UNIVERSIDADE VILA VELHA – ES

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

ESTUDO COMPARATIVO DE VIABILIDADE CELULAR ENTRE DIFERENTES MÉTODOS DE ACONDICIONAMENTO E TEMPO APÓS O PREPARO DE CÉLULAS TRONCO MESENQUIMAIS PARA UTILIZAÇÃO EM TERAPIA CELULAR

CARLOS HENRIQUE TAVARES MATHIAS

VILA VELHA – ES

JANEIRO / 2016

UNIVERSIDADE VILA VELHA – ES

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

ESTUDO COMPARATIVO DE VIABILIDADE CELULAR ENTRE DIFERENTES MÉTODOS DE ACONDICIONAMENTO E TEMPO APÓS O PREPARO DE CÉLULAS TRONCO MESENQUIMAIS PARA UTILIZAÇÃO EM TERAPIA CELULAR

Dissertação apresentada a Universidade Vila Velha, como pré-requisito do Programa de Pós-graduação em Ciência Animal, para a obtenção do grau de Mestre em Ciência Animal.

CARLOS HENRIQUE TAVARES MATHIAS

VILA VELHA - ES

JANEIRO / 2016

Catalogação na publicação elaborada pela Biblioteca Central / UVV-ES

M431e Mathias, Carlos Henrique Tavares.

Estudo comparativo de viabilidade celular entre diferentes métodos de acondicionamento e tempo após o preparo de células tronco mesenquimais para utilização em terapia celular / Carlos Henrique Tavares Mathias. – 2016.
33 f. : il.

Orientadora: Betânia Souza Monteiro.

Dissertação (Mestrado em Ciência Animal) – Universidade Vila Velha, 2016.
Inclui bibliografias.

1. Células-tronco. 2. Células. 3. Transplante. 4. Terapia celular. 5. Veterinária I. Monteiro, Betânia Souza. II. Universidade Vila Velha. III. Título.

CDD 571.6

CARLOS HENRIQUE TAVARES MATHIAS

ESTUDO COMPARATIVO DE VIABILIDADE CELULAR ENTRE DIFERENTES MÉTODOS DE ACONDICIONAMENTO E TEMPO APÓS O PREPARO DE CÉLULAS TRONCO MESENQUIMAIS PARA UTILIZAÇÃO EM TERAPIA CELULAR

Dissertação apresentada a Universidade Vila Velha, como pré-requisito do Programa de Mestrado em Ciência Animal, para obtenção do grau de Mestre em Ciência Animal.

Aprovada em 28 de Janeiro de 2016,

Banca Examinadora:

Carlos Eduardo Tadokoro (UVV)

Frederico Jacob Eutrópio (ICCA)

Betânia Souza Monteiro (UVV - Orientadora)

DEDICATÓRIA*:*

Primeiramente a Deus, aos meus pais, noiva e irmão, que sempre estiveram ao meu lado em todos os momentos importantes da minha vida, responsáveis por todo apoio, carinho e amor fundamentais para conclusão desta etapa. Vocês são o meu verdadeiro exemplo de vida!

AGRADECIMENTOS

Primeiramente agradeço a Deus, por me guiar sempre me dando força e sabedoria par concluir essa grande etapa da minha vida.

À Fundação de Amparo à Pesquisa do Espírito Santo (FAPES) e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa que viabilizou meus estudos, dedicação e apoio financeiro ao projeto.

À minha noiva Laura, companheira de todo tempo, que é a grande responsável por eu ter ingressado no mestrado, por me fazer uma pessoa melhor e por melhorar a minha vida, soube suportar meu nervosismo dos dias decisivos, e ao mesmo tempo retribuir com amor, carinho e dedicação. Te amo mais que tudo!

A minha família, em especial, agradeço à minha mãe Luciana, que me incentivou muito, sempre, meu pai Rubens, meu irmão Rafael, minha avó Izaura, minha sogra Aline Poyart. Muito obrigado por todo carinho desprendido, por serem meu porto seguro e por estarem sempre do meu lado me dando o apoio e ajuda necessário. Agradeço também a todos os primos e tios por todos momentos de felicidade e descontração.

À minha orientadora, Dra. Betânia Souza Monteiro, que se tornou uma grande amiga. Difícil expressar em palavras o agradecimento por ter viabilizado o meu sonho orientando tanto o meu trabalho de mestrado como minha carreira. Finalizo esta etapa com a certeza de que com ela sempre poderei contar, muito obrigado!

A minha amiga/irmã Viviane Raposo Fortunato, exemplar e incansável companheira! Que fez desses dois anos de mestrado os melhores, sempre do meu lado me fazendo “rir” e sempre disposta a fazer tudo sempre com um sorriso no rosto e por todos: “...né Carlos!”, “eu não sei, mas o Carlos sabe!”

Aos meus verdadeiros amigos e companheiros, Juliano Izidoro, Karina Coelho, Gabriel Vicente, Monique Lázaro, Juliana Barros, Liz, Julia Piccoli, Alvaro, Renan Carvalho, Vinícius Vaneli, Jullia Cabidelli, Dariele Gonçalves, Marcel Avanza, Clarisse Simões, Daniela Campagnol, Eduardo Raposo, Odael

Spadeto,Tatiana Champion. Vocês foram excepcionais em todos os dias dessa jornada, tanto nos momentos difíceis, quanto nas horas de brincadeira e lazer. Estavam sempre por perto para dar aquele apoio especial.

À coordenação do Hospital Veterinário pela autorização para utilização das instalações.

A todos os professores do programa de pós-graduação em Ciência animal que fizeram parte de toda a minha trajetória.

Aos funcionários do HV-UVV, Terezinha, Lurdinha, tia Lúcia, Antônio, Adriano, Dhyego, Jukleber, Felipe, o meu agradecimento por todo apoio.

Aos membros da banca examinadora Prof. Dr. Carlos Eduardo Tadokoro e Dr. Frederico Jacob Eutrópio pela disponibilidade e contribuições.

A todos, muito obrigado!!!!!!

SUMÁRIO

CAPITULO 2 - EFFECT OF CONDITIONING METHODS IN MESENCHYMAL STROMAL CELLS VIABILITY FOR CELLULAR THERAPY PROTOCOLS

LISTA DE FIGURAS

CAPITULO 2 - EFFECT OF CONDITIONING METHODS IN MESENCHYMAL STROMAL CELLS VIABILITY FOR CELLULAR THERAPY PROTOCOLS

LISTA DE TABELAS

CAPITULO 2 - EFFECT OF CONDITIONING METHODS IN MESENCHYMAL STROMAL CELLS VIABILITY FOR CELLULAR THERAPY PROTOCOLS

RESUMO

MATHIAS, Carlos Henrique Tavares, MSc., Universidade Vila Velha – ES, janeiro de 2016. **Estudo comparativo de viabilidade celular entre diferentes métodos de acondicionamento e tempo após o preparo de células tronco mesenquimais para utilização em terapia celular**: Orientadora: Betânia Souza Monteiro.

As células tronco mesenquimais (CTM) são consideradas uma linhagem de células tronco somáticas e estão presentes em regiões perivasculares de todos os tecidos adultos, incluindo a medula óssea (MO), tecido adiposo, periósteo, tecido muscular, parede dos vasos que compõem o cordão umbilical e os órgãos parenquimatosos. O crescente interesse nesse grupo celular para utilização em pesquisas e aplicação terapêuticas advém de sua capacidade de interagir com sistema imune do hospedeiro, imunoestimulando e/ou imunodeprimindo as células de defesa e reparadoras, em prol de um processo de cicatrização do organismo. Com o advento da terapia celular, a busca por esse tipo de terapia cresceu exponencialmente, porém, nem sempre temos laboratórios especializados perto das clínicas para o fornecimento de células, tendo assim, a necessidade de transportar essas células sem que ocorra perda na viabilidade. Atentando-se as necessidades atuais para comercialização desse tipo celular, o estudo objetivou realizar um comparativo de viabilidade celular entre diferentes métodos de acondicionamento e tempo após o preparo de células tronco mesenquimais, para utilização em terapia celular. Foram utilizadas 10 amostras de células tronco mesenquimais para quantificação de células totais, células viáveis e inviáveis, sendo as amostras acondicionadas em 72 seringas estéreis contendo 0,30mL de solução PBS 1x mais células. As seringas foram dividas aleatoriamente em três ambientes: Geladeira, Isopor com gelo, Isopor sem gelo, e em tempos predeterminados foram realizados teste de viabilidade usando o método de Azul de tripan. Foi observado que no ambiente “Geladeira” e “Isopor com gelo” a viabilidade celular permaneceu superior a 90% em todos os momentos com exceção do momento T48, já no ambiente “Isopor” a viabilidade permaneceu inferior a 90% nos momentos T8, T10, T12, T24 e T48. Identificou-se que temperaturas de 2ºC à 8ºC são consideradas melhores para o acondicionamento das células durante um transporte, mantendo boa viabilidade celular durante 24 horas.

Palavras-chave: Protocolo de cultivo, caracterização, diferenciação, citometria, antígenos de superfície, logística, transplante celular, Azul de Tripan, diferenciação celular.

ABSTRACT

MATHIAS, Carlos Henrique Tavares, MSc, University Vila Velha - ES, January 2016. **Comparative cell viability study between different conditioning methods and times after mesenchymal stem cell preparation for use in cellular therapy**: Supervisor: Betânia Souza Monteiro.

Mesenchymal stem cells (MSCs) are considered a lineage of somatic stem cells and are present in perivascular regions of all adult tissues, including bone marrow (BM), adipose tissue, periosteum, muscle tissue, walls of vessels which make up the umbilical cord, and parenchymal organs. The growing interest for this cell group for use in research and therapeutic applications comes from its ability to interact with the host's immune system, immune-stimulating and/or immune-de With the advent of cellular therapy, search for this type of therapy has grown exponentially, however specialized laboratories aren't always found near the clinics for cell supplying, thus having the need to transport these cells without occurring viability loss. Keeping in mind the current needs for commercialization of this cell type, the study aimed to conduct a cell viability comparative between different conditioning methods and times after the preparation of mesenchymal stem cells, for use in cellular therapy. Ten samples of mesenchymal stem cells were used for quantification of total cells, viable and nonviable cells, and the samples conditioned in 72 sterile syringes containing 0.30 ml of 1x PBS, plus cells. The syringes were randomly divided into three environments: Refrigerator, Styrofoam box with ice, Styrofoam box without ice, and at predetermined times viability tests were performed using the Trypan blue method. It was observed that in the environments "Refrigerator" and "Styrofoam box with ice" cell viability remained higher than 90% at all times with the exception of moment T48, already, in the environment "Styrofoam box" viability remained below 90% in moments T8, T10, T12, T24, and T48. It was found that temperatures from 2ºC to 8ºC are considered best for conditioning the cells during transportation, maintaining good cell viability for 24 hours.

