marrow and placental [36]. On the other hand, considering length and width in culture, UC-MSCs exhibited were longer and narrower than MSCs from other sources.

MSC migration capacity is essential for the homing process and could increase therapeutic competence. Cells must acquire a polarized morphology to migrate [47]. Cell polarity analysis may be a suggestive parameter of MSC motility capacity, considering that more elongated cell morphology is usually associated with better migratory performance [48-49]. The polarity index of MSCs may be affected by the presence of drugs and in certain conditions of injury [48-49]. According to data from the present study, UC-MSCs would have increased migration capacity, while A-MSCs had the least mobility.

MSCs from different sources are known to have different proliferation rates [25-26]. The greatest difference in proliferation potential between MSC sources in the present study was the lower growth capacity of A-MSCs, which is consistent with other reports [25]. These lower growth rates are related to the heterogeneous population obtained in A-MSCs cultures [35]. This contradicts studies that were able to maintain A-MSC cultures in proliferation for several passages [21,50]. In preliminary studies, using another isolation protocol, we were able to obtain A-MSCs with a better proliferation potential and lifespan (data not shown). In this experiment, UC-MSCs exhibited decreased proliferation from P8. A decline in UC-MSC growth potential after P10 has been reported in a study [23], while another reported proliferation capacity beyond P25 [42]. Similar findings for UC-MSC and D-MSC growth being greater than C-MSC growth have been reported [25]. D-MSCs and C-MSCs maintained a similar expansion capacity until P9, which contradicts previous studies that compared the same sources and reported superiority of D-MSCs [25] or C-MSCs [26]. It is important to observe that in first passages (the time at which MSCs are used for cell therapy), no significant difference in population doubling between C-MSCs, D-MSCs and UC-MSCs was observed, while A-MSCs showed significantly lower proliferation ($p<0.001$).

In this study, maternal and fetal tissues were obtained from discarded material, with similar frequencies, since these tissues compose all placentas eliminated at birth. However, the amount of tissue available from each donor is different. Amniotic membrane is the most quantitatively limited tissue, followed by chorionic membrane.

Umbilical cord is available in larger quantities, while placental decidua is the most abundant tissue in terms of weight.

In view of our findings, we believe that amniotic membrane was the worst MSC source. On comparison between maternal and fetal tissues, immature cells have been deemed to provide several advantages, such as superior cellular activity, lower possibility of mutations [14], greater differentiation, homing and engraftment potency, and lower immunogenicity [30]. Therefore, chorionic membrane and umbilical cord were considered good options for future use in cell therapy. However, results on the best source of MSCs are controversial. Indumathi et al. (2013) suggested that A-MSCs were superior to C-MSCs and D-MSCs [26], while Soncini et al. (2007) observed that A-MSCs and C-MSCs were similar [21]. González et al. (2015) agreed that chorion was the best source of MSCs when compared with placental decidua and umbilical cord [25].

## Conclusion

Our findings indicate that both fetal and maternal tissues serve as source of multipotent stem cells, as isolation of MSCs (with differentiation capacity and characteristic immunophenotype) from all four analyzed tissues was possible. Given the findings of this study, we consider that C-MSCs and UC-MSCs warrant further research with a view to future use as an alternative for cellular therapy, while A-MSCs are less promising.

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## Disclosure of Interest:

The authors declare no conflicts of interest.

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Table 1. Immunophenotype comparison of MSCs derived from amniotic membrane (A-MSC), chorionic membrane (C-MSC), placental decidua (D-MSC) and umbilical cord (UC-MSC), analyzed by flow cytometry (mean percentage ± standard deviation). Undifferentiated cells were collected and marked with monoclonal antibodies (P3-P5).

