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Camila Vieira Pinheiro

COMPARAÇÃO ENTRE VIAS DE ADMINISTRAÇÃO DE CÉLULAS-TRONCO EM CAMUNDONGOS NEONATOS

Porto Alegre
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Orientador: Prof. Dr. Guilherme Baldo
Co orientadora: M.ª Édina Poletto

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota".

(Madre Teresa de Calcutá)

RESUMO

Células-tronco são amplamente estudadas para a busca de novas formas de terapias para diversas doenças devido a sua capacidade de auto renovação e de se diferenciar em vários outros tipos celulares. As células tronco melhor caracterizadas são as células-tronco hematopoiéticas (CTH) e células-tronco mesenquimais (CTM), ambas sendo encontradas principalmente na medula óssea. No entanto, não existem estudos em que se observe como é a biodistribuição dessas células em camundongos neonatos nem por qual via de administração esses transplantes seriam mais eficazes, sendo que esses dados seriam importantes visando a busca por novas formas terapêuticas, principalmente de doenças em que a progressão é rápida e uma intervenção precoce seja relevante. O objetivo do presente trabalho foi avaliar a biodistribuição das CTH e CTM injetadas em camundongos neonatos de dois dias de vida, avaliando órgãos como fígado, pulmão, baço, coração, rim, córtex cerebral e medula óssea. As células foram injetadas por três vias de administração distintas (seio venoso retro orbital, veia temporal e via intraperitoneal) e os animais foram avaliados em diferentes tempos (48 horas e 30 dias pós-transplante). Para isso, foram utilizados camundongos C57BL6-GFP como doadores de células e camundongos 129SV como receptores, pois dessa forma se torna viável a detecção das células transplantadas. As células provenientes da medula óssea dos doadores foram purificadas e contadas em câmera de Neubauer para realização dos transplantes. Os órgãos dos animais transplantados foram analisados por imunohistoquímica utilizando anticorpo anti-GFP. As CTM não foram encontradas em quantidades significativas em nenhum dos tecidos analisados. O principal resultado encontrado foi a determinação da via da veia temporal (VT) como a mais eficiente. CTH estavam presentes após 48 horas no fígado e no baço e, em 30 dias, na medula óssea e no baço, em animais que havam sido imunossuprimidos pré-transplante. Embora tenha sido observada diferença estatística somente na medula óssea e no baço em 30 dias e pela via VT, os demais órgãos também apresentaram células GFP+ e, mesmo que em pouca quantidade, talvez seja o suficiente para auxiliar no tratamento de determinas condições.

Palavras-chave: Células-tronco hematopoiéticas. Células-tronco mesenquimais. Biodistribuição de células-tronco. Vias de administração. Camundongo neonatos.

ABSTRACT

Stem cells are widely studied aiming new forms of therapies for various diseases due to their ability to self renew and differentiate into several other cell types. The most characterized stem cells are hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), both of which are mainly found in the bone marrow. However, there are not studies analysing the biodistribution of these cells in newborn mice, nor testing different routes of administration in which the transplants would be more effective, and such data are important for developing new therapeutic approaches, mainly for diseases with rapid progression and where early intervention is relevant. The aim of this study was to evaluate the biodistribution of HSCs and MSCs in two-days-old newborn mice in organs such as liver, lung, spleen, heart, kidney, cerebral cortex and bone marrow. Cells were injected by three different administration routes (venous retro-orbital sinus, temporal vein and intraperitoneal route) and engraftment was evaluated at different times (48 hours and 30 days post-injection). C57BL6-GFP mice were used as cell donors and 129SV as recipients, in order to detect the transplanted cells. Cells from donor's bone marrow purified and counted in a Neubauer camera to perform the transplants. The organs from transplanted animals were analyzed by immunohistochemistry using anti-GFP antibody. We did not find MSC at any organs analysed. The temporal vein (TV) was the most efficient route and the bisdistribution of HSC in 48 hours was more concentrated in the liver and spleen; and in 30 days in the bone marrow and spleen, in animals that suffered immunosuppression before transplant. Although there was statistical difference only in the bone marrow and spleen in 30 days and via the TV route, other organs also presented few GFP+ cells which may be sufficient to assist in the treatment of certain conditions.

Keywords: Hematopoietic stem cells. Mesenchymal stem cells. Biodistribution of stem cells. Routes of administration. Newborn mice.

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1 INTRODUÇÃO COMPREENSIVA

1.1 CÉLULAS-TRONCO

Células-tronco (CT) são células caracterizadas por sua capacidade de auto renovação e de se diferenciar em outros tipos celulares. Elas são amplamente utilizadas em estudos científicos como uma alternativa para a terapia celular de diversas doenças, seja na substituição de células já existentes no tecido ou lhes fornecendo suporte, produzindo compostos necessários para o tratamento de determinadas condições. A classificação das CT pode ser feita da seguinte forma: (a) células-tronco pluripotentes induzidas; (b) células-tronco embrionárias; e (c) células-tronco fetal e de adultos – ou somáticas – (BUZHOR et al., 2014) (figura 1).

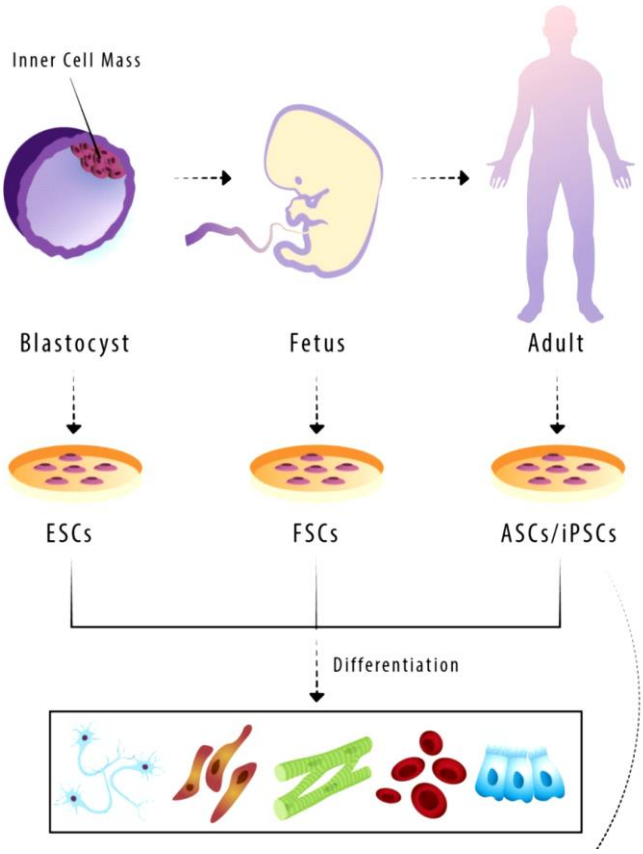

Figura 1: As diferentes origens das células-tronco (embrionárias, pluripotente induzidas ou fetal e de adultos). Adaptado de (BUZHOR et al., 2014).

As células-tronco pluripotentes induzidas, mais conhecidas pela sigla iPS (do inglês *induced pluripotent stem cell*), são células somáticas reprogramadas em laboratório para tornarem-se células pluripotentes (capazes de se transformarem em todas as células dos folhetos

germinativos, como ectoderma, mesoderma e endoderma; com exceção apenas da placenta e do tecido extra-embrionário, sendo essas últimas incluídas na categoria de células totipotentes, ou seja, além dos três folhetos germinativos). As iPS foram descritas pela primeira vez em 2006, no Japão, pelo pesquisador Yamanaka, o qual utilizou quatro fatores de transcrição – Oct3/4, Sox2, c-Myc e Klf4 – para reprogramar células de fibroblastos, fatores esses que hoje são mundialmente conhecidos como "fatores de Yamanaka" (TAKAHASHI et al., 2007). Apesar dessas células serem muito semelhantes às células-tronco embrionárias, há algumas diferenças no nível de metilação do DNA e na expressão de determinados genes endógenos, mostrando um potencial cancerígeno e teratogênico no uso dessas células (TAKAHASHI et al., 2007). O uso dessas células tem sido focado como uma ferramenta para estudar o epigenoma de células cancerígenas (SEMI; YAMADA, 2015) e também para produzir células cancerígenas induzidas, para melhor estuda-las (OSHIMA et al., 2014).

As células-tronco embrionárias, também pluripotentes, são derivadas da massa celular interna de embriões na fase de blastocisto, 4 a 5 dias após a fecundação (THOMSON et al., 2009). Devido as questões éticas envolvidas no uso dessas células, seu uso terapêutico se resume basicamente a estudos em animais. Um exemplo de seu potencial científico, através do desenvolvimento de um protocolo padrão (HEUER et al., 1993), consiste em testes de embriotoxicidade, onde se utiliza células-tronco embrionárias murinas para testar o potencial tóxico de diversos compostos, como: a embriotoxicidade de ervas comumente usadas por mulheres grávidas na China (LI et al., 2015), a toxicidade de Bisfenol A – componente de plásticos o qual é lixiviado para a comida – e de genisteína – fitoestrógeno derivado da soja e outras leguminosas – (KONG et al., 2013) e também na triagem de embriotoxicidade de nanopartículas, visto que essa é uma técnica em crescente estudo em engenharias biomédicas (CAMPAGNOLO et al., 2013).

Por último, encontram-se as células-tronco somáticas, provenientes de tecido fetal ou de adultos de diversas partes do corpo, como: medula óssea, tecido adiposo, sangue periférico, sangue de cordão umbilical, placenta, polpa dentária e dos respectivos órgãos do corpo humano – para que assim a manutenção e renovação de todos os órgãos ocorra quando necessário durante a vida de um indivíduo – (EHNINGER; TRUMPP, 2011). Diferente dos outros dois tipos de CTs citadas anteriormente, as somáticas são consideradas multipotentes, pois são células ligeiramente mais diferenciadas que as demais, capazes de originarem apenas células de um mesmo folheto germinativo. Mas, apesar dessa pequena limitação quanto ao seu poder de diferenciação e auto renovação, essas células apresentam vantagens por possuírem facilidades quanto às questões éticas de aquisição, manutenção do cultivo em laboratórios e,

principalmente, às suas perspectivas terapêuticas (como o uso das células-tronco dos próprios órgãos e tecidos para as diversas patologias que acometem cada órgão, tornando a pesquisa mais direta para tratamentos mais específicos).

Logo, vem crescendo o número de estudos que procuram entender melhor as CTs provenientes de cada órgão, principalmente as células-tronco neurais (GALLI et al., 2003) e células-tronco cardíacas (JOHNSON; SINGLA, 2017), por serem de órgãos de suma importância e cujo os índices de mortes por enfermidades que acometem esses órgãos serem altos em toda a população. Porém, existem dois tipos de CTs somáticas que são estudadas por mais tempo, as células-tronco hematopoiéticas (CTH) e células-tronco mesenquimais (CTM). Apesar de já se ter muito conhecimento sobre essas células, muitas pesquisas ainda devem ser conduzidas para investigar seu potencial de plasticidade (OGAWA; LARUE; MEHROTRA, 2015; WANG et al., 2014), uma vez que elas podem ser aplicadas em terapias de doenças de vários órgãos ou sistemas e são, então, uma possível alternativa mais abrangente que as CT órgão-específicas. Deste modo, essas células foram escolhidas para a realização deste trabalho e, portanto, uma maior abordagem sobre elas será desenvolvida abaixo.