Keywords: Culture protocol, characterization, differentiation, cytometry, surface antigens, logistics, cellular transplantation, Trypan Blue, cellular differentiation.

CAPÍTULO 1

ATUALIZAÇÕES EM CÉLULAS TRONCO MESENQUIMAIS: ESTUDO DA ARTE

CARLOS HENRIQUE TAVARES MATHIAS[1*], VIVIANE FORTUNATO RAPOSO[1], LAURA MONTEIRO DE CASTRO CONTI[2], BRUNA LOPES DE ALMEIDA[1], BETÂNIA SOUZA MONTEIRO[3]
*carloshenriquetm@gmail.com

[1]Mestrando em Ciência Animal, Laboratório de Células Tronco e Terapia Celular, Universidade Vila Velha (UVV), Vila Velha, ES, cep 29.102-920, Brazil. [2]Médico Veterinário, Mestre, Laboratório de Células Tronco e Terapia Celular, UVV. [3]Professora do Programa de Pós-Graduação em Ciência Animal, Coordenadora do grupo de pesquisa, Laboratório de Células Tronco e Terapia Celular, UVV.

Resumo

As células tronco mesenquimais (CTM) são consideradas uma linhagem de células tronco somáticas e estão presentes em regiões perivasculares de todos os tecidos adultos, incluindo a medula óssea (MO), tecido adiposo, periósteo, tecido muscular, parede dos vasos que compõem o cordão umbilical e os órgãos parenquimatosos. O crescente interesse nesse grupo celular para utilização em pesquisas e aplicação terapêuticas advém de sua capacidade de interagir com sistema imune do hospedeiro, imunoestimulando e/ou imunodeprimindo as células de defesa e reparadoras, em prol de um processo de cicatrização do organismo. A presente revisão teve como objetivo reunir conceitos atuais sobre as células tronco e demonstrar a forma utilizadas para caracterização e diferenciação das CTM.

Palavras chave: Protocolo de cultivo, caracterização, diferenciação, citometria.

1. INTRODUÇÃO

As células tronco (CT) são células progenitoras e indiferenciadas, capazes de auto renovação e diferenciação em múltiplas linhagens de células e tecidos responsáveis pela reparação tecidual. O termo CT, outrora chamado de células estromais, foi instituído para facilitar o entendimento do que são essas células, fazendo uma analogia entre uma árvore e o organismo; uma vez que os galhos, ramos e folhas de uma árvore se originam do tronco, enquanto que diversos tipos celulares especializados originam–se estas células progenitoras (RAO, 2004)

Durante o desenvolvimento embrionário, encontram-se células totipotentes as quais são chamadas de células tronco embrionárias (CTE), que são derivadas da massa celular interna do blastocisto (uma fase de embrião precoce) e têm uma alta capacidade proliferativa, podendo gerar todas as três camadas germinativas (MANGANELLI et al., 2011). Após o processo de diferenciação celular, originam-se células adultas pertencentes a uma linhagem somática, portanto passam a ser chamadas de células tronco somáticas (CTS) ou adultas, que são multipotentes (DEL CARLO et al., 2008). No grupo das CTS incluem as células-tronco hematopoiéticas (CTH) e mesenquimais (CTM). Recentemente, em 2007, um novo tipo de célula tronco foi criado pela comunidade cientifica e nomeada de CT pluripotentes induzidas (iPS) (TAKAHASHI et al., 2007).

As CTH são de origem mesenquimal e, durante o período de vida intrauterino, se encontram presentes no saco vitelino embrionário e, posteriormente, deslocam-se por via hematógena para o fígado, baço, linfonodos e timo fetal. Em um determinado período de vida intrauterino, a hematopoiese passa a ocorrer na medula óssea, que se torna o principal sítio hematopoiético. Elas exibem potencial proliferativo, porém com capacidade de se diferenciar apenas em células do sistema linfohematopoético (NAKAGE & SANTANA, 2006).

As CTM foram identificadas inicialmente por Friedenstein et al. (1970), que descreveram uma população de células aderentes com morfologia similar a fibroblastos, as quais ele referenciou como células precursoras osteogênicas (ALHADLAQ & MAO, 2004). Elas estão presentes em regiões perivasculares de

todos os tecidos adultos, em pequenas quantidades, incluindo a medula óssea, tecido adiposo, órgão parenquimatosos (ZUCCONI et al., 2010), tecidos fetais, periósteo e tecido muscular (ZAGO & COVAS, 2006; BYDLOWSKI et al., 2009; FOSSUM & SCHULZ, 2015; PEREIRA et al., 1995; MEIRELLES et al., 2008; JESUS et al., 2011; BARRIGA et al., 2013; PINTO FILHO et al., 2013). Caracterizam-se por ser uma população de células multipotentes capazes de se diferenciar e produzir tipos celulares necessários num processo de reparação (PITTENGER et al., 1999). Tais características de plasticidade sugerem que esse tipo celular é o responsável pelo “turnover” e manutenção de todos os tecidos do organismo (CAPLAN, 2009).

As células iPS são geneticamente modificadas e reprogramadas, que se originam de células somáticas ou de CTS, por meio de mecanismos de transfecção celular gerando exemplares similares as células embrionárias. Esta reprogramação envolve a incorporação de fatores transcripcionais que são altamente expressados pelas CTE, como, por exemplo, os fatores Oct3/4, Sox2, c-Myc e Klf4, gerando células semelhantes as embrionárias em relação a morfologia e funcionalidade (TAKAHASHI & YAMANAKA, 2006)

2. CÉLULAS TRONCO MESENQUIMAIS

As CTM são classificadas como “multipotentes” pelo fato de já terem passado por um processo de diferenciação e agora seguem uma linhagem específica, porém ainda podem se diferenciar em tecidos e células distintas dentro dessa linhagem (ABDALLAH & KASSEM, 2008).

Estudos mostram que as CTM apresentam alta plasticidade, pois podem sofrer transdiferenciação, diferenciação e fusão, pois expressam receptores para fatores de crescimento (FC), que são peptídeos produzidos pelo nicho, sofrendo então estímulos bioquímicos e se diferenciando de acordo com essa sinalização do ambiente (TAKAHASHI et al., 2007). Ademais, sabe-se atualmente, que um dos principais mecanismos de ação delas é sob efeitos parácrino e telécrino, nos quais as CTM liberam citocinas que agem nas células ao seu redor, fazendo com que as células do nicho, também se diferenciem em outras linhagens (TAKAHASHI et al., 2007). Sabe-se ainda que essas citocinas modulam respostas inflamatórias,

promovem angiogênese e mitose de células na reparação tecidual (WAN et al., 2008).

A transdiferenciação pode ser direta, onde há alteração do seu citoesqueleto, ou indireta, onde há um processo no qual a célula assume sua forma primitiva novamente e depois se diferencia em outro tipo celular. Já a fusão, é o processo no qual a célula tronco se une a uma outra célula adulta e assume sua expressão gênica (HERZOG et al., 2003; MEIRELLES et al., 2006). Portanto, dependendo do local onde as CTM situam-se, podem se transformar em osteoblastos (LIMA et al., 2012), neurócitos (KOPEN, 1999), cardiomiócitos (CARVALHO, 2012) etc.

Segundo DEL CARLO et al. (2008) as células podem ser transplantadas para o local de lesão tecidual, ainda indiferenciadas, para que sofram a diferenciação posterior por ação do nicho, ou podem ser transplantadas após sofrerem diferenciação *in vitro*. Podem também ser aplicadas *in situ* (diretamente na lesão), por via intravenosa. Por infusão será necessário um maior volume celular para a aplicação, já que as células se distribuem por diversos locais e não se restringem apenas a lesão (HORWITZ et al., 2002), contudo uma forma fácil de administrar.

Ainda há a opção da aplicação de células autógenas ou alógenas, e devem-se considerar as vantagens e desvantagens de cada uma. Por exemplo, utilizando a técnica do aspirado de medula, para células autógenas, ela se mostra pouco eficiente em pacientes idosos, visto que a quantidade de CTM na medula óssea diminui com a idade avançada. Outra forma de utilização de CTM autógenas é por isolamento, cultivo e expansão *in vitro* que se mostra a mais eficaz no processo de regeneração, porém requer um maior tempo para esse cultivo não permitindo uma utilização imediata (ALVES, 2013). O uso de CTM alógenas é uma alternativa que fornece uma grande quantidade de células em um período de tempo menor, já que elas são retiradas de banco de culturas celulares (ARINZEH et al., 2003).

As CTM tornaram-se foco de atenção terapêutica devido seu potencial imunomodulatório (WAN et al., 2008). Mediante o contato direto das CTM com o tecido (alógeno ou autógeno) ou mediante a interação destas células com o interferon-gama (INF-γ) produzido pelas células imunes do organismo, as CTM desencadeiam a liberação de diversos fatores solúveis. Estes fatores atuarão sobre as células do sistema imunológico (linfócitos e células dendríticas e demais células apresentadoras de antígeno) modulando a resposta imune (CORCIONE et al., 2006; NAUTA & FIBBE, 2007).

Dentre esses fatores, estão as prostaglandinas (PGE2), interleucinas (IL-4, IL-6, IL-8, IL-10), fator de crescimento transformador beta (TGF-ß), fator de crescimento do hepatócito (HGF) e a enzima indoleamine 2,3-dioxygenase (IDO) (NAUTA & FIBBE, 2007). A liberação de TGF-ß e HGF suprime a proliferação dos linfócitos T e B (Di NICOLA et al., 2002). Enquanto, a liberação de IDO, PGE2 e TGF-ß induzem a perda do potencial citotóxico das células *natural killer* (NK), uma vez que suprimem a produção de IL-2, IL-15 e INF-γ pelas mesmas (SOTIROPOULOU et al., 2006).