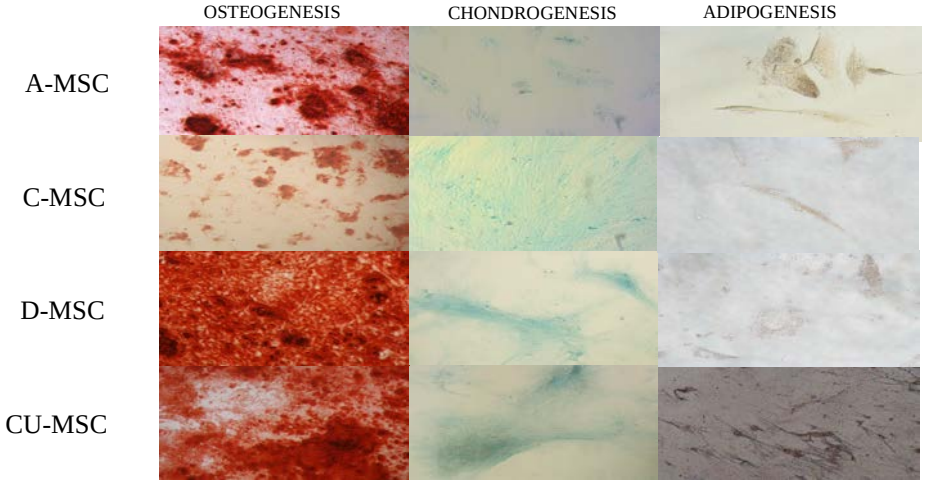
Supplementary Figure S1. Differentiation potential (P3-P5) of A-MSCs, C-MSCs, D-MSCs and UC-MSCs into three mesodermal lineages: osteocytes (Alizarin Red stain, magnification 100x), chondrocytes (Alcian Blue stain, magnification 100x) and adipocytes (Oil Red stain, magnification 200x).

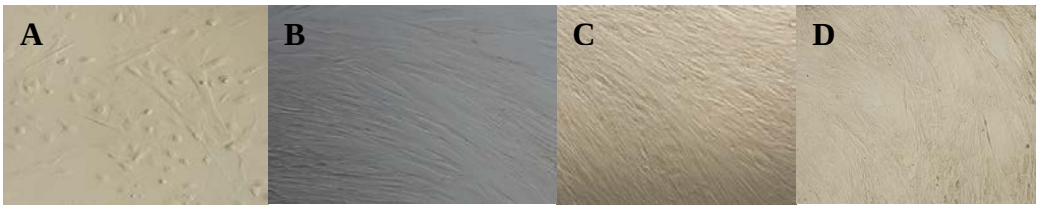
Supplementary Figure S2. Morphology of MSCs obtained from fetal adnexa (P3-P5). (A) A-MSCs: heterogeneous cultures containing fibroblast-like cells and circular/polygonal cells. (B) C-MSCs, (C) D-MSCs and (D) UC-MSCs presented a homogeneous population of fibroblast-like cells. Magnification 100x.

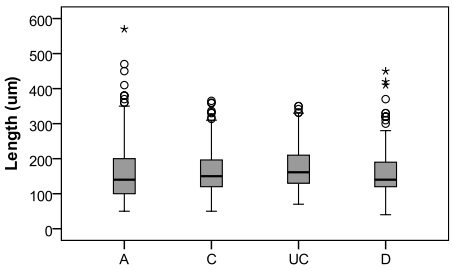
Figure 1. Analysis of morphological characteristics of A-MSCs (n=319), C-MSCs (n=384), D-MSCs (n=498) and UC-MSCs (n=330) after 24h in culture (P3-P5). (A) Length (B) Width (C) Cell polarity index. Values were obtained from four independent cultures of each tissue. UC-MSCs showed larger length, smaller width and higher polarity index, with a more elongated morphology. A-MSCs were wider and had a lower polarity index, which is consistent with more rounded cell shape.

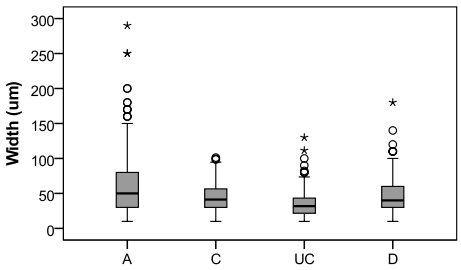
Figure 2. Comparison of population doublings of MSCs from four human fetal adnexa sources: A-MSCs (n=4), C-MSCs (n=4), D-MSCs (n=4) and UC-MSCs (n=3). Values presented are the mean of analyzed samples. (A) Growth kinetics until P10. Each point represents the passage day. Cells were passaged once weekly. (B) Survival of MSCs until P15.