1.1.1 Células-tronco hematopoiéticas

As células-tronco hematopoiéticas (CTH) são células responsáveis pela constante produção de componentes sanguíneos e imunes, podendo ser eles de origem mieloide (eritrócitos, plaquetas, eosinófilos, monócitos, basófilos e neutrófilos) ou de origem linfoide (linfócitos T e B); sendo esse processo denominado hematopoese (figura 2), o qual ocorre majoritariamente na medula óssea. Porém, as CTH também podem ser encontradas no sangue de cordão umbilical e em sangue periférico, embora que em pouca quantidade (COPELAN, 2006).

O estudo sobre essas células se iniciou durante a segunda guerra mundial, principalmente devido aos ataques com bombas nucleares, onde se tinha grande emissão de radiação à população, a qual começou a desenvolver diversos sintomas e complicações que os levavam a morte por insuficiência medular (COPELAN, 2006). Na época, na tentativa de remediar a situação, utilizava-se sangue placentário armazenado para transfusão, porém a melhora era quase que insignificante. Somente anos mais tarde que se descobriu que o motivo dessa baixa eficiência era devido à falta de histocompatibilidade (LORENZ, E; UPHOFF, D; REID, TR; SHELTON, R; SHELTON, 1951).

Após a segunda guerra é que essas células foram melhor estudadas. Estudos com animais intencionalmente expostos à radiação mostraram que a proteção prévia dos órgãos hematopoiéticos com chumbo – como baço ou algum osso longo – evitava o óbito do animal, pois parte do tecido responsável pela atividade hematopoiética se mantinha íntegro; já os animais não protegidos com chumbo desenvolviam sintomas similares aos humanos durante a segunda guerra. Anos mais tarde, foi observado que, além da radiação, alguns fármacos também tinham esse poder destrutivo em relação às CTH (HO; PUNZEL, 2003).

Então, no início da década de 60, os genes do complexo HLA (*Human leucocyte antigen*) foram estudados, tornando possível o transplante alogênico de medula óssea, onde as células transplantadas não precisam ser geneticamente idênticas às células do receptor (COPELAN, 2006). Outro fato importante foi a imunofenotipagem dessas células, para que assim se conhecesse quais moléculas e anticorpos eram expressos nas CTH para, então, separa-las das demais, enriquecendo o número de CTH para um tratamento mais eficaz, sendo o principal marcador a molécula CD34 (CHEVALLIER et al., 2013). A partir disso, as CTH começaram a ser fortemente utilizadas como terapia celular de diversas doenças como: linfomas, leucemias, imunodeficiências congênitas, anemias e erros inatos do metabolismo (COPELAN, 2006).

Logo, faz mais de 50 anos que as CTH são usadas na prática clínica, o que aumentou significativamente a expectativa de vida dos pacientes. Porém, complicações em decorrência dos transplantes também aumentaram, principalmente pelo fato de os pacientes precisarem passar por processos de ablação de sua medula óssea para melhor receber as células transplantadas. Além desse condicionamento, o paciente deve fazer uso de tratamento com imunossupressores durante toda a vida, o que previne rejeição contra o transplante mas que pode implicar no desenvolvimento da Doença do Enxerto Contra Hospedeiro (DECH) ou outras complicações sistêmicas (DEAN et al., 2018; HOSPITAL, 2004).

Outra característica que vem sendo bastante estudada é a possível aplicação dessa terapia em outras doenças não hematológicas, cujos estudos mostram um potencial dessas células de se diferenciarem em outros tipos celulares, como células endoteliais, hepatócitos e fibroblastos (PILAT; UNGER; BERLAKOVICH, 2013).

Assim sendo, é importante continuar estudando cada vez mais essas células, principalmente pela hipótese delas servirem de tratamentos para outras doenças as quais ainda não possuem uma terapia eficaz e, também, para fazer com que o transplante de CTH, para as doenças nas quais essa terapia já é aplicada, se torne cada vez mais segura com consequente diminuição dos efeitos adversos.

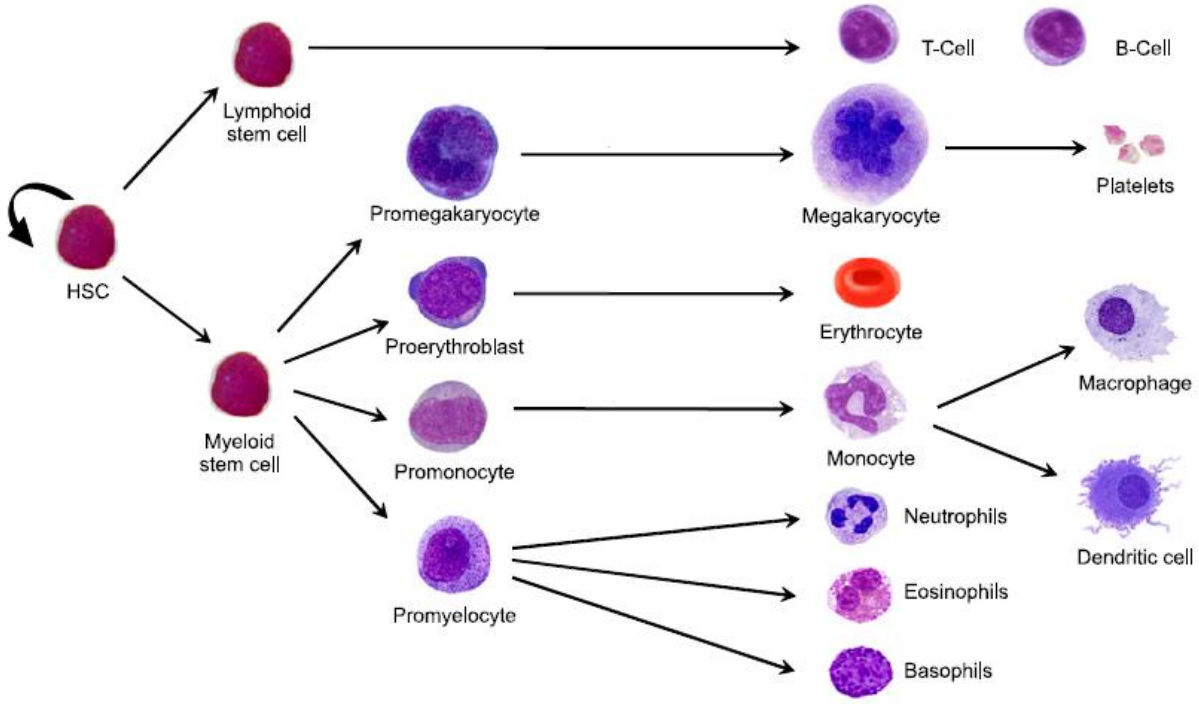

Figura 2: Representação da hematopoese a partir das células-tronco hematopoiéticas com seus respectivos progenitores e células diferenciadas. Adaptado de (SARVOTHAMAN et al., 2015).

1.1.2 Células-tronco mesenquimais

Assim como as CTH, as células-tronco mesenquimais (CTM) também podem ser encontradas na medula óssea, porém podem também ser provenientes de outros tecidos, como músculo, tecido adiposo e derme (CAPLAN, 2007; CAPLAN; PH, 2005; GARCÍA-CASTRO et al., 2008). Têm por principais características a aderência ao plástico, a capacidade de diferenciar-se em linhagens osteogênica, condrogênica e adipogênica e expressar marcadores de superfície como CD105, CD73 e CD90 (DOMINICI et al., 2006). As CTM foram observadas pela primeira vez em 1970, onde os cientistas acreditavam ser fibroblastos, principalmente por apresentarem aderência à placa de cultura e possuirem uma forma fusiforme (FRIEDENSTEIN; CHAILAKHJAN; LALYKIN, 1970). Só em 2006 que se convencionou que, para ser uma CTM, ela deve apresentar as três características citadas anteriormente.

Ainda não se sabe muito bem o papel dessas células. No início, acreditava-se que era somente na diferenciação e manutenção das respectivas células as quais elas podem dar origem (como osteócito, condrócito e adipócito – figura 3), mas vem crescendo o número de pesquisas onde se investiga o papel das CTM como coadjuvantes da imunomodulação do microambiente

a qual se encontram, exercendo um efeito parácrino. Já foi observado que as CTMs são capazes de secretar moléculas antiapoptóticas, neoangiogênicas e pró-mitóticas (MELIEF et al., 2013; NAGAYA, 2004; ORTIZ et al., 2003); e também que as CTM favorecem a liberação de substâncias anti-inflamatórias como IL-4 e IL-10 e inibem citocinas pró-inflamatórias como IFN-γ e TNF-α (MARIGO; DAZZI, 2011; WATERMAN et al., 2010).

Apesar dos tratamentos utilizando as CTM, como uma alternativa à terapia celular convencional, ainda não terem sido aprovados pela FDA *(Food and Drug Administration)* ou outros órgãos de igual importância, estudos têm sido conduzidos para aplicação dessas células em doenças como infarto agudo do miocárdio, doenças renais, doenças pulmonares, diabetes e também para a Doença Enxerto Contra Hospedeiro (DECH); onde se associa a terapia celular das CTH com as CTM para uma melhor resposta ao transplante, diminuindo os efeitos adversos (LE BLANC et al., 2008). Logo, nota-se o quanto é promissor continuar estudando as CTM, tanto isoladas quanto associadas às CTH.