Outra importante característica das CTM é a baixa imunogenicidade exibida por essas células (UCHIBORI et al., 2014). Várias pesquisas evidenciaram que as CTM podem exercer a função imunossupressora para vários tipos de células imunitárias seja imunidade inata ou adaptativa, como células T, células B, células NK e células dentríticas (SILVA et al., 2009; YANG et al., 2013). Porém, os mecanismos de imunossupressão da resposta inflamatória, assim como os mecanismos de rejeição ao transplante não foram completamente esclarecidos (PATEL et al., 2008).

Alguns autores referiram que as CTM expressam poucas quantidades de complexo de histocompatibilidade principal do tipo I (MHC-I), além de níveis negligenciáveis de MHC-II em sua superfície (LE BLANC, 2003). Dificultando o reconhecimento das CTM, uma vez que as células de defesa do organismo utilizam o sistema MHC para diferenciar as demais células como próprias ou não-próprias. Portanto na ausência do MHC, esse processo de seleção de não-próprio não acontece, conferindo o impedimento da rejeição (MAJUMDAR et al., 2003).

Vários estudos já realizados demonstraram que o transplante de CTM não possui a capacidade de desenvolver neoplasias, contudo essa característica é observada nas CTE por apresentar maior indiferenciação, aumentando sua capacidade mitótica e podendo haver formação de teratomas ou outros tumores (ALVES, 2013).

3. CARACTERIZAÇÃO CELULAR

É importante saber que, as culturas de células tronco mesenquimais são compostas por uma população de células heterogêneas. Segundo CARVALHO et al. (2012) é necessário fazer a caracterização das células, visto que apenas a aderência ao plástico não é suficiente para definir uma população de CTM como pura, pois linhagens hematopoiéticas, fibroblastos e macrófagos também apresentam essa capacidade de adesão e uma morfologia semelhante a elas.

Essa caracterização é feita por meio do reconhecimento de antígenos de superfície da membrana celular, por meio da utilização de anticorpos monoclonais, esse processo é denominado imunofenotipagem (MEIRELLES et al., 2006).

Uma dificuldade na Medicina Veterinária é a baixa disponibilidade de anticorpos monoclonais específicos (CARVALHO et al., 2009), sendo então a caracterização feita identificando marcadores específicos e não específicos (ALHADLAQ & MAO, 2004; MEIRELLES et al., 2006). A citometria de fluxo se mostra um método rápido, fácil e eficaz para a caracterização, e vem sendo bastante utilizada na Medicina Veterinária (ZUCCONI et al., 2010).

Esses marcadores de superfície podem variar de acordo com o tecido de origem (KOLF et al., 2007). Células derivadas de medula óssea apresentam positividade para CD13, CD29, CD54, CD73, CD90, CD106 e Stro-1, e células derivadas de tecido adiposo apresentam marcação positiva para CD13, CD29, CD44, CD54, CD73, CD90, CD105, CD144 e CD166 (CARVALHO, 2011). Já os marcadores de células hematopoiéticas CD34, CD45, não são expressos por CTM (SEO et al., 2009).

A Sociedade Internacional de terapia celular *(International Society for Cellular Therapy – ISCT)* padronizou alguns critérios básicos para que uma célula possa ser considerada célula tronco mesenquimal. Estabeleceu-se que deveria possuir morfologia semelhante a um fibroblasto, possuir capacidade de aderir ao substrato plástico e de se diferenciar em no mínimo três tipos celulares: osteoblastos, condrócitos e adipócitos (ZAGO & COVAS, 2006; DOMINICI et al., 2006), deveriam ainda possuir proteínas de superfície onde são expressos os marcadores CD105, CD73 e CD90 e não os marcadores de células hematopoiéticas CD45 e CD34 (FOSSUM & SCHULZ, 2015; JESUS et al., 2011; DOMINICI et al., 2006).

4. DIFERENCIAÇÃO CELULAR

Como a ISCT definiu, uma cultura celular só poderá ser considerada CTM quando forem demonstradas propriedades de proliferação e diferenciação das células *in vitro.* A indução da diferenciação das CTM *in vitro* ocorre por cultivo das mesmas em meio de cultura específico, enriquecido com agentes indutores, por um determinado período de tempo e, mais comumente são fornecidos estímulos para três tipos celulares: osteoblastos, condrócitos e adipócitos (ZAGO & COVAS, 2006; DOMINICI et al., 2006).

A diferenciação condrogênica, por exemplo, ocorre por meio de adição de *Transforming Growth Factor*-β (TGB- β) (DENNIS et al., 2002). O TGB- β e outras citocinas indutoras direcionam a condrogenese por meio de ativação da via de sinalização intracelular, a via de ativação das quinases é iniciada pelos mitógenos (mitogen-activated protein – MAP kinases), tais como: ERK-1, p38, PKC e Jun (SEKIYA et al., 2002). Para confirmação dessa diferenciação são realizados testes histológicos que detectam a presença de proteoglicanos na matriz extracelular e cadeias de colágeno tipo II, típico de cartilagem articular (PITTENGER et al., 1999).

A adipogênese advém do tratamento das CTM com dexametasona, isobutil metil xantina (IBMX) e indomectina (DENNIS et al., 2002). A diferenciação será comprovada através da coloração de Oil Red, mediante a expressão de

proteínas, como receptor ativado de proliferação peroxisoma γ 2 (PPARγ2), lipoproteína lipase e proteína ligante de ácido graxo (PITTENGER et al., 1999).

Outra forma de caracterizar as CTM é através da indução de uma diferenciação osteogênica *in vitro*, suplementando o meio de cultura DMEM (Dulbecco's modified Eagle médium) com dexametasona, ácido ascórbico e β-glicerofosfato. A dexametasona atua alterando a via de sinalização dos fatores de crescimento e dos hormônios, influenciando então na proliferação e diferenciação das células em linhagem osteogênica (LIMA et al., 2012). Já o ácido ascórbico e o β-glicerofosfato atuam tardiamente, um sendo cofator indispensável na síntese do colágeno e outro fornecendo o fosfato necessário para mineralização da matriz extracelular (ALVES, 2013). Para obter a confirmação de que as CTM de fato sofreram uma diferenciação osteogênica, as células são coradas com Alizarin Red, o qual cora a matriz mineralizada em vermelho (BRAVO et al., 2012).

5. CONSIDERAÇÕES FINAIS

As CTM possuem potencial de diferenciação em várias linhagens celulares e podem ser cultivadas objetivando a expansão em número. Esta plasticidade faz com que esse tipo celular possuam um grande potencial para várias aplicações terapêuticas, com a finalidade de reestabelecer o funcionamento do organismo. Outra vantagem para a utilização destas é a sua disponibilidade em todos os tecidos adultos, como por exemplo, medula óssea, tecido adiposo e órgãos parenquimatosos, e a sua relativa facilidade de obtenção e cultivo. A padronização de técnicas para o processamento, identificação, caracterização e diferenciação destas células permitirá o desenvolvimento de ensaios experimentais e clínicos de forma que seja possível sua utilização com segurança e eficácia em pacientes animais e humanos.

6. REFERÊNCIA BIBLIOGRÁFICA

ABDALLAH, B.M.; KASSEM, M. Human mesenchymal stem cells: from basic biology to clinical applications. **Gene Therapy**, v.15, n.2, p.109-116, 2008.

ALHADLAQ, A.; MAO, J.J. Mesenchymal stem cells: isolation and therapeutics. **Stem Cells Development**, v.13, n.4, p.436-448, 2004.

ALVES, E.G.L. **Células tronco mesenquimais de cães associadas ou não à vidro bioativo, diferenciação osteogênica in vitro e tratamento de defeito ósseo crítico.** 2013. 108p. Tese de Doutorado em Ciência Animal – Universidade Federal de Minas Gerais.

ARINZEH, T.L. et al. Allogeneic mesenchymal stem cells regenerate bone in a critical-size canine segmental defect. **The Journal of Bone & Joint Surgery**, v.85, n.10, p.1927-1935, 2003.

BARRIGA, A.; MEDRANO, M.; DE-JUAN, J. et al. Infusión intravenosa de células madre adultas procedentes del tejido adiposo para la reparación de lesiones medulares isquêmicas. Estudio experimental em conejos. **Revista Española de Cirugía Ortpédica y Traumatología**, v.57, n.2, p.89-94, 2013.

BRAVO, M.O.; MORAES, J.M.; DUMMONT, C.B.S. et al. Isolamento, expansão e caracterização de células-tronco do tecido adipose de equinos. **ARS VETERINARIA**, v.28, n.2, P.66-74, 2012.

BYDLOWSKI, S.P.; DEBES, A.A.; DUARTE, S.A. et al. Células-tronco do líquido amniótico. **Revista Brasileira de Hematologia e Hemoterapia**, v.31, n.1, p.45-52, 2009.

CAPLAN, A.I. Why are MSCs therapeutic? New data: new insight. **The Journal of Pathology**, v.217, n.2, p. 318-324, 2009.

CARVALHO, A.M.; ALVES, A.L.; GOLIM, M.A. et al**.** Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue. **Veterinary Immunology and Immunopathology**, v.132, n.2-4, p.303-306, 2009.

CARVALHO, P.H.; DAIBERT, A.P.F.; MONTEIRO, B.S. et al. Diferenciação de Células Tronco Mesenquimais Derivadas do Tecido Adiposo em Cardiomiócitos. **Arquivos Brasileiros de Cardiologia**, v.100, n.1, p.82-89, 2013.

CARVALHO, PH. **Avaliação neurológica e histological de lesão compressive da medulla espinhal de ratos wistar, tratados com células-tronco mesenquimais**. 2011. 79p. Dissertação de Mestrado em Medicina Veterinária - Universidade Federal de Viçosa.

CORCIONE, A.; BENVENUTO, F.; FERRETTI, E. et al. Human mesenchymal stem cells modulate B-cell functions. **Blood**, v.107, n.1, p.367-372, 2006.

DEL CARLO, R.J.; MONTEIRO B.S.; ARGÔLO NETO, N.M. Células tronco e fatores de crescimento na reparação tecidual. **Ciência Veterinária nos Trópicos**, v.11, supl.1, p.167-169, 2008.

DENNIS, J.E.; CHARBORD, P. Origin and differentiation of human murine stroma. **Stem Cells**, v. 20, n.3, p. 205-214, 2002.

