UNIVERSIDADE FEDERAL DA BAHIA
INSTITUTO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA

TRABALHO DE TESE

BRYSA MARIANA DIAS SILVEIRA

ASPECTOS REGENERATIVOS E IMUNOMODULATÓRIOS DAS CÉLULAS ESTROMAIS MESENQUIMAIS DO TECIDO ADIPOSO EM MODELO MURINO DE ANEMIA FALCIFORME

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Tese apresentada ao Programa de Pós-graduação em Imunologia, da Universidade Federal da Bahia como requisito para obtenção do título de Doutor em Imunologia.

Orientador: Prof. Dr. Vitor Fortuna
Coorientadora: Profª. Drª Songeli Menezes Freire

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ATA DA SESSÃO PÚBLICA DO COLEGIADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA PARA JULGAMENTO DO TRABALHO DE TESE **INTITULADO "ASPECTOS REGENERATIVOS E IMUNOBIOLÓGICOS DAS CÉLULAS ESTROMAIS MESENQUIMAIS DO TECIDO ADIPOSO EM MODELOS MURINO DE CICATRIZAÇÃO"**DA DOUTORANDA **BRYSA MARIANA DIAS SILVEIRA**.

Ao oitavo dia do mês de agosto do ano de dois mil e vinte e três, na sala de reunião vinculada ao Programa de Pós-graduação em Imunologia da Universidade Federal da Bahia (PPGIm-UFBA), a Banca Examinadora composta pelos Professores: **Dr. Vitor Antônio Fortuna** Orientador**, Dr. Bruno Solano de Freitas Souza, Dr. Jaime Ribeiro Filho, Dra. Elisângela Vitória Adorno** e **Dra. Natalia Machado Tavares**se reúniram com a finalidade de discutir, avaliar e julgar o trabalho de tese intitulado**"Aspectos regenerativos e imunológicos das Células Estromais Mesenquimais do tecido adiposo em modelos murino de cicatrização"**da doutoranda **Brysa Mariana Dias Silveira.** Após a apresentação, argüição e comentários dos membros da Banca Examinadora ficam determinados que as sugestões discutidas sejam implementadas na versão final da tese. Havendo cumprido as exigências do Programa quanto à defesa do trabalho final, a Banca Examinadora conclui que mediante a entrega do exemplar final pelo aluno com as devidas modificações no prazo de 60 dias, a Pós-Graduanda está habilitada à obtenção do título de Doutora em Imunologia. Adicionalmente, os pareceres individuais dos membros da Banca Examinadora serão anexados à ata. Nada mais havendo a tratar se encerra a sessão da qual é lavrada a presente ata que após lida e aprovada vai assinada pelos componentes da Banca examinadora, pela doutoranda e pela Coordenadora do Programa de Pós-Graduação. Salvador, oito de agosto do ano de dois mil e vinte e três.

Dr. Vitor Antônio Fortuna
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Dedico este trabalho aos meus pais, Mara Gleide e José Silveira. Sem vocês nada disso seria possível!

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“E num campo de batalha um guerreiro brilhou
Era Ogum me ensinando a lutar e vencer”
Música Ogum (Rosa Amarela)

RESUMO

Úlceras crônicas de perna (CLU) são complicações microvasculares comuns em pacientes com anemia falciforme. CLUs são recalcitrantes e na ausência de tratamento podem levar à amputação dos membros inferiores. As células estromais do tecido adiposo (ASC) representam uma alternativa para o tratamento de CLUs, devido a sua capacidade de secretar mediadores solúveis envolvidos no reparo tecidual. Desta forma, avaliamos os efeitos do secretoma de ASC em modelo *in vitro* de células endoteliais do cordão umbilical (HUVECs) e em modelo *in vivo* com camundongos C57BL/6 transgênicos e camundongos Towness falciformes. Nosso trabalho observou que o secretoma de ASC pré-condicionado em normóxia (condição com 20% de oxigênio) e hipóxia (condição com 5% de oxigênio) apresentou em sua composição marcadores de regeneração tecidual (ex: VEGF, MCP1, IL-8 e Angiogenina). A análise *in vitro* demonstrou que ambos os meios condicionados (MCs) exerceram ação anti-apoptótica e angiogênica em HUVECs, com melhor desempenho das células tratadas com MC em normóxia. Nosso modelo *in vivo* utilizando camundongos transgênicos Nestin-GFP+/NG2-DsRed+ revelou aceleração do processo cicatricial de feridas tratadas com os MCs. Considerando que o nosso produto terapêutico apresentou melhor desempenho em condições de normóxia, analisamos o potencial terapêutico do MC normóxia em modelo de cicatrização com camundongos falciformes. Neste trabalho, buscamos avaliar o efeito pró-angiogênico e imunomodulatório em peles tratadas com o MC através das técnicas de imunohistoquímica, imunofluorescência e RT-PCR. Nossos resultados revelaram que a administração do MC foi capaz de estimular o processo de cicatrização através da redução da inflamação local, aumento do número de fibroblastos e maior produção de colágeno, bem como através da redução de transcritos inflamatórios, evidenciando o efeito terapêutico do MC sobre a imunomodulação.