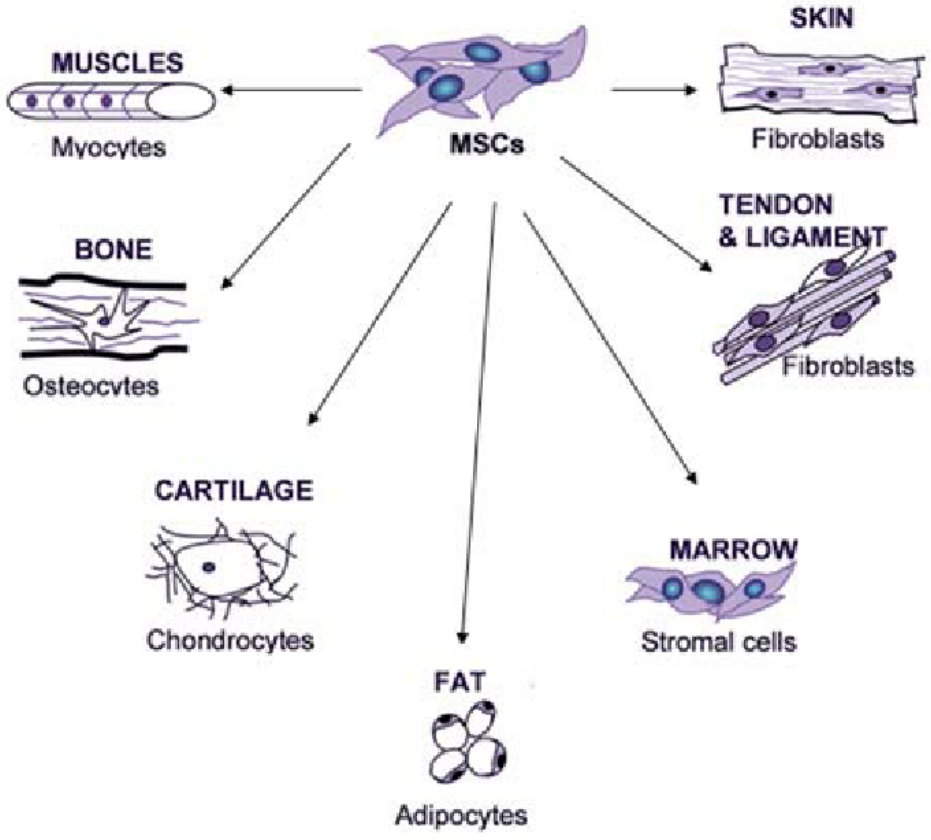

Figura 3: Potencial de diferenciação das células-tronco mesenquimais. (DAS; SUNDELL; KOKA, 2013).

1.2 TERAPIA CELULAR ASSOCIADA À TERAPIA GÊNICA

A terapia gênica consiste na aplicação de diferentes métodos para tentar corrigir e reparar genes de células que não estejam cumprindo sua função devidamente, que, dependendo do gene em questão, pode resultar no desenvolvimento de diversas doenças. Essa manipulação de genes pode ser *in vivo*¸ quando vetores são administrados diretamente no paciente; ou *ex vivo*, quando se faz uso de células retiradas do próprio paciente (normalmente células-tronco), para a então manipulação gênica *in vitro* e posterior reimplantação dessas células de volta ao organismo – sendo essa uma associação da terapia celular com a terapia gênica (COTRIM; BAUM, 2008).

No início da década de 60, questionou-se sobre a possibilidade dos vírus serem utilizados na terapia gênica, devido a sua capacidade natural de carregar e introduzir seu material genético infectante nas células (FRIEDMANN, 1997). Depois de estudar essa possibilidade, foram desenvolvidos vetores virais sem fatores de virulência, mas que mantinham sua capacidade de transduzir células eucarióticas (MACHIDA, 2002). Eles são os vetores mais eficientes na questão de entrega do material genético às células, porém podem gerar eventos adversos relacionados ao sistema imune.

Os vetores não virais, como, por exemplo, os plasmídeos (VOSS, 2007) e as nanoestruturas (FENSKE; CHONN; CULLIS, 2008), surgiram como alternativas mais seguras para a entrega de ácidos nucleicos às células. Além da utilização de sequências que contanham o cDNA do gene de interesse junto a um promotor para expressão, pode-se lançar mão de técnicas de edição gênica, que utilizam nucleases guiadas por domínios proteicos – como *Zinc Finger Nucleases* (ZFN) e *Transcription Activator-Like Effector Nucleases* (TALEN) – ou por sequências de RNA – como *Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein* (CRISPR/Cas) (figura 4) (MAEDER; GERSBACH, 2016). Logo, torna-se interessante relacionar a terapia celular das células-tronco (tanto hematopoiéticas quanto mesenquimais) com a terapia gênica para desenvolver tratamentos para as mais diversas doenças de forma mais específica.

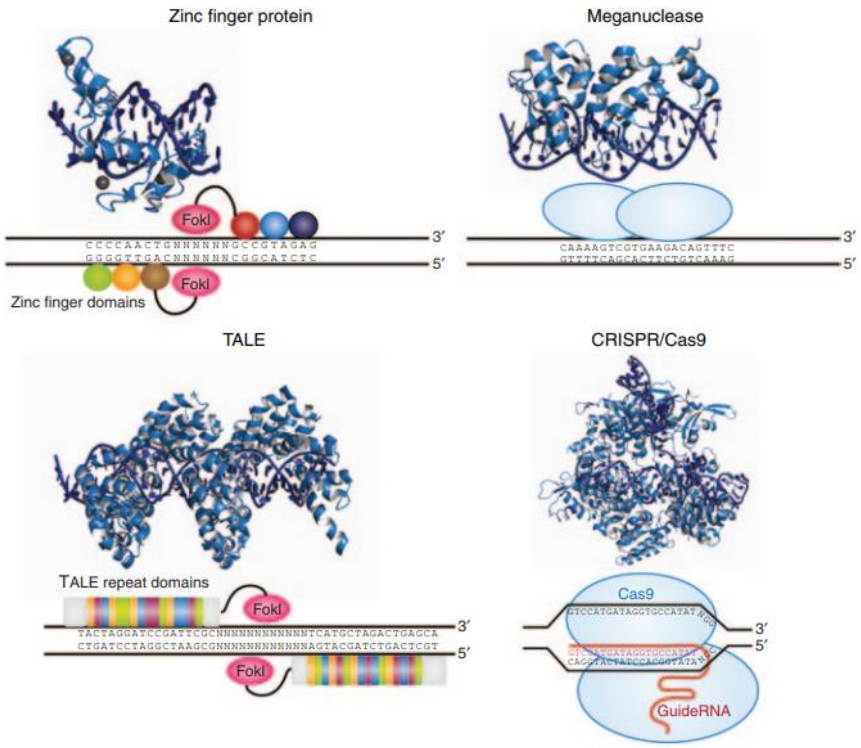

Figura 4: Técnicas utilizadas para proporcionar a manipulação gênica. (MAEDER; GERSBACH, 2016).

1.3 MODELO ANIMAL E VIAS DE ADMINISTRAÇÃO

O desenvolvimento de novas terapias é um processo longo, que requer numerosos passos para a comprovação de segurança e eficácia do tratamento. Inicialmente, estudos que demonstrem o mecanismo de ação e a toxicidade de novos tratamentos costumam ser realizados em cultivo celular. Se aprovado nos primeiros testes, o tratamento é testado em animais de pequeno porte, para avaliação da distribuição e da interação entre os sistemas do organismo. Muitas vezes, tratamentos promissores em cultivo de células são contestados nos testes com animais, por serem metabolizados e degradados, por não chegarem ao tecido alvo ou por gerarem muitos efeitos adversos, por exemplo. Só após a comprovação da eficiência e da segurança em animais é que o tratamento é conduzido para ensaios clínicos (NORMAN, 2016). Dessa forma, embora haja uma mobilização para redução da experimentação animal, o uso de modelos animais ainda se faz muito necessário.

Em camundongos, existem vários estudos que procuram entender melhor como funciona o transplante de células-tronco, quais mecanismos estão envolvidos, visando o aperfeiçoamento do procedimento na prática clínica. Dentre alguns mecanismos encontrados

na literatura, podemos citar os seguintes trabalhos: o uso do fármaco FTY720, um imunomodulador que depleta os linfócitos T naive e com isso diminui a incidência da DECH (LAKSHMIKANTH et al., 2016) e; o bloqueio de Notch2 – uma molécula de sinalização importante para as CT – que aumenta a mobilização de CTH e suas progenitoras e proporciona o homing dessas células (LAKSHMIKANTH et al., 2016).

Além desses estudos – e de vários outros – que procuram por formas de aprimorar os transplantes de CT com base no uso ou no bloqueio de moléculas ou fármacos (tanto para direcionar essas células, purificar melhor um tipo celular, quanto para diminuir o ataque pelo sistema imune), existe outro ponto importante de se avaliar que é a administração em si dessas células. A escolha da via de administração é um passo significativo e deve mimetizar o procedimento feito em humanos, tanto para a adminsitração de células quanto de outros compostos, pois dessa forma se pode favorecer determinados sistemas, ainda mais considerando que as CT podem se diferenciar em diferentes tipos celulares.

Em camundongos adultos, há estudos comparando diferentes vias de administração (KUSHIDA et al., 2001; LEON-RICO et al., 2015). A via intravenosa é normalmente a de escolha e possui dois acessos principais: a veia caudal lateral e o seio venoso retro-orbital. Ambos os acessos conferem distribuição semelhante para injeção de células (LEON-RICO et al., 2015), de anticorpos (SCHOCH et al., 2014) e de fármacos (STEEL et al., 2008), embora alguns autores sugiram que o seio venoso retro-orbital é o mais acessível e o que requer menor treinamento, além de causar menos estresse ao animal. Outra via intravenosa bastante utilizada é a da veia temporal, cuja utilização só é possível em camundongos neonatos devido a sua localização superficial (FLORES et al., 2010; PIEVANI et al., 2015). Além das vias intravenosas citadas acima, existe ainda outra via que é bastante utilizada para a administração de vários compostos: a via intraperitoneal. Essa, por sua vez, mostrou-se mais viável em comparação à via intravenosa em alguns trabalhos (GUICHARD; LOCHON; PHARMACOLOGIE, 1998; YOUSE et al., 2013).

Em camundongos neonatos, de até dois dias de vida, não há trabalhos que comparem as diferentes vias de administração. Sendo esse um protocolo importante de ser estabelecido, principalmente quando se pretende estudar formas de terapias para patologias que necessitam de tratamento precoce, devido à rápida progressão de sintomas.

2 JUSTIFICATIVA

Justamente por haver esta falta de informação sobre qual a melhor via de administração em neonatos – ou seja, a que comporta maior volume administrado, que distribui mais uniformemente os compostos injetados e que representa menor desconforto aos animais – é importante que se realize uma análise comparativa entre as rotas mais utilizadas e, assim, que se crie e se otimize um protocolo de injeção. Dessa maneira, podemos estabelecer um protocolo definitivo de transplante de CTH e CTM em camundongos neonatos, visando à utilização desse modelo em conjunto com protocolos de terapia gênica *ex vivo* para o tratamento de doenças hereditárias, principalmente as de progressão rápida que requerem intervenção precoce.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Estabelecer um protocolo de injeção de células-tronco hematopoiéticas e mesenquimais em camundongos neonatos.

3.2 OBJETIVOS ESPECÍFICOS

a) Injetar células-tronco hematopoiéticas e mesenquimais pelas três principais vias de administração (seio venoso retro-orbital, veia temporal e intraperitoneal) em camundongos neonatos de até dois dias de vida;

b) Observar qual via apresenta maior facilidade de injeção pelo manipulador;

c) Avaliar a distribuição das células transplantadas em diversos tecidos, como medula óssea, fígado, baço, rim, pulmão, coração e córtex cerebral.

4 ARTIGO CIENTÍFICO

Periódico: Laboratory Animals.

Título: Comparison between routes of administration of stem cells in newborn mice.

Normas da revista: Disponível em: https://us.sagepub.com/en-us/sam/journal/laboratory-animals#submission-guidelines e no anexo A.