DI NICOLA, M.; CARLO-STELLA, C.; MAGNI, M. et al. Human bone marrow stromal cells suppress T lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. **Blood**, v.99, n.10, p.3838-3843, 2002.

DOMINICI, M. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. **Cytotherapy**, v. 8, n.4, p.315-317, 2006.

FOSSUM, T.W.; SCHULZ, K.S. Medicina Regenerativa e Terapia com Célula-Tronco. In: FOSSUM, T.W. **Cirurgia de Pequenos Animais**. Rio de Janeiro: Elsevier, 2015, cap.14, p.167-175.

FRIEDENSTEIN, A.J.; CHAILAKHJAN, R.K.; LALYKINA, K.S. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. **Cell Proliferation**, v.3, n.4, p.393-403, 1970.

HERZOG, E.L.; CHAI, L.; KRAUSE, D.S. Plasticity of marrow-derived stem cells. **Blood**, v.102, n.10, p. 3483-3493, 2003.

HORWITZ, E.M.; GORDON, P.L.; KOO, W.K. et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implication for cell therapy of bone. **Proceedings of the Nacional Academy of Sciences of the United States of America**, v.99, n.13, p.8932-8937, 2002.

JESUS, A.A.; SOARES, M.B.P.; SOARES, A.P. et al. Coleta e cultura de células-tronco obtidas da polpa de dentes decíduos: técnica e relato de caso clínico. **Dental Press Journal of Orthodontics**, v.16, n.6, p.111-118, 2011.

KOLF, C.M.; CHO, E.; TUAN, R.S. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. **Arthritis Research & Therapy**, v.9, n.1, p.204-213, 2007.

KOPEN, G.C.; PROCKOP, D.J.; PHINNEY, D.G. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. **Proceedings of the Nacional Academy of Sciences of the United States of America**, v.96, n.19, p. 10711-10716, 1999.

LE BLANC, K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. **Cytotherapy**, v. 5, n.6, p. 485-489, 2003.

LIMA, S.A.F.; WODEWOTZKY, T.I.; LIMA-NETO, J.F et al. Diferenciação in vitro de células-tronco mesenquimais da medula óssea de cães em precursores osteogênicos. **Pesquisa Veterinária Brasileira**, v.32, n.5, p.463-469, 2012.

MAJUMDAR, M.K.; KEANE-MOORE, M.; BUYANER, D. et al. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. **Journal of Biomedical Science**, v.10, n.2, p.228-241, 2003.

MANGANELLI, G.; FICO, A.; FILOSA, S. Embryonic Stem Cells: from Blastocyst to in vitro Differentiation. In: ATWOOD, C. **Methodological Advances in the Culture, Manipulation and Utilization of Embryonic Stem Cells for Basic and Practical Applications**. Croácia: Intech, 2011, cap.1, p.3-24.

MEIRELLES, L.S.; CAPLAN, A.I.; NARDI, N.B. In Search of the In Vivo Identity of Mesenchymal Stem Cells. **Stem Cells**, v.26, n.9, p.2287-2299, 2008.

SILVA MEIRELLES, L.; CHAGASTELLES, P.C.; NARDI, N.B. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. **Journal of Cell Science**, v.119, n.11, p.2204-2213, 2006.

NAKAGE, A.P.M.; SANTANA, A.E. Células-tronco hematopoéticas em cães. **Ciência Rural**, v.36, n.1, p.325-329, 2006.

NAUTA, A.J.; FIBBE, W.E. Immunomodulatory properties of mesenchymal stroma cells. **Blood**, v.110, n.10, p.3499-3506, 2007.

PATEL, S.A.; SHERMAN, L.; MUNOZ, J. et al. Immunological properties of mesenchymal stem cells and clinical implications. **Archivum Immunologiae et Therapiae Experimentalis**. v.56, n.1, p.1-8, 2008.

PEREIRA, R.F.; HALFORD, K.W.; O'HARA, M.D. et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in

irradiated mice. **Proceedings of the Nacional Academy of Sciences of the United States of America**, v.92, n.11, p.4857-4861, 1995.

PINTO FILHO, S.T.L.; TREICHEL, T.L.E.; ARAMBURÚ JUNIOR, J.S. et al. Células-tronco mesenquimais adultas: características e aplicações experimentais em animais. **Veterinária e Zootecnia**, v. 20, p.49-59, 2013.

PITTENGER, M.F.; MACKAY, A.M.; BECK, S.C. et al. Multilineage potential of adult human mesenchymal stem cells. **Science**, v.284, n.5411, p.143-147, 1999.

RAO, M.S. Stem sense: a proposal for the classification of stem cells. **Stem Cells and Development**, v.13, n.5, p.452-455, 2004.

SEO, M.; JEONG, Y.; PARK, J.; et al. Isolation and characterization of canine umbilical cord blood-derived mesenchymal stem cells. **Journal of Veterinary Science**, v.10, n.4, p.181-187, 2009.

SEKIYA, I.; LARSON, B.R.; SMITH, J.R. et al. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. **Stem Cells***,* v. 20, n.6, p. 530-541, 2002.

SILVA, C. L. **Estudos moleculares de células tronco mesenquimais cultivadas in vitro.** 2009. 159p. Tese doutorado – Instituto Nacional de Câncer.

SOTIROPOULOU, P. A.; PEREZ, P.A.; GRITZAPIS, A.D. et al. Interactions between human mesenchymal stem cells and natural killer cells. **Stem Cells**, v.24, n.1, p.74-85, 2006.

TAKAHASHI, K.; TANABE, K.; OHNIKI, M. et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. **Cell**, v.131, n.5, p.861-872, 2007.

TAKAHASHI, K.; YAMANAKA, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. **Cell**, v.126, n.4, p.663-676, 2006.

UCHIBORI, R.; TSUKAHARA, T.; OHMINE, K. et al. Cancer gene therapy using mesenchymal stem cells. **International Journal of Hematology**, v.99, n.4, p.377-382, 2014.

WAN, C.D.; CHENG, R.; WANG, H.B. et al. Immunomodulatory effects of mesenchymal stem cells derived from adipose tissues in a rat orthotopic liver transplantation model. **Hepatobiliary & Pancreatic Diseases International**, v.7, n.1, p.29-33, 2008.

YANG, X.; HOU, J.; HAN, Z. et al. One cell, multiple roles: contribution of mesenchymal stem cells to tumor development in tumor microenvironment. **Cell & Bioscience**, v.3, n.1, p.1-10, 2013.

ZAGO, M.A. Células-tronco: origens e propriedades. In: ZAGO, M.A.; COVAS, D.T. (Eds). **Células-tronco a nova fronteira da medicina**. São Paulo: Atheneu, 2006. Cap.1, p.3-20.

ZUCCONI, E.; VIEIRA, N.M.; BUENO, D.F. et al. Mesenchymal stem cells derived from canine umbilical cord vein: A novel source for cell therapy studies. **Stem Cells and Development**, v.19, n.3, p.395-402, 2010.

CAPÍTULO 2

EFFECT OF CONDITIONING METHODS IN MESENCHYMAL STROMAL CELLS VIABILITY FOR CELLULAR THERAPY PROTOCOLS

CARLOS HENRIQUE TAVARES MATHIAS[1*], VIVIANE FORTUNATO RAPOSO[1], LAURA MONTEIRO DE CASTRO CONTI[2], ALVARO DE PAULA LAGE OLIVEIRA[2,] JULLIA FERREIRA CABIDELLI[3], JULIANA STELZER ZARDO[1], BETÂNIA SOUZA MONTEIRO[4]
*carloshenriquetm@gmail.com

[1]Master student of Animal Science, Stem Cells and Cell Therapy Laboratory, Vila Velha University (UVV), Vila Velha, ES, zip code 29.102-920, Brazil. [2]DVM, Master in Animal Science, Stem Cells and Cell Therapy Laboratory, UVV. [3]Graduate student of Veterinary Medicine, Scientific Initiation Scholarship, Stem Cells and Cell Therapy Laboratory, UVV. [4]Professor of Animal Science Postgraduate Program, Group Research Coordinator, Stem Cells and Cell Therapy Laboratory, UVV.

ABSTRACT

Background. With the advent of cell therapy the search for this type of therapy has grown exponentially, however specialized laboratories are not always found near the clinics, thus having the need to transport these cells without occurring viability loss. Keeping in mind the current needs for commercialization of this cell type, the study aims to conduct a cell viability comparative between different conditioning methods and times after the preparation of mesenchymal stromal cells, for use in cellular therapy. ***Methods.*** Ten samples of mesenchymal stromal cells were used for quantification of total cells, viable and nonviable cells, and the samples conditioned in 72 sterile syringes containing 0.30 ml of PBS 1x plus cells. The syringes were randomly divided into three environments: Refrigerator, Styrofoam box with ice, Styrofoam box without ice, and at predetermined times viability tests were performed using the Trypan blue method. ***Results.*** It was observed that in the environments "Refrigerator" and "Styrofoam box with ice" cell viability remained higher than 90% at all times with the exception of moment T48, already in the environment "Styrofoam box" viability remained below 90% in moments T8, T10, T12, T24 and T48. ***Conclusion.*** It was found that temperatures from 2ºC to 8ºC are considered best for conditioning the cells during transportation, maintaining good cell viability for 24 hours.

Keywords. Surface antigens, Logistics, Cell Transplantation, Trypan Blue, Cell Differentiation.

1. Introduction

Stem cells are undifferentiated cells that have a high capacity for proliferation, self-renewal, generation of progenitor cells, regeneration of various tissues, and *in vivo* reconstruction of specific tissue (1-5).

They've been standing out as a renewable source of cells and replacement tissues for the treatment of some diseases that are the leading causes of disability and death in society,

such as neoplasia, pulmonary diseases and genetic diseases (2), heart disease, diabetes mellitus (2,4), spinal cord injury, burns, osteoarthritis, and rheumatoid arthritis (4).

Mesenchymal stromal cells (MSC) can be isolated from bone marrow, adipose tissue, fetal tissue, periosteum, muscle tissue, and parenchymal organs (2-4, 6-10). To identify the MSC some immunophenotypic markers are used, however, these markers aren't specific and can be found in other various cell types, and therefore analysis of the set of antibodies that are most expressed in the cells in question should be performed (2, 4).