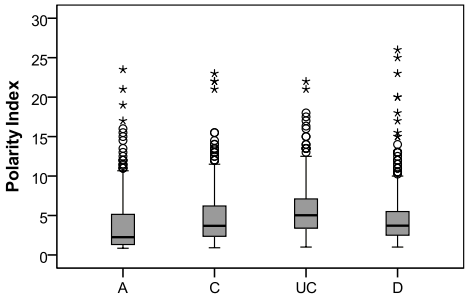
| | CD45CD34CD19CD11bHLA-DR | HLA-G | CD14 | CD3 | CD73 | CD90 | CD105 | CD29 | CD44 | CD56 |
|---|---|---|---|---|---|---|---|---|---|---|
| A-MSC | 0,4±0,4 | 0,9±0,2 | 3,2±2,8 | 1,0±0,8 | 99,0±0,5 | 98,6±0,6 | 45,0±12,9 | 83,6±0,5 | 85,6±1,0 | 3,2±1,5 |
| C-MSC | 0,2±0,1 | 1,6±0,6 | 0,4±0,3 | 0,3±0,3 | 99,2±0,6 | 96,6±0,4 | 92,8±7,0 | 97,1±0,5 | 96,0±2,3 | 7,6±4,9 |
| D-MSC | 0,3±0,1 | 0,5±0,0 | 0,4±0,4 | 0,1±0,1 | 99,2±0,4 | 97,3±1,2 | 98,0±1,0 | 99,0±0,5 | 98,5±0,8 | 4,7±3,3 |
| UC-MSC | 0,1±0 | 0,1±0 | 0,5±0,2 | 0,2±0,2 | 99,2±0,3 | 99,3±0,3 | 80,7±1,6 | 97,6±1,1 | 91,7±6,7 | 9,3±1,9 |

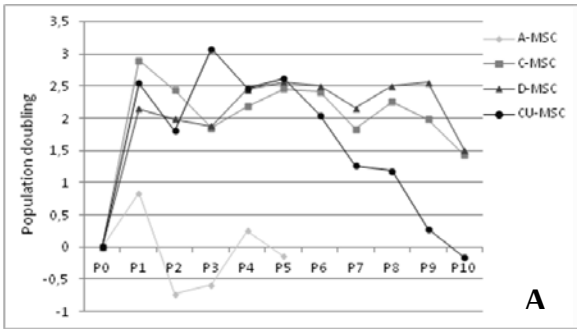


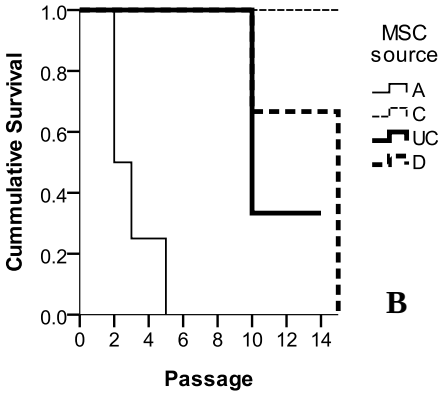




3,5
3
2,5
2
1,5
1
0,5
0
-0,5
-1
Population doubling
P0 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10
A-MSC
C-MSC
D-MSC
CU-MSC
A


1.0
0.8
0.6
0.4
0.2
0.0
Cummulative Survival
0 2 4 6 8 10 12 14
Passage
MSC source
A
C
UC
D
B

## 8. CONSIDERAÇÕES FINAIS

- Foi possível a obtenção e caracterização das células-tronco mesenquimais nos quatro tecidos estudados: membrana amniótica, membrana coriônica, decídua da placenta e cordão umbilical, sendo que todas apresentaram maior potencial de diferenciação na linhagem osteogênica e menor potencial de diferenciação na linhagem adipogênica. CTMs de membrana amniótica demonstraram menor expressão dos marcadores de superfície caracteristicamente positivos para CTMs.

- Foi estabelecida a cultura de células-tronco mesenquimais isoladas dos anexos embrionários.

- Dentre os protocolos analisados, não foi possível padronizar um protocolo ideal para isolar células-tronco mesenquimais da membrana amniótica.

- Células-tronco mesenquimais foram isoladas da membrana coriônica com protocolos de digestão enzimática com colagenase tipo I isolada ou associada à tripsina, sendo o protocolo com uso exclusivo de colagenase considerado o melhor.

- Células-tronco mesenquimais foram obtidas da decídua da placenta utilizando protocolo de digestão enzimática usando somente colagenase tipo I.

- Células-tronco mesenquimais podem ser isoladas de fragmento da parede do cordão umbilical utilizando protocolos com digestão enzimática associando tripsina e colagenase tipo I.

- Não foi possível padronizar um único protocolo como o ideal para as quatro fontes teciduais estudadas.

- Morfologicamente, culturas de C-CTMs, D-CTMs e CU-CTMs apresentaram-se homogêneas, com células fibroblastóides. Culturas de membrana amniótica apresentaram-se heterogêneas, com presença de células fibroblastóides e arredondadas.

- C-CTMS foram consideradas as células de menor tamanho, enquanto D-CTMs as de maior tamanho.