COMPARISON BETWEEN ROUTES OF ADMINISTRATION OF STEM CELLS IN NEWBORN MICE

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ABSTRACT

Stem cells are widely studied aiming new forms of therapies for various diseases due to their ability to self renew and differentiate into several other cell types. The most characterized stem cells are hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), both of which are mainly found in the bone marrow. However, there are not studies analysing the biodistribution of these cells in newborn mice, nor testing different routes of administration in which the transplants would be more effective, and such data are important for developing new therapeutic approaches, mainly for diseases with rapid progression and where early intervention is relevant. The aim of this study was to evaluate the biodistribution of HSCs and MSCs in two-days-old newborn mice in organs such as liver, lung, spleen, heart, kidney, cerebral cortex and bone marrow. Cells were injected by three different administration routes (venous retro-orbital sinus, temporal vein and intraperitoneal route) and engraftment was evaluated at different times (48 hours and 30 days post-injection). C57BL6-GFP mice were used as cell donors and 129SV as recipients, in order to detect the transplanted cells. Cells from donor's bone marrow purified and counted in a Neubauer camera to perform the transplants. The organs from transplanted animals were analyzed by immunohistochemistry using anti-GFP antibody. We did not find MSC at any organs analysed. The temporal vein (TV) was the most efficient route and the bisdistribution of HSC in 48 hours was more concentrated in the liver and spleen; and in 30 days in the bone marrow and spleen, in animals that suffered immunosuppression before transplant. Although there was statistical difference only in the bone marrow and spleen in 30 days and via the TV route, other organs also presented few GFP+ cells which may be sufficient to assist in the treatment of certain conditions.

Keywords: Hematopoietic stem cells. Mesenchymal stem cells. Biodistribution of stem cells. Routes of administration. Newborn mice.

INTRODUCTION

Hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are cells that are self-renewing and have the ability to differentiate into several cell types. Both can be found in the bone marrow, but the HSCs can be also found in peripheral blood and umbilical cord blood and the MSCs in muscle, adipose tissue and dermis, among other tissues (1–4). These cells can be used as cell therapy for a variety of diseases such as lymphomas, leukemias, congenital immunodeficiencies, anemias, and inborn errors of metabolism (1), to name a few.

In an animal model, there are many ways to improve the efficacy of hematopoietic stem cell transplantation (HSCT), from the targeting of these cells to some specific organ, enrichment of cellular niches and in the attempt to reduce the attack by the immune system. However, there is a significant step which is not very studied, related to the route of administration of these cells and their biodistribution. Considering that these cells can differentiate into many cell types, it is interesting to know if any route of administration favors any organ or system, which organ is more receptive to HSCs and MSCs and whether this is pathway dependent.

Regarding adult mice, there are studies comparing different routes of administration (5,6). The intravenous route is usually the one of choice and has two main accesses: the lateral caudal vein and the retro-orbital venous sinus. Both approaches provide a similar distribution for injection of cells (6), antibodies (7) and drugs (8), although they suggest that the retro-orbital venous sinus is the most accessible and requires less training, in addition to causing less stress to the animal (6,7,8). Another commonly used intravenous access is the temporal vein, where it is only possible to usein newborn because of its superficial location (9,10). In addition to the intravenous routes mentioned, there is another route that is also widely used for the administration of various compounds: the intraperitoneal route. There are studies comparing these routes (intraperitoneal and intravenous) and, in these studies, the intraperitoneal route was a more viable option (11,12).

However, in the case of newborn mice up to two days old, there are no studies comparing the different routes of administration. This is an important protocol to be established, especially when one intends to study therapies for pathologies in which early treatment is necessary.

Therefore, the objective of the present study was to inject HSCs and MSCs by the three main administration routes (retro-orbital venous sinus, temporal vein and intraperitoneal) in 2

days old mice and to evaluate the biodistribution of transplanted cells to several tissues, such as bone marrow, liver, spleen, kidney, lung, heart and cerebral cortex.

MATERIALS AND METHODS

Animals

This research project was approved by the Ethics Committee for the Use of Animals (CEUA) of the Hospital de Clínicas of Porto Alegre under number 16-0260. C57BL6-GFP and 129SV mice, as donors and recipients, respectively, were used. After weaning at 21 days of age, animals from the same litter were kept in plastic boxes with a maximum of 5 animals/box in a controlled environment (temperature 20-24ºC, 40-60% relative humidity and air exhaust systems) with cycles of 12 hours of light and 12 hours of dark, standard commercial feed for the species and water ad libitum. Euthanasia was performed using isoflurane inhalation anesthetic overdose.

Treatment

Three large groups were created: one group of animals was injected with MSCs in the concentration of $1x10^5$ cells/50uL without myeloablation; a second group received HSCs at same regimen and dose. In the third group, animals were injected with HSCs at a concentration of $1x10^6$ cells/50uL with a myeloablative regimen (busulfan 20mg/kg administered intraperitoneally 24 hour before transplant). These groups were divided into the three administration routes tested: retro-orbital venous sinus (RO), temporal vein (TV) and intraperitoneal (IP); and then each route was subdivided at different euthanasia times: 48 hours and 30 days after cell injections (figure 1). The negative control group was composed of adult 129SV animals without any intervention, while the positive control group was composed of adult GFP+ animals. Three to five animals were used for each group analyzed.

Obtaining HSCs and MSCs

Cells from donor animals were obtained from bone marrow of the femur and tibia of adult GFP mice by flush extraction with saline solution. For HSCs obtention, total bone marrow was processed with the Lineage Cell Depletion Kit (Miltenyi Biotec) – which removes the differentiated cells and enriches the HSCs population through different antibodies expressed in these cells – according to manufacturer's protocol. For MSCs obtention, homogenized whole

bone marrow was cultured in DMEM medium, supplemented with 20% FBS and 1% penicillin/streptomycin. The culture medium was changed every 72 hours for the removal of cells in suspension, to enrich the MSCs population. Cells were expanded and transplanted between passages 3 and 6. After obtention of cells, they were counted in Neubauer's camera at the previously mentioned concentrations in a final volume of 50 uL. The cell suspension was aspirated into BD Ultrafine II 0.5mL 6mm needle syringes and injected slowly into manually immobilized animals by experienced veterinarians.

Immunohistochemistry

To evaluate the cellular biodistribution, the immunohistochemistry technique was performed using anti-GFP antibody, to detect cells from the donor. The organs were processed in paraffin blocks, cut in thin sections and placed in an oven for 1 hour at 75ºC. After dewaxing, antigen retrieval was performed by incubation with 10mM citrate buffer pH 6 for 35 minutes at 94 °C. Slides were incubated with primary anti-GFP antibody (1:600 dilution; rabbit polyclonal IgG from Santa Cruz Biotchnology), overnight at 4 °C in a dark camera. Finally, slides were incubated with peroxidase-conjugated goat anti-rabbit IgG (1: 200; Santa Cruz Biotchnology) secondary antibody for 90 minutes at room temperature on a dark camera and then developed through the DAB Kit (Dako) through chromogen 3-3'-diaminobenzidine (DAB). After immunohistochemistry, slides were analyzed by the ImageJ software, through the Color Deconvolution plugin, resulting in the expression of the results in percentage of area occupied by GFP+ cells in the field, analyzing 3 fields of each organ.

Statistical analysis

The results obtained were categorized, and the following comparisons were made: Difference of migration to the same organ, at the same time, by different routes; and difference of migration to the same organ at different times. The data were analyzed by two-way ANOVA, comparing differences between the groups with Tukey test as post hoc when the values had normal distribution. The level of significance was 5%.

RESULTS

Injection of HSCs and MSCs without myeloablation

We performed injections of HSCs or MSCs at the concentration of $1x10^5$ cells/50uL without performing myeloablation, and the results of this first step are shown in table 1.

Under these conditions, we did not detect any GFP+ cell (from the donor) in most of the organs, indicating that the transplantation was not efficient. As we can also see in table 1 and figure 2, in some organs we could observe GFP+ cells but they were very few, without statistical difference from untreated mice. In this way, it was not possible to make any correlation between the administration routes with the times in which we analyzed, nor even a tendency of one of the cellular types to be migrating for any organ.

Injection of HSCs with myeloablation

After the initial results, we decided to increase the concentration of cells injected to $1x10^6$ cells/50uL and to perform myeloablation prior to the tranplant, through the immunosuppressant drug Busulfan, which destroys the bone marrow cells of the recipient animal to increase the possibility of homing of the injected cells. We started with HSCs and we will continue this work with the MSCs in a future study.

In 48 hours, there was no statistical difference between groups (figure 3-A), but a considerably larger number of GFP+ cells were found compared to the group of animals that were injected with HSCs without myeloablation (1/3 compared to immunosuppressed animals). Another result observed was that the IP pathway was the only route in which no GFP+ cells were found in any organ. Among the analyzed organs, the heart was the only organ that did not present positive cells in any of the routes; and in the cerebral cortex only few positive cells were visualized by RO route, although the result was almost equal to the negative control (RO = 0.092, and negative = 0.054 %GFP+/area). However, the organs that stood out the most in 48 hours were the spleen and the liver; and for the spleen the best route was TV (equivalent to 44% of the positive control) and for the liver the RO pathway (65% in relation to the positive control). These results have no statistical difference but show a trend of biodistribution of HSCs to these organs analyzed in a short time.

In 30 days, no positive cells were found in the cerebral cortex and very little in the heart (less than 1% in relation to the positive control). The organs that had the most cells in 30 days (long time) were the spleen and the bone marrow, which obtained statistical difference in relation to the negative control through the TV pathway. Although the RO and IP pathways had no statistical difference for the spleen and bone marrow, their results were not very different from the TV pathway. In the case of the bone marrow of the transplanted animals, the number

of GFP+ cells were higher than in the positive control. The other organs did not show statistical difference, but it is important to note that: for the liver, the three routes and positive control were very similar between them (although the RO route was slightly better), showing that even if it had few GFP+ cells in the liver, the same result was achieved regardeless of the administration route; for the lung, the RO route was highlighted from the others, presenting results equivalent to 60% of the positive control; for the kidney, the result was more discrete, but GFP+ cells were found in the three routes, the TV being slightly better (14% of the positive control) (Figures 3-B and 4).

Another data observed was the ease of manipulation of the animals for each route of injection analyzed, which were performed by experienced veterinarians. According to them, the IP route was the easiest, followed by the RO route and later the TV route. Although the IP route was the easiest, in the first injections it was seen that there was reflux of the administered volume, which was solved after standardizing the needle gauge and application plane. The RO route was the second easier to apply and not a single lesion to the eyes were observed, though it was doubtful if the whole volume had been administered correctly. The TV route was the one that demanded more training and which the veterinarians had the most difficulty performing, as the vessel's caliber is almost as thick as the needle it self. However, the success of administration via TV was easily assessed, once immediate subcutaneous edema is formed when the solution is injected outside the vessel, leaving no room for doubt.