Stem cell differentiation occurs by specific stimulation (3,9), by means of pro and anti-inflammatory cytokine secretion, as well as growth factors, leading to modulation of the local inflammatory response, promoting tissue repair by cell differentiation of the tissue in question (11,12). The use of these cells for replacement or even partial function restoration of damaged tissues or organs has increased, since demand for tissue donated for transplantation is low (4).

The *International Society for Cellular Therapy* standardized some basic criteria so that a cell can be considered a mesenchymal stromal cell. It was established that the cell should have a fibroblast-like morphology, have the capacity to adhere to the plastic substrat, and differentiate into at least three cell types: osteoblasts, chondrocytes, and adipocytes (2,13), they must also have surface proteins where the markers CD105, CD73 and CD90 are expressed and not the hematopoietic cell markers CD45 and CD34 (4,8,13).

Research with stem cells is increasing, thus resulting in a great interest in the use of these cells as a form of therapy for diseases (7). Through this increased demand for cells, the emergence of specialized laboratories has increased. It is known that, in Brazil, there is a normative ANVISA (No. 02/2012-GETOR/GGSTO), from 28/03/2012, aimed to standardize the national transport of hematopoietic progenitor cells, adult, embryonic, and other cells and derivatives, in which standardizes the technical shipping notification stating that the storage

conditions of biological materials should be sufficient to withstand a maximum transportation time which does not compromise the treatment's effectiveness. However, since there is no specific conditioning recommendation: chilled, frozen, or room temperature, each company selling the cellular product adopts a form of shipment, still, the technical literature does not direct the best way of shipping and conditioning.

Keeping in mind the current needs for commercialization of this cell type and/or the best form of conservation of MSC for an *in vivo* experimentation, this study aimed to conduct a cell viability comparison between different conditioning methods and times after the preparation of mesenchymal stromal cells, for use in cellular therapy.

2. Materials and Methods

The study was conducted according to the standards of the "Colégio Brasileiro de Experimentação Animal" (COBEA) and was approved by the Ethics Committee, Bioética e Bem-Estar Animal (CEUA) of the Vila Velha University (UVV), process 295/2013.

The collection of the subcutaneous adipose tissue fragments (SCAD) were performed in the operating room of the veterinary hospital "Professor Ricardo Alexandre Hippler" and the cell cultures were performed at the Stem Cell and Cell Therapy Laboratory (LCET-UVV) of the University Vila Velha (HV-UVV). The flow cytometry was performed in Cellular Immunology and Biomolecular Laboratory located at the Institute of Biological Sciences - ICB, at the Federal University of Minas Gerais.

2.1. Patient selection and tissue collection

Animals of the canine species showing normal blood count, erythrocyte, urea, creatinine, coagulation time, alkaline phosphatase, alanine aminotransferase, and which would be undergoing elective routine surgery in the HV-UVV were selected.

The animals were submitted to inhalation anesthesia, according to the evaluation of the team from the anesthesia sector of the HV-UVV and the adipose tissue fragments were collected from the abdominal region, always seeking places with lower presence of blood vessels. Adipose tissue samples of approximately 1.0 cm^3 (1 gram) were collected and placed in a 15 ml falcon tube containing the cell culture medium DMEM (Dulbecco's Eagle Medium Modified - low glucose) plus 10% FBS (fetal bovine serum), at room temperature and referred to the LCET-UVV.

2.2. Mesenchymal stromal cell culture

Immediately after collection, in a laminar flow chamber, the tissue fragments were washed 3 times with Phosphate-Buffered Saline 1X (1X PBS) to remove blood and cellular debris. The tissue was transferred to a second 15 ml falcon tube containing 10 ml of 1.5% type 1 collagenase solution (Sigma, St Louis MO, USA) with and then incubated at 37ºC and 5% carbon dioxide (CO_2) for 45 minutes, and this solution homogenized every 10 minutes. After this period, the sample was centrifuged at 22°C and 694G (2000 rpm) for 10 minutes. The supernatant was discarded and the pellet resuspended in 10 ml 1X PBS, the sample was centrifuged once again at 22 ° C and 694G (2000 rpm) for 10 minutes.

After centrifugation the supernatant was again discarded and the pellet resuspended in 10 ml of 20% DMEM. A 50 ml aliquot of the medium with the resuspended cells was transferred to a 96 well plate and 50 µL of Trypan Blue was added, to determine the concentration of cells present in the solution, where cells not stained in blue were considered viable. The remaining solution was then transferred to cell culture flasks of 75 cm^2. The flasks were kept in an incubator at 37°C in 5% CO_2 and 95% humidity. The cells were monitored daily, using an inverted optical microscope, and the culture medium being changed every 2 to 3 days according to cell metabolism.

As soon as 80 to 90% confluency was reached, the cultures were subjected to a trypsinization process. The culture medium was completely discarded and the flasks washed 3 times with 10 ml of 1X PBS to remove remaining culture medium. After washing, 4 ml of 1X trypsin-EDTA was added so that the cellular bonds with the extracellular matrix were undone, allowing cellular detachment from the plastic surface of the culture flasks. The flasks were incubated for 4 minutes at 37ºC and 5% CO_2. After the trypsin's action period the same was inactivated with 8 ml of DMEM supplemented with 10% FBS and centrifuged for 10 minutes at 694G. Following, the supernatant was discarded and the pellet resuspended in DMEM supplemented with 10% FBS. One 50 ml aliquot was separated for verification of viable cell concentration present in the solution. The remaining solution was placed in flasks containing 8 ml of DMEM supplemented with 10% FBS and a 2 ml aliquot of medium with the resuspended cells, and these were incubated at 37ºC and 5% CO_2. This procedure was repeated every 2 to 3 days taking into consideration cell confluency in each plate. With every new confluence of at least 80% the procedure was performed again until reaching the third cell passage.

Four culture medium flasks containing the cells of fourth passage were kept incubated at 37°C and 5% CO_2 for photographic documentation of *in vitro* morphology in inverted microscope and for evaluation in scanning electron microscopy (SEM); induction of osteogenic differentiation; induction of adipogenic differentiation, and for performing flow cytometric immunophenotyping. The remaining flasks were subjected to trypsinization and the cell samples were counted and evaluated for cell viability using the Trypan Blue technique.

2.3. Osteogenic differentiation induction in MSC cultures

A culture flask containing fourth passage cells were trypsinized and divided between six wells belonging to a growth plate. The culture medium in 3 wells was supplemented with

10-8 mol/ml of dexamethasone, 5.0 µg/ml of ascorbic acid 2-phosphate, 10.0 mmol/L of β-glycerophosphate and incubated at 37°C for four weeks. The remaining three wells were kept as a control, containing only complete DMEM.

At the end of this period the wells of the culture flask were washed in PBS and stained for five minutes with Alizarin red at room temperature, washed with distilled water and observed under an optical microscope for calcium deposition.

2.4. Immunophenotyping

A culture flask containing fourth passage cells was trypsinized, resuspended in 1.0 ml of PBS and plated at a concentration of $1x10^5$ cells/well in a growth plate containing 96 wells. The samples were characterized by immunophenotyping by means of incubation with antibodies anti-CD90, CD29, CD45 and CD34 and processed in a FACScan flow cytometer (Fluorescence Activated Cell Analyser) using the Cell Quest software. This stage was performed in partnership with the Animal Biology and Cellular and Molecular Immunogenetics Laboratory of the Federal University of Minas Gerais.

2.5. Experimentation: Cell conditioning

Two flasks of cultures with 80% confluence containing third passage cells were trypsinized and cellular pellets of the two flasks were placed in a 50 ml falcon tube containing 22 ml of PBS. After homogenization, the initial concentration and cell viability was established by quantification in a Neubauer chamber.

This total volume was divided among 72 sterile syringes of 1 mL with a volume of 0.30 ml. The syringes were placed in different environments for the purpose of simulating transportation of cells for use as a form of therapy. The conditioning types were: Styrofoam box with ice, Styrofoam box without ice, and refrigerator, and in each of these locations 24 syringes were placed (Figure 1A, 1B, 1C).

At each predetermined evaluation time, syringe triplicates were removed from the established locations and again subjected to quantification and cell viability. The determined times were: Initial Moment (T0), 1 hour after the initial moment (T1), 5 hours after the initial moment (T5), 8 hours after the initial moment (T8), 10 hours after the initial moment (T10), 12 hours after the initial moment (T12), 24 hours after the initial moment (T24), and 48 hours after the initial moment (T48).

2.6. Determination of cell viability

In each evaluation period three syringes were removed, their content were homogenized and a 50 ml aliquot of the solution separated from each one of the three syringes being deposited in separate wells of a plate containing 96 wells, where 50 µl of Trypan Blue was added in each well, homogenized and subsequently put into a Neubauer chamber for determining the concentration of viable and non-viable cells (Figure 1D, 2B).

The entire experimental trial was repeated with 10 different cultures and all of the presented values were equivalent to the obtained means.

2.7. Statistical analysis

Analysis of the quantitative variables were performed using the Graphpad Prism 5.3® program and the data analyzed for normality through the Kolmogorov-Smirnov test, considering normal distribution for samples with the value of $p > 0.05$. The variables were compared within each group regarding the different times of evaluation, and the normal distribution data analyzed by analysis of variance (ANOVA) with a post Tukey test and, the nonparametric using the Kruskal-Wallis test with a post Dunn test, both considering $p < 0.05$ for significant difference. For qualitative analysis, descriptive statistics were performed.