Palavras-chave: Anemia falciforme. Úlceras crônicas de pernas. Angiogênese. Imunomodulação. Células estromais mesenquimais.

ABSTRACT

Chronic leg ulcers (CLU) are common microvascular complications in patients with sickle cell anemia. CLUs are recalcitrant and the absence of treatment can lead to lower limb amputation. Adipose tissue stromal cells (ASC) represent an alternative for the treatment of CLUs, due to their ability to secrete soluble mediators involved in tissue repair. Thus, we evaluated the effects of the ASC secretome *in vitro* model of umbilical cord endothelial cells (HUVECs) and *in vivo* model with transgenic Nestin-GFP+/NG2-DsRed+ mice and sickle Towness mice. Our work found that the secret of ASC preconditioned in normoxia (condition with 20% oxygen) and hypoxia (condition with 5% oxygen) presented in its composition markers of tissue regeneration (eg: VEGF, MCP1, IL-8 and angiogenin). *In vitro* analysis demonstrated that both conditioned media (CMs) exerted anti-apoptotic and angiogenic action on HUVECs, with better performance of cells treated with CM in normoxia. Our *in vivo* model using C57BL/6 transgenic mice revealed radiation from the healing process of wounds treated with MCs. Considering that our therapeutic product performed better under conditions of normoxia, we analyzed the therapeutic potential of MC normoxia in wound healing model with sickle cell mice. In this work, we sought to evaluate the pro-angiogenic and immunomodulatory effect in skins treated with CM using immunohistochemistry, immunofluorescence and RT-PCR techniques. Our results revealed that CM administration was able to stimulate the healing process by reducing local inflammation, increasing the number of fibroblasts and increasing collagen production. Just as we identified reduced expression of inflammatory transcripts, evidencing the therapeutic effect of MC on immunomodulation.

Keywords: Sickle cell anemia. Chronic leg ulcers. Angiogenesis. Immunomodulation. Mesenchymal stromal cells.

LISTAS

Tabelas

Figuras

Abreviaturas

AF	Anemia Falciforme
ALT	Alamina aminotransferase
Ang-1	Angiogenina 1
ARG-1	Arginina 1
ASC	Do inglês Adipose derived Stromal Cells
α-SMA	Alfa actina de músculo liso
BNDF	Fator neurotrófico derivado do cérebro
BrdU	5-Bromo-2'-deoxyuridine
Bregs	Linfócitos B reguladores
CCL2	Ligante de quimiocina 2

CCL4	Ligante de quimiocina 2
CCR1	Receptor de quimiocina C-C tipo 1
CCR2	Receptor de quimiocina C-C tipo 2
CD105	Endoglina
CD14	Glicoproteina de membrana expressa em leucócitos
CD146	Molécula de adesão celular de melanoma
CD206	Receptor da manose
CD29	Integrina beta 1
CD34	Marcador de células hematopoiéticas
CD40	Proteína transmembrana tipo 1
CD45	Proteína tirosina fosfatase
CD73	5'-nucletidase
CD90	Gliproteína ligada a GPI
células NK	Células Natural Killer
CM	Do inglês conditioned Medium
CMMO	Células mononucleares da medula óssea
DCs	Células dentríticas
DF	Doença Falciforme
DNA	Ácido desoxirribonucleico
ECM	Matriz extracelular
EGF	Fator de crescimento epidermal
Evs	Vesículas extracelulares
FBS	Do inglês fetal bovine serum
FGF	Fator de crescimento de fibroblastos
FVS	Fração vascular estromal
GM-CSF	Fator estimulante de colônia de granulócitos-macrófagos
hASC	Do inglês human adipose tissue-derived stem cells
Hb	Hemoglobina
HbA	Hemoglobina A
HbC	Hemoglobina C
HbD	Hemoglobina D
HbF	Hemoglobina Fetal
HbS	Hemoglobina S
HCM	Meio condicionado hipóxia
HE	Do inglês hematoxylin-eosin
HGF	Fator de crescimento de hepatócitos
HIF-1	Fator induzivel por hipóxia 1
HLADR	Receptor de superfície celular MHC classe II
HUVECs	Do ingês human umbilical vein endothelial cells
IDO	Indoleamina 2,3-dioxigenase 1
IFN-γ	Interferon gama
IGF1	Fator de crescimento semelhantes à insulina
IGF2	Fator de crescimento semelhantes à insulina
IGFBP3	Proteína ligadora IGF-1 tipo 3
IL-10	Interleucina 10
IL-12	Interleucina 12
IL-17	Interleucina 17
IL-1α	Interleucina 1 alfa
IL-1β	Interleucina 1 beta
IL-2	Interleucina 2
IL-4	Interleucina 4
IL-6	Interleucina 6
IL-8	Interleucina 8
LPS	Lipopolissacarídeo
LTBR4	Leucotrieno B4
LY6C	Do inglês lymphocyte antigen 6 family member C1
MC	Meio condicionado
MCP-1	Proteína quimioatraente de monócitos 1