DISCUSSION

Some studies discuss wheter to perform myeloablative conditioning or not before hematopoietic stem cell transplantation (HSCT), both using drugs and irradiation. Conditioning is usually done because it is believed that this frees up space so that cells from the donor can establish themselves and proliferate. For Zhong et al. (2002) (14) ablated or non-ablated animals harbor the donor cells in the same way, but non-ablated animals do not proliferate these cells as much as the ablated animals, because they do not have the stimulation from the drug/irradiation that destroys the recipient cells and then stimulates the proliferation of donor cells. However, in our study, we saw that not only the proliferation of these cells was compromised, but also the egraftment of these cells in the bone marrow, inferring that the myeloablative conditioning made a difference so that the graft remained in the donor.

Analyzing the data from groups transplanted without myeloablation (table 1), although without statistical difference, the HSCs showed a tendency to be more bio-distributable among

the organs than the MSCs; for this reason, we conducted another set of experiments to evaluate the biodistribution of HSC by different routes using myeloblation. We did a pilot group with myeloablative conditioning and same number of HSCs ($1x10^5$ cells/50uL), but the distributions was also unsatisfactory, therefore we increased the dose of cells to $1x10^6$/50uL, which finally generated more pronounced results.

Regarding the biodistribution of these cells, for the spleen and the bone marrow, we already expected them to present positive results, since both are hematopoietic organs (1). It has been shown that HSCs are not normally found in the spleen (15), but under certain conditions, such as myeloablation, this organ begins to perform extramedullary hematopoiesis (16). In the case of the liver, it has been shown that HSCs are able to originate hepatocytes (17) but the author of the study mentioned that in cases of HSC this is a very rare event. This corroborates our findings, because although the liver has presented a moderate number of GFP+ cells (much alike the positive control), these cells do not have hepatocyte's morphological characteristics but rather of cells of the immune system, such as Kupffer' cells. In fact, these findings apply to the other organs, where the GFP+ cells found do not correspond to the cell types of each organ, but to cells of the immune system. We expected to find GFP+ cells in the cerebral cortex, as the blood brain barrier (BBB) is not yet completely closed in newborn mice (18), but this was not observed in our study.

Regarding the analyzed times, we saw that in 48 hours the organs that stood out most were the liver and the spleen (although without statistical basis). We believe that in the short term these cells have not had enough time to reach the bone marrow, hitting the liver and spleen first by the very anatomy of the site of the injections, reaching those organs more easily through the bloodstream. But also, and especially, because these two organs are considered hematopoietic organs. The spleen we have already seen in the previous paragraph and the liver is described as the main hematopoietic organ during the fetal period, ceasing to be after birth. However in cases of marrow damage, the liver can also perform extramedullary hematopoiesis in newborn mice (19). In 30 days, we have seen - with statistical difference - enough cells in the bone marrow, indicating that the homing of injected HSCs occured and derived cells (lymphoid and myeloid) were possibly produced and migrated to the other organs.

Considering the routes of administration, TV route was the one that needed more training by the veterinarians, but it was the one that obtained the best results. We believe that the best performance of this route was because it was the way we were most sure of the success of the injections, where it was seen that all the volume administered entered the vessel and did not form edema (which was an indication that part of the volume injected had come out of the

vase). Therefore, investing in the improvement of technique is important and should be taken into account.

In conclusion, the TV pathway was the route that best biodistributed the HSCs in newborn mice, and in 30 days there was a statistical difference for the bone marrow and spleen, although GFP+ cells were also observed in the other organs such as liver, kidney and lung; only in the heart and in the cerebral cortex that this finding was extremely low or null.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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REFERENCES

1. Copelan EA. Hematopoietic Stem-Cell Transplantation. N Engl J Med [Internet]. 2006;354(17):1813–26. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMra052638

2. Caplan AI, Ph D. Mesenchymal Stem Cells : Cell-Based Reconstructive Therapy. 2005;11(7).

3. Caplan AI. Adult Mesenchymal Stem Cells for Tissue Engineering Versus Regenerative Medicine. 2007;(June):341–7.

4. García-castro J, Trigueros C, Madrenas J, Pérez-simón JA, Ag L. Mesenchymal stem cells and their use as cell replacement therapy and disease modelling tool. 2008;12(6):2552–65.

5. Kushida T, Inaba M, Hisha H, Ichioka N, Esumi T, Ogawa R, et al. Intra-bone marrow injection of allogeneic bone marrow cells: A powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood. 2001;97(10):3292–9.

6. Leon-Rico D, Fernández-García M, Aldea M, Sánchez R, Peces-Barba M, Martinez-Palacio J, et al. Comparison of haematopoietic stem cell engraftment through the retro-orbital venous sinus and the lateral vein: alternative routes for bone marrow transplantation in mice. Lab Anim. 2015;49(2):132–41.

7. Schoch A, Thorey IS, Engert J, Winter G, Emrich T. Comparison of the lateral tail vein and the retro-orbital venous sinus routes of antibody administration in pharmacokinetic studies. Lab Anim (NY) [Internet]. 2014;43(3):95–9. Available from: http://dx.doi.org/10.1038/laban.481

8. Steel CD, Stephens AL, Hahto SM, Singletary SJ, Ciavarra RP. Comparison of the lateral tail vein and the retro-orbital venous sinus as routes of intravenous drug delivery in a transgenic mouse model. Lab Anim (NY). 2008;37(1):26–32.

9. Flores C, De Vries TJ, Moscatelli I, Askmyr M, Schoenmaker T, Langenbach GEJ, et al. Nonablative neonatal bone marrow transplantation rapidly reverses severe murine osteopetrosis despite low-level engraftment and lack of selective expansion of the osteoclastic lineage. J Bone Miner Res. 2010;25(9):2069–77.

10. Pievani A, Azario I, Antolini L, Shimada T, Patel P, Remoli C, et al. Neonatal bone marrow transplantation prevents bone pathology in a mouse model of mucopolysaccharidosis type I. Blood. 2015;125(10):1662–71.

11. Guichard S, Lochon I, Pharmacologie L De. Comparison of the pharmacokinetics and ef ® cacy of irinotecan after administration by the intravenous versus intraperitoneal route in mice. 1998;165–70.

12. Youse F, Ebtekar M, Soleimani M, Soudi S, Mahmoud S. International Immunopharmacology Comparison of in vivo immunomodulatory effects of intravenous and intraperitoneal administration of adipose-tissue mesenchymal stem cells in experimental autoimmune encephalomyelitis (EAE). 2013;17:608–16.

13. Zhong JF, Zhan Y, French Anderson W, Zhao Y. Murine hematopoietic stem cell distribution and proliferation in ablated and nonablated bone marrow transplantation. Blood. 2002;100(10):3521–6.

14. Morita Y, Iseki A, Okamura S, Suzuki S, Nakauchi H, Ema H. Functional

characterization of hematopoietic stem cells in the spleen. Exp Hematol [Internet]. 2011;39(3):351–359.e3. Available from: http://dx.doi.org/10.1016/j.exphem.2010.12.008

15. Morrison SEANJM, Wright DOEW. Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. 1997;94(March):1908–13.

16. Pilat N, Unger L, Berlakovich GA. Implication for bone marrow derived stem cells in hepatocyte regeneration after orthotopic liver transplantation. Int J Hepatol [Internet]. 2013;2013(Figure 1):310612. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24109514%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3784276

17. Ek CJ, D'angelo B, Baburamani AA, Lehner C, Leverin AL, Smith PLP, et al. Brain barrier properties and cerebral blood flow in neonatal mice exposed to cerebral hypoxia-ischemia. J Cereb Blood Flow Metab. 2015;35(5):818–27.

18. Wolber FM, Leonard E, Michael S, Orschell-Traycoff CM, Yoder MC, Srour EF. Roles of spleen and liver in development of the murine hematopoietic system. Exp Hematol. 2002;30(9):1010–9.

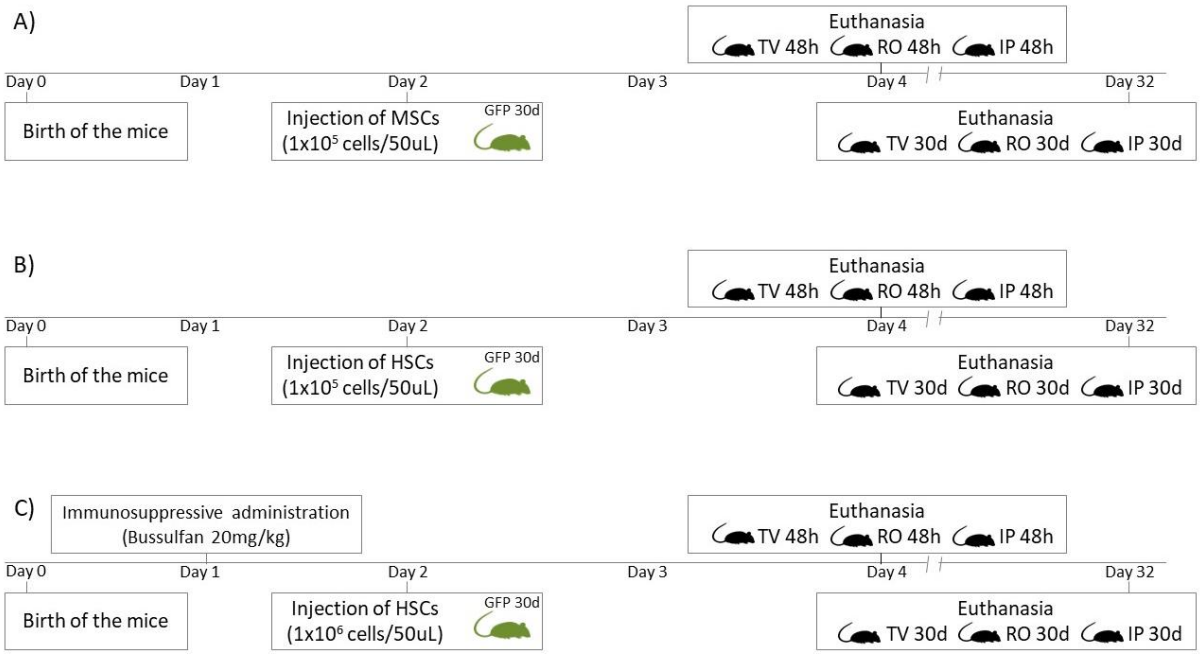

Figure 1: Representation of the methodology used. A) Two-day-old animals were injected with MSCs at a concentration of $1x10^5$ cells/50ul and then divided into groups with administration routes (TV, RO and IP) and at different times (48 hours and 30 days). B) Same conditions as in "A" but with HSCs. C) Only HSCs were injected with an additional stage of myeloablation with the drug busulfan – 24 hours before the injections of the cells – and with an increase in the number of cells injected to $1x10^6$ cells/50ul. Key: TV- Temporal vein; RO- Retro orbital; IP-Intraperitoneal. HSCs- Hematopoietic stem cells; MSCs- Mesenchymal stem cells.

Table 1: Results of injections of HSCs and MSCs without myeloablation. Results shown as "% GFP+ area / field" *.