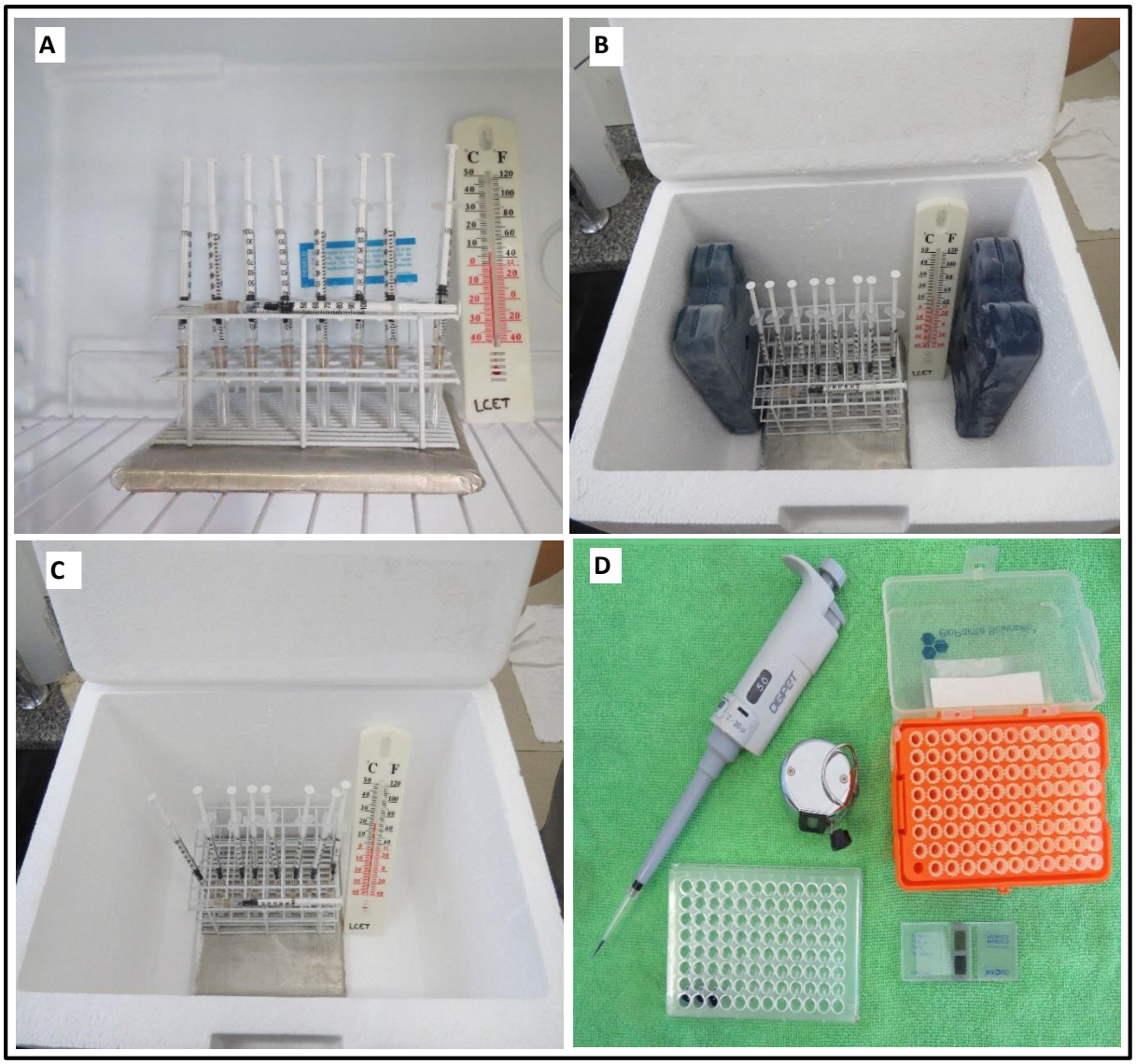

Figure 1 – Locations for conditioning the 1 mL syringes filled with 0.30 ml of MSC/PBS. A) Refrigerator. B) Styrofoam box with ice. C) Styrofoam box without ice. D) An aliquot of cell solution added to Trypan Blue was deposited in separate wells of a plate containing 96 wells and then, put into a Neubauer chamber for determining the concentration of viable and non-viable cells.

3. Results

In the first 24 hours, the adipose tissue yielded a heterogeneous culture, comprising non adherent rounded cells and lipid micelles in the supernatant. After the third day, the cellular population appeared more homogeneous, with the predominance of adherent cells that had a fibroblastoide morphology and were organized in colonies (Figure 2A).

The phenotypic characterization obtained by flow cytometry showed low expression of CD45 (6.11 %) and CD34 (3.47 %) hematopoietic cells markers and high expression of CD90

(99.0 %) and CD29 (95.8 %) mesenchymal stromal cells markers (Values were obtained through 10 culture evaluations) (Figure 2).

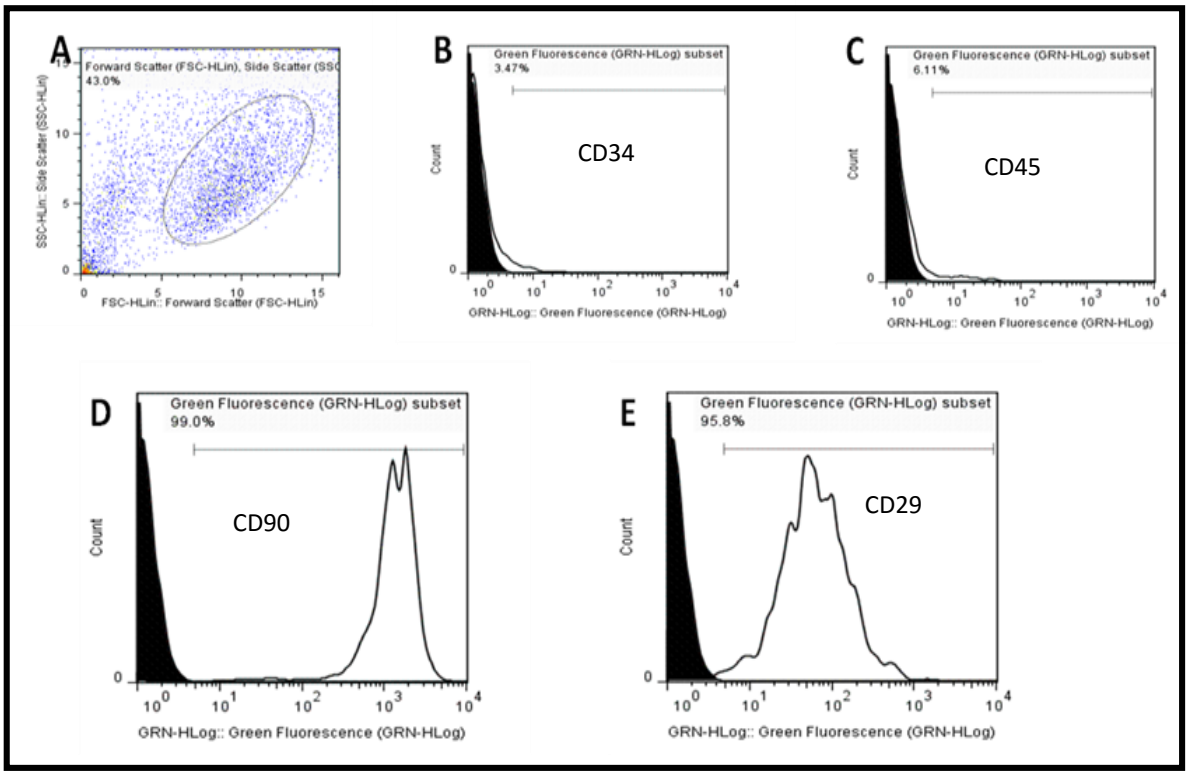

Figure 2 - Evaluation of the frequency of CD34, CD45, CD90 and CD29 by flow citometry on adipogenic derived mesechymal stromal cells (ADSC) of dogs. The fluorescence intensity of each surface marker in undifferentiated ADSC (White graphics) is compared with isotyp controls (Black graphics). The X-axis represents the fluorescence scale, the cells being positive when exceeding 10^1. The Y-axis indicates the number of cells evaluated during the event. A) a dot plot showing the cell population selected for the study (R1) which represented 43.0% homogeneity. The culture sample showed a negative expression of CD34 at 96.53% (b) and CD45 at 93.89% (c) while positive expression. of CD90 at 99,0% (D) and CD29 at 95,8% (E).

Cultures submitted to osteogenic differentiation presented calcification points of the matrix from the seventh day on. Mineralized nodules containing cells with rounded morphology, such as osteoblast cells, were observed since day 14. However, on day 21 of the culture in osteogenic environment, these nodules were more evident.

Temperatures obtained in the refrigerator ranged from 1°C to 5°C; in the Styrofoam box with ice there was an initial variation of 12ºC and final of 22ºC and; in the Styrofoam box without ice temperatures maintained in the range of 22 to 24ºC. The experimental room maintained temperatures close to 24°C and the external temperature ranged from 29 to 31°C.

The values obtained for total cell count and viable cells from the refrigerator and Styrofoam box with ice environments showed normal distribution in the homoscedasticity test, while, when analyzed viable cell count in the Styrofoam box without ice; non-viable cell count in refrigerator, Styrofoam box with ice and Styrofoam box without ice, as well as total cell count in the Styrofoam box without ice environment, the figures showed non-parametric distribution.

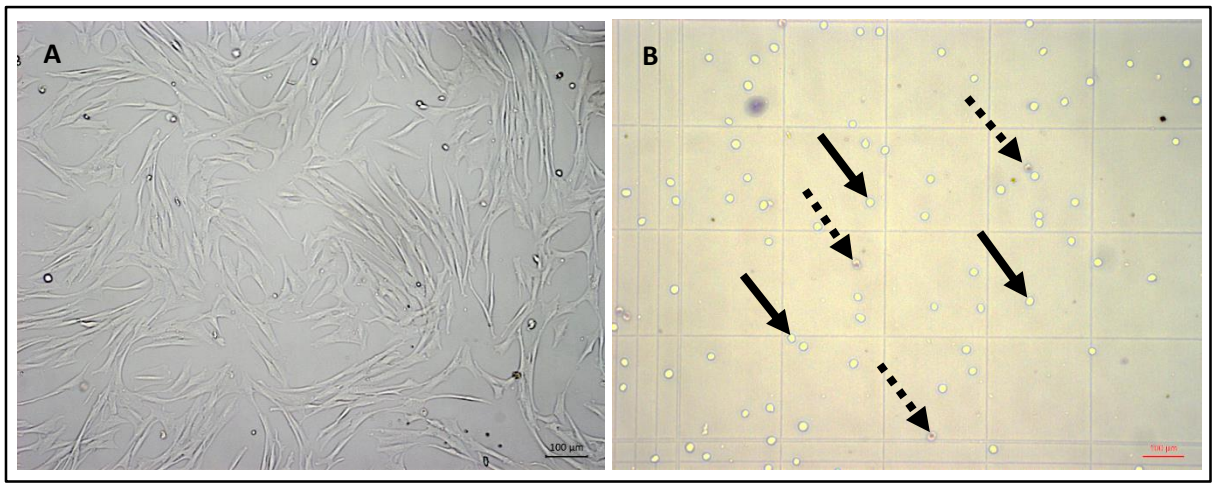

Figure 3 – A) Homogeneous cellular population with predominance of fibroblastoide adherent cells. B) Viable (Black Arrow) and non-viable (Dotted Black Arrow) cells visualized on Neubauer chamber to determinate concentration and viability.