MHC	Complexo principal de histocompatibilidade
MMP-9	Metaloproteinase 9
MSC	Do inglês Mesenchymal Stromal Cells
NCM	Meio condicionado normoxia
NG2	Antígeno neuronal glial 2
NOS-2	Óxido nítrico sintetase 2
OMS	Organização Mundial de Saúde
Osm	Oncostatina M
PDGF	Fator de crescimento derivado de plaquetas
PDGFRB	Receptor beta do fator de crescimento derivado de plaquetas
PFA	Paraformaldeido
PGE2	Mediadores prostaglandina E2
PI	Do inglês propidium iodide
PIGF	Proteína de classe F da biossíntese de fosfatidilinositol glicano
PTX3	Pentraxina 3
RT-PCR	Transcrição reversa da reação em cadeia da polimerase
SCA	DO inglês sickle cell anemia
Serpins F1 and E1	Serpinas F1 e E1
SIPR5	Marcador esfingosina-1-fosfato 5
SM22	Calponina de músculo liso
STAT-1	Transdutor de sinal e ativador da transcrição 1
SVF	Do inglês stromal vascular fraction
Tbx18	Fator de transcrição T-box 18
TCD4+	Linfócito T auxiliar (linfócito TCD4+)
TCD8+	Linfócito T citotóxico (linfócito TCD8+)
TCLE	Do inglês informed consent form
TGF	Fator de transformação do crescimento
TGF-alfa	Fator de transformação do crescimento alfa
TGF-b	Fator de transformação do crescimento beta
TGF-β	Fator de transformação do crescimento beta
TIMP-1	Inibidor tecidual da metaloproteinase 1
TLR4	Receptores tipo toll-liker 4
TNF	Fator de necrose tumoral
TNF-alfa	Fator de necrose tumoral alfa
TNF-α	Fator de necrose tumoral alfa
Tregs	Linfócitos T reguladores
UCL	Do inglês chronic leg ulcers
VEGF	Fator de crescimento endotelial vascular
β-Actin	Beta actina

Símbolos

β	Beta
α	Alfa
γ	Gama
µg	Micrograma
µM	Micrômetro
µL	Microlitro

SUMÁRIO

1. INTRODUÇÃO

Os portadores da Doença Falciforme (DF) desenvolvem um quadro clínico heterogêneo, podendo apresentar retardo do crescimento, maior susceptibilidade a infecções, icterícia, alterações oftalmológicas e crises de dor intensa. A presença da hemoglobina (Hb) S mutante é determinante para o desenvolvimento destas manifestações, provocando eventos moleculares e celulares que influenciam no curso da doença (INUSA et al., 2019). Uma das hemoglobinopatias de maior relevância mundial é a Anemia Falciforme (AF), caracterizada pela produção da HbS em homozigose. Eventos de vaso-oclusão e hemólise presentes em pacientes com AF são responsáveis por problemas vasculares, isquemia e dano tecidual em vários órgãos como cérebro, baço, pulmão e fígado (PICCIN et al, 2019).

A úlcera cutânea de membros inferiores é uma complicação vascular altamente debilitante, de prevalência elevada entre pacientes jovens e adultos com AF. Esta condição dificulta a socialização dos indivíduos, uma vez que promove dor local intensa, problemas de locomoção, odor fétido e maior susceptibilidade a infecções, podendo levar à amputação dos membros inferiores. Neste contexto, o manejo e tratamento das úlceras cutâneas é uma questão de saúde pública e visa o restabelecimento de uma melhor qualidade de vida para os pacientes e familiares envolvidos (PEART, 2015). As causas que levam ao desenvolvimento das úlceras cutâneas falciformes ainda não foram totalmente elucidadas, mas dados da literatura sugerem que a dificuldade de movimentação dos eritrócitos falcêmicos pelos capilares, devido ao seu fenótipo de foice e maior rigidez de sua estrutura, associado ao aumento da polimerização destas células em locais de baixa pressão de oxigênio, influenciam em crises vaso-oclusivas que levam ao estabelecimento de um microambiente propício para o desenvolvimento das feridas (ALDALLAL, 2019).