		Cerebral Cortex	Kidney	Spleen	Liver	Lung	Heart	Bone Marrow
CONTROLS	+	0.69 ± 0.24	7.68 ± 1.90	7.64 ± 0.23	0.99 ± 0.13	5.18 ± 1.06	23.9 ± 4.53	7.76 ± 9.45
	-	0.054 ± 0.071	0.071 ± 0.035	0.76 ± 0.69	0.01 ± 0.01	1.01 ± 0.89	0.04 ± 0.03	0.65 ± 0.24
WT + MSCs	TV 48h	-	-	-	-	1.43 ± 0.43	-	1.27 ± 1.08
	TV 30d	-	-	1.582 ± 1.43	-	-	-	-
	RO 48h	0.078 ± 0.042	-	-	-	-	-	-
	RO 30d	-	0.34 ± 0.47	-	-	-	0.05 ± 0.03	-
	IP 48h	-	-	-	-	-	-	-
	IP 30d	-	-	-	-	-	0.20 ± 0.29	-
WT + HSCs	TV 48h	0.049 ± 0.01	-	-	0.014 ± 0.015	-	-	0.47 ± 0.19
	TV 30d	-	-	-	0.02 ± 0.02	-	-	-
	RO 48h	0.28 ± 0.40		0.85 ± 0.16	0.08 ± 0.12	-	-	-
	RO 30d	0.022 ± 0.04	-	-	-	1.40 ± 0.68	0.064 ± 0.028	-
	IP 48h	-	-	1.03 ± 0.47	-	-	-	-
	IP 30d	-	-	-	-	1.12 ± 0.21	-	-

- : No GFP+ cells found. * mean ± standard deviation

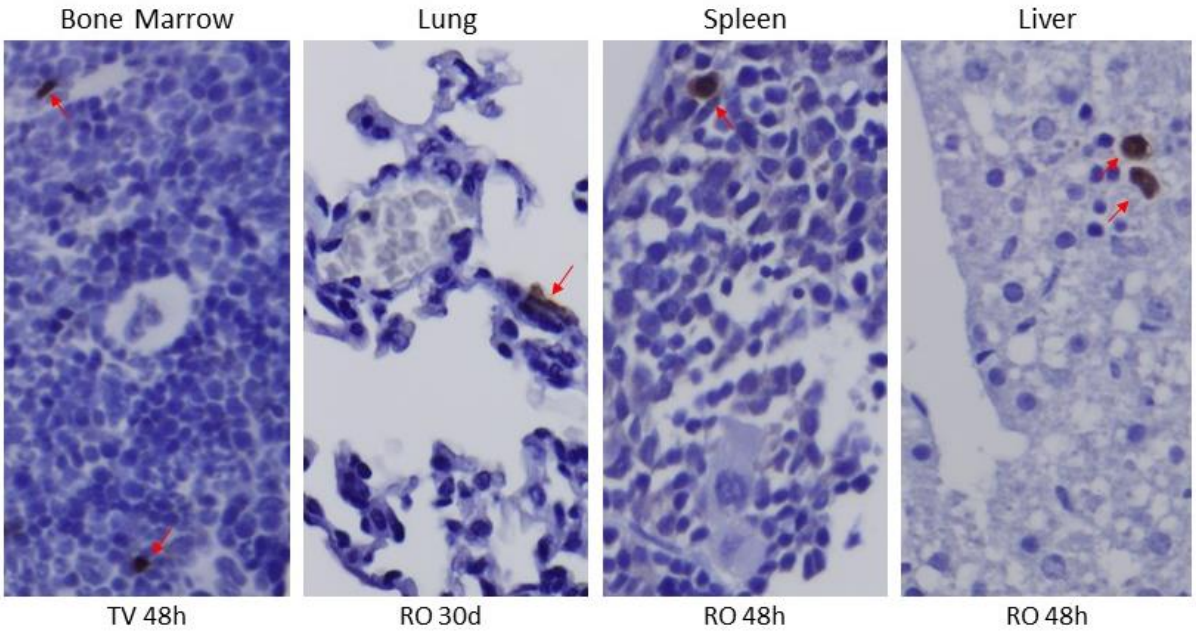

Figure 2: Immunohistochemistry images of transplanted animals without myeloablation. These images represent injections with HSCs ($1x10^5$ cells/50uL). We can observe that very few GFP+ cells were found (red arrows). Magnification 400x.

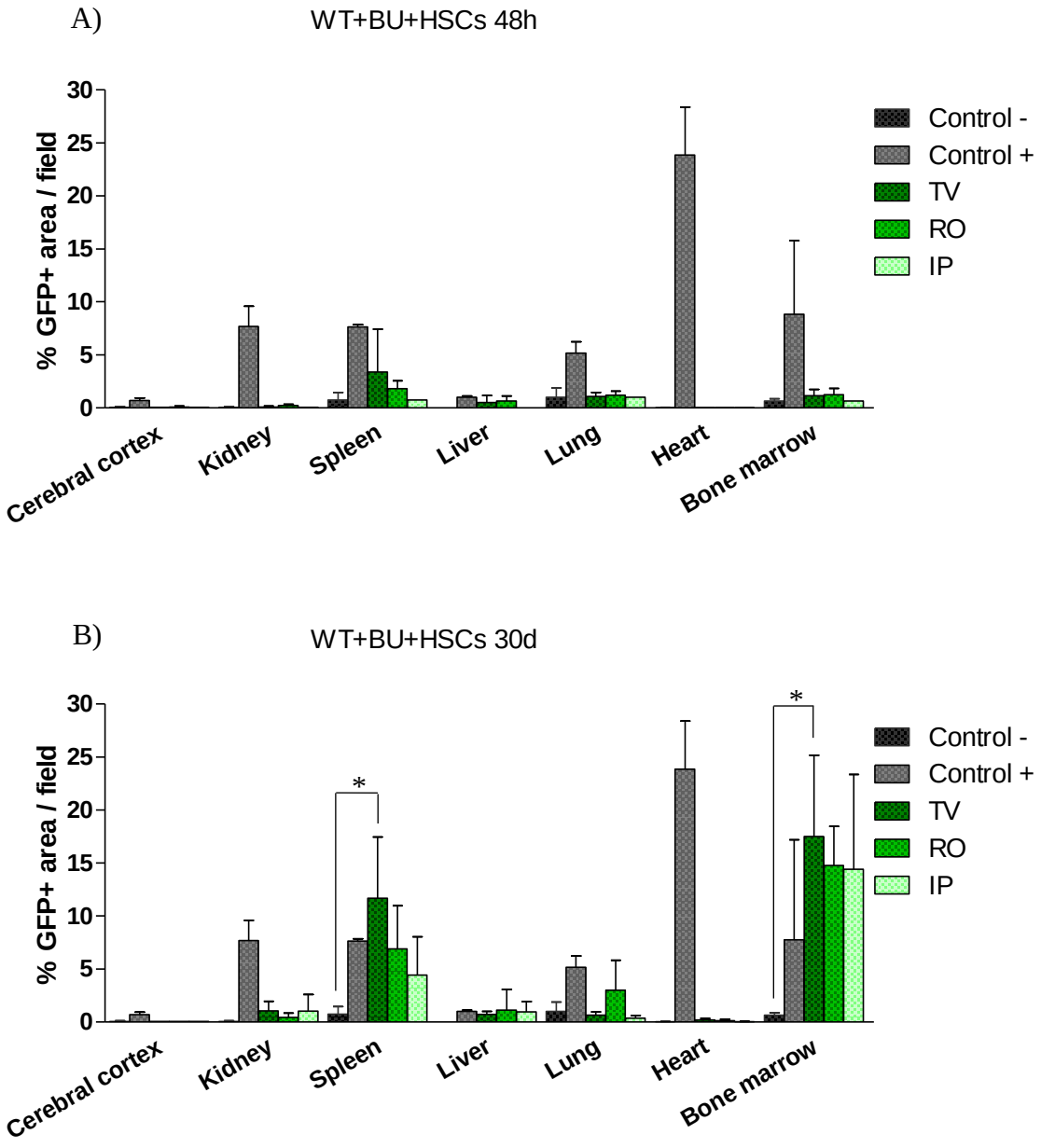

Figure 3: Biodistribution of HSCs (1x10^6 cells/50uL) in organs of transplanted mice, after immunosuppression with busulfan. A) Observed data in 48 hours; and B) Data observed in 30 days. TV- Temporal vein; RO- Retro orbital; IP- Intraperitoneal. BU- Busulfan. * p <0.05.

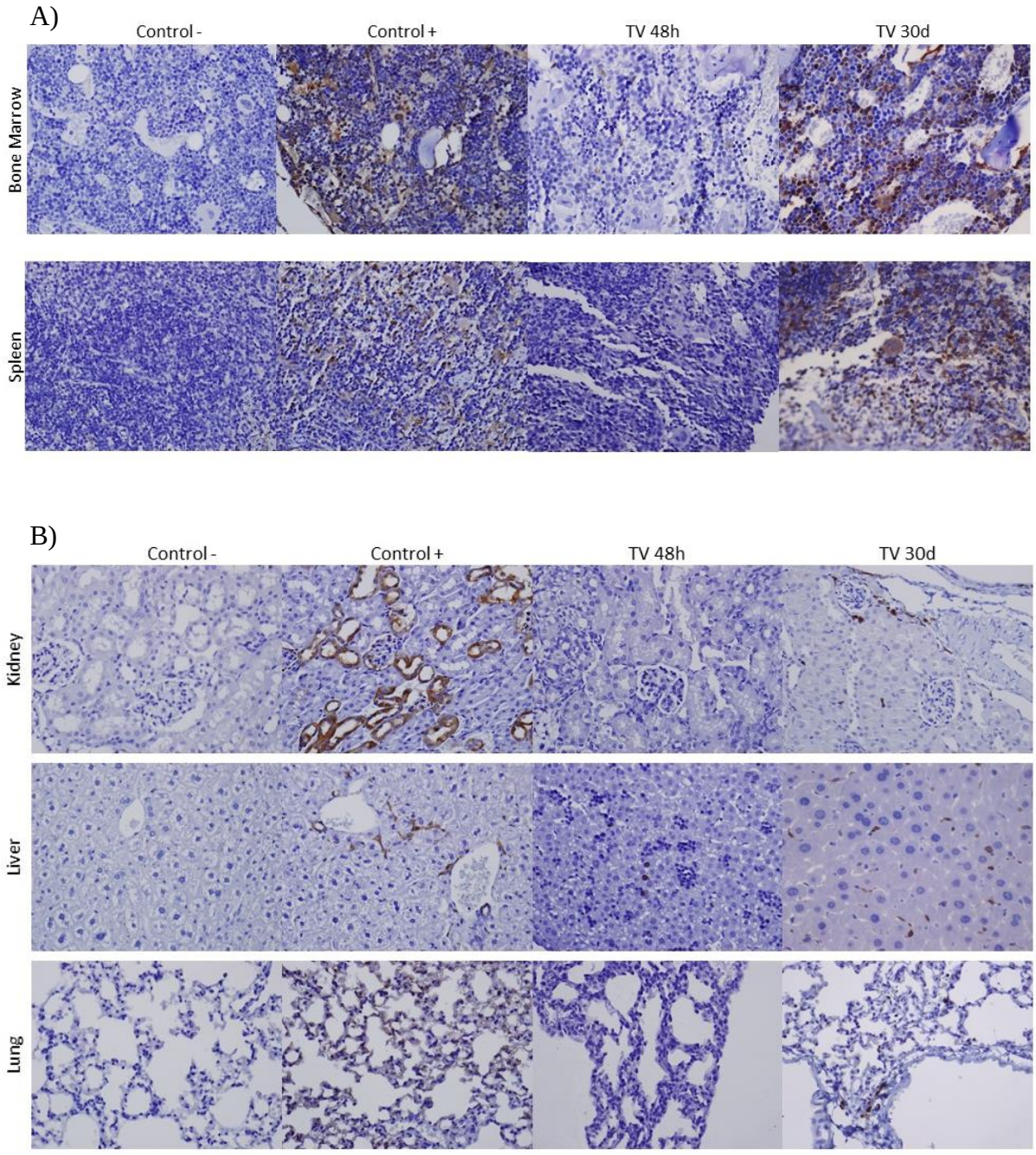

Figure 4: Immunohistochemistry images of animals transplanted with myeloablation and HSCs (1x10^6 cells/50uL). A) The organs that obtained statistical difference of p <0.05, bone marrow and spleen. B) The other organs, although not giving statistical difference, presented some GFP+ cells. Magnification 400x.

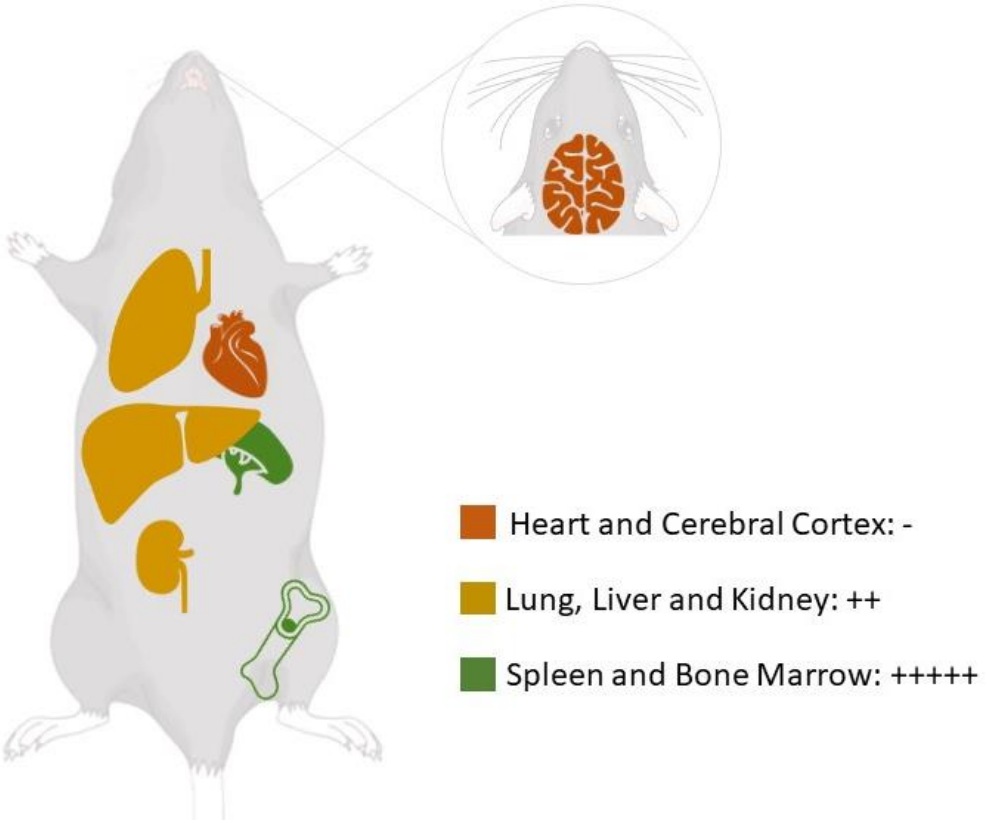

Figure 5: Summary of the results found in relation to the biodistribution of HSCs in newborn mice. Where much of these cells were observed in the spleen and bone marrow; a moderate amount in the lung, liver and kidney; and very little or no GFP+ cells in the cerebral cortex and heart.

5 CONCLUSÕES E PERSPECTIVAS

Como principais conclusões deste trabalho, temos que: a) a via de administração que melhor biodistribuiu as células tronco hematopoiéticas para os órgãos analisados – fígado, baço, medula óssea, pulmão, rim, coração e córtex cerebral – foi a via da veia temporal, por mais que seja uma das vias que mais requer treinamento, foi observado que dessa forma se garante que as injeções sejam todas corretas e padronizadas, pois era possível analisar se parte do volume injetado havia extravasado para fora do vaso; e b) em relação à biodistribuição das CTH, em 48 horas os órgãos onde se detectou uma maior quantidade de células GFP+ provenientes do transplante, foram o fígado e o baço; já em 30 dias foi a medula óssea e o baço.

Por mais que só tenha sido observado diferença estatística na medula óssea e no baço na via TV em 30 dias, é importante ressaltar que talvez o pouco de células GFP+ encontradas nos demais órgãos já seja o suficiente para auxiliar no tratamente de determinadas patologias.

Para o estudo das perspectivas, é importante considerar as limitações do trabalho. Sob esta ótica, é essencial ressaltar que não foram encontradas CTM nos tecidos listados. No entanto os dados de CTM e CTH não são diretamente comparáveis, pois foi realizado um teste adicional com as CTH, com dose maior e mielossupressão. Experimentos subsequentes serão realizados para verificar a distribuição das CTM nas mesmas condições. Outro ponto a ser considerado é a caracterização completa das CTM, que será realizada através de imunofenotipagem e testes de diferenciação.

Este trabalho faz parte de um projeto maior, no qual objetiva desenvolver um tratamento de terapia gênica *ex vivo* para mucopolissacaridose tipos I e II – utilizando o modelo animal da doença e mimetizando um transplante autólogo de células tronco geneticamente modificadas.

Logo, outras perspectivas deste trabalho são: prosseguir os experimentos com a edição gênica *in vitro* das células tronco e seu transplante em animais com mucopolissacaridose tipo I e tipo II para avaliação da eficácia do tratamento.

REFERÊNCIAS

BUZHOR, E. et al. Cell-based therapy approaches: The hope for incurable diseases. **Regenerative Medicine**, [s. l.], v. 9, n. 5, p. 649–672, 2014.

CAPLAN, A. I. Adult Mesenchymal Stem Cells for Tissue Engineering Versus Regenerative Medicine. [s. l.], n. June, p. 341–347, 2007.

CAPLAN, A. I.; PH, D. Mesenchymal Stem Cells : Cell-Based Reconstructive Therapy. [s. l.], v. 11, n. 7, 2005.

CHEVALLIER, P. et al. Characterization of various blood and graft sources: A prospective series. **Transfusion**, [s. l.], v. 53, n. 9, p. 2020–2026, 2013.

COPELAN, E. A. Hematopoietic {Stem}-{Cell} {Transplantation}. **The New England Journal of Medicine**, [s. l.], p. 14, 2006.

COTRIM, A. P.; BAUM, B. J. Gene Therapy: Some History, Applications, Problems, and Prospects. **Toxicologic Pathology**, [s. l.], v. 36, n. 1, p. 97–103, 2008.

DAS, M.; SUNDELL, I. B.; KOKA, P. S. Adult mesenchymal stem cells and their potency in the cell-based therapy. **J Stem Cells**, [s. l.], v. 8, n. 1, p. 1–16, 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24459809>

DEAN, C. et al. for. [s. l.], v. 68, n. 5, p. 1129–1135, 2018.

DOMINICI, M. et al. Minimal criteria for defining multipotent mesenchymal stromal cells . The International Society for Cellular Therapy position statement. **Cytotherapy**, [s. l.], v. 8, n. 4, p. 315–317, 2006. Disponível em: <http://dx.doi.org/10.1080/14653240600855905>

EHNINGER, A.; TRUMPP, A. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. **The Journal of Experimental Medicine**, [s. l.], v. 208, n. 3, p. 421–428, 2011. Disponível em: <http://www.jem.org/lookup/doi/10.1084/jem.20110132>

FENSKE, D. B.; CHONN, A.; CULLIS, P. R. Liposomal Nanomedicines: An Emerging Field. **Toxicologic Pathology**, [s. l.], v. 36, n. 1, p. 21–29, 2008.

FLORES, C. et al. Nonablative neonatal bone marrow transplantation rapidly reverses severe murine osteopetrosis despite low-level engraftment and lack of selective expansion of the osteoclastic lineage. **Journal of Bone and Mineral Research**, [s. l.], v. 25, n. 9, p. 2069–2077, 2010.

FRIEDENSTEIN, A. J.; CHAILAKHJAN, R. K.; LALYKIN, K. S. The development of fibroblast colonies in marrow and spleen cells. **Cell Tissue Kinet.**, [s. l.], v. 3, p. 393–403, 1970.

FRIEDMANN, T. The road toward human gene therapy - A 25-year perspective. **Annals of Medicine**, [s. l.], v. 29, n. 6, p. 575–577, 1997.

GALLI, R. et al. Neural Stem Cells. [s. l.], p. 598–608, 2003.

GARCÍA-CASTRO, J. et al. Mesenchymal stem cells and their use as cell replacement therapy and disease modelling tool. [s. l.], v. 12, n. 6, p. 2552–2565, 2008.

GUICHARD, S.; LOCHON, I.; PHARMACOLOGIE, L. De. Comparison of the pharmacokinetics and ef ® cacy of irinotecan after administration by the intravenous versus intraperitoneal route in mice. [s. l.], p. 165–170, 1998.

HEUER, J. et al. Development of an in vitro embryotoxicity test using murine embryonic stem cell cultures. **Toxicology in Vitro**, [s. l.], v. 7, n. 4, p. 551–556, 1993.

HO, A. D.; PUNZEL, M. Hematopoietic stem cells : can old cells learn new tricks ? **Journal of Leukocyte Biology**, [s. l.], v. 73, n. 5, p. 547–555, 2003.

HOSPITAL, M. G. Follow-up 26 Years after Treatment for Acute Myelogenous Leukemia. **New England Journal of Medicine**, [s. l.], p. 2456–2457, 2004.

JOHNSON, T. A.; SINGLA, D. K. Chapter 20 Therapeutic Application of Adult Stem Cells in the Heart. [s. l.], v. 1553, 2017.

KONG, D. et al. Individual and combined developmental toxicity assessment of bisphenol A and genistein using the embryonic stem cell test in vitro. **Food and Chemical Toxicology**, [s. l.], v. 60, p. 497–505, 2013. Disponível em: <http://dx.doi.org/10.1016/j.fct.2013.08.006>

KUSHIDA, T. et al. Intra-bone marrow injection of allogeneic bone marrow cells: A powerful

new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. **Blood**, [s. l.], v. 97, n. 10, p. 3292–3299, 2001.

LAKSHMIKANTH, T. et al. In vivo engineering of mobilized stem cell grafts with the immunomodulatory drug FTY720 for allogeneic transplantation. **European Journal of Immunology**, [s. l.], v. 46, n. 7, p. 1758–1769, 2016.

LE BLANC, K. et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. **The Lancet**, [s. l.], v. 371, n. 9624, p. 1579–1586, 2008.

LEON-RICO, D. et al. Comparison of haematopoietic stem cell engraftment through the retro-orbital venous sinus and the lateral vein: alternative routes for bone marrow transplantation in mice. **Laboratory Animals**, [s. l.], v. 49, n. 2, p. 132–141, 2015.

LI, L. I. N. Y. A. N. et al. Assessment of the embryotoxicity of four Chinese herbal extracts using the embryonic stem cell test. [s. l.], n. 12, p. 2348–2354, 2015.

LORENZ, E; UPHOFF, D; REID, TR; SHELTON, R; SHELTON, E. Modification of Irradiation in- Jury in Mice and Guinea Pigs By Bone Marrow Injections. [s. l.], n. July, p. 1–3, 1951.

MACHIDA, C. A. Viral vectors for gene therapy: methods and protocols. In: **Viral vectors for gene therapy: methods and protocols.** [s.l: s.n.]. p. 608.

MAEDER, M. L.; GERSBACH, C. A. Genome-editing Technologies for Gene and Cell Therapy. **Official journal of the American Society of Gene & Cell Therapy Single**, [s. l.], n. 1, 2016.

MARIGO, I.; DAZZI, F. **The immunomodulatory properties of mesenchymal stem cells**, 2011.

MELIEF, S. M. et al. C ELL -B ASED D RUG D EVELOPMENT , S CREENING , AND T OXICOLOGY Adipose Tissue-Derived Multipotent Stromal Cells Have a Higher Immunomodulatory Capacity Than Their Bone Marrow-Derived Counterparts. [s. l.], p. 455–463, 2013.

NAGAYA, N. Intravenous administration of mesenchymal stem cells improves cardiac

function in rats with acute myocardial infarction through angiogenesis and myogenesis. **AJP: Heart and Circulatory Physiology**, [s. l.], v. 287, n. 6, p. H2670–H2676, 2004. Disponível em: <http://ajpheart.physiology.org/cgi/doi/10.1152/ajpheart.01071.2003>

NORMAN, G. A. Van. Drugs, Devices, and the FDA: Part 1: An Overview of Approval Processes for Drugs. **JACC: Basic to Translational Science**, [s. l.], v. 1, n. 3, p. 170–179, 2016. Disponível em: <http://dx.doi.org/10.1016/j.jacbts.2016.03.002>

OGAWA, M.; LARUE, A. C.; MEHROTRA, M. Best Practice & Research Clinical Haematology Plasticity of hematopoietic stem cells. **Best Practice & Research Clinical Haematology**, [s. l.], v. 28, n. 2–3, p. 73–80, 2015. Disponível em: <http://dx.doi.org/10.1016/j.beha.2015.10.003>

ORTIZ, L. A. et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. **Proceedings of the National Academy of Sciences**, [s. l.], v. 100, n. 14, p. 8407–8411, 2003. Disponível em: <http://www.pnas.org/cgi/doi/10.1073/pnas.1432929100>

OSHIMA, N. et al. Induction of Cancer Stem Cell Properties in Colon Cancer Cells by Defined Factors. [s. l.], v. 9, n. 7, 2014.

PIEVANI, A. et al. Neonatal bone marrow transplantation prevents bone pathology in a mouse model of mucopolysaccharidosis type I. **Blood**, [s. l.], v. 125, n. 10, p. 1662–1671, 2015.

PILAT, N.; UNGER, L.; BERLAKOVICH, G. A. Implication for bone marrow derived stem cells in hepatocyte regeneration after orthotopic liver transplantation. **International journal of hepatology**, [s. l.], v. 2013, n. Figure 1, p. 310612, 2013. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/24109514%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3784276>

SARVOTHAMAN, S. et al. Apoptosis: role in myeloid cell development. **BLOOD RESEARCH**, [s. l.], v. 50, 2015.

SCHOCH, A. et al. Comparison of the lateral tail vein and the retro-orbital venous sinus routes of antibody administration in pharmacokinetic studies. **Lab Animal**, [s. l.], v. 43, n. 3, p. 95–99, 2014. Disponível em: <http://dx.doi.org/10.1038/laban.481>

SEMI, K.; YAMADA, Y. Induced pluripotent stem cell technology for dissecting the cancer epigenome. [s. l.], v. 106, n. 10, 2015.

STEEL, C. D. et al. Comparison of the lateral tail vein and the retro-orbital venous sinus as routes of intravenous drug delivery in a transgenic mouse model. **Lab Animal**, [s. l.], v. 37, n. 1, p. 26–32, 2008.

TAKAHASHI, K. et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. **Cell**, [s. l.], v. 131, n. 5, p. 861–872, 2007.

THOMSON, J. A. et al. Embryonic Stem Cell Lines Derived from Human Blastocysts. **Science**, [s. l.], v. 1145, n. 1998, p. 1145–1148, 2009.

VOSS, C. Production of plasmid DNA for pharmaceutical use. **Biotechnology Annual Review**, [s. l.], v. 13, n. 07, p. 201–222, 2007.

WANG, Y. et al. review Plasticity of mesenchymal stem cells in immunomodulation : pathological and therapeutic implications. [s. l.], v. 15, n. 11, p. 1009–1016, 2014.

WATERMAN, R. S. et al. A new mesenchymal stem cell (MSC) paradigm: Polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. **PLoS ONE**, [s. l.], v. 5, n. 4, 2010.

YOUSE, F. et al. International Immunopharmacology Comparison of in vivo immunomodulatory effects of intravenous and intraperitoneal administration of adipose-tissue mesenchymal stem cells in experimental autoimmune encephalomyelitis (EAE). [s. l.], v. 17, p. 608–616, 2013.

APÊNDICE A

APROVAÇÃO DO COMITÊ DE ÉTICA

HOSPITAL DE CLÍNICAS PORTO ALEGRE RS

UFRGS UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

GRUPO DE PESQUISA E PÓS GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificamos que o projeto abaixo, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) e pelas áreas de apoio indicadas pelo pesquisador.

Projeto: 160260 **Data de Aprovação do Projeto:** 12/07/2016

Título: Comparação entre vias de administração de células-tronco em camundongos neonatos **Data de Término:** 01/06/2018

Pesquisador Responsável: GUILHERME BALDO

Equipe de pesquisa:

CAMILA VIEIRA PINHEIRO EDINA POLETTO MARINA HENTSCHKE LOPES TALITA GIACOMET DE CARVALHO

Submissão	Documento	Espécie/Linhagem	Sexo/Idade	Qtd.	Data Reunião	Situação
05/07/2016	APROVAÇÃO	CAMUNDONGO - C57BL/6 GFP	-/2meses	48	28/06/2016	APROVADO
05/07/2016	APROVAÇÃO	CAMUNDONGO - 129SvEv Acidemia Glutárica	M/2meses	4	28/06/2016	APROVADO
05/07/2016	APROVAÇÃO	CAMUNDONGO - 129SvEv Acidemia Glutárica	F/2meses	4	28/06/2016	APROVADO
05/07/2016	APROVAÇÃO	CAMUNDONGO - 129SvEv Acidemia Glutárica	-/2dias	63	28/06/2016	APROVADO
19/02/2018	EMENDA	CAMUNDONGO - C57BL/6 GFP	-/2meses	10	23/01/2018	APROVADO
19/02/2018	EMENDA	CAMUNDONGO - 129SvEv Acidemia Glutárica	M/2meses	4	23/01/2018	APROVADO
19/02/2018	EMENDA	CAMUNDONGO - 129SvEv Acidemia Glutárica	F/2meses	4	23/01/2018	APROVADO
19/02/2018	EMENDA	CAMUNDONGO - 129SvEv Acidemia Glutárica	-/2dias	30	23/01/2018	APROVADO

Total de Animais: 167

MICHAEL EVERTON ANDRADES
Vice-Coordenador
Comissão de Ética no Uso de Animais

- Os membros da CEUA/HCPA não participaram do processo de avaliação onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

ANEXO A

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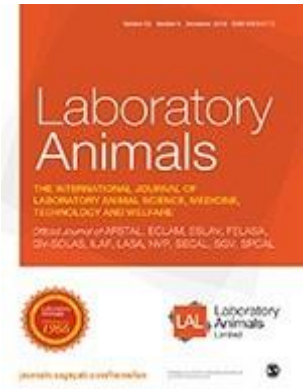

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Word count should not exceed 3,000 words, including abstract and references. Word count for the abstract should be 250 words. No more than 4 displays (tables and/or figures). Up to 30 references.

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2. Drafted the article or revised it critically for important intellectual content,
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The journal requires detailed information on the animals and their conditions of husbandry (see *Laboratory Animals* 1985;19:106–108). The methodology for the euthanasia of animals should be consistent with recommendations in previously published reports (see *Laboratory Animals* 1996;30:293–316 and 1997;31:1–32). The journal recommends referring to the American Veterinary Medical Association document on euthanasia also (https://www.avma.org/KB/Policies/Documents/euthanasia.pdf). The protocols and studies

involving fish should be reported in the manner detailed in *Laboratory Animals* 2000;34:131–135. Please read the Editorial entitled 'You and your research report: implementing the ARRIVE Guidleines' for further clarification.

Of particular note, the source and full strain nomenclature of any laboratory animal stock must be specified according to international recommendations. Authors should note this information is available from source laboratories and animal vendors. A brief statement describing the legislative controls on animal care and use should be provided. Measures to refine experimental techniques to benefit animal welfare can be described in detail and the disposition and fate of the animals at the end of the experiment should be clear. Products used (e.g. drugs, equipment, feed, bedding) should be described in the format “generic description (trade name, vendor name, city and country where vendor located)".
The experimental design and the statistical analysis should be detailed, particularly in relation to using only the appropriate numbers of animals (see Festing M et al. The Design of Animal Experiments: Reducing the use of animals in research through better experimental design 2nd Edition, available from SAGE). Pre-test power analyses should be presented in justification of sample size or number of animals required whenever possible. Power analyses for many common statistical procedures both parametric and non-parametric are given in Zar J. Biostatistical Analysis, 4th edn. When reporting variability about the mean, variances, and/or discussing significance or non-significance of statistically derived values, the Zar recommendations should be considered, and claims of statistical non-significance should be accompanied by post-test power analyses whenever possible.

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