Total (Figure 3), viable, and non-viable cell counts from each group and their viabilities in each moment, are described in Tables 1, 2 and 3.

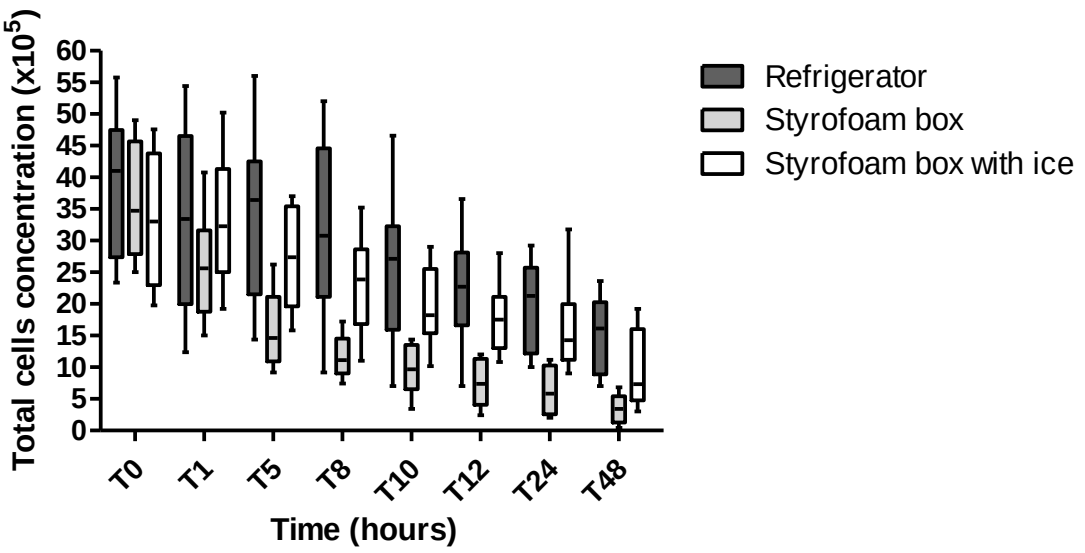

Figure 4. Demonstration of total cell concentration (cells/ml) when conditioned in the environments "refrigerator", "Styrofoam box with ice" and "Styrofoam box" in moments T0, T1, T5, T8, T10, T12, T24, and T48.

Table 1. Description of total, viable, and unviable cells on average and standard deviation, as well as cell viability in percent, in the environment "refrigerator" at all evaluation moments.

	Total cells (Cells/ml) (x10^5)	**Viable Cells (Cells/ml) (x10^5)**	**Unviable Cells (Cells/ml) (x10^4)**	**Cellular Viability**
T0	39 ± 11 [a]	37 ± 9.9 [a]	18 ± 78	95.47%
T1	34 ± 14 [a]	32 ± 10 [a]	18 ± 81	94.66%
T5	34 ± 14 [a]	33 ± 7.3 [a]	12 ± 49	96.46%
T8	31 ± 13 [a]	30 ± 7.6 [a]	12 ± 36	96.08%
T10	26 ± 11 [a]	24 ± 5.2 [a]	19 ± 43	92.81%
T12	22 ± 8.5 [b]	20 ± 4.7 [b]	17 ± 38	92.26%
T24	20 ± 7.3 [b]	18 ± 6.3 [b]	11 ± 48	94.19%
T48	15 ± 5.6 [b]	14 ± 5.7 [b]	17 ± 22	88.64%

Different letters in the same column differ statistically ($p < 0.05$) in the Variance Analysis test with post Tukey test.

Table 2. Description of total, viable, and unviable cells on average and standard deviation, as well as cell viability in percent, in the environment "Syrofoam box with ice" at all evaluation moments.

	Total cells (Cells/ml) (x10^5)	**Viable Cells (Cells/ml) (x10^5)**	**Unviable Cells (Cells/ml) (x10^4)**	**Cellular Viability**
T0	33 ± 10 [a]	31 ± 9.9[a]	18 ±5.9	94.44%
T1	34 ± 10 [a]	31 ± 10[a]	21 ± 11	93.69%
T5	27 ± 7.8 [a]	26 ± 7.3 [a]	13 ± 11	95.14%
T8	23 ± 7.7 [b]	22 ± 7.6 [a]	8.8 ± 6.1	96.17%
T10	19 ± 5.8 [b]	18 ± 5.2 [b]	14 ± 9.9	92.77%
T12	18 ± 5.0 [b]	16 ± 4.7 [b]	13 ± 6.9	92.57%
T24	16 ± 6.8 [b]	15 ± 6.3 [b]	14 ± 6.7	91.43%
T48	9.5 ± 5.9 [b]	8.0 ± 5.7 [b]	15 ± 4.1	84.33%

Different letters in the same column differ statistically ($p < 0.05$) in the Variance Analysis test with post Tukey test.

Table 3. Description of total, viable, and unviable cells on average and standard deviation, as well as cell viability in percent, in the environment "Styrofoam box" at all evaluation moments.

	Total cells (Cells/ml) (x10^5)	**Viable Cells (Cells/ml) (x10^5)**	**Unviable Cells (Cells/ml) (x10^4)**	**Cellular Viability**
T0	34 ± 8.1^a	33 ± 7.7^a	13 ± 10	96.14%
T1	25 ± 9.1^a	23 ± 9.4^a	13 ± 7.4	94.71%
T5	15 ± 5.4^a	14 ± 5.2^a	9.3 ± 4.5	93.82%
T8	12 ± 3.2^b	10 ± 3.4^b	15 ± 10	87.07%
T10	9.7 ± 3.7^b	8.6 ± 4.0^b	11 ± 4.7	88.47%
T12	7.6 ± 3.9^b	6.6 ± 3.8^b	10 ± 3.1	86.78%
T24	6.1 ± 3.7^b	5.3 ± 3.4^b	8.3 ± 4.5	86.37%
T48	3.5 ± 2.4^b	3.1 ± 2.2^b	4.5 ± 3.5	87.08%

Different letters in the same column are statistically different ($p < 0.05$) in the Kruskal-Wallis test with post Dunn's test.

A significant difference was obtained when total cell count groups were analyzed in all environments, but in different moments. In the environment "refrigerator" significant difference ($p < 0.05$) was obtained between the moment T0 and moments T12, T24, and T48, and; in environments "Styrofoambox with ice" ($p < 0.05$) and "Styrofoam box" ($p < 0.05$) differences were observed when comparing moments T8, T10, T12, T24, and T48 to moment T0.

In the viable cell count groups the environment "refrigerator" obtained significant difference ($p < 0.05$) when compared moment T0 to T12, T24, and T48; the environment "Styrofoam box with ice" significant difference ($p < 0.05$) was observed in moments T0 with T10, T12, T24, and T48 and; in the "Styrofoam box", a significant difference ($p < 0.05$) was obtained when comparing the moment T0 to moments T8, T10, T12, T24, and T48.

The non-viable cell count in the group "refrigerator" showed no significant difference ($p = 0.069$) between evaluation moments, however, the environment "Styrofoam box with ice" showed significant difference ($p < 0.05$) when comparing moments T0 and T8 and, the environment "Styrofoam box" there was significant difference ($p < 0.05$) when comparing moment T0 with moment T48.

Cell viability was described in percentage for each evaluation moment within each group, being observed at all moments of the environment "Refrigerator" and "Styrofoam box with ice" viability greater than 90%, except in moment T48. In the "Styrofoam box" environment, cell viability found was less than 90% in moments T8, T10, T12, T24, T48 and superior in T1 and T5.

4. Discussion

The therapeutic use of mesenchymal stromal cells in various diseases, both in small and large animals as well as in humans, have been increasingly reported, demonstrating the benefits of cellular therapy in promoting improvement in life quality and/or resolution of disorders. For example, cellular applications in injured tendons of athletic horses (14) and in the treatment of equine laminitis (15), acupoint applications for hip dysplasia in dogs (16) therapies in spinal cord injuries in dogs (17) and transplant in failures in order to increase the repair process (18).

However, so that cellular treatments can provide satisfactory results and be considered successful, some factors should be considered, because, they may affect the quality of the cells that are going to be infused, damaging the interaction of these with the receptor and, thereby preventing the body's immunomodulatory and reparative functions (19, 20). These factors highlight the amount of viable cells to be infused into the patient for cell therapy. Garvican et al. (14) alerted that the amount of cells may be affected during transportation from the cultivation laboratory to the clinic in which transplantation will occur, since no determination of the best transportation conditions exists to ensure cell viability until the final destination moment.

Despite the ideal method of conditioning and transportation for stem cell not yet not yet been defined by any specific legislation, in Brazil a normative, ANVISA, exists intended for different kinds of human tissues, stating that the conditioning form should be sufficient so

that the biological material can reach its destination without the cells losing their therapeutic power, although it does not determine what would be the ideal conditioning method so that the transportation is performed.

Among the various forms available for determining cell viability, the Trypan Blue staining method was opted on. This test, as highlighted by some authors, is often used to detect the integrity of the cell membrane as it is a colorimetric method of exclusion, in which the dead cells are stained blue due to the fact that the dye penetrates the damaged cell membrane (19, 21-23). Moreover, this viability technique was considered easily accessible, inexpensive, and of fast execution, being routinely used in research and service laboratories (19). The dilution used for the study followed the method described by Garvican et al. (14) in the ratio 1:1.

During the selection of the three environments that would be used for conditioning of the MSC, locations commonly used for commercial transportation of laboratory products were chosen. The Styrofoam box without any addition of other forms of temperature preservation, maintaining an average temperature of 23°C, which is used often for short distances; the Styrofoam box with addition of ice, which for a few hours is able to maintain low temperatures near 14ºC and due to being a widely used form of transporting various biological materials for an extended period, and; maintenance in refrigerator that guarantees the smallest variation in temperature, keeping close to 4°C, and is used with the aid of proper packaging and refrigerated, for transportation that require low temperatures (24).