A Hemoglobina Fetal (HbF) inibe a formação de polímeros HbS, atuando como moduladora do fenótipo da anemia falciforme. Desta forma, alguns medicamentos utilizados para o tratamento da AF, como hidroxiuréia e 5-azacitidina, tem como princípio ativo a estimulação da síntese de HbF, diminuindo assim os efeitos desencadeados pela polimerização dos eritrócitos. Contudo, nem todos os pacientes apresentam boa resposta ao tratamento, apresentando efeitos colaterais e permanência do quadro clínico (GRIESSHAMMER et al., 2021).

Devido à ausência de um tratamento eficaz, a medicina regenerativa tem como um dos principais focos a compreensão da fisiopatogênese associada ao desenvolvimento de úlceras cutâneas em pacientes com AF, visando a estimulação da regeneração tecidual através da terapia celular. O uso das Células Estromais Mesenquimais (MSC – do inglês Mesenchymal Stromal Cell) possui papel central na terapia celular, sendo estudada em diversas patologias degenerativas. O papel promissor das MSC se relaciona com sua capacidade de auto renovação, diferenciação celular e modulação do microambiente, atuando no recrutamento, migração e proliferação de células-alvo (ex: células endoteliais e pericitos), influenciando assim na formação de vasos sanguíneos e em processos de reparo tecidual (FU et al., 2017).

Nosso grupo de pesquisa desde 2016 vem propondo terapias alternativas no tratamento das complicações cutâneas, na intenção de amenizar os efeitos da Anemia Falciforme e melhorar a qualidade de vida de indivíduos com AF no Estado da Bahia (MENEZES et al., 2016). Os resultados clínicos obtidos em nosso grupo são corroborados por observações experimentais e suportam os efeitos benéficos do transplante autólogo de células mononucleares da medula óssea (CMMO) em pacientes com vasculopatias e úlceras cutâneas falcêmicas (MENEZES et al., 2016). No entanto, apesar do transplante autólogo ter sido seguro e melhorar a cicatrização, a eficácia desta terapia não foi observada em todos os pacientes, sendo necessária a otimização da alternativa terapêutica.

Hoje sabe-se que boa parte dos efeitos terapêuticos das MSCs é proveniente da sua ação parácrina, pela liberação de mediadores solúveis, microvesículas e microRNAs (HASSAN, GREISER & WANG, 2014; AN et al., 2021). No contexto do estudo da funcionalidade das MSC pela terapia celular, a utilização de MSC de tecido adiposo vem ganhando destaque devido ao fácil acesso de seu material biológico de origem (ex: lipoaspirado), isolamento simples e elevado potencial angiogênico (MAZINI et al., 2020). Desta forma, este trabalho visou investigar os efeitos do secretoma de ASC de tecido adiposo humano no tratamento de lesões cutâneas de camundongos com anemia falciforme. O Estudo em questão será de grande valor para a busca da alternativa terapêutica mais segura e eficaz para o tratamento de úlceras cutâneas em indivíduos falcêmicos.

2. REVISÃO DE LITERATURA

2.1 Doença falciforme

A doença falciforme é um grupo de distúrbios hereditários caracterizado pela presença de uma hemoglobina mutante, a hemoglobina HbS. A síntese desta hemoglobina é proveniente de uma mutação de substituição do ácido glutâmico pela valina, na posição 6 do cromossomo 11 que codifica a cadeia β da hemoglobina (região rs334). Esta mutação de substituição tem origem em uma única mudança de base nitrogenada, adenina por timina (A>T) (WILLIAMS & THEIN, 2018). Em heterozigose, a HbS pode vir associada com outras variantes, tais como HbC (HbSC), HbD (HbSD), talassemias (HbS/β0, HbS/β+ E HbS/α) e com a hemoglobina normal HbA (HbSA), gerando a condição de traço falciforme. Quando a hemoglobina é expressa em homozigose, a condição recebe o nome de Anemia Falciforme (KATO et al., 2018).

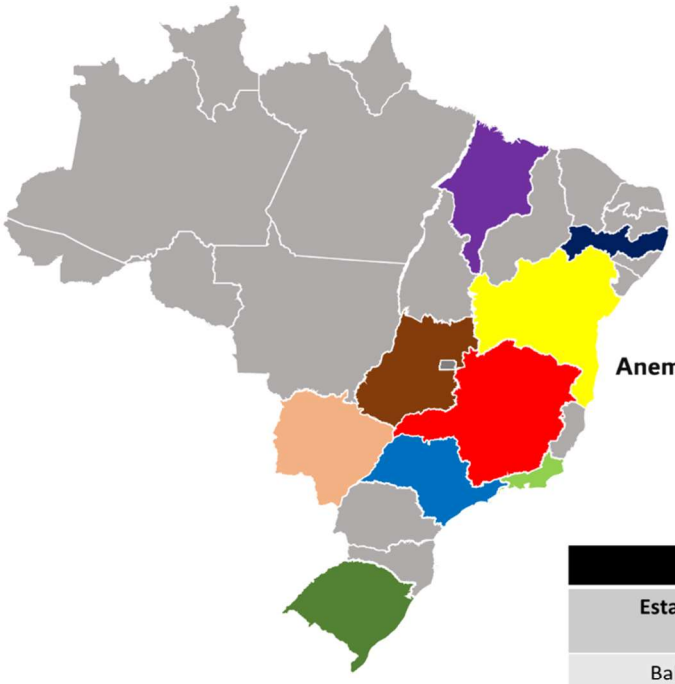
De acordo com a Organização Mundial de Saúde (OMS), a cada ano cerca de 250.000 a 300.000 pessoas nascem com Doença Falciforme em todo o mundo. No Brasil, cerca de 30.000 a 50.000 brasileiros são acometidos pela DF, com incidência de aproximadamente 3.500/ano entre os nascidos vivos e maior prevalência nos estados sudeste e nordeste, sendo mais frequente entre indivíduos de origem afrodescendente (Figura 1) (OMS, 2017). Estudos sugerem que a Hemoglobina S surgiu entre os períodos Paleolítico e Mesolítico, nas regiões da África Subsaariana, leste da Ásia e Índia e o motivo de sua alta prevalência está associada à proteção que a condição de traço falciforme (HbAS) garantiu contra o desenvolvimento da malária grave e de suas complicações secundárias. Os fenômenos de migração populacional e tráfico de africanos no período da escravidão contribuíram para a distribuição da HbS pelo mundo, tornando assim uma condição com alta prevalência mundial e de grande relevância clínica (ONDEI, ZAMARO & BONINI-DOMINGOS, 2005).

A Hemoglobina é uma metaloproteína composta por quatro subunidades de globina associadas ao grupamento heme, responsável pelo transporte de uma molécula de oxigênio. Quando comparada a HbA, a hemoglobina S apresenta menor afinidade ao oxigênio, o que contribui para a polimerização dos eritrócitos falciformes

e resulta na alteração das propriedades físicas e fenotípicas destas células, reduzindo mais ainda a afinidade da HbS ao oxigênio. Este processo de polimerização está diretamente associado à concentração da HbS nos eritrócitos, mas também pode ser influenciada pela variação de temperatura, pH, pela concentração intracelular de 2,3-difosfoglicerato e pela presença de outras variantes da Hb. A alteração estrutural e funcional das membranas contribui para o desequilíbrio da homeostase catiônica e por sua vez para a desidratação celular, aumentando a concentração da HbS e os eventos de polimerização. Além disso, os eritrócitos falciformes são mais frágeis e apresentam envelhecimento prematuro (tempo de vida de 16 dias) e estes fatores contribuem para a hemólise eritrocitária e o aumento do estresse oxidativo local (SUNDD, GLADWIN & NOVELL, 2019).

Desta forma, o conjunto de eventos que tem como ponto de partida a deformação dos eritrócitos é responsável pelas alterações clínicas dos pacientes falcêmicos. Os episódios de vaso-oclusão desencadeados pela dificuldade de movimentação dos eritrócitos falcêmicos, bem como os episódios de hemólise e o estresse oxidativo contribuem para quadros clínicos de hipoxemia tecidual local, hipertensão pulmonar, crise álgica, osteonecrose, acidente vascular cerebral isquêmico, priapismo e úlcera de membros inferiores (DARBARI, SHEEHAN & BALLAS, 2020; OFORI-ACQUAH, 2020).

Uma das complicações mais comuns e altamente debilitantes em pacientes com DF são as úlceras cutâneas de membros inferiores. Esta manifestação cutânea tem característica recidivante e de difícil cicatrização, com maior prevalência em pacientes jovens e homozigotos para a HbS. Para além da dor intensa e cronicidade provocadas pelas úlceras cutâneas, esta complicação interfere na qualidade de vida dos pacientes portadores, prejudicando as interações sociais e relações conjugais, estando associadas a casos de marginalização social e depressão (UMEH et al., 2017; MONFORT & SENET, 2020).



Anemia falciforme atinge cerca de 8% da população afrodescendente no Brasil

Anemia Falciforme no Brasil		
Estados	**Nascidos vivos/ano**	**Traço Falciforme**
Bahia	1:650	1:17
Rio de Janeiro	1:1.300	1:21
Pernambuco, Maranhão, Minas Gerais e Goiás	1:1.400	1:23
São Paulo	1:4.000	1:35
Mato Grosso do Sul	1:5.850	-
Rio Grande do Sul	1:11.000	1:65

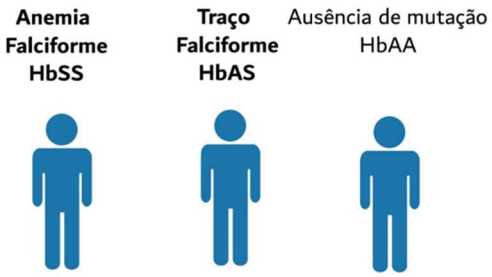


Figura 1: Distribuição da hemoglobina S no Brasil. Frequência do número de nascidos/vivos com Anemia Falciforme e de indivíduos com Traço Falciforme em regiões com maior prevalência no território brasileiro. Adaptado de (CANÇADO & JESUS, 2007).

2.2 Úlceras cutâneas na Anemia Falciforme

As úlceras cutâneas de pacientes falciformes são lesões profundas, dolorosas, com bordas regulares e de caráter não cicatrizante. Cerca de 5% a 10% dos indivíduos adultos com AF desenvolvem úlceras cutâneas, geralmente presentes nos membros inferiores, com proximidade à região do tornozelo (ANTWI-BOASIAKO et al., 2020). Pacientes portadores necessitam de demanda ambulatorial contínua e auxílio de uma equipe de profissionais especializada, visto que a resposta terapêutica é variável e a ausência de tratamento pode levar à amputação dos membros inferiores e morte. Devido aos gastos relacionados ao manejo e tratamento das úlceras e por toda a problemática social que envolve os portadores, as úlceras cutâneas falcêmicas

representam uma questão de saúde pública (GREEN et al., 2014; MONFORT & SENET, 2020).

Os mecanismos moleculares que levam à insuficiência de cicatrização das úlceras em pacientes falciformes ainda não foram totalmente elucidados. A complexidade do processo de cicatrização envolve diferentes tipos celulares locais e sistêmicos. As células e as citocinas pró-inflamatórias são as mesmas nas úlceras falciformes. Entretanto, nos pacientes com AF a etapa de inflamação é mais do que uma resposta transitória (SUNDD, GLADWIN & NOVELL, 2019).

A baixa tensão de oxigênio contribui para a polimerização da HbS e a falcização dos eritrócitos. A repetição do processo de polimerização da HbS aumenta a concentração de Ca2+ intracelular, ativando os canais K+ dependentes de Ca2+ que por sua vez promove a desidratação dos eritrócitos. As mudanças na estrutura dos eritrócitos prejudicam sua capacidade de deformação, que é essencial para sua livre movimentação pelos vasos sanguíneos de menor calibre. Desta forma, o fenótipo falciforme e o aumento da rigidez dos eritrócitos contribuem para a obstrução da microcirculação (CONRAN & BELCHER, 2018). Como consequência, a vaso-oclusão estimula a externalização de elementos de adesão, promovendo ativação do endotélio e interação com células do sistema imune, como monócitos, neutrófilos e plaquetas ativadas, que liberam citocinas pró-inflamatórias e formam agregados celulares que intensificam o processo de obstrução, podendo gerar lesão tecidual isquêmica e necrose (CONRAN & BELCHER, 2018).

A soma dos processos de polimerização da HbS, associado a desidratação dos eritrócitos, disfunção endotelial e estabelecimento de um microambiente inflamatório, promovem a mudança irreversível dos eritrócitos para o formato de foice, tornando-os mais rígidos e frágeis. Os eritrócitos falciformes tendem a sofrer hemólise intracelular, evento que resulta na liberação de hemoglobina na corrente sanguínea. A hemoglobina oxigenada sequestra o óxido nítrico (ON) para a formação de nitrato (NO_3^-) e metemoglobina (Fe^{3+}), assim como pode reagir com peróxido de hidrogênio (H_2O_2) para a formação do radical livre hidroxila (OH•) e metemoglobina (Fe^{3+}) (SUNDD, GLADWIN & NOVELL, 2019). A metemoglobina é degradada e gera heme livre, o que aumenta o estresse oxidativo. Estes eventos promovem a vasoconstrição, hipóxia e dor nos pacientes, podendo levar a lesão e isquemia tecidual (KATO et al., 2018; NADER, ROMANA & CONNES, 2020) (Figura 2).

Um quadro clínico muito comum em pacientes portadores da AF com alta taxa de hemólise celular é o desenvolvimento de monocitose. A interação célula-célula entre as hemácias e as células endoteliais, associado a produção da proteína de classe F da biossíntese de fosfatidilinositol glicano (PIGF) pelas hemácias promove a migração e ativação dos monócitos, aumentando a expressão do antígeno CD11b, interleucina 1 beta (IL-1b) e o fator de necrose tumoral alfa (TNF-alfa) (WONGTONG et al., 2015; SLOMA et al., 2004). O aumento do número de neutrófilos também é comum nestes pacientes, estando associado à gravidade da doença. A ativação das propriedades adesivas dos neutrófilos contribuem para a agregação heterocelular com plaquetas e células endoteliais, aumentando a formação de trombos (ANYAEGBU CC et al., 1998).

A elevação dos níveis extracelulares de Hb e consequentemente do heme também é responsável pela ativação de receptores tipo toll-liker 4 (TLR4), influenciando na mudança do perfil fenotípico de macrófagos (redução dos marcadores HO-1, FPN e CD163 e aumento da expressão de ferritina) que adquirem a polaridade pró-inflamatória M1 (VINCHI F et al., 2016) Desta forma, a manutenção do microambiente inflamatório crônico corrobora com um ciclo fisiopatológico deletério de origem multifatorial que impede um prognóstico favorável ao tratamento e perpetua a não cicatrização das úlceras.

O tratamento mais comum para a AF é a administração oral de hidroxiureia, fármaco capaz de inibir a enzima ribonucleotídeo redutase, responsável pela síntese de DNA. A hidroxiureia age aumentando os níveis de hemoglobina fetal, diminuindo o número de neutrófilos, plaquetas e citocinas inflamatórias. Entretanto, cerca de 30% dos pacientes não reagem bem ao tratamento e mais de 50% das úlceras cutâneas reincidem em um determinado período, destacando a necessidade de alternativas terapêuticas mais eficazes (GRIESSHAMMER et al., 2021).