- O índice de polaridade indicou um perfil mais alongado nas CTMs isoladas de cordão umbilical e um perfil mais arredondado das culturas de CTMs isoladas da membrana amniótica.

- C-CTMS, D-CTMs e CU-CTMs apresentaram potencial de crescimento semelhante até P8, enquanto A-CTMs apresentaram baixo potencial de crescimento.

- Membrana amniótica foi considerada a pior fonte de CTMs na metodologia utilizada por este estudo.

- C-CTMs e CU-CTMs foram consideradas as melhores fontes de CTMs, visto terem as vantagens associadas a tecidos neonatais, superiores às D-CTMs.

# 9. PERSPECTIVAS FUTURAS

Em seguimento à linha de pesquisa instituída neste trabalho de doutorado, investigando a utilização de células-tronco mesenquimais obtidas de tecidos perinatais, pretende-se investigar outras características das células isoladas deste material que possam interferir na sua ação terapêutica, sendo estas:

- atividade migratória randômica
- atividade migratória em direção a um quimioatrator
- secretoma das CTMs destes tecidos
- potencial imunomodulatório de CTMs *in vitro*.

# 10. ANEXOS

## ANEXO 1

### TERMO DE CONSENTIMENTO INFORMADO

Eu, abaixo assinado, autorizo a equipe médica e assistentes, no Hospital de Clinicas de Porto Alegre, a realizarem o procedimento de coleta das células do sangue do cordão umbilical e da placenta do meu bebê. Estas células serão armazenadas e poderão ser utilizadas para o tratamento de diversas doenças sangüíneas e/ou imunológicas em pacientes que não tem familiar em condições de doar. Este programa é parte da Rede Brasilcord, vinculado ao Ministério da Saúde, realizado no Hospital de Clínicas de Porto Alegre (HCPA) e não acarretará custo ou qualquer remuneração, compensação ou privilégio pela doação do sangue do cordão umbilical e placentário.

A equipe explicou-me de forma clara a natureza e o objetivo deste procedimento e me foi dada oportunidade de fazer perguntas, sendo todas elas respondidas completa e satisfatoriamente.

Estou ciente que a doação deste material biológico, que habitualmente é descartado, não trará qualquer risco ao meu bebê e além disso estarei ajudando no tratamento de pessoas portadoras de doenças graves, como leucemias e algumas anemias hereditárias, que em muitos casos podem ser fatais. O sangue do cordão umbilical e da placenta possui células que são capazes de desenvolver uma nova medula óssea nestes pacientes, que irá produzir sangue.

Fui informada que a coleta do sangue de cordão umbilical e placentário ocorre após o nascimento do bebê e a retirada da placenta, não tendo qualquer interferência no parto, nem no cuidado da mãe ou do bebê. Este material será coletado, processado e, quando considerado em condições de ser utilizado para tratamento, será armazenado no Banco de Sangue de Cordão Umbilical e Placentário (BSCUP) do HCPA, por longo período de tempo. Quando algum paciente necessitar destas células para tratamento, o material armazenado será utilizado.

Concordo que para participar deste programa, além da coleta do sangue do cordão umbilical e placentário, será necessário:

- consultar as informações contidas no meu prontuário e no do meu bebê;
- realizar uma entrevista breve com perguntas sobre minha gravidez e história clínica;
- coletar amostras do meu sangue, para fazer exames considerados necessários.

Fui informada também que no meu sangue e no sangue do cordão umbilical e placentário serão realizados testes para verificar a possibilidade de utilizar este material em pacientes que poderão se beneficiar com este tipo de tratamento. Estes testes incluem contagens de células e testes para verificar a presença de infecções e de anemias hereditárias graves, sendo que parte deste material será armazenada para testes futuros. Caso eu tenha interesse em saber os resultados dos exames, os mesmos estarão disponíveis no Banco de Sangue do HCPA. Os resultados destes testes serão mantidos de forma confidencial.

Caso o material biológico coletado não esteja em condições de ser utilizado para tratamento, eu ( ) concordo ( ) não concordo que ele possa ser utilizado em projetos de pesquisa, sem qualquer dado de identificação pessoal associado.

Estou ciente que a minha decisão em participar ou não deste programa não irá influenciar de forma alguma na qualidade do meu atendimento e do meu bebê e caso tenha aceitado e queira desistir da doação, estarei livre para fazê-lo até o momento da coleta. Em caso de dúvidas poderei ainda entrar em contato com o BSCUP, pelo telefone 33597654.

Data: / /

Nome (em letra de forma) do paciente ou responsável:

Documento de identificação:

Assinatura:

Responsável pela obtenção do Termo: