UNIVERSIDADE ESTADUAL PAULISTA ‘’JULIO DE MESQUITA FILHO’’
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

POTENCIAL DE TRANSDIFERENCIAÇÃO *IN VITRO* DE CÉLULAS TRONCO MESENQUIMAIS EQUINAS EM CÉLULAS *SCHWANN-LIKE*

LUCAS VINÍCIUS DE OLIVEIRA FERREIRA

Botucatu – SP
2022

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Orientador: Prof.Dr. Rogério Martins Amorim

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Palavras-chave: Células *Schwann-like*; Fatores neurotróficos; Lesão de nervo periférico; Nervo facial; Regeneração nervosa.

Título: POTENCIAL DE TRANSDIFERENCIAÇÃO *IN VITRO* DE CÉLULAS TRONCO MESENQUIMAIS EQUINAS EM CÉLULAS *SCHWANN-LIKE*

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LISTA DE SIGLAS E ABREVIATURAS

ACTB – beta-actina, do inglês *beta-actin*
AR – ácido all-trans- retinóico
BDNF – fator neurotrófico derivado do cérebro, do inglês *brain-derived neurotrophic factor*
bFGF – fator de crescimento de fibroblasto básico
BME – beta-mercaptoetanol
B2M – beta -2- microglobulina, do inglês *beta -2- microglobulin*
cAMP - monofosfato cíclico de adenosina intracelular
CD – grupamento de diferenciação, do inglês *cluster of differentiation*
cDNA – DNA complementar, do inglês *complementary DNA*
CEUA - Comitê de Ética no Uso de Animais, do inglês *Ethics Committee in the Use of Animals*
CNTF – fator neurotrófico ciliar
CS- células de Schwann, do inglês *Schwann cells* (SCs)
CSL- células *Schwann-like*, do inglês *Schwann-like cells* (SLCs)
CTM – células tronco mesenquimais, do inglês *mesenchymal stem cells* (MSCs)
CTM – MO – células tronco mesenquimais derivadas da médula óssea, do inglês *mesenchymal stem cells derived from bone marrow* (BM-MSCs)
CTM – TA – células tronco mesenquimais derivadas do tecido adiposo, do inglês *mesenchymal stem cells derived from adipose tissue* (AT-MSCs)
CTM– CU – células tronco mesenquimais derivadas do cordão umbilical, do inglês *mesenchymal stem cells derived from umbilical cord* (UC-MSCs)
DMEM- *Dulbecco's Modified Eagle's Medium*
DPBS- *Dulbecco's Phosphate Buffered Saline*
FGF-2 – fator de crescimento dos fibroblastos -2
FSK – forskolina
GAPDH – gliceraldeído 3- fosfato desidrogenase, do inglês *glyceraldehyde 3-phosphate dehydrogenase*
GDNF – fator neurotrófico derivado da glia, do inglês *glial cell-derived neurotrophic factor*
GFAP – proteína ácida fibrilar da glia, do inglês *glial fibrillary acidic protein*
GGF2 – fator de crescimento glial -2
HGF – fator de crescimento dos hepatócitos
HPRT – hipoxantina-guanina fosforibosiltransferase, do inglês *hypoxanthine-guanine phosphoribosyltransferase*
HRG- heregulina
IDO – indoleamina 2,3-dioxigenase
IGF – fator de crescimento semelhante a insulina
IL- interleucina
LIF – fator inibidor de leucemia
MCP-1 – proteína quiomiotática de monócitos – 1
MHC-II – complexo de histocompatibilidade tipo II
NGF – fator de crescimento do nervo, do inglês *nerve growth factor*
NT3- neurotrofina 3, do inglês *neurotrophin-3*
NT4- neurotrofina 4
NT5- neurotrofina 5
ON – óxido nítrico
P3-P4- passagem das CTM após o isolamento (passagem 3-4)

$P75^{NTR}$- receptor de baixa afinidade p75
PBS - *phospate buffered saline*
PDGF- fator de crescimento derivado de plaquetas
PGE2 – prostaglandina E2
qPCR – reação em cadeia da polimerase em tempo real, do inglês *real-time polymerase chain reaction*
SNC- sistema nervoso central
SNP- sistema nervoso periférico, do inglês *peripheral nervous system* (PNS)
TGF-β – fator de crescimento transformador β
TNF-α – fator de necrose tumoral - α
Trks – receptores de alta afinidade tirosina quinase
VEGF-A – fator de crescimento endotelial vascular - A

SUMÁRIO

FERREIRA, L.V.O. **Potencial de transdiferenciação *in vitro* de células tronco mesenquimais equinas em células *schwann-like***. Botucatu, 2022. 90p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

RESUMO

As lesões do sistema nervo periférico (SNP) são condições clínicas comuns que podem desencadear déficits funcionais, afetando a qualidade de vida de humanos e equinos. As lesões do nervo facial são comumente observadas nestas espécies. Para o processo de regeneração, as células de Schwann (CS) são fundamentais, pois proliferam e produzem fatores neurotróficos. Contudo, a sua utilização como terapia celular é limitada devido à disponibilidade destas células e do tempo de cultivo. Neste contexto, busca-se desenvolver tecnologias ou métodos alternativos para a geração de CS. Diversos estudos demonstram a capacidade das células tronco mesenquimais (CTM) se transdiferenciarem em células de *Schwann-like* (CSL) utilizando a co-cultura com CS ou protocolos químicos. Entretanto, são métodos de difícil execução e com custos elevados. Uma abordagem mais prática para induzir a transdiferenciação *in vitro* das CTM foi descrita com sucesso utilizando meio de cultura condicionado com fatores neurotróficos secretados do nervo isquiático de ratos. O objetivo deste estudo foi avaliar o potencial de transdiferenciação *in vitro* de CTM equinas derivadas do tecido adiposo (TA), medula óssea (MO) e cordão umbilical (CU) em CSL. Neste estudo, o nervo facial de um equino adulto foi coletado após o procedimento de eutanásia. O nervo foi seccionado em fragmentos e incubado em meio de cultura completo por 48 horas. Este meio foi filtrado e utilizado como meio de indução para transdiferenciação das CTM em CSL. As CTM foram previamente caracterizadas imunofenotípicamente pela expressão dos marcadores CD44, CD90, CD105 e ausência da expressão dos marcadores CD34 e MHC-II; e pela capacidade de diferenciação em linhagem mesenquimal osteogênica, condrogênica e adipogênica. As CTM-TA, CTM-MO, CTM-CU equinas foram incubadas com o meio de indução por cinco dias. Após este período foi avaliado a morfologia, a expressão gênica dos marcadores glias S100β e GFAP (proteína ácida fibrilar da glia), do fator de crescimento do nervo (NGF), fator neurotrófico derivado do cérebro (BDNF), fator neurotrófico derivado da glia (GDNF) e realizado a imunofluorescência de S100 e GFAP. As CTM-TA e CTM-MO incubadas com o meio de indução exibiram morfologia bipolar e/ou tripolar com grandes núcleos, processos citoplasmáticos alongados, semelhantes à morfologia SC. Já nas CTM-CU não foram observadas alterações morfológicas. Houve aumento significativo na expressão gênica de BDNF, GDNF, GFAP e S100β nas CTM-TA, de GDNF, GFAP e S100β nas CTM-MO e de GDNF nas CTM-CU pós-diferenciação. Não verificamos a expressão gênica de GFAP em nenhuma das amostras, mas a expressão de S100β foi observada em todas as amostras após o processo de transdiferenciação das CTM-CU. A imunofluorescência revelou a expressão proteica de GFAP nas CTM-TA, CTM-MO e CTM-CU equinas indiferenciadas e diferenciadas. Entretanto, S100 foi expresso apenas em CTM-TA e CTM-MO equinas diferenciadas. Nossos resultados indicam a transdiferenciação das CTM-TA e CTM-MO equinas em CSL. Portanto, o protocolo de transdiferenciação de CTM-TA e CTM-MO equinas proposto neste estudo mostrou-se eficiente. Contudo, nossos dados não foram consistentes em relação as CTM-CU equinas e não verificamos a capacidade de transdiferenciação dessas células em CSL.

Palavras-chave: Células *Schwann-like*, fatores neurotróficos, lesão de nervo periférico, nervo facial, regeneração nervosa.

FERREIRA, L.V.O. ***In vitro*** **transdifferentiation potential of equine mesenchymal stem cells into schwann-like cells**. Botucatu, 2022. 90p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

ABSTRACT

Peripheral nerve system (PNS) injuries are common clinical conditions that can trigger functional deficits, affecting the quality of life of humans and horses. Facial nerve injuries are commonly seen in these species. For the regeneration process, Schwann cells (SCs) are essential, as they proliferate and produce neurotrophic factors. However, its use as cell therapy is limited due to the availability of these cells and the time of cultivation. In this context, we seek to develop alternative technologies or methods for the generation of SCs. Several studies demonstrate the ability of mesenchymal stem cells (MSCs) to transdifferentiate into Schwann-like cells (SLCs) using co-culture with SCs or chemical protocols. However, these are complicated and expensive methods. A more practical approach to induce MSCs transdifferentiation *in vitro* was successfully described using culture medium conditioned with neurotrophic factors secreted from the sciatic nerve of rats. The aim of this study was to evaluate the *in vitro* transdifferentiation potential of equine MSCs derived from adipose tissue (AT), bone marrow (BM) and umbilical cord (UC) into SLCs. In this study, the facial nerve of an adult horse was collected after the euthanasia procedure. The nerve was cut into fragments and incubated in complete culture medium for 48 hours. This medium was filtered and used as an induction medium to transdifferentiate MSCs into SLCs. MSCs were previously immunophenotypically characterized by the expression of markers CD44, CD90, CD105 and absence of expression of markers CD34 and MHC-II; and by the ability to differentiate into osteogenic, chondrogenic and adipogenic mesenchymal lineages. The equine AT-MSCs, BM-MSCs, UC-MSCs were incubated with the induction medium for five days. After this period, the morphology, gene expression of glial markers S100β and GFAP (glial fibrillary acidic protein), and nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic fator (GDNF) and immunofluorescence of S100 and GFAP was performed. AT-MSCs and BM-MSCs incubated with the induction medium exhibited bipolar and/or tripolar morphology with large nuclei, elongated cytoplasmic processes, similar to SCs morphology. In the UC-MSCs, no morphological changes were observed. There was a significant increase in the gene expression of BDNF, GDNF, GFAP and S100β in AT-MSCs, of GDNF, GFAP and S100 in BM-MSCs and of GDNF in UC-MSCs post-differentiation. We did not verify the gene expression in any of the GFAP samples, but the expression of S100β was observed in all samples after the UC-MSCs transdifferentiation process. Immunofluorescence revealed GFAP protein expression in undifferentiated and differentiated equine AT-MSCs, BM-MSCs and UC-MSCs. However, S100 was expressed only in differentiated equine AT-MSCs and BM-MSCs. Our results indicate the transdifferentiation of equine AT-MSCs and BM-MSCs into SLCs. Therefore, the equine TA-MSCs and BM-MSCs transdifferentiation protocol proposed in this study proved to be efficient. However, our data were not consistent in relation to equine UC-MSCs and we did not verify the ability of these cells to transdifferentiate into SLCs.

Keywords: Schwann-like cells, neurotrophic factors, peripheral nerve injury, facial nerve, nerve regeneration.

1 INTRODUÇÃO

A lesão do sistema nervoso periférico (SNP) é uma condição clínica comum em humanos e animais que representa um grande problema econômico para a sociedade (WOJTKIEWICZ et al., 2015; RHODE et al., 2021). As causas são múltiplas e distintas, mas são comumente atribuídas à eventos traumáticos (CAILLAUD et al., 2019; PIOVESANA et al., 2021). A disfunção motora, sensorial ou autonômica é a principal consequência associada à este tipo de lesão e geralmente desencadeia déficits funcionais, afetando a qualidade de vida do paciente (RICCIO et al., 2019; ZHANG et al., 2021).

Embora o SNP apresente um potencial regenerativo, atribuído predominantemente à plasticidade das células de Schwann (CS), a reparação espontânea é na maioria das vezes insatisfatória (XIE et al., 2017; PIOVESANA et al., 2021). Assim o desenvolvimento de novos tratamentos que auxiliem na regeneração de nervos periféricos lesionados apresenta grande relevância clínica (FARONI et al., 2015).

A terapia celular tem sido proposta como uma estratégia promissora para a regeneração de nervos periféricos (YU et al., 2021). As CS são responsáveis pela síntese e expressão de fatores neurotróficos, por orientar a regeneração nervosa e promover a reimielinização (LOPATINA et al., 2011). Estudos tem demonstrados que o transplante dessas células apresenta efeitos benéficos em lesões do SNP (LEVI et al., 2016; HAN et al., 2019), contudo, sua aplicação clínica apresenta limitações relacionadas à necessidade de sacrifício de um nervo funcional para realizar o cultivo e o tempo necessário para expandir as CS pode retardar o tratamento (LIU et al., 2020; PIOVESANA et al., 2021).

Devido à estas limitações, as células tronco mesenquimais (CTM) se tornaram o foco de investigações pela sua capacidade de se diferenciar em várias linhagens celulares (RAMLI et al., 2019). Diversos estudos demonstraram a capacidade das CTM provenientes de diferentes fontes e espécies em se transdiferenciarem *in vitro* em células *Schwann-like* (CSL), geralmente por meio da co-cultura com CS ou utilizando protocolos químicos (LI et al., 2009; WAKAO et al., 2010; WANG et al., 2011; VILLAGRÁN et al., 2016; FU et al., 2019; JUAN et al., 2020; HOPF et al., 2020). Além disso, foi observado que o transplante de CSL *in vivo* pode auxiliar na regeneração de nervos periféricos (GEORGIOU et al., 2015; HEI et al., 2016).

Uma abordagem mais prática foi descrita com sucesso utilizando meio de cultura condicionado com fatores neurotróficos secretados do nervo isquiático de ratos para induzir a transdiferenciação *in vitro* das CTM derivadas do tecido adiposo (CTM-TA) e

da medula óssea (CTM-MO) de ratos em CSL (LIU et al., 2013; TAWAB et al., 2018; GALHOM et al., 2018).

Na espécie equina, há um estudo descrevendo a capacidade das CTM-MO se transdiferenciarem em CSL, no qual CTM-MO equinas foram submetidas à condições específicas *in vitro* com uma combinação de produtos químicos para realizar a transdiferenciação (VILLAGRÁN et al., 2016).

As CTM são obtidas principalmente da medula óssea ou do tecido adiposo (MATHOT et al., 2020), contudo as CTM-TA apresentam uma série de vantagem como a facilidade de isolamento, grande disponibilidade e a alta taxa de proliferação *in vitro* (QOMI; SHEYKHHASAN, 2017; HOPF et al., 2020). Estudos recentes estão utilizando células de outras fontes como as derivadas do cordão umbilical (CTM-CU), pois podem ser obtidas de forma não invasiva, são abundantes e facilmente acessíveis (MERLO et al., 2018).

Em nosso conhecimento, não há estudos descrevendo a capacidade de transdiferenciação das CTM-TA e CTM-CU equinas em CSL *in vitro* e não há relatos sobre a elaboração de um meio de cultura condicionado com fatores secretados de nervo periférico de equinos e da sua utilização para realizar o processo de transdiferenciação. Sendo assim, o presente trabalho tem como objetivo avaliar o potencial de transdiferenciação *in vitro* de CTM-TA, CTM-MO e CTM-CU equinas em CSL com intuito de utilizá-las futuramente para auxiliar na reparação de nervos periféricos lesionados nessa espécie.

2 REVISÃO DE LITERATURA

2.1 Lesão do Sistema Nervoso Periférico

As lesões no SNP são relativamente frequentes e podem desencadear dor neuropática, perdas da função motora, sensorial ou autonômica nos segmentos corporais desnervados e consequentemente, perda na qualidade de vida em animais e humanos (CAMPBELL, 2008; NAVARRO, 2016; VILLAGRÁN et al., 2016; ALVITES et al., 2020; HUSSAIN et al., 2020; WILCOX et al., 2020; SAFFARI et al., 2021).

Em equinos, essas lesões ocorrem por processos traumáticos, doenças metabólicas, tóxicas, enfermidades infecciosas e degenerativas (SCHWARZ et al., 2008; VILLAGRÁN et al., 2014). Uma das neuropatias mais comum nesta espécie é a paralisia do nervo facial, associada à eventos traumáticos na maioria dos casos (BOORMAN et al., 2020). Esta é frequentemente caracterizada por assimetria da musculatura facial, ptose auricular, palpebral e labial em equídeos (BOORMAN et al., 2020).

Em humanos, estudos epidemiológicos estimam uma taxa de incidência de lesão do SNP entre 13 e 23 indivíduos por 100.000 habitantes por ano nos países desenvolvidos (LI et al., 2014). Sendo a paralisia do nervo facial um problema comumente observado em humanos, causando grande impacto na qualidade de vida (LUIJMES et al., 2017; GEORGE et al., 2020).

2.1.1 Classificação das Lesões

O pesquisador Herbert Seddon descreveu pela primeira vez a classificação das lesões de nervo periférico, propondo o estabelecimento de três diferentes graus de lesão em 1943 (SEDDON, 1943). A neuropraxia é o primeiro grau, caracterizada pela desmielinização focal sem danos aos axônios ou tecidos conjuntivos circundantes. Nas lesões de segundo grau, conhecida como axonotmese, há uma perda na continuidade do axônio além da desmielinização focal e pôr fim a neurotmese, em que ocorre a secção completa do nervo.

Subsequentemente, em 1951, Sydney Sunderland ampliou a classificação para cinco graus (I a V) com intuito de diferenciar a extensão das lesões nos tecidos conjuntivos (SUNDERLAND, 1951). Os graus I e V correspondem à neuropraxia e neurotmese, respectivamente (na classificação de Seddon). Os graus II e IV representam

subdivisões da axonotmese, contudo diferem de acordo com o acometimento do tecido conjuntivo. No grau II é observado lesão no axônio sem o acometimento do tecido conjuntivo, no grau III, é observado lesão no axônio e no endoneuro, já no IV os axônios, o endoneuro e o perineuro estão danificados.

2.1.2 Degeneração Nervosa

Após a lesão do SNP, se inicia o processo de degeneração Walleriana, o qual é caracterizado pela degeneração distal do nervo lesionado e a subsequente eliminação dos detritos celulares inibitórios, estabelecendo um microambiente pró-regenerativo para o crescimento de novos axônios (DEFRANCESCO-LISOWITZ et al., 2015; SUMARWOTO et al., 2021). Dentro de 12 a 24 horas da lesão, os microtúbulos axonais começam a se tornar desorganizados e se inicia a dissolução do citoesqueleto axonal (ALVITES et al., 2020). Nos primeiros dias após a lesão, um influxo de íons de cálcio ativa as proteases de calpaína, que degradam os neurofilamentos axonais, liberando detritos granulares (CARROLL; WORLEY, 2017).

Apenas algumas horas após uma lesão, as CS se convertem nas chamadas CS de reparo (NOCERA; JACOB, 2020), as quais auxiliam na degradação de restos de mielina e detritos celulares, além da expressão de citocinas que atraem macrófagos para auxiliar no processo de reparação (JESSEN; MIRSKY, 2019). Este evento é de extrema importância para a regeneração, pois a mielina contém moléculas que inibem o crescimento axonal (RICCIO et al. 2019).

A expressão de citocinas como fator de necrose tumoral (TNF) -α, interleucina (IL) -1α, -1β, fator inibidor de leucemia (LIF) e proteína quimiotática de monócitos (MCP) -1 é regulada positivamente (ROTSHENKER, 2011; JESSEN; MIRSKY, 2016). Dessa forma há a estimulação da proliferação das CS e o recrutamento de macrófagos e outras células do sistema imune para o local da lesão (RHODE et al., 2021). Após fagocitose dos restos de mielina e dos detritos, os macrófagos são eliminados por meio da apoptose local ou por reentrada na circulação (ALVITES et al., 2020).

No segmento proximal do nervo lesionado, ocorre em menor extensão, o processo de degeneração retrógrada até o próximo nódulo Ranvier preservado (MENORCA et al., 2013; ALVITES et al., 2020). As mudanças neste segmento variam de acordo com a sua gravidade e a localização da lesão, podendo ocorrer apoptose em lesões próximas ao

corpo neuronal ou em determinados casos, cromatólise reversível (MENORCA et al., 2013; RISHAL; FAINZILBER et al., 2014).

2.1.3 Envolvimento das Células de Schwann na Regeneração Nervosa

Em contraste ao SNC, o SNP é capaz de se regenerar com eficiência após uma lesão (MAHAR; CAVALLI, 2018). Paralelamente ao processo de degeneração que ocorre após a lesão, diversos eventos celulares e moleculares se iniciam na tentativa de reparação do nervo lesionado (DE LA ROSA et al., 2018). A integridade do corpo neuronal e das CS são um pré-requisito para esse processo (SCHEIB; HÖKE, 2013). Estas células proliferam, sofrem alongamento e alinham-se dentro de sua lâmina basal para formar as bandas de Büngner, as quais servem de guia e suporte para o recrescimento axonal (JESSEN; MIRSKY, 2019).

Além disso, as CS são responsáveis por sintetizar fatores neurotróficos fundamentais para a orientação e o crescimento dos axônios, como fator de crescimento do nervo (NGF), fator neurotrófico derivado do cérebro (BDNF), fator neurotrófico derivado da glia (GDNF) neurotrofinas -3, -4 e -5 (NT-3/4/5), fator de crescimento dos fibroblastos 2 (FGF-2), neuroregulinas, fator neurotrófico ciliar (CNTF), que interagem seletivamente em receptores tirosina quinases (Trks) e receptores de neurotrofinas p75 ($P75^{NTR}$) (CARROLL; WORLEY, 2017; CAILLAUD et al., 2019; GONÇALVES et al., 2019; HOPF et al., 2020).

As CS por meio de autofagia/mielinofagia e pelo recrutamento de macrófagos após a lesão são cruciais para proporcionar um ambiente adequado (GOMEZ-SANCHEZ et al., 2015; BALAKRISHNAN et al., 2021). Os macrófagos secretam moléculas associadas à neovascularização como o fator de crescimento endotelial vascular A (VEGF-A), o qual contribui para o fornecimento de oxigênio no local lesionado e orientam as CS de reparo no processo regenerativo (CATTIN et al., 2015; HUANG et al., 2020).

Adicionalmente, as CS produzem proteínas como a laminina e a fibronectina que são incorporadas a matriz extracelular e promovem a extensão dos axônios regenerantes (CAILLAUD et al., 2019).

Embora o SNP apresente capacidade intrínseca de reparação, diversos fatores podem ser limitantes para esse processo como o crescimento desorganizado dos axônios em regeneração que não atingem os órgãos-alvo, dificuldade de obter um grande número

de axônios do coto proximal para o distal, perda da conexão nervo-músculo que desencadeia a perda de fibras musculares. Além disso, quando ocorre a perda crônica do contato das CS com os axônios, as lâminas basais e a as bandas de Büngner não são mantidas, criando um ambiente que não suporta a regeneração (SCHEIB; HÖKE, 2013; JESSEN; MIRSKY, 2019; RHODE et al., 2021).

2.2 Células Tronco Mesenquimais

As CTM são células progenitoras multipotentes de origem não hematopoiética isoladas a partir de diversos tecidos adultos. Essas células são caracterizadas por sua capacidade de proliferação e de diferenciação *in vitro* em inúmeras linhagens mesenquimais a partir de um estímulo adequado, incluindo adipócitos, condrócitos e osteócitos (BARBERINI et al., 2014; MACDONALD; BARRETT, 2020).

Apesar de controverso, estudos indicam que as CTM sob condições especificas também podem se diferenciar em células derivadas das três camadas germinativas, como neurônios, células da glia e hepatócitos (REED; JOHNSON, 2008; WU; TAO, 2012; VILLAGRÁN et al., 2014; PENNINGTON et al., 2016; VILLAGRÁN et al., 2016; ANDRZEJEWSKA et al., 2019).

A Sociedade Internacional de Terapia Celular estabeleceu critérios mínimos para a caracterização das CTM em humanos. As condições para identificar as CTM são a aderência ao plástico, expressão ou não de marcadores de superfície (*Clusters Differentiation – CD*) e a capacidade de se diferenciar nas três linhagens mesodermais osteogênica, adipogênica e condrogênica após estímulos específicos (DOMINICI et al., 2006).

Um conjunto de padrões não foi definido para as CTM em equinos, sendo que diversos marcadores de superfície estão sendo avaliados e utilizados conforme a sua expressão ou não, mas ainda falta consenso estabelecido nesta espécie. As propriedades disponíveis mais confiáveis para a caracterização imunofenotípica das CTM em equinos são a aderência ao plástico, a diferenciação nas linhagens osteogênica, condrogênica e adipogênica, a expressão dos marcadores CD29, CD44, CD90, CD105, e a ausência dos marcadores CD14, CD34, CD79α e MHC-II (CARVALHO et al., 2011; DE SCHAUWER et al., 2012; BARBERINI et al., 2014).

Diversas fontes podem ser utilizadas para obtenção de CTM equinas, como tecido adiposo, medula óssea (COLBATH et al., 2017), membrana sinovial, liquido sinovial

(MURATA et al., 2014; PRADO et al., 2015), sangue periférico (DHAR et al., 2012), sangue ou tecidos do cordão umbilical, membrana amniótica, geléia de Wharton (IACONO et al., 2012; IACONO et al., 2015; MERLO et al., 2018), endométrio (RINK et al., 2017), tendão (BURK et al., 2013), polpa dentária (ISHIKAWA et al., 2017), gengiva e ligamento periodontal (MENSING et al., 2011).

As CTM-TA e as CTM-MO são as mais investigadas em equinos (BURK et al., 2013; GONZÁLEZ-FERNÁNDEZ et al., 2016). As CTM-TA apresentam vantagens como facilidade no isolamento, alta disponibilidade, abordagem mais segura, potencial de proliferação e podem ser obtidas em grandes quantidades (GUTIERREZ-NIBEYRO, 2011; HOPF et al., 2020). Além disso, as CTM-CU são uma alternativa promissora de células, pois podem ser obtidas de forma não invasiva e são facilmente isoladas em grandes quantidades (MERLO et al., 2018).

2.3 Potencial Mecanismo de Ação das Células Tronco Mesenquimais na Regeneração Nervosa

A facilidade de expansão em cultura, a capacidade de diferenciação em diversos tipos celulares *in vitro*, produção de fatores neurotróficos, anti-inflamatórios, imunomoduladores e pró-angiogênicos, fazem das CTM uma abordagem promissora (STEWART; STEWART, 2011; DE SCHAUWER et al., 2012; CASEIRO et al., 2016).

O potencial terapêutico do transplante de CTM em lesões do nervo periférico já foi demostrado em diversas espécies como humanos, ratos, cães e ovinos (CASAÑAS et al., 2014; SANCHEZ et al., 2017; CUI et al., 2018; YOUSEFI et al., 2019). Os principais mecanismos de ação das CTM baseiam-se em sua capacidade de integração no microambiente e na sua ação parácrina de produzir complexas moléculas pró-regenerativas (LOPATINA et al., 2011). As CTM conferem neuroproteção por meio da produção de NGF, BDNF, GDNF, FGF-2, fator de crescimento semelhante a insulina (IGF) e fator de crescimento de hepatócitos (HGF) (SALGADO et al., 2010; CASEIRO et al., 2016; RODRÍGUEZ-SÁNCHEZ et al., 2021; SUMARWOTO et al., 2021).

Além disso, em geral, os efeitos imunomodulatórios das CTM estão relacionados com a secreção do fator de crescimento transformador beta (TGF- β), prostaglandina E2 (PGE2), indoleamina 2,3-dioxigenase (IDO), óxido nítrico (ON), IL-6 e IL-10 e efeitos angiogênico principalmente por meio do VEGF (CARRADE; BORJESSON, 2013; RIBEIRO et al., 2015; KOTA et al., 2017; ELEUTERI; FIERABRACCI, 2019).

Entretanto, o potencial de expressar os fatores imunomodulatórios pode variar entre as espécies e as células de diferentes fontes (CARRADE et al., 2012; CASSANO et al., 2018).

2.4 Células *Schwann-like*

As CS desempenham um papel fundamental durante a regeneração do SNP, mas para a sua aquisição e utilização na prática clínica é necessário o sacrifício de um nervo funcional saudável do doador, além de que a proliferação dessas células *in vitro* está associada à um longo período de cultivo e expansão (SAFFARI et al., 2021). Devido à essas limitações, a transdiferenciação das CTM em CSL tem sido proposta como uma estratégia promissora para o tratamento de lesões periféricas (DE LA ROSA et al., 2018; HUANG et al., 2020).

Há diversos estudos que demonstram essa capacidade de transdiferenciação por meio da estimulação com diferentes fatores (KINGHAM et al., 2007; LADAK, et al., 2011; FARONI et al., 2016; JUNG et al., 2016) e da co-cultura com CS (LI et al., 2009).

Dezawa e colaboradores descreveram o primeiro protocolo desenvolvido para transdiferenciar as CTM em CSL *in vitro* no ano de 2001 (DEZAWA et al., 2001). CTM-MO de ratos foram submetidas a uma combinação de fatores como o beta-mercaptoetanol (BME), ácido all-trans retinóico (AR), forskolina (FSK), fator de crescimento de fibroblasto básico (bFGF), fator de crescimento derivado de plaquetas (PDGF) e Heregulina (HRG) para realizar a transdiferenciação (DEZAWA et al., 2001). O BME é um agente redutor utilizado nas CTM para promover a diferenciação em células de linhagem neural (WOODBURY et al., 2000). O AR atua como fator desencadeante que induz a alterações morfológicas, regula a expressão de diversos fatores de transcrição durante a diferenciação neuronal e aumentam a capacidade de resposta à neurotrofinas (TAKAHASHI; PALMER; GAGE, 1999; PAN; CAI, 2012). FSK eleva o nível de monofosfato cíclico de adenosina intracelular (cAMP), resultando no aumento das respostas mitogênicas e da capacidade de resposta das células aos fatores neurotróficos (GAO et al., 2015). PDGF, bFGF e HRG são neurotrofinas envolvidas na diferenciação, sobrevivência e proliferação das células gliais. Além disso, bFGF e PDGF são considerados potentes mitógenos para as CTM (HOPF et al., 2020).

Com base neste protocolo, diversos outros foram desenvolvidos ao longo do tempo utilizando CTM de diferentes fontes e espécies. O primeiro relato com sucesso na

transdiferenciação de CTM-TA *in vitro* foi descrito por Kingham e colaboradores utilizando células de ratos. Os pesquisadores aumentaram o tempo de indução e da concentração de FSK e acrescentaram o fator de crescimento glial – 2 (GGF2) no procedimento previamente estabelecido por Dezawa e colaboradores (KINGHAM et al., 2007).

Outras abordagens alternativas de transdiferenciação são relatadas como a adição de fatores neurotróficos como BDNF (GAO et al., 2015) e NGF (XIAO; WANG, 2015), de glicocorticoides, insulina e progesterona (KANG et al., 2019) no meio de diferenciação. Na literatura cientifica o método mais descrito é a transdiferenciação utilizando produtos químicos (MATHOT et al., 2019), no entanto, esses métodos de indução apresentam desvantagens como protocolos de difícil execução e custos elevados (LIU et al., 2013; TAWAB et al., 2018).

Atualmente um novo método *in vitro* foi proposto baseado na utilização de meio de cultura condicionado com fatores neurotróficos secretados do nervo isquiático de ratos, para realizar a transdiferenciação das CTM-TA e das CTM-MO de ratos em CSL (LIU et al., 2013; TAWAB et al., 2018; GALHOM et al., 2018). O sucesso dessa abordagem foi confirmado pela mudança morfológica em que as células exibiram uma morfologia bipolar e/ou tripolar com grandes núcleos, os processos citoplasmáticos tornaram-se finos e alongados, e observou-se a expressão dos marcadores gliais S100 e GFAP (proteína ácida fibrilar da glia).

Além disso, foi demonstrado que o transplante de CSL *in vivo* obtidas por esta nova abordagem pode auxiliar na regeneração de nervo periférico em um modelo de lesões por esmagamento em nervo isquiático de ratos (TAWAB et al., 2018).

Estudos utilizando CTM de ratos e humanas demonstram que a secreção de fatores neurotróficos como BDNF, GDNF, NGF e VEGF-A aumentam nas CTM-TA diferenciadas em relação as CTM indiferenciadas (TOMITA et al., 2012; KINGHAM et al., 2014). O aumento do potencial neurotrófico auxilia na neurorregeneração em modelos *in vitro* quanto *in vivo*, evidenciando o potencial dessas células (HOPF et al., 2020; PIOVESANA et al., 2021).

A maioria dos estudos descrevem o sucesso da transdiferenciação *in vitro* baseada nas alterações morfológicas e na expressão de marcadores específicos de CS (LIU et al., 2013; TAWAB et al., 2018; MATHOT et al., 2019). Embora não tenha sido estabelecido nenhum consenso sobre quais marcadores utilizar, a expressão destes é considerada o

critério mais importante para identificação da transdiferenciação em CSL (LIU et al., 2015; RAMLI et al., 2019).

Os marcadores gliais S100 e GFAP são frequentemente utilizados para caracterizar a transdiferenciação *in vitro* (LIU et al., 2013; TAWAB et al., 2018; HASELBACH et al., 2016; VILLAGRÁN et al., 2016; XIE et al et al., 2017; FU et al., 2019). As proteínas S100 foram implicadas em uma variedade de funções intracelulares e extracelulares, sendo que a sua presença está associada à proteção do tecido nervoso e à nutrição das CS (PAN et al., 2012; DONATO et al., 2013). Já a GFAP consiste em uma proteína de filamento intermediário responsável pela estrutura do citoesqueleto da maioria das células gliais, sendo de grande importância na proliferação das CS (ENG; GHIRNIKAR; LEE, 2000; TRIOLO et al., 2006; KAMIL et al., 2020).

Em nosso conhecimento, não há estudos descrevendo a capacidade de transdiferenciação das CTM equinas em CSL utilizando um meio de cultura condicionado com fatores secretados de nervo periférico de equinos, sendo assim, mais estudos são necessários para esclarecer o potencial de transdiferenciação das CTM equinas em CSL.

3 HIPÓTESE

A hipótese deste estudo é que as CTM equinas quando submetidas a estímulos apropriados *in vitro,* apresentam capacidade de transdiferenciação em CSL.

4 OBJETIVO

4.1 Objetivo Geral

Avaliar o potencial de transdiferenciação *in vitro* das CTM equinas em CSL.

4.2 Objetivos Específicos

- Padronizar um protocolo de transdiferenciação das CTM-TA, CTM-MO e CTM-CU equinas em CSL;
- Avaliar a morfologia celular após a indução da transdiferenciação das CTM-TA, CTM-MO e CTM-CU equinas em CSL;
- Avaliar a viabilidade celular das CTM-TA e CTM-MO equinas indiferenciadas e diferenciadas;
- Avaliar a expressão gênica dos marcadores gliais S100β e GFAP e dos fatores neurotróficos NGF, BDNF e GDNF nas CTM-TA, CTM-MO e CTM-CU equinas indiferenciadas e diferenciadas;
- Avaliar a expressão proteica dos marcadores gliais S100 e GFAP nas CTM-TA, CTM-MO e CTM-CU equinas indiferenciadas e diferenciadas por meio da imunofluorescência.

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CAPÍTULO 2 – TRABALHO CIENTÍFICO

‘’Tabalho a ser enviado para a revista Stem Cells and Development’’

In Vitro Transdifferentiation Potential of Equine Mesenchymal Stem Cells into Schwann-Like Cells

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Abstract

In peripheral nerve injuries, Schwann cells (SCs) are essential for the regenerative process. However, its use as cell therapy has limitations. In this context, we seek to develop new technologies and alternative methods that mimic the effects of SCs. Several studies have demonstrated the ability of mesenchymal stem cells (MSCs) to transdifferentiate into Schwann-like cells (SLCs) using chemical protocols or co-culture with SCs. However, they are complicated and expensive methods. The aim of this study was to evaluate the *in vitro* transdifferentiation potential of MSCs derived from equine adipose tissue (AT) and equine bone marrow (BM) into SLCs. In this study, the facial nerve of an adult horse was collected, cut into fragments and incubated in complete culture medium for 48 hours. This medium was used to transdifferentiate MSCs into SCLs. Equine AT-MSCs and BM-MSCs were incubated with the induction medium for five days. After this period, the morphology, gene expression of glial markers S100β and GFAP (glial fibrillary acidic protein) and nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and protein expression of S100 and GFAP were evaluated in undifferentiated and differentiated cells. MSCs from the two sources incubated with the induction medium exhibited bipolar and/or tripolar morphology with large nuclei and elongated cytoplasmic processes, similar to SCs morphology. There was a significant increase in the gene expression of BDNF, GDNF, GFAP and S100β in equine AT-MSCs and of GDNF, GFAP and S100β in equine BM-MSCs post-differentiation. Immunofluorescence revealed GFAP protein expression in undifferentiated and differentiated cells derived from equine AT-MSCs and BM-MSCs. However, S100 was only expressed in differentiated cells from both sources. Based on our results we conclude that equine AT-MSCs and BM-MSCs have a great transdifferentiation potential into SLCs, and are promising strategy for cell-based therapy for peripheral nerve regeneration.

Keywords: Facial nerve, nerve regeneration, neurotrophic factors, peripheral nerve injury, Schwann-like cells.

Introduction

Peripheral nervous system (PNS) lesions are common in humans and animals [1,2]. Persistent sensorimotor dysfunction is the main consequence associated with injuries, affecting the patient's quality of life [3,4].

In equine the causes of PNS injury are multiple and can occur due to traumatic processes, metabolic and toxic diseases and infectious and degenerative diseases [5,6]. One of the most common neuropathies in horses is facial nerve paralysis, which is associated with traumatic events in most cases [7].

Despite the regenerative potential of the PNS, predominantly attributed to the plasticity of Schwann cells (SCs), spontaneous repair is mostly unsatisfactory [8,9]. Although the continued introduction of new treatments, nerve functional recovery is associated with a series of limitations [10,11]. In this context, cell-based therapy is an approach that attracts interest in regenerative medicine and has been proposed as a promising strategy for the regeneration of injured peripheral nerves [12]. SCs are fundamental in the regeneration process, as they provide support and guide the growth of axons through the secretion of neurotrophic factors, cytokines and phagocytosis of myelin debris [13,14].

SCs transplantation has shown beneficial effects in PNS lesions [15,16], however, for its acquisition and use in clinical practice, it is necessary to sacrifice a healthy functional nerve from the donor, in addition to the fact that the proliferation of these cells *in vitro* is associated with a long period of cultivation and expansion [9,17,18]. To overcome these limitations, several studies have demonstrated the ability of Mesenchymal Stem Cells (MSCs) from different sources and species to transdifferentiate *in vitro* into Schwann-like cells (SLCs), usually through co-culture with SCs or using chemical protocols [14,19-24]. However, these induction methods have disadvantages such as complicated protocols and high costs [25,26].

A practical approach was successfully described using culture medium conditioned with neurotrophic factors secreted from the sciatic nerve of rats to induce *in vitro* transdifferentiation of rat MSCs derived from adipose tissue (AT-MSCs) and bone marrow (BM-MSCs) into SLCs [25-27].

In equine species, there is a study describing the ability of MSCs to transdifferentiate into SLCs, in which BM-MSCs were subjected to specific *in vitro* conditions with a combination of chemicals to perform transdifferentiation [22]. Equine

MSCs are obtained mainly from AT and BM [28,29]. However, AT-MSCs have a number of advantages such as ease of isolation, wide availability and high *in vitro* proliferation rate [14,30].

To our knowledge, there are no studies describing the ability to transdifferentiate equine AT-MSCs into SLCs *in vitro* and there are no reports on the development of a conditioned culture medium with trophic factors secreted from the peripheral nerve of horses and its use to perform transdifferentiation.

Therefore, the present study aims to evaluate the *in vitro* transdifferentiation potential of equine AT-MSCs and BM-MSCs into SLCs using a conditioned medium obtained from equine facial nerve explant culture, in order for future cell-based therapy for neuroregeneration of peripheral nerve injuries.

Materials and methods

Animal Ethics

All stages of this study were conducted in accordance with the Ethical Principles in Animal Experimentation and approved by the Ethics Committee for the Use of Animals (CEUA) of São Paulo State University (UNESP) – Botucatu, according to protocol number 0039/2021.

Facial Nerve Collection and Preparation of Induction Medium

An equine female, 3 years old, Mangalarga, 300 kg, was submitted to the euthanasia procedure due to musculoskeletal injuries and permanent recumbency. The procedure was performed by sedation with 10% Xylazine Hydrochloride intravenously (1 mg/kg) (Equisedan®, JA Saúde Animal, São Paulo, Brazil), subsequently, intravenous sodium thiopental was administered (1 g/150 kg) (Thiopentax®, Cristália, São Paulo, Brazil). A facial nerve (approximately 10 cm) from the horse was collected immediately after euthanasia, under aseptic conditions and placed in a sterile 50 ml conical tube containing a solution of *Dulbecco's phosphate buffered Saline* (DPBS) (LGC Biotecnologia, São Paulo, Brazil), 3% penicillin/streptomycin and 0.5% amphotericin (both from Gibco, Grand Island, New York, USA).

The procedure for preparing the induction medium and the method for transdifferentiation MSCs into SLCs was carried out according to the methodology first

described by Liu et al. [25], with modifications. The facial nerve was washed with DPBS (LGC Biotecnologia, São Paulo, Brazil) containing 1% penicillin/streptomycin (Gibco, Grand Island, New York, USA). All excess tissue adhered to the nerve was removed and the nerve was cut into 1 cm fragments within the laminar flow under sterile conditions. Each fragment was soaked in 15 ml of complete culture medium containing 90% Dulbecco's Modified Eagle's Medium (DMEM)/F12 (LGC Biotecnologia, São Paulo, Brazil), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1 % amphotericin (all Gibco, Grand Island, New York, USA) and incubated at 37.5°C in a humidified atmosphere containing 95% air and 5% CO2 for 48 hours. After this period, the culture medium was aspirated and filtered (BD Falcon cell filter, 70 µm, San Jose, CA, USA) to remove the nerve fragments. Subsequently, the induction medium was filtered with a 0.22 µm syringe filter (Sarstedt, Nümbrecht, Germany) and cryopreserved at -80ºC for the next steps (Fig. 1A).

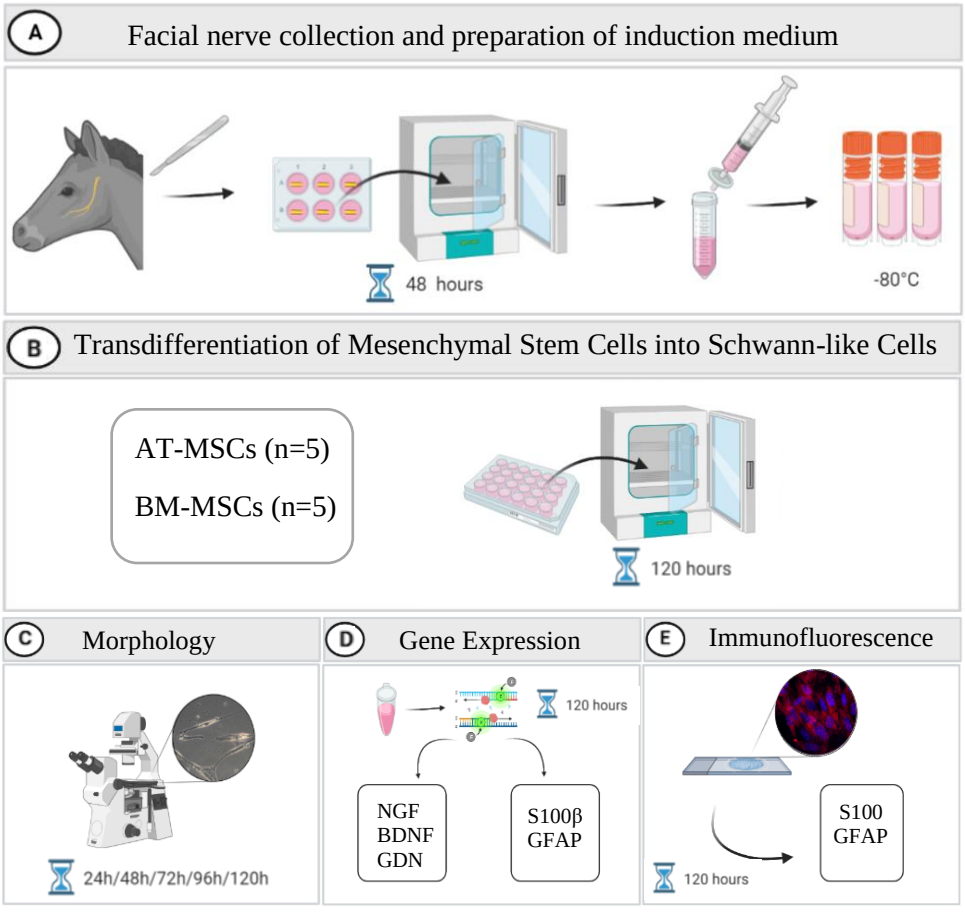


FIG. 1. A summary of the experimental design. (A) The facial nerve from the horse was collected immediately after euthanasia. The nerve was cut into 1 cm fragments. Each fragment was soaked in 15 ml of complete culture medium and incubated for 48 hours. After this period, the culture medium was aspirated, filtered and cryopreserved at -80ºC. (B) Equine AT-MSCs (n=5) and equine BM-MSCs (n=5) were seeded in 24-well plates to perform transdifferentiation into SLCs. Cells were incubated with the induction medium for five days. (C) the morphology of equine MSCs was evaluated by inverted microscopy during the period of transdifferentiation. After this period, (D) the gene expression of glial markers and neurotrophic factors was quantified in undifferentiated and differentiated equine AT-MSCs and BM-MSCs. Real-time polymerase chain reaction (qPCR) was performed. (E) Protein expression of glial markers was analyzed by immunofluorescence staining. Images created with biorender (https://biorender.com/).

Mesenchymal Stem Cells

Equine MSCs (P3) derived from AT (n=5) and BM (n=5) were obtained from the cell bank of our research group. MSCs were previously characterized (differentiation into osteogenic, chondrogenic and adipogenic mesenchymal lineages and evaluation of surface markers). Immunophenotypic analysis performed by flow cytometry revealed high expression of the markers CD44, CD90 and CD105 and absence of the markers CD34 and MHC-II [31].

Transdifferentiation of Mesenchymal Stem Cells into Schwann-like Cells

MSCs were thawed and resuspended in complete culture medium. Equine AT-MSCs and BM-MSCs were seeded at a density of $1x10^4$ cells per well in 24-well plates (Kasvi, São José do Pinhais, PR, Brazil) with a 0.5 ml volume of complete culture medium containing 90% Dulbecco's Modified Eagle's Medium (DMEM)/F12 (LGC Biotecnologia, São Paulo, Brazil), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1 % amphotericin (all Gibco, Grand Island, New York, USA).

To perform transdifferentiation into SLCs, the culture medium was removed from the subconfluent MSCs cultures and replaced with 0.5 ml of prewarmed induction medium in each well. Cells were incubated in this condition for five days at 37.5ºC in a humidified atmosphere containing 95% air and 5% CO2. The induction medium was

changed every 2-3 days. Equine AT-MSCs and BM-MSCs were divided into 2 groups: cells cultured in complete culture medium (undifferentiated cells) and cells cultured in induction medium (differentiated cells) (Fig. 1B).

Morphology Evaluation

The morphology of equine MSCs was evaluated by inverted microscopy (LEICA DMIRB). Photomicrographs of undifferentiated and differentiated cells were captured during the transdifferentiation period (Fig. 1C).

Cell viability

After the transdifferentiation period (120 h), cell viability of undifferentiated and differentiated equine AT-MSCs and BM-MSCs was performed using the exclusion test was performed with 0.4% trypan blue. The MSCs were homogenized in a microcentrifuge tube with 0.4% Trypan Blue and placed in a Neubauer chamber to count (the number of viable cells, dead cells and total cells).

Gene Expression - qPCR

The gene expression of glial markers, glial fibrillary acidic protein (GFAP) and S100β and neurotrophic factors brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF) was quantified in undifferentiated and differentiated cells derived from equine AT-MSCs and BM-MSCs after the transdifferentiation period (120 h) (Fig. 1D). Cells were lysed and homogenized with TRIzol reagent (TRIzol™, Invitrogen™, ThermoFisher Scientific, São Paulo, Brazil), and RNA extraction was performed with the same reagent according to the manufacturer's instructions. The RNA was eluted with RNA-free water, quantified and analyzed by spectrophotometry with Thermo Scientific NanoDrop 2000 equipment (ThermoFisher Scientific, Wilmington, USA) for absorbance ratios of 260/280 nm and 260/230 nm. The total RNA extracted from the cells was of high quality and purity, indicating that the extraction method was efficient. cDNA synthesis was performed with the reagents of the High-Capacity cDNA Reverse Transcription Kit (Applied

Biosystems™, Life Technologies Corporation, Carlsbad, USA), according to the manufacturer's instructions, and reverse transcription was performed to obtain cDNA with the Veriti 96 Well Thermal Cycler (Applied Biosystems™, ThermoFisher Scientific), under the following thermal cycling conditions: 10 minutes at 25ºC; 12 minutes at 37°C; and 5 minutes at 85°C. The cDNA samples were cryopreserved at -80 ºC and used as templates for PCR reactions.

Reactions were performed in duplicate using cDNA produced previously with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, Life Technologies, Carlsbad, CA, USA), RNA-free water, and equine primers (Thermo Fisher Scientific, São Paulo, Brazil), which were designed using Primer Express™ Software v3.0.1 (Applied Biosystems™, Thermo Fischer Scientific), as shown in Table 1.

Samples were tested with four reference genes, beta-actin (ACTB), beta -2-microglobulin (B2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT). The mean cycle threshold (CT) of the four reference genes was used. The real-time polymerase chain reaction (qPCR) method was performed with the QuantStudio™ 12K Flex Real-Time PCR System thermocycler (Applied Biosystems™, Thermo Fischer Scientific) with the following parameters: 50°C for 2 minutes, 95 °C for 2 minutes and 40 cycles of 95 °C for 1 second and 60 °C for 30 minutes with a subsequent dissociation curve. The relative quantification of the expression of the genes of interest was performed using the ΔΔCt method [32].

Table 1. The Primer Sets Used for Quantitative Polymerase Chain Reaction

Gene	*Forward primer*	*Reverse primer*
BDNF	TTGGATGAGGGCCAGAAAGT	CAAGTCCGCGTCCTTACTGTT
GDNF	CAGGGACTCTTCCTCCATCCT	TGGGCACGAGCATGTTTCT
NGF	CCAACGGAGCAGCTTTCTGT	AACAACATGGACATTACGCTATGC
GFAP	GAAACCAGCCTGGACACCAA	TCACCACGATGTTCCTCTTGAG
S100β	CGCTCATGGCTATTGGTGAA	TCGTGGATACAAGAGCAAAAGTT
ACTB	CGGCGGCTCCATTCTG	CTGCTTGCTGATCCACATCTG
B2M	CACCCAGCAGAGAATGGAAAG	CGGATGGAACCCAGAGACA
GAPDH	GGCAAGTTCCATGGCACAGT	GGGCTTTCCGTTGATGACAA
HPRT	GCTCGAGATGTGATGAAGGAGAT	CCCCCTTGAGCACACAGAGT

BDNF: brain-derived neurotrophic factor; GDNF: glial cell-derived neurotrophic factor; NGF: nerve growth factor; GFAP: glial fibrillary acidic protein; ACTB: beta-actin; B2M: beta -2-microglobulin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HPRT: hypoxanthine-guanine phosphoribosyltransferase.

Immunofluorescence

The protein expression of glial markers was determined by immunofluorescence staining (Fig. 1E). Briefly, equine AT-MSCs and BM-MSCs were seeded at a density of $1x10^4$ cells/cm^2 on 13 mm round coverslips (Kasvi, São José do Pinhais, PR, Brazil) previously treated with Poly-L-Lysine (Sigma-Aldrich®, Saint Louis, MO, USA) in an adherent 24-well plate (Kasvi, São José do Pinhais, PR, Brazil) and the transdifferentiation process was performed. After the transdifferentiation period, undifferentiated and differentiated equine MSCs were fixed with phospate buffered saline (PBS) + 2% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 10 min at room temperature and blocked with 2% bovine serum albumin for 20 min (all from Sigma-Aldrich®, Saint Louis, MO, USA).

Cells were incubated overnight at 4°C with antibodies against S100 (polyclonal rabbit anti-S100, DAKO® GA504, 1:200) and GFAP (polyclonal rabbit anti-GFAP, DAKO® GA524, 1:200). Subsequently, the cells were washed with PBS and incubated with goat anti-rabbit IgG H&L (Alexa Fluor® 594) (Abcam, ab150080) secondary antibody for 1 hour at room temperature. Vectashield mounting medium with DAPI reagent (Vector Laboratories, Burlingame, CA, USA) was used for mounting.

In the qualitative analysis of S100 and GFAP immunofluorescence, representative images of undifferentiated and differentiated cells were obtained using a LEICA TCS SP8 confocal microscope with LAS X software.

Each slide was analyzed in three different fields and the images were imported for the determination of the integrated pixel density that represented the intensity of labeling using ImageJ software, (version 1.33u, National Institutes of Health, USA).

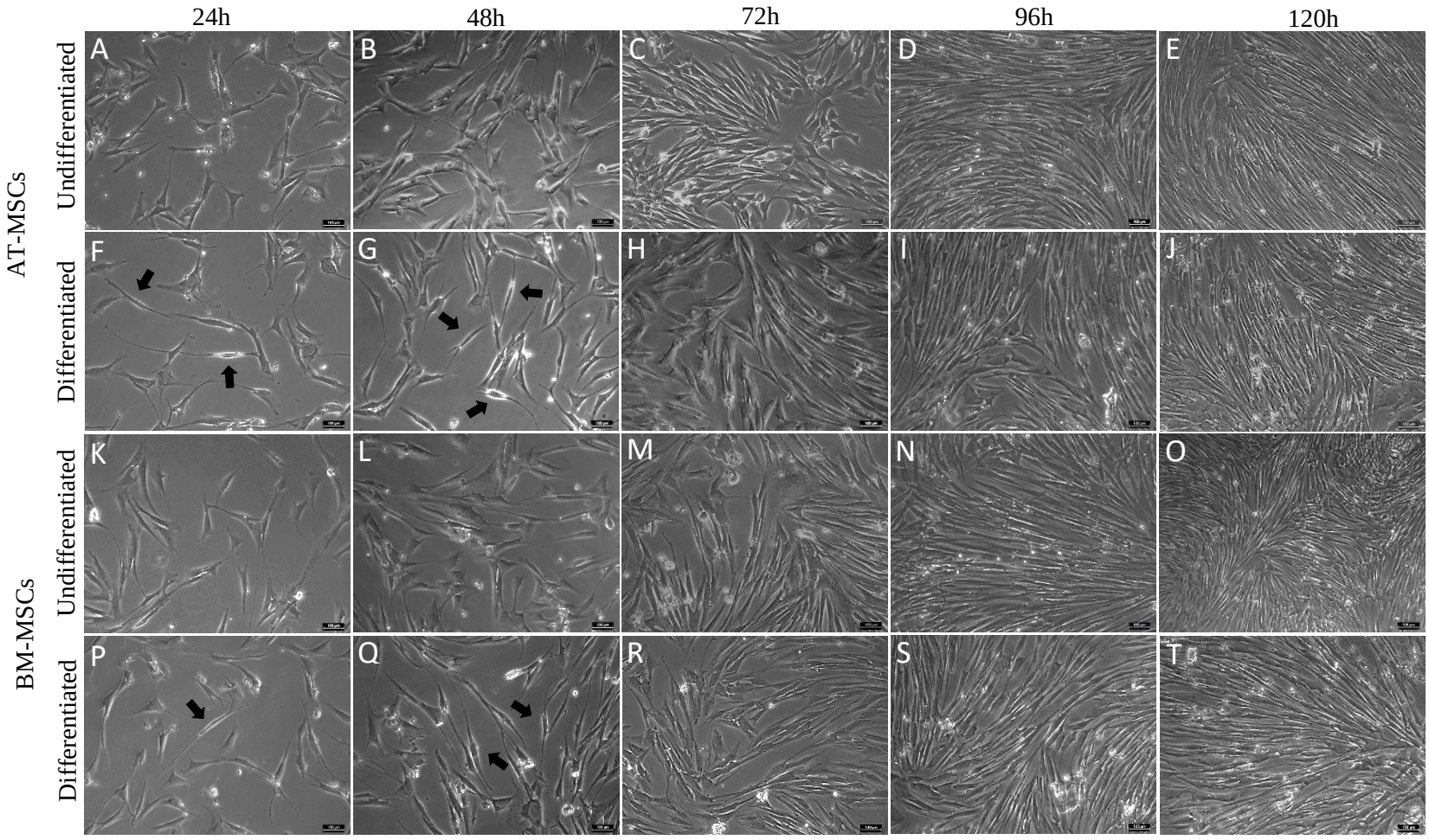
Statistical analysis

The variables cell viability, relative quantification and integrated pixel density were assessed for normality using statistical tests (Shapiro–Wilk), descriptive statistics, and graphic analysis. For parametric data, the t-test was performed with unpaired samples. For non-parametric data, the Mann-Whitney test was performed for unpaired samples. The level of significance between the groups was set at $p < 0.05$. The differences are denoted by a single asterisk ($p < 0.05$) (GraphPad Prism version 8 for Mac, San Diego, USA).

Results

Morphological changes

Fusiform bipolar cells began to appear 24 hours after induction of transdifferentiation. Cells exhibited a bipolar and/or tripolar morphology with large nuclei and the cytoplasmic processes became thin and elongated compared to undifferentiated equine AT-MSCs and equine BM-MSCs during incubation with the induction medium (Fig. 2A-T).


24h
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AT-MSCs
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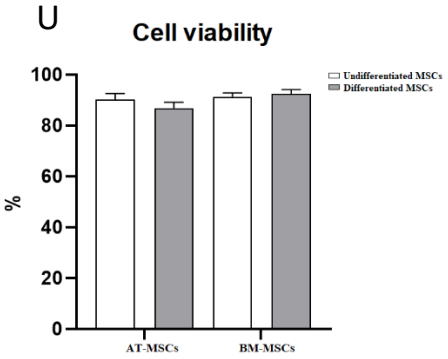
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Cell viability
100
80
60
40
20
0
%
AT-MSCs
BM-MSCs
Undifferentiated MSCs
Differentiated MSCs

FIG. 2. Characterization of morphological differentiation and viability of equine MSCs. Undifferentiated equine AT-MSCs and BM-MSCs at 24 h, 48 h, 72 h, 96 h, 120 h of culture (**A**, **B**, **C**, **D**, **E**, **K**, **L**, **M**, **N**, **O**, respectively) showing fibroblastoid morphology without cytoplasmic extensions. Differentiated equine AT-MSCs and BM-MSCs at 24 h, 48 h, 72 h, 96 h, 120 h after induction of transdifferentiation (**F**, **G**, **H**, **I**, **J**, **P Q R S T**, respectively). Morphological changes were more evident in areas with lower cell density in horses AT-MSCs and BM-MSCs at 24 and 48 hours after induction of transdifferentiation (**F**, **G**, **P**, **Q**, respectively), cells showed a fusiform bipolar to tripolar morphology with large nuclei and elongated, thin cytoplasm (black arrows). Magnification, x200. Scale bar = 100µm. Cell viability of undifferentiated and differentiated equine AT-MSCs and BM-MSCs (**U**). Data are represented as the mean ± standard deviation.

Cell viability

Using the trypan blue exclusion test, we observed cell viability means of 86.8% and 92.4% in the differentiated equine AT-MSCs and BM-MSCs, respectively (Fig. 2U).

Gene expression of glial markers and neurotrophic factors

The gene expression of the neurotrophic factors, BDNF, GDNF, NGF and the glial markers GFAP and S100β are represented in Fig. 3.

In equine AT-MSCs there was a significant increase in the gene expression of BDNF, GDNF, GFAP and S100β in differentiated cells compared to undifferentiated cells.

For equine BM-MSCs there was a significant difference in the gene expression of GDNF, GFAP and S100β, which was greater in differentiated cells than in undifferentiated cells. Furthermore, in two samples of equine BM-MSCs the expression of S100β was observed only in the differentiated cells, while in the other three samples there was the expression of this marker in both groups (undifferentiated and differentiated), being higher in the differentiated cells.

Comparing the equine MSCs sources in this study, the expression of glial markers was significantly higher in differentiated AT-MSCs than in differentiated BM-MSCs.

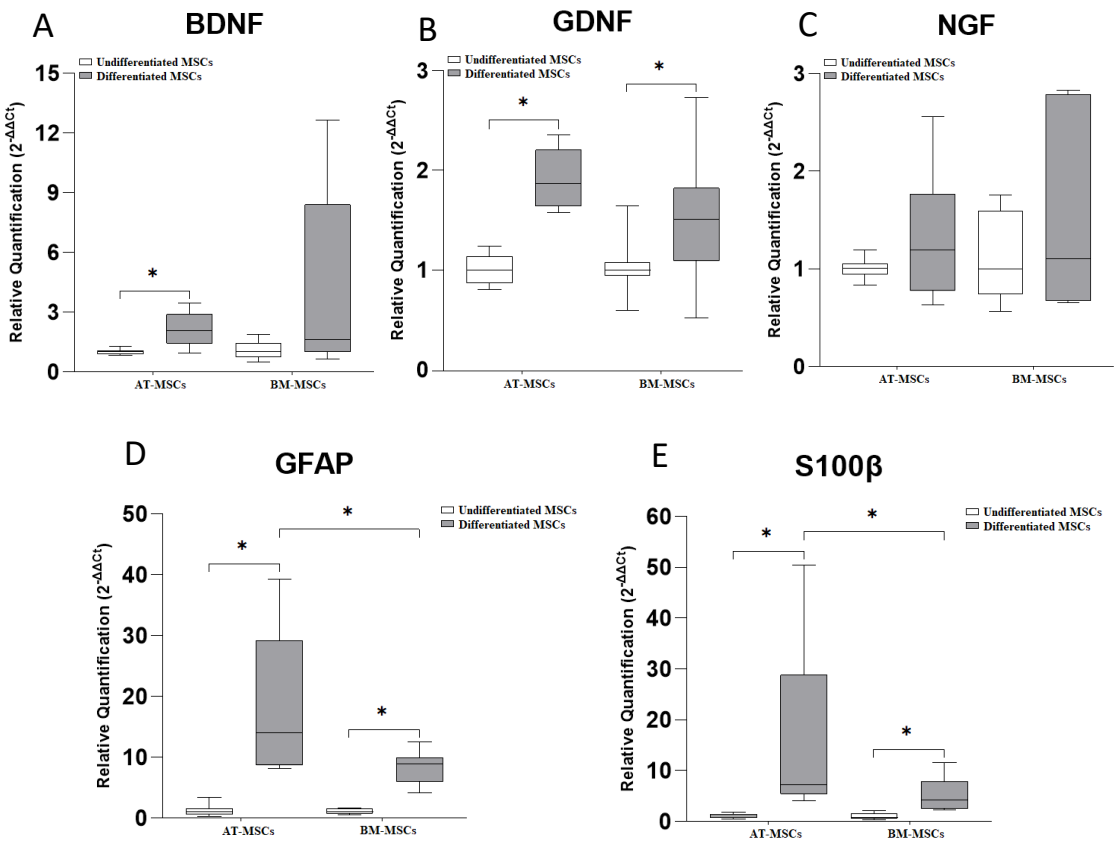
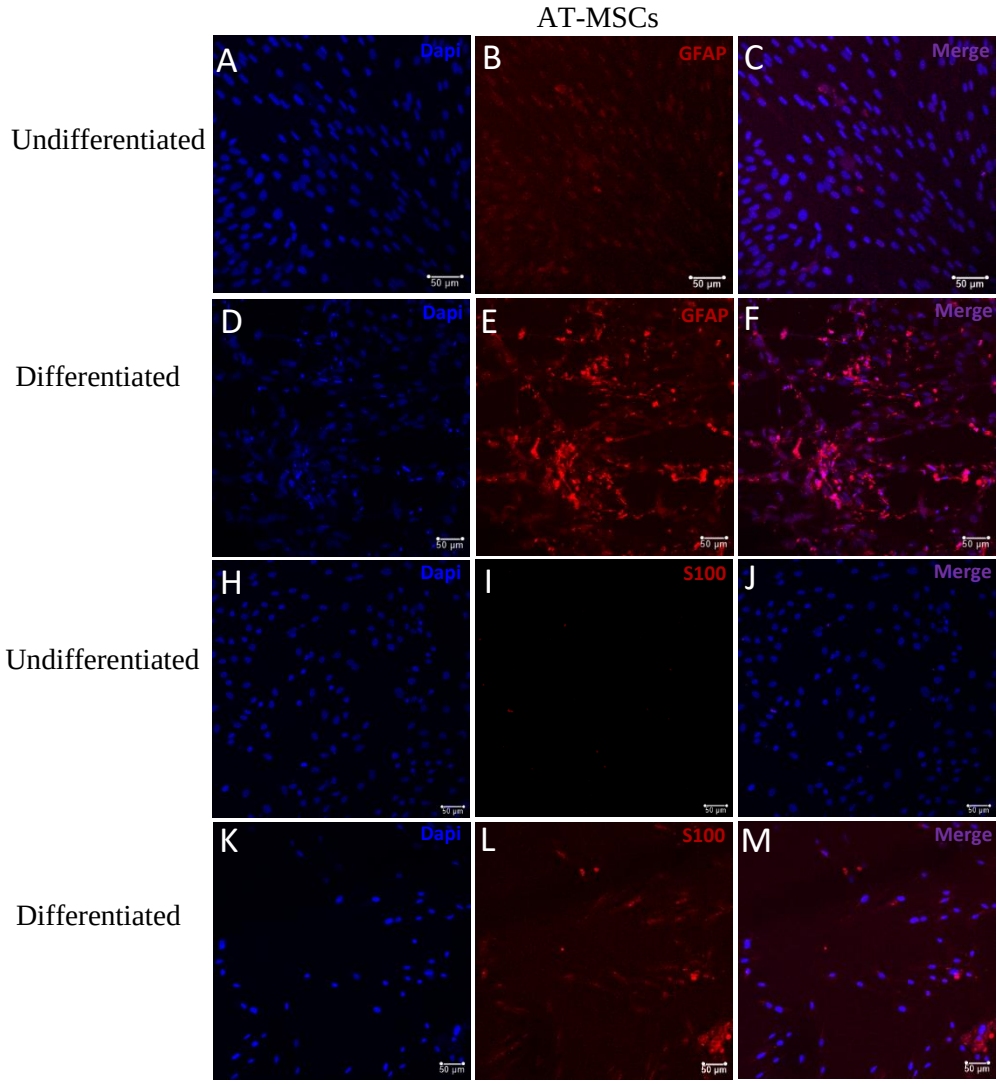
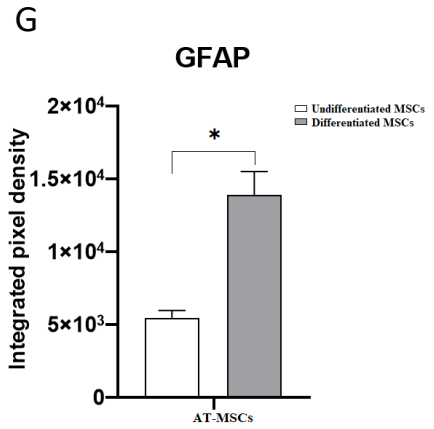


FIG. 3. Relative expression of the neurotrophic factors, BDNF, GDNF, NGF and glial markers GFAP and S100β in undifferentiated and differentiated equine AT-MSCs and BM-MSCs. BDNF **(A)**, GDNF **(B)**, NGF **(C)**, GFAP **(D)** and S100β **(E)**. Data are represented as medians, interquartile ranges and minimum and maximum values ($p < 0,05$*).

Immunofluorescence

To analyze the transdifferentiation process, we performed immunostaining of GFAP and S100 in equine AT-MSCs and BM-MSCs, represented in Figs. 4 and 5, respectively. Immunofluorescence staining revealed GFAP expression in undifferentiated and differentiated cells, with a significant increase in integrated pixel density in differentiated AT-MSCs and BM-MSCs. We observerd that glial marked S100 was expressed in differentiated cells in both sources.


AT-MSCs
Undifferentiated
Differentiated
Undifferentiated
Differentiated
A
Dapi
B
GFAP
C
Merge
D
Dapi
E
GFAP
F
Merge
H
Dapi
I
S100
J
Merge
K
Dapi
L
S100
M
Merge
50 µm


G
GFAP
Integrated pixel density
2×10⁴
1.5×10⁴
1×10⁴
5×10³
0
*
Undifferentiated MSCs
Differentiated MSCs
AT-MSCs

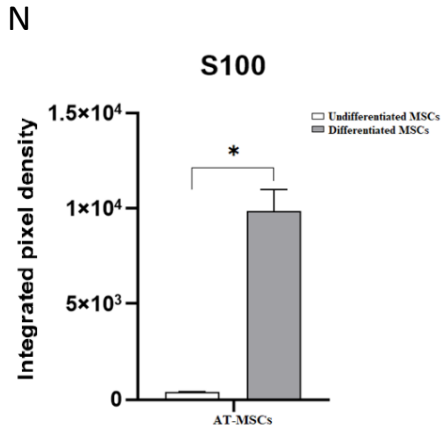
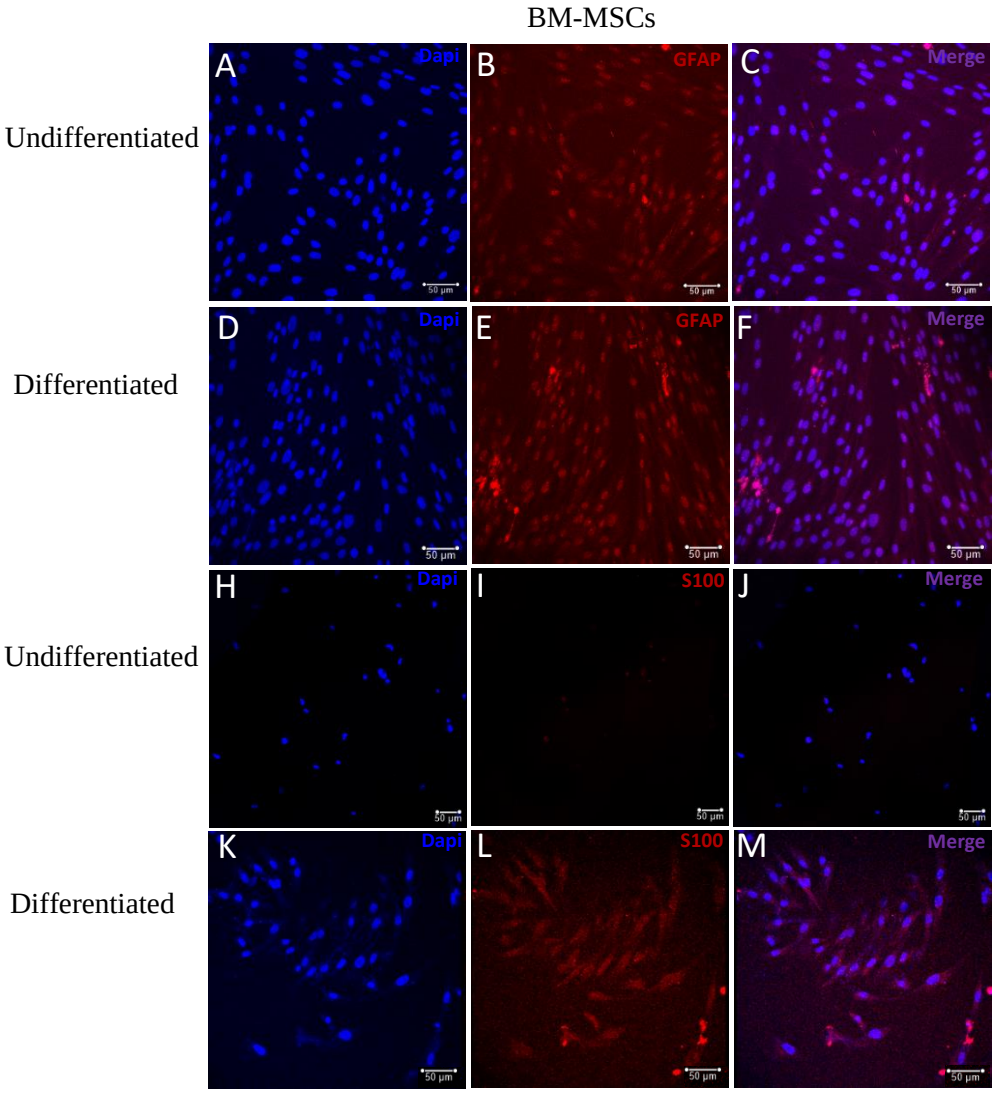
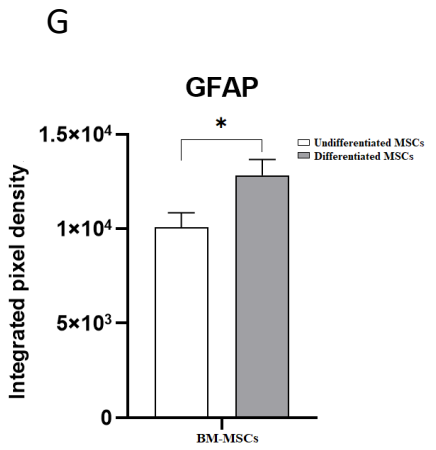

N
S100
Integrated pixel density
1.5×10⁴
1×10⁴
5×10³
0
*
Undifferentiated MSCs
Differentiated MSCs
AT-MSCs

FIG. 4. Immunofluorescence staining of glial markers on undifferentiated and differentiated equine AT-MSCs. Representative images of triple labeling with DAPI (blue) anti GFAP (red) in undifferentiated (**A**, **B**, **C**), differentiated (**D**, **E**, **F**) AT-MSCs and integrated pixel density analysis (**G**). Representative images of triple labeling with DAPI (blue) anti S100 (red) in undifferentiated (**H**, **I**, **J**), differentiated (**K**, **L**, **M**) AT-MSCs and integrated pixel density analysis (**N**). The glial marker S100 was expressed only in differentiated AT-MSCs. The values of the integrated pixel density are represented as the mean ± standard deviation ($p < 0.05$*). Scale bar: 50 µm.


BM-MSCs
Undifferentiated
Differentiated
Undifferentiated
Differentiated
A
Dapi
B
GFAP
C
Merge
D
Dapi
E
GFAP
F
Merge
H
Dapi
I
S100
J
Merge
K
Dapi
L
S100
M
Merge
50 µm


G
GFAP
*
Undifferentiated MSCs
Differentiated MSCs
Integrated pixel density
1.5×10⁴
1×10⁴
5×10³
0
BM-MSCs

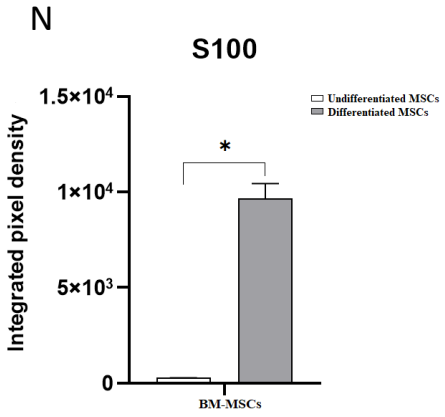

N
S100
*
Undifferentiated MSCs
Differentiated MSCs
Integrated pixel density
1.5×10⁴
1×10⁴
5×10³
0
BM-MSCs

FIG. 5. Immunofluorescence staining of glial markers on undifferentiated and differentiated equine BM-MSCs. Representative images of triple labeling with DAPI (blue) anti GFAP (red) in undifferentiated (**A**, **B**, **C**), and differentiated (**D**, **E**, **F**) BM-MSCs and integrated pixel density analysis (**G**). Representative images of triple labeling with DAPI (blue) anti S100 (red) in undifferentiated (**H**, **I**, **J**), differentiated (**K**, **L**, **M**) BM-MSCs and integrated pixel density analysis (**N**). The glial marker S100 was expressed only in differentiated BM-MSCs The values of the integrated pixel density are represented as the mean ± standard deviation ($p < 0.05$*). Scale bar: 50 μm.

Discussion

Cell-based therapies such as SCs transplantation are promising for the treatment of peripheral nerve injuries [16]. SCs are the main glial cells in the PNS and play a crucial role in nerve regeneration after injury [13]. However, there are two important issues that need to be addressed in SCs transplantation in humans and animals. First, the source of SCs is limited. Second, although extraction and purification of autologous SCs is possible, obtaining SCs in sufficient quantity and of good quality for efficacy is limited [9,17,18]. To solve this issues, previous studies have demonstrated the ability of MSCs to transdifferentiate into cells from the three germ layers, including glial cells [22,33].

Dezawa et al. [34] described the first protocol developed to successfully transdifferentiate rat BM-MSCs into SLCs using chemical methods. Based on this transdifferentiation protocol, several others were developed with some modifications over time using MSCs from different sources and species, such as rats, humans and equines [14,22,35,36]. However, these transdifferentiation procedures are usually complicated and have high costs [25,26].

In the present study, we used a practical approach first reported by Liu et al. [25]. After peripheral nerve injury, several neurotrophic factors such as BDNF, GDNF, NGF and neurotrophin-3 (NT-3) are synthesized by SCs to promote neuroregeneration [10,14,37,38]. In this context, we simulated peripheral nerve injury by sectioning an equine facial nerve into small fragments. These were incubated in culture medium for two days to obtain conditioned medium with factors secreted from the nerve.

After incubation with the induction medium, equine AT-MSCs and BM-MSCs exhibited bipolar to tripolar morphology, thin cytoplasmic extensions and large nuclei, similar to the morphology of SCs. Our results agree with other studies that have used conditioned medium with secreted factors from the sciatic nerve of rats for *in vitro* transdifferentiation of MSCs into SLCs [25-27] as well as with studies that have submitted MSCs, derived from different sources and species to chemical protocols [22,39,40].

SCs secrete several neurotrophic factors that are important molecules that act synergistically, stimulating the proliferation of SCs and promoting neuronal survival and axonal regeneration [41]. Due to the importance of neurotrophic factors for the regeneration of the PNS, we performed the gene expression of BDNF, GDNF and NGF. We observed different gene expression of neurotrophic factors in the two sources of

equine MSCs after the transdifferentiation protocol. While differentiated equine AT-MSCs showed a significant increase in BDNF and GDNF expression, similar to other studies with human AT-MSCs [42,43], differentiated equine BM-MSCs showed a significant difference in the gene expression of GDNF.

Regarding NGF, we did not observe a significant difference in the gene expression of the differentiated cells derived from equine AT-MSCs and BM-MSCs, as well as Faroni et al. [43] in their study with human AT-MSCs after 18 days of transdifferentiation. Some studies report that these results may be attributed to the time of analysis after submitting the cells to different stimuli [43,44].

We observed the gene expression of the glial markers S100β and GFAP in all equine AT-MSCs samples of both groups (undifferentiated and differentiated). However, differentiated cells showed a significant increase in the expression of these markers. These data corroborate experiments carried out with human and rat AT- MSCs [39,45]. Similar results were observed in equine BM-MSC, however in two cell-samples, S100β was expressed only in differentiated cells. Comparing the sources of equine MSCs submitted to the induction medium, we observed that the gene expression of glial markers was significantly higher in equine AT-MSCs, being the best candidates for cell-based therapy in the context of peripheral nerve injuries.

Immunofluorescence staining revealed GFAP expression in undifferentiated and differentiated cells, with a significant increase in the integrated pixel density in the differentiated cells from equine AT-MSCs and BM-MSCs and S100 was only expressed in differentiated equine MSCs from both sources. These results were similar to another study with equine BM-MSCs, in which they observed protein expression of GFAP in undifferentiated and differentiated cells and of S100 only in SLCs [22]. GFAP protein expression in undifferentiated equine MSCs has already been demonstrated by our study group (unpublished data) and is in agreement with another study with equine BM-MSCs [6].

Previous studies using conditioned medium with factors secreted from the rat sciatic nerve to carry out the process of transdifferentiation of rat AT-MSCs and BM-MSCs into SLCs are in agreement with our data regarding the protein expression of S100 only in differentiated cells, but differ in relation to GFAP expression, as they did not observe the expression of this glial marker in undifferentiated cells, only in SLCs [25-27].

Most of the undifferentiated equine MSCs from the two sources used in this study expressed some glial markers, without being subjected to any type of induction, suggesting their potential for differentiation into extra-mesodermal lineages [22]. Similar findings have been reported previously [22,40,46-49].

The expression and/or increase in glial markers in differentiated equine MSCs demonstrates the ability of AT-MSCs and BM-MSCs to transdifferentiate into SLCs. This is a promising strategy to obtain these cells for cell-based therapy in the context of peripheral nerve injuries. Furthermore, after the transdifferentiation process, the differentiated equine AT-MSCs and BM-MSCs showed cell viability means of 86.8% and 92.4%, respectively. This Result that differs from a previous study using a chemical method to transdifferentiate equine BM-MSCs in SLCs that obtained low viability of differentiated cells, 25% [22].

The *in vitro* transdifferentiation success of this study was based on morphological analysis and the expression of SCs markers. Data that corroborate several studies performed with MSCs derived from AT and BM of rats, humans, rabbits and horses submitted to different protocols of transdifferentiation into SLCs [21-23,25,26,39,50].

The expression of glial markers is considered the most relevant indicator to confirm the transdifferentiation of MSCs into SLCs and although no consensus on which to use has yet been established [40,51], S100 and GFAP markers are commonly used to characterize *in vitro* transdifferentiation [8,22,23,25-27,52].

S100 family proteins have several extracellular and intracellular functions, and their presence is associated with the protection of nervous tissue and the nutrition of SCs [53,54]. GFAP is responsible for the cytoskeleton structure of most glial cells and is involved in SCs proliferation [55-57].

To the best of our knowledge, the present study was the first to use a conditioned medium with factors secreted by the equine peripheral nerve to transdifferentiate equine MSCs into SLCs. The lack of identification of the glial markers proteins S100 and GFAP in differentiated equine MSCs using the Western blotting technique and the lack of identification of which factors secreted by the equine facial nerve were fundamental to promote transdifferentiation are the limitations of this study. We have demonstrated the potential *in vitro* for equine AT-MSCs and BM-MSCs transdifferentiation into SLCs, and further studies should be carried out exploring the functionality and homing of these SLCs, in addition to the ability to develop 3D-printed nerve guidance conduits (NGC) functionalized with equine MSCs-derived SLCs for peripheral nerve tissue engineering.

Conclusion

The approach using conditioned medium obtained from equine facial nerve explant culture altered the morphology of equine AT-MSCs and BM-MSCs and upregulated the gene expression of BDNF, GDNF, GFAP and S100β in equine AT-MSCs and GDNF, GFAP and S100β in equine BM-MSCs, as well as the expression and/or increased glial markers protein expression of GFAP and S100. Based on our results we conclude that equine AT-MSCs and BM-MSCs have great transdifferentiation potential into SLCs and are a promising strategy for cell-based therapy for peripheral nerve regeneration.

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Data Availability Statement

The datasets used and/or analyzed during the currentstudy are available from the corresponding author on rea-sonable request.

Author Disclosure Statement

The authors declare no potential conflicts of interest.

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Capítulo 3 – TRABALHO CIENTÍFICO

‘’Tabalho a ser enviado para a revista**:** International Journal of Stem Cells’’

In Vitro Transdifferentiation Potential of Equine Mesenchymal Stem Cells Derived from Umbilical Cord into Schwann-Like Cells: A Preliminary Study

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Abstract

Injuries to the peripheral nervous system of equines can trigger a drop in performance and even death in these animals. Schwann cells (SCs) are essential for the regenerative process. However, its clinical use has limitations. Thus, alternative cellular systems for generating SCs are desired. Umbilical cord-derived mesenchymal stem cells (UC-MSCs) are candidate proliferative cells to transdifferentiate into Schwann-like cells (SLCs), as they can be obtained noninvasively and are abundant and easily accessible. In this context, the aim of this preliminary study was to analyze the transdifferentiation potential of equine UC-MSCs into SLCs. The equine facial nerve explant was cultured in standard DMEM for two days. After this period, the conditioned medium was used as an induction medium. Equine UC-MSCs were incubated with the induction medium for five days. Then, morphological analysis, gene expression of the glial markers S100β and GFAP (glial fibrillary acidic protein), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and protein expression of S100 and GFAP were performed. No morphological changes were observed in cells submitted to the induction medium. There was a significant increase in GDNF gene expression, and none of the samples expressed GFAP, but S100β gene expression was observed in all samples post-differentiation. Immunofluorescence revealed GFAP protein expression in undifferentiated and differentiated cells. However, S100 expression was not observed in either group. Therefore, our results were not consistent and equine UC-MSCs did not transdifferentiate into SLCs using this protocol.

Keywords: Facial nerve, Nerve regeneration, Regenerative medicine, Schwann-like cells

Introduction

Peripheral nervous system (PNS) injuries are relatively frequent and can trigger neuropathic pain, loss of motor, sensory or autonomic function in denervated body segments and, consequently, loss of quality of life in animals and humans (1-4).

In humans, epidemiological studies estimate an incidence rate of PNS injury between 13 and 23 individuals per 100.000 inhabitants per year in developed countries (5).

In horses, these injuries occur due to traumatic, metabolic, toxic, infectious and degenerative diseases (6,7) resulting in a drop in performance and even the death of these animals (3). Facial nerve paralysis is one of the main peripheral neuropathies in horses, with 31% of cases resulting from traumatic events (8).

After PNS injury, Schwann cells (SCs) proliferate, differentiate into a repair phenotype, and produce a variety of neurotrophic factors and cytokines to provide a suitable microenvironment for neuroregeneration (9). However, for its acquisition and use in clinical practice, it is necessary to sacrifice a healthy functional nerve from the donor, in addition to the fact that the proliferation of these cells *in vitro* is associated with a long period of cultivation and expansion (4).

In this context, the ability to use highly proliferative and accessible cells to produce cells with SCs properties is of great clinical relevance (10). Positive results have been previously demonstrated using co-culture with SCs or chemical protocols for transdifferentiation *in vitro* of rat, human, equine mesenchymal stem cells (MSCs) into Schwann-like cells (SLCs) (3,11-14). However, these transdifferentiation methods are complex and have high costs (15,16).

Currently, a practical method has been proposed based on the use of a conditioned culture medium with neurotrophic factors secreted from the sciatic nerve of rats, to

perform the transdifferentiation of rat MSCs derived from adipose tissue (AT-MSCs) and bone marrow (BM-MSCs) into SLCs (15-17).

Umbilical cord-derived MSCs (UC-MSCs) are proliferative cells that are candidates to differentiate into SLCs, as they can be obtained noninvasively, are abundant and easily accessible, and are one of the most practical sources for cell therapy (10,18).

Thus, the aim of this study was to evaluate the *in vitro* transdifferentiation potential of equine UC-MSCs into SLCs using a conditioned medium obtained from equine facial nerve explant culture, in order for future cell-based therapy for neuroregeneration of peripheral nerve injuries.

Materials and Methods

Animal ethics

All stages of this study were approved by the Ethics Committee on Animal Use of São Paulo State University (UNESP) - Botucatu (Protocol 0039/2021-CEUA).

Facial nerve collection and preparation of induction medium

A facial nerve of an equine, Mangalarga, 3-year-old, female was collected immediately after the animal was submitted to the euthanasia procedure due to permanent recumbency. Under aseptic conditions, the nerve was collected and placed in a 50 ml sterile conical tube containing *Dulbecco's Phosphate Buffered Saline* (DPBS) solution (LGC Biotecnologia, São Paulo, Brazil), 3% penicillin/streptomycin and 0.5% amphotericin (both from Gibco, Grand Island, New York, USA) and used in the preparation of the induction medium.

We used the methodology described by Liu et al. (15) for preparation of the induction medium, with some modifications. The facial nerve was washed with DPBS

(LGC Biotecnologia, São Paulo, Brazil) containing 1% penicillin/streptomycin (Gibco, Grand Island, New York, USA). Excess tissue adhered to the nerve was removed and the nerve was cut into 1 cm fragments within the laminar flow. Each fragment was soaked in 15 ml of complete culture medium containing 90% Dulbecco's Modified Eagle's Medium (DMEM)/F12 (LGC Biotecnologia, São Paulo, Brazil), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% amphotericin (all Gibco, Grand Island, New York, USA) at 37.5°C and 5% CO2 for 48 hours. After this period, the culture medium was aspirated and filtered with a 70 µm cell filter (BD Falcon, San Jose, CA, USA) and with a 0.22 µm syringe filter (Sarstedt, Nümbrecht, Germany) and cryopreserved at -80ºC for the next steps (Fig. 1).

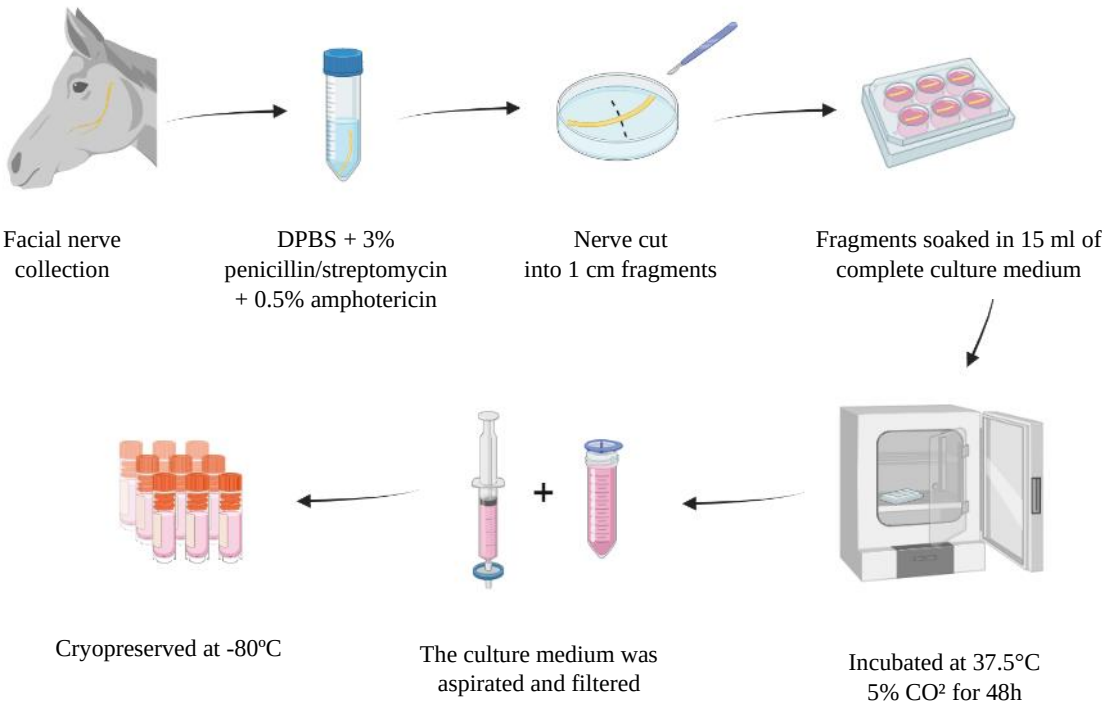

Fig. 1. Facial nerve collection and preparation of induction medium. Images created with biorender (https://biorender.com/).

Mesenchymal stem cells

Low passage equine MSCs (P3-P4) derived from UC (n=4) obtained from the cell bank of our research group were used. These cells were previously immunophenotypically characterized by the expression of markers CD44, CD90, CD105, lack of expression of markers CD34 and MHC-II and by the ability to differentiate into osteogenic, chondrogenic and adipogenic mesenchymal lineages (19).

Transdifferentiation of mesenchymal stem cells into Schwann-like cells

For transdifferentiation, samples of UC-MSCs were thawed and resuspended in complete culture medium. UC-MSCs were seeded at a density of $1x10^4$ cells per well in 24-well plates (Kasvi, São José do Pinhais, PR, Brazil) with a 0.5 ml volume of complete culture medium containing 90% Dulbecco's Modified Eagle's Medium (DMEM)/F12 (LGC Biotecnologia, São Paulo, Brazil), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1 % amphotericin (all Gibco, Grand Island, New York, USA).

Culture medium was removed from the subconfluent UC-MSCs cultures and replaced with 0.5 ml of prewarmed induction medium in each well. Cells were incubated in this condition for five days at 37.5ºC and 5% CO2. The induction medium was changed every 2-3 days. UC-MSCs cultivated in complete culture medium were called undifferentiated, and those cultivated with induction medium, differentiated.

Morphology evaluation

The morphology of equine MSCs was evaluated by inverted microscopy (LEICA DMIRB). Photomicrographs of control cells and cells cultured with induction medium were captured throughout the transdifferentiation period.

Gene expression - qPCR

The gene expression of neurotrophic factors (BDNF, GDNF) and glial markers (S100β and GFAP) was quantified in undifferentiated and differentiated equine UC-MSCs. Cells were lysed and homogenized with TRIzol reagent (TRIzol™, Invitrogen™, ThermoFisher Scientific, São Paulo, Brazil), and RNA extraction was performed with the same reagent according to the manufacturer's instructions. The RNA was eluted with RNA-free water, quantified and analyzed by spectrophotometry with Thermo Scientific NanoDrop 2000 equipment (ThermoFisher Scientific, Wilmington, USA) for absorbance ratios of 260/280 nm and 260/230 nm. The total RNA extracted from the cells was of high quality and purity, indicating that the extraction method was efficient. cDNA synthesis was performed with the reagents of the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Life Technologies Corporation, Carlsbad, USA), according to the manufacturer's instructions, and reverse transcription was performed to obtain cDNA with the Veriti 96 Well Thermal Cycler (Applied Biosystems™, ThermoFisher Scientific), under the following thermal cycling conditions: 10 minutes at 25ºC; 12 minutes at 37°C; and 5 minutes at 85°C. The cDNA samples were cryopreserved at -80 ºC and used as templates for PCR reactions.

Reactions were performed in duplicate using cDNA produced previously with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, Life Technologies, Carlsbad, CA, USA), RNA-free water, and equine primers (Thermo Fisher Scientific, São Paulo, Brazil), which were designed using Primer Express™ Software v3.0.1 (Applied Biosystems™, Thermo Fischer Scientific), as shown in Table 1.

Samples were tested with four reference genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT), beta -2-microglobulin (B2M) and beta-actin (ACTB). The mean cycle threshold (CT) of

the four reference genes was used. The real-time polymerase chain reaction (qPCR) method was performed with the QuantStudio™ 12K Flex Real-Time PCR System thermocycler (Applied Biosystems™, Thermo Fischer Scientific) with the following parameters: 50°C for 2 minutes, 95 °C for 2 minutes and 40 cycles of 95 °C for 1 second and 60 °C for 30 minutes with a subsequent dissociation curve. The relative quantification of the expression of the genes of interest was performed using the ΔΔCt method (20).

Table 1. Primers used in qPCR reactions

Gene	Forward primer	Reverse primer
BDNF[a]	TTGGATGAGGGCCAGAAAGT	CAAGTCCGCGTCCTTACTGTT
GDNF[b]	CAGGGACTCTTCCTCCATCCT	TGGGCACGAGCATGTTTCT
S100β	CGCTCATGGCTATTGGTGAA	TCGTGGATACAAGAGCAAAAGTT
GFAP[c]	GAAACCAGCCTGGACACCAA	TCACCACGATGTTCCTCTTGAG
ACTB[d]	CGGCGGCTCCATTCTG	CTGCTTGCTGATCCACATCTG
B2M[e]	CACCCAGCAGAGAATGGAAAG	CGGATGGAACCCAGAGACA
HPRT[f]	GCTCGAGATGTGATGAAGGAGAT	CCCCCTTGAGCACACAGAGT
GAPDH[g]	GGCAAGTTCCATGGCACAGT	GGGCTTTCCGTTGATGACAA

[a]Brain-derived neurotrophic fator; [b]Glial cell-derived neurotrophic fator; [c]Glial fibrillary acidic protein; [d]Beta-actin; [e]Beta-2-microglobulin; [f]Hypoxanthine-guanine phosphoribosyltransferase; [g]Glyceraldehyde 3-phosphate dehydrogenase.

Immunofluorescence

The protein expression of glial markers was determined by immunofluorescence staining. Equine UC-MSCs were seeded at a density of $1x10^4$ cells/cm^2 on 13 mm round coverslips (Kasvi, São José do Pinhais, PR, Brazil) previously treated with Poly-L-Lysine (Sigma-Aldrich®, Saint Louis, MO, USA) in na adherent 24-well plate (Kasvi, São José

do Pinhais, PR, Brazil) and the transdifferentiation process was performed. After five days of transdifferentiation, undifferentiated and differentiated equine MSCs were fixed with phospate buffered saline (PBS) + 2% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 10 min at room temperature and blocked with 2% bovine serum albumin for 20 min (all from Sigma-Aldrich®, Saint Louis, MO, USA).

Cells were incubated overnight at 4°C with antibodies against S100 (polyclonal rabbit anti-S100, DAKO® GA504, 1:200) GFAP (polyclonal rabbit anti-GFAP, DAKO® GA524, 1:200). Subsequently, the cells were washed with PBS and incubated with goat anti-rabbit IgG H&L (Alexa Fluor® 594) (Abcam, ab150080) secondary antibody for 1 hour at room temperature. Vectashield mounting medium with DAPI reagent (Vector Laboratories, Burlingame, CA, USA) was used for mounting.

In the qualitative analysis of S100 and GFAP immunofluorescence, representative images of undifferentiated and differentiated cells were obtained using a LEICA TCS SP8 confocal microscope with LAS X software.

Statistical analysis

The relative quantification variables was assessed for normality using statistical tests (Shapiro–Wilk), descriptive statistics, and graphic analysis. For parametric data, the t-test was performed with unpaired samples. The level of significance between the groups was set at $p < 0.05$. The differences are denoted by a single asterisk ($p < 0.05$). (GraphPad Prism version 8 for Mac, San Diego, USA).

Results

Morphological changes

No morphological changes were observed when comparing undifferentiated and differentiated equine UC-MSCs with induction medium at any time during the five days of the transdifferentiation process (Fig. 2).

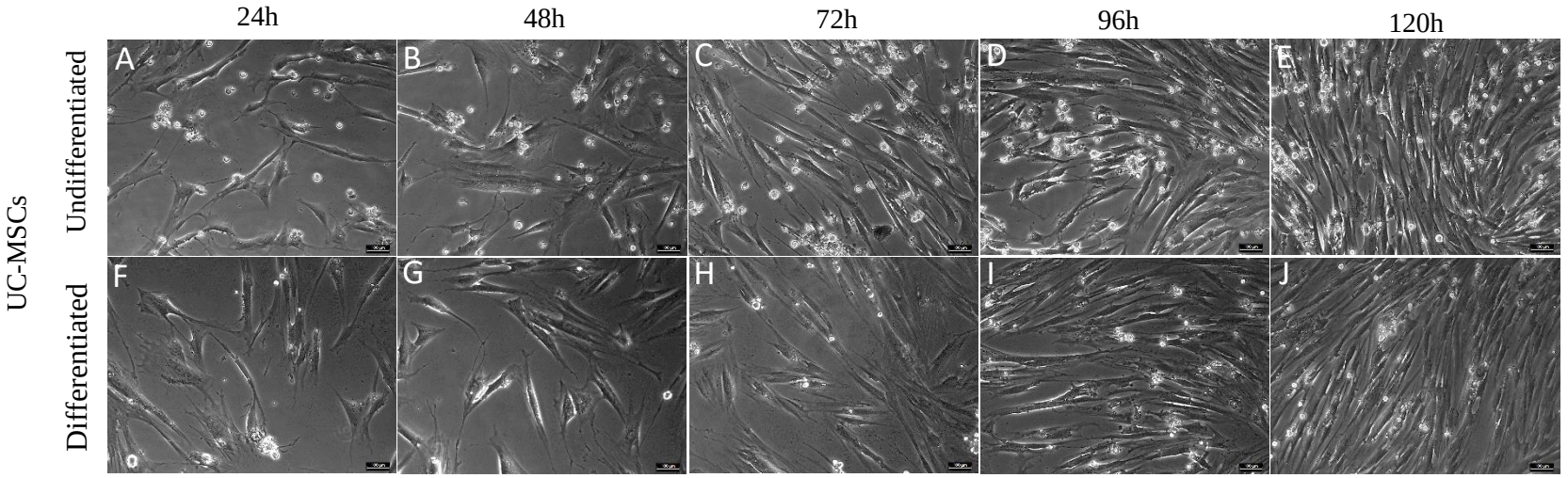

Fig. 2. Morphological characterization of equine UC-MSCs. Undifferentiated equine UC-MSCs after 24 h, 48 h, 72 h, 96 h and 120 h of culture (**A**, **B**, **C**, **D**, **E**, respectively), showing fibroblastoid morphology. Differentiated equine UC-MSCs cultured with induction medium after 24 h, 48 h, 72 h, 96 h and 120 h (**F**, **G**, **H**, **I**, **J**, respectively), in which no morphological changes were observed in relation to undifferentiated UC-MSCs. Magnification, x200. Scale bar = 100µm.

Gene expression of neurotrophic factors and glial markers

The gene expression of the neurotrophic factors BDNF and GDNF is represented in Fig. 3. No significant difference was observed in the evaluation of BDNF between undifferentiated and differentiated UC-MSCs. However, there was a significant increase in the expression of the GDNF gene in the differentiated UC-MSCs cultured with induction medium compared to the undifferentiated UC-MSCs.

Regarding glial markers, we did not observe the expression of S100β in undifferentiated UC-MSCs cells, however, all differentiated cells expressed this marker. GFAP was not expressed in any of the samples from either group.

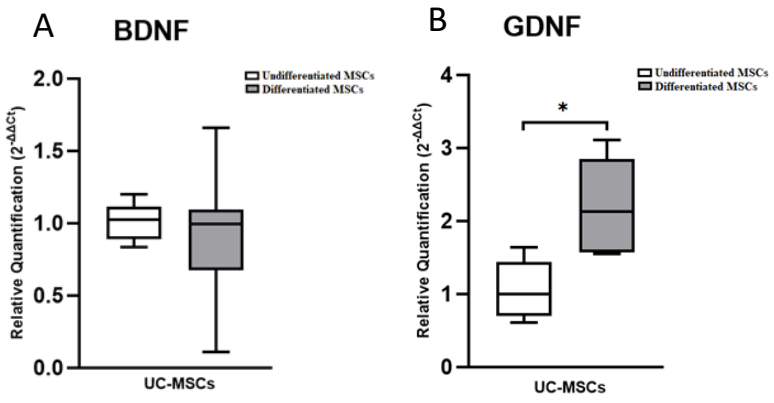

Fig. 3. Relative expression of BDNF and GDNF in undifferentiated and differentiated equine UC-MSCs. BNDF (**A**) and GDNF (**B**). Relative quantification values are represented as the mean, interquartile ranges and minimum and maximum values ($p < 0.05$*).

Immunofluorescence

Protein expression of glial markers by immunofluorescence staining in UC-MSCs is represented in Fig. 4. Immunofluorescence staining revealed GFAP expression in

undifferentiated and differentiated UC-MSCs. However, S100 expression was not observed in any of the samples from either group.

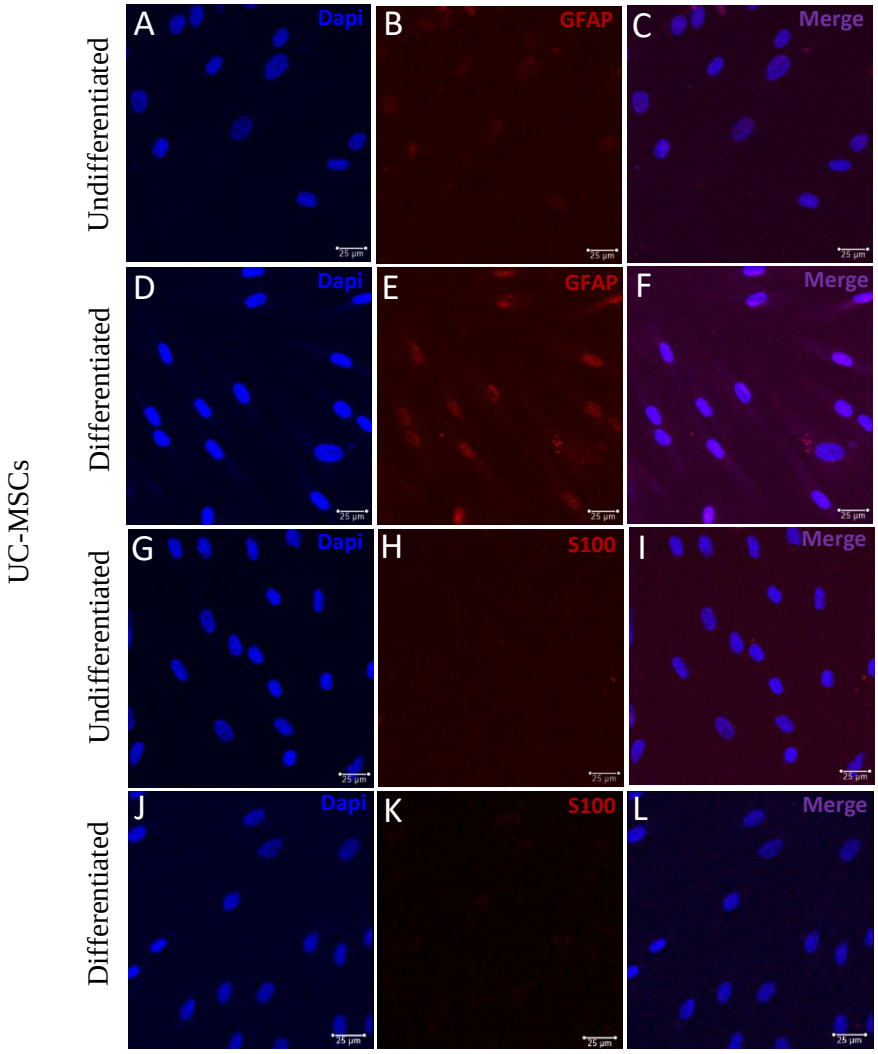

Fig. 4. Immunofluorescence staining of glial markers in undifferentiated and differentiated equine UC-MSCs. Representative images of triple labeling with DAPI (blue) anti GFAP (red) in undifferentiated (**A**, **B, C**) and differentiated UC-MSCs (**D, E, F**). The glial marker GFAP was expressed in both groups. Representative images of triple labeling with DAPI (blue) anti S100 (red) in undifferentiated (**G**, **H, I**) and differentiated UC-MSCs (**J**, **K**, **L**). S100 expression was not observed in any of the groups. Scale bar: 25 µm.

Discussion

UC-MSCs are being used in several studies, as they can be obtained noninvasively, are abundant and have the potential to differentiate into other types of cells (10,18,21).

Previous studies have demonstrated the ability to transdifferentiate rat AT-MSCs and BM-MSCs into SLCs using conditioned medium with neurotrophic factors secreted from the sciatic nerve of rats (15-17). The present study is the first description of the application of a similar method with equine facial nerve to induce equine UC-MSCs into SLCs *in vitro*.

No morphological changes were observed in UC-MSCs cultured with induction medium compared to undifferentiated cells. These results differ from those of other studies that used chemical protocols (10,22) and from reports using conditioned medium with factors secreted from the sciatic nerve of rats to perform transdifferentiation (15-17).

qPCR revealed no significant difference in BDNF gene expression when comparing the two groups in this study, however there was a significant increase in the expression of GDNF in differentiated equine UC-MSCs. These results differ from those of other studies that observed an increase in the expression of both neurotrophic factors after transdifferentiation (23). GDNF is an important neurotrophic factor secreted by SCs after peripheral nerve injury, and is responsible for aiding neuronal survival, axon regeneration and SCs proliferation (24).

Regarding glial markers, we found different results for gene and protein expression. qPCR revealed the gene expression of S100β in all equine UC-MSCs submitted to the induction medium. However, GFAP expression was not observed in any of the samples in either group. These results that differ from our previous studies that

demonstrated GFAP gene expression in undifferentiated and differentiated equine AT-MSCs and BM-MSCs from both sources (unpublished data).

Both glial markers are commonly used to characterize transdifferentiation into SLCs *in vitro* and can be identified by immunostaining (3,15,22, 25). However, in immunofluorescence staining, we observed only the expression of GFAP in both groups, but we did not observe the expression of S100, considered a classic marker of SCs (26).

We used qPCR and immunofluorescence staining to confirm that UC-MSCs cultured with induction medium could produce protein indicators characteristic of SCs. However, our results were not consistent and demonstrated that UC-MSCs did not transdifferentiate into SLCs using conditioned medium from equine facial nerve explant culture. Data that differ from other studies using similar conditioned medium to perform the transdifferentiation of rat AT-MSCs and BM-MSCs (15-17).

To the best of our knowledge, the present study was the first to use a conditioned medium with factors secreted by the equine peripheral nerve to transdifferentiate equine UC-MSCs into SLCs. However, our results were not consistent and did not demonstrate the transdifferentiation of equine UC-MSCs into SLCs. The lack of identification of glial marker proteins S100 and GFAP in equine MSCs differentiated using the Western blotting technique and morphological analysis of equine cells differentiated using scanning electron microscopy are the limitations of this study. Further studies should be performed to evaluate the transdifferentiation potential of equine UC-MSCs into SLCs.

Conclusion

The approach using conditioned medium obtained from equine facial nerve explant culture did not change the morphology of equine UC-MSCs, upregulated the gene expression of GDNF and showed the gene expression of S100β in all samples. The

evaluation of protein expression revealed the expression of GFAP in undifferentiated and differentiated cells. However, S100 expression was not observed in either group. Therefore, our results were not consistent and did not demonstrate the transdifferentiation of equine UC-MSCs into SLCs.

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Potential Conflict of Interest

The authors declare no potential conflicts of interest.

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CAPÍTULO IV

DISCUSSÃO GERAL

As lesões do SNP são condições clínicas comuns em equinos desencadeadas por processos traumáticos, doenças metabólicas, tóxicas, enfermidades infecciosas e degenerativas (SCHWARZ et al., 2008; VILLAGRÁN et al., 2014). A paralisia do nervo facial é considerada uma das neuropatias mais comum nesta espécie, sendo 31% dos casos decorrentes de eventos traumáticos (BOORMAN et al., 2020).

A queda do desempenho, o comprometimento na apreensão alimentar, a ocorrência de alterações permanentes no órgão-alvo, como atrofia muscular, lesões oculares e até mesmo a morte dos animais, tornam a pronta recuperação essenciais (VILLAGRÁN et al., 2016; BOORMAN et al., 2020). Atualmente, a conduta terapêutica nesta espécie baseia-se principalmente na utilização de antiinflamatórios, reabilitação e eletroacupuntura (FOURMESTRAUX et al., 2014; JEONG et al., 2015; VILLAGRÁN et al., 2016), entretanto muitas vezes não apresentam resultados satisfatórios (VILLAGRÁN et al., 2016).

Nesse contexto o desenvolvimento de novos tratamentos que auxiliem na regeneração de nervos periféricos lesionados apresenta grande relevância clínica (FARONI et al., 2015). A terapia celular é uma abordagem que desperta interesse na medicina regenerativa e tem sido proposta como uma estratégia promissora para a neurorregeneração (YU et al., 2021). Nas lesões de nervos periféricos, as CS são essenciais no auxílio da regeneração, pois proliferam e secretam diversos fatores neurotróficos como o NGF, BDNF, GDNF, neurotrofina- 3 (NT-3), criando um microambiente favorável para a regeneração (DE LA ROSA et al., 2018; CAILLAUD et al., 2019; GONÇALVES et al., 2019; HOPF et al., 2020), contudo sua aquisição e utilização clínica está associada à uma série de limitações (PIOVESANA et al., 2021).

Com intuito de superar essas limitações, diversos estudos demonstraram a capacidade de transdiferenciação de CTM de diferentes espécies e fontes em CSL, geralmente utilizando protocolos químicos ou por meio de co-cultura com CS (LI et al., 2009; VILLAGRÁN et al., 2016; FU et al., 2019; JUAN et al., 2020; HOPF et al., 2020). No entanto, esses métodos de transdiferenciação em geral, são complicados e apresentam custos elevados (LIU et al., 2013; TAWAB et al., 2018).

No presente estudo, utilizamos uma metodologia mais prática para verificar o potencial de transdiferenciação das CTM equinas em CSL. Incubamos os fragmentos de nervo facial equino em meio de cultura completo para formar um meio condicionado com fatores secretados do nervo facial e utilizamos este meio de indução para realizar a transdiferenciação.

Observamos mudanças morfológicas nas CTM-TA e CTM-MO equinas assim como descrito em outros estudos utilizando meio condicionado semelhante com fatores secretados do nervo isquiático de ratos para realizar o processo de transdiferenciação (LIU et al., 2013; GALHOM et al., 2018; TAWAB et al., 2018). No entanto, não verificamos alterações morfológicas nas CTM-CU equinas submetidas ao meio de indução.

Em relação a expressão gênica dos fatores neurotróficos, as CTM-TA equinas diferenciadas mostraram um aumento significativo na expressão de BDNF e GDNF, semelhante a estudos anteriores (KINGHAM et al., 2014; FARONI et al., 2016). Contudo, as CTM-MO e CTM-CU diferenciadas apresentaram aumento significativo apenas na expressão de GDNF. Após lesão de nervos periféricos, os fatores neurotróficos BDNF e GDNF são moléculas importantes que atuam em sinergismo, promovem a proliferação de CS, influenciam a sobrevivência neuronal e a regeneração axonal (ALLODI et al., 2012).

Verificamos a expressão gênica dos marcadores gliais S100β e GFAP em todas as CTM-TA equinas indiferenciadas e diferenciadas. Contudo, as células diferenciadas apresentaram um aumento considerável na expressão gênica desses marcadores. Dados que estão de acordo com outros estudos (RAZAVI et al., 2012; FU et al., 2016). Resultados semelhantes foram encontrados ao analisarmos as CTM-MO equinas, porém em duas amostras S100β foi expresso apenas em células diferenciadas. Já nas CTM-CU equinas, constatamos a expressão gênica de S100β em todas as amostras submetidas ao meio de indução. No entanto, a expressão de GFAP não foi observada em nenhuma das amostras.

A coloração por imunofluorescência revelou expressão de GFAP em células indiferenciadas e diferenciadas nas CTM-TA, CTM-MO e CTM-CU equinas. S100 é considerado um marcador clássico de SC (PAN et al., 2012) e observamos a sua expressão apenas nas CTM-TA e CTM-MO equinas diferenciada, resultado não observado nas CTM-CU equinas diferenciadas.

Comparando as fontes de CTM equinas neste estudo, a expressão gênica dos marcadores gliais GFAP e S100β foi significativamente maior nas CTM-TA equinas diferenciadas. Nossos resultados demonstram que as CTM-TA equinas submetidas ao meio de indução são as melhores candidatas para estudos futuros.

Apesar da abordagem realizada no presente estudo tenha sido eficiente para promover a transdiferenciação das CTM-TA e CTM-MO equinas em CSL, mais estudos devem ser realizados com intuito de identificar quais fatores secretados do nervo periférico são responsáveis por promover a transdiferenciação.

Além disso, no nosso estudo, utilizamos um nervo facial equino saudável para obter o meio de indução e realizar a transdiferenciação. Mas sabe-se que após uma lesão de nervo periférico, as CS se convertem nas chamadas CS de reparo (NOCERA; JACOB, 2020) e promovem a degradação de restos de mielina e detritos celulares, além da secreção de diversas citocinas e fatores neurotróficos (JESSEN; MIRSKY, 2019). Dessa forma, uma alternativa para próximos estudos seria a utilização de fragmentos de nervos periféricos equinos lesionados para cria um meio condicionado com fatores secretados desse nervo.

CONCLUSÕES GERAIS

O protocolo de transdiferenciação de CTM-TA e CTM-MO equinas mostrou-se eficiente, com base nos resultados observados de alteração morfológica, aumento da expressão gênica de BDNF, GDNF, GFAP e S100β nas CTM-TA e GDNF, GFAP e S100β nas CTM-MO, bem como expressão e/ou aumento da expressão proteica de marcadores gliais GFAP e S100 em ambas as fontes. Diferentemente das CTM-CU equinas, as quais não apresentaram o mesmo potencial. Nossos resultados demonstram que as CTM-TA equinas submetidas ao meio de indução são as melhores candidatas para estudos futuros explorando a funcionalidade e homing dessas CSL, além da capacidade de desenvolver condutos de orientação nervosa (NGC) impressos em 3D funcionalizados com CSL derivadas de CTM-TA equinas para engenharia de tecido nervoso periférico.

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ATESTADO

Atesto que o Projeto "Potencial de diferenciação in vitro de células tronco mesenquimais equinas em células Schwann-like" **Protocolo CEUA 0039/2021** , a ser conduzido por Lucas Vinícius de Oliveira Ferreira, responsável/orientador Rogério Martins Amorim , para fins de pesquisa científica/ensino - encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 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.

Finalidade	PESQUISA CIENTÍFICA
Vigência do projeto	03/05/2021 a 28/02/2022
Nome Comum / Espécie / Linhagem	/ /
Raça	
Nº de animais machos	0
Nº de animais fêmeas	0
Nº de animais sexo indefinido	0
Peso médio de animais machos	0
Peso médio de animais fêmeas	0
Peso médio de animais sexo indefinido	0
Idade	ano(s) e 0 mes(es) e 0 dia(s).
Procedência	Células do Professor Rogério Martins Amorim

Projeto de Pesquisa aprovado em reunião da CEUA em 15/04/2021

JOSÉ NICOLAU PRÓSPERO PUOLI FILHO
Presidente da CEUA da FMVZ, UNESP - Campus de Botucatu