Analogously to the present study, Antonenas et al. (25) also evaluated the viability of hematopoietic stem cells undergoing transportation in different climate conditionings, with temperature ranging between 1°C and 24°C. In which the refrigerated temperature remained between 2 and 8°C and room temperature ranged from 18ºC to 24ºC, in which they suggested

that the optimal temperature for transportation and storage of hematopoietic cells for maintenance of cell viability is from 2°C to 8°C for up to 72 hours.

The conditioning and the viability of cells undergoing freezing methods were not evaluated during this study because, according to Garvican et al. (14) this method is not suitable when the goal is the immediate cell application, since it becomes necessary to unfreeze and re-culture cells prior to transplantation.

Using refrigerated conditionings with temperatures between 1°C and 5°C, difference between the total number of cells, as well as the number of viable cells from moment T0 to moment T12, T2,4 and T48 could be observed, however, the cellular viability was maintained above 90% until the T24, reducing viability only in T48. Similarly, Garvican et al. (14) found no difference in cell viability when conditioning at 4°C to 8°C, between baseline, 12 hours and 24 hours later, observing only a reduction in cell viability when evaluated at 72 hours.

In Brazil there is no standardization regarding the lowest viability rate that would make unfeasible a cellular transplant with stem cells, while in the United States, the Food and Drug Administration (FDA) recommends a rate of 70% viability as the minimum acceptable. However, despite the recommendation of 70% (19, 26), it was noted that in clinical and preclinical studies, researchers opt to transplant cultures demonstrating cellular viability greater than 90%. Following this rationality, by the data found in the present study, and considering cell viability and total number of cells, it is indicated that cells kept refrigerated would be the most suitable for transplant, obeying periods of up to 5 hours for cells conditioned in Styrofoam boxes with ice (2.7×10^6 cells/ml, 95.14%) and up to 10 hours for cells conditioned in a refrigerator (2.6×10^6 cells/ml, 92.81%).

At room temperature (ranging from 18 to 24ºC), Antonenas et al. (25) could conclude that, regarding refrigerated temperatures, there is a greater loss of viable cells over time at all evaluation moments (24, 48, and 72 hours), analogously to that observed in the present study,

we found higher viable cell loss in the Styrofoam box (temperature varying between 22ºC and 24ºC) and Styrofoam box with ice (with a variation of the initial temperature at 12°C and the final at 22°C) environments in relation to the refrigerator.

Furthermore, in this study a greater loss of viable cells over time when cells were conditioned in all 48 hours at 22ºC to 24°C than when conditioned in an initial temperature of 12ºC and final temperature of 22ºC was observed, difference also observed for cell viability, since the viability was maintained above 90% in the Styrofoam box with ice until 24 hours, and in the Styrofoam box without ice viability was maintained above 90% only until the initial 12 hours.

The non-viable cell count varied over time in each environment, not showing a proportionate increase to the loss of viable cells in the cell count, a fact that may have been caused by the Trypan Blue staining, wherein, when there is early cellular apoptosis, the coloring may not penetrate the cell and therefore not dyeing it, requiring more sensitive methods to detect them (21). Another possibility for this fact, can be based on some studies in which there was a description of after dilution 1:1 with Trypan Blue, a wait of 2 (14) to 5 minute (21) took place before performing the count and, in the present study, after full homogenization of the dilution, the cells were immediately submitted to counting.

Another insight throughout the experiment was that the total number of cells decreased over time, without, however, the cellular solution presenting increase in non-viable cells and/or cell debris. This fact leads us to infer that the MSC may have adhered to the syringe's plastic biomaterial, like they do when they are in cell culture.

5. Conclusion

The present study identified that temperatures of 2°C to 8°C are considered better for the conditioning of the cells during transportation, maintaining quantity and high cell viability

for 24 hours. It is suggested that when the time between preparation of the cells and the transplant is greater than 24 hours, it's better that the patient dislocates to a location close to the culture laboratory to optimize the success of the therapy, because the tested methods do not ensured good viability and appropriate cell quantity for conditioning periods longer than 24 hours.

6. Acknowledgments

The authors thank the Nacional Council of Technological and Scientific Development (CNPq – Process 552488/2011-9 and 483518/2011-5) and Fundação de Amparo à Pesquisa do Espírito Santo (FAPES) for granting the fellowships and financial support that permitted us to conduct this study.

7. Disclosure of interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

8. References

1. Bonad MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B. Aging of mesenchymal stem cell in vitro. BMC Cell Biol 2006;7:1-7
2. Zago MA, Covas DT. Células-Tronco: Origens e Propriedades. In: Células-Tronco: A nova fronteira da medicina. São Paulo, BR: Editora Atheneu; 2006;1:3-20.
3. Bydlowski SP, Debes AA, Duarte SA, Janz FL, Cavaglieri RC, Maselli LMF. Células-tronco do líquido amniótico. Rev Bras Hematol Hemoter 2009;31:45-52.
4. Fossum TW, Schulz KS. Medicina Regenerativa e Terapia com Célula-Tronco. In: Fossum TW. Cirurgia de Pequenos Animais. Rio de Janeiro, BR: Editora Elsevier; 2015;14:167-75.
5. Konala VBR, Mamidi MK, Bhonde R, Das AK, Pochampally R, Pal R. The current landscape of the mesenchymal stroma cell secretome: A new paradigm for cell-free regeneration. Cytotherapy 2015;18:13-24.
6. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci 1995;92:4857-61.

7. Meirelles LS, Caplan AI, Nardi NB. In Search of the In Vivo Identity of Mesenchymal Stem Cells. Stem Cells 2008; 26:2287-99.
8. Jesus AA, Soares MBP, Soares AP, Nogueira RC, Guimarães ET, Araújo TM, et al. Coleta e cultura de células-tronco obtidas da polpa de dentes decíduos: técnica e relato de caso clínico. Dental Press J Orthod 2011;16:111-18.
9. Barriga A, Medrano M, De-Juan J, Burgos J. Infusión intravenosa de células madre adultas procedentes del tejido adiposo para la reparación de lesiones medulares isquêmicas. Estudio experimental em conejos. Rev Esp Cir Ortop Traumatol 2013;57:89-94.
10. Pinto Filho STL, Treichel TLE, Aramburú Junior JS, Rosa MB, Dalmlin F, Brun MV, Krause A, et al. Células-tronco mesenquimais adultas: características e aplicações experimentais em animais. Vet Zootec 2013:20:49-59.
11. Del Carlo RJ, Monteiro BS, Argôlo Neto NM. Avanço no estudo de células-tronco no Brasil e suas implicações. Rev Ceres 2009;56:446-50.
12. Monteiro BS, Argôlo Neto NM, Del Carlo RJ. Células-tronco mesenquimais. Cienc. Rural 2009;40:238-45.
13. Dominici M, Le Blanc K, Muller I, Slaper-Cortenbach I, Marini FC, Krause DS, et al. Minimal criteria for defining multipotente mesenchymal stromal cells. Cytotherapy 2006;8:315-17.
14. Gravican ER, Cree S, Bull L, Smith RKW, Dudhia J. Viability of equine meseschymal stem cells during transport and implantation. **Stem Cell Res Ther** 2014;5:1-10.
15. Morrison S, Dryden VC, Bras R, Morrell S. How to use stem cells in clinical laminitis cases. AAEP Proceedings 2014;60:499-03.
16. Marx C, Silveira MD, Selbach I, Silva AS, Braga LMGM, Camassola, et al. Acupoint injection of autologous stromal vascular fraction and allogeneic adipose-derived stem cells to treat hip dysplasia in dogs. Stem Cells Int 2014;1-6.
17. Lim JH, Byeon YE, Ryu HH, Jeong YH, Lee YW, Kim WH, et al. Transplantation of canine umbilicalcord blood-derived mesenchymal stem cells in experimentally induced spinal cord injured dogs. J Vet Sci 2007;8:275-82.
18. Monteiro B, Favarato L, Carvalho P, Okano B, Menegatti MA, Oliveira A, et al. Clinical and radiographic characterization of xenotransplantation of rat bone marrow-derived mesenchymal stem cell for repair of radial defects of rabbit. Turk J Vet Anim Sci 2015;39:1-9.
19. Chen Y, Yu B, Xue G, Zhao J, Li RK, Lui Z, Niu B. Effects of storage solutions on the viability of human umbilical cord mesenchymal stem cells for transplantation. *Cell Transplant* 2013;22:1075-86.
20. Park SS, Byeon YE, Ryu HH, Kang BJ, Kim YS, Kim WH, et al. Comparison of Canine Umbilical Cord Blood-Derived Mesenchymal Stem Cell Transplantation Times: Involvement of Astrogliosis, Inflammation, Intracellular Actin Cytoskeleton Pathways, and Neurotrophin-3. *Cell Transplant* 2011;20:1867-80.

21. Martin-Piedra MA, Garzon I, Oliveira AC, Alfonso-Rodriguez CA, Sanchez-Quevedo MC, Campos A, et al. Average cell viability lavels of human dental pulp stem cells: an accurate combinatorial index for quality control in tissue engineering. Cytotherapy 2013;15:507-18.
22. Patricio LFL, Rebelatto CLK, Brofman PRS, Beltrame OC, Brito HFV, Locatelli-Dittrich R. Isolamento e caracterização de células mesenquimais do tecido adiposo de cães. Arq Bras Med Vet Zootec 2013;65:946-54.
23. Reissis Y, García-Gareta E, Korda M, Blunn GW, Hua J. The effect of temperature on the viability of human mesenchymal stem cells. **Stem Cell Res Ther** 2013;4:1-11.
24. Godwin EE, Young NJ, Dudhia J, Beamish IC, Smith RKW. Implantation of boné marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon. Equine Vet J 2012;44:25-32.
25. Antonenas V, Garvin F, Webb M Sartor M, Bradstock KF, Gottlieb D. Fresh PBSC harvest, but not BM, show temperature-related loss of CD34 viability during storage and transport. Cytotherapy 2006;8:158-65.
26. Guidance for industry. Potency tests for cellular and gene therapy products. Rockville (MD): Center for Biologics Evaluation and Research (CBER); 20Available from:http://www.federalregister.gov/articles/2011/02/16/2011–3462/guidance-for-industry-potency-tests-for-cellular-and-genetherapy-products-availability.