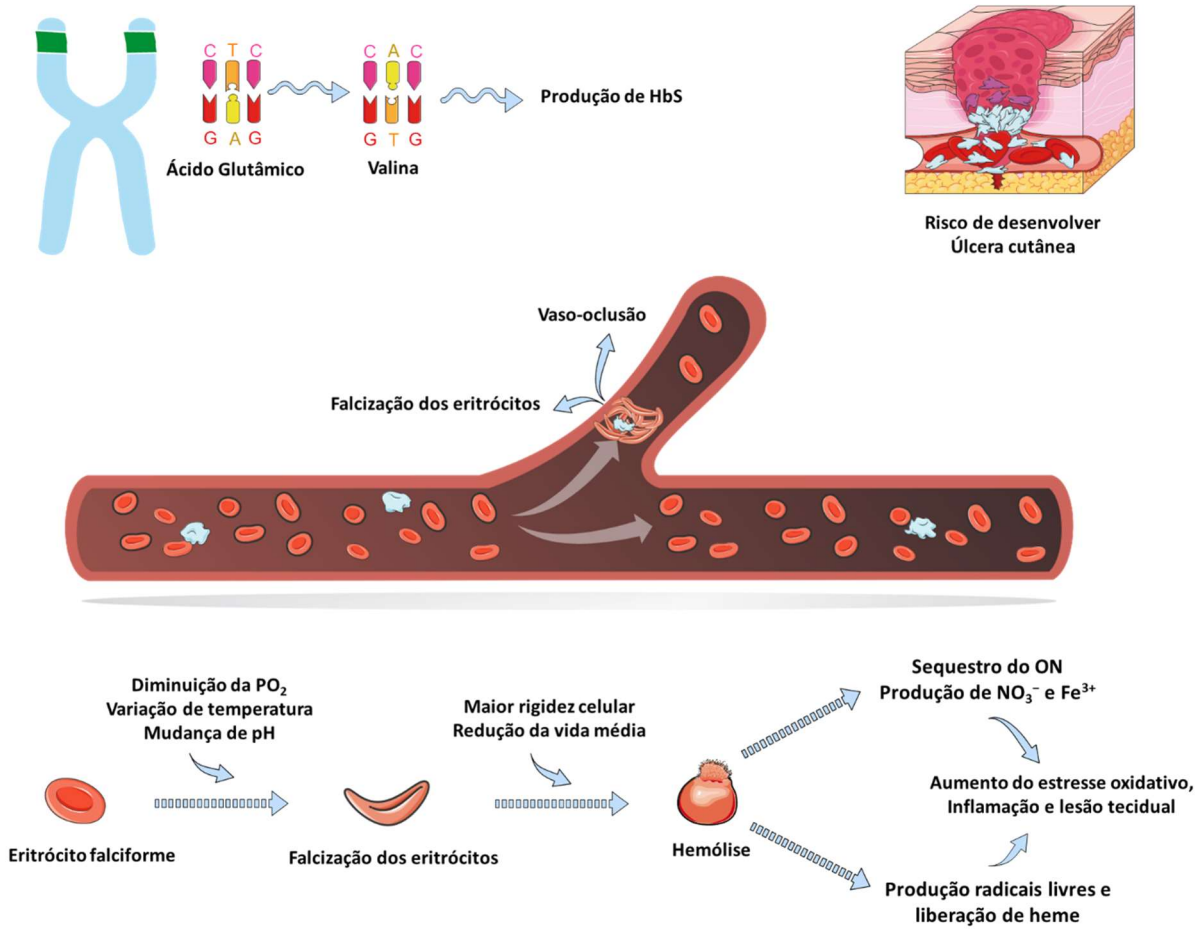


Figura 2: Fisiopatologia da anemia falciforme. A mutação de substituição do ácido glutâmico pela valina leva a produção de uma hemoglobina mutante (HbS) em pacientes com anemia falciforme. Os eritrócitos que apresentam a HbS em seu interior, ao serem expostos a variações de temperatura, de pH e da pressão de oxigênio, sofrem mudança em seu fenótipo, adquirindo formato de foice. Por sua vez, a falcização dos eritrócitos prejudica sua livre movimentação e sua capacidade de deformação ao passar pelos vasos de menor calibre, promovendo a vaso-oclusão dos capilares, hemólise e aumento do estresse oxidativo. O processo inflamatório local pode levar a lesão tecidual e isquemia, o que favorece o surgimento de algumas complicações, como por exemplo as úlceras cutâneas falcêmicas.

2.3 Processo de cicatrização tecidual

A pele atua como uma barreira protetora contra microrganismos, traumas mecânicos e químicos, sendo responsável pela regulação da temperatura corporal, percepção de estímulos dolorosos e manutenção do equilíbrio eletrolítico. Portanto, a pele possui papel essencial para a sobrevivência do indivíduo e quando a sua

integridade é afetada, uma ação coordenada de eventos é crucial para seu restabelecimento.

O processo cicatricial envolve uma sequência de eventos moleculares e celulares que interagem em prol do reparo tecidual. Quatro fases distintas compõem este processo: hemostasia, inflamação, proliferação e remodelação do tecido. Estas fases são integradas por cascatas de fatores de crescimento, citocinas e quimiocinas, todos desempenhando papéis que se sobrepõem. Fatores como: fator de crescimento derivado de plaquetas (PDGF), fator de crescimento de fibroblastos (FGF), fator de crescimento endotelial vascular (VEGF), fatores de crescimento semelhantes à insulina (IGF1, IGF2), fator de crescimento epidermal (EGF), fator de transformação do crescimento beta (TGF-b), são os principais envolvidos no processo de cicatrização das lesões cutâneas (GUO & DIPIETRO, 2010).

O extravasamento sanguíneo e preenchimento da área afetada com plasma e elementos celulares dá início a fase da hemostasia. A agregação plaquetária e a formação do coágulo de fibrina atuam como um tampão, restabelecendo a hemostasia, formando uma barreira física contra a invasão de microrganismos e organizando a matriz provisória necessária para a migração celular. Uma vez controlada a hemorragia, as células inflamatórias são recrutadas para a ferida (quimiotaxia) iniciando a fase inflamatória. A infiltração sequencial de neutrófilos, macrófagos e linfócitos caracteriza esta fase (CAMPOS et al., 2008). Os macrófagos são as principais células efetoras do processo de reparo tecidual, desempenhando papéis múltiplos: (1) liberação de citocinas que induzem a resposta inflamatória por meio do recrutamento e ativação de leucócitos adicionais; (2) degradação e remoção de componentes do tecido conjuntivo danificado; (3) indução e remoção de células apoptóticas; (4) Produção de fatores de crescimento como VEGF, PDGF, TGF-beta e FGF, importantes para a formação do tecido de granulação, indução da angiogênese e regeneração tecidual (MESZAROS et al., 2000; MOSSER & EDWARDS, 2008).

A terceira fase (proliferativa) é responsável pelo fechamento da lesão, compreendendo as etapas de reepitelização (movimentação de células epiteliais oriundas da margem e do centro da lesão), fibroplasia e angiogênese, que compõem o tecido de granulação. A formação de novos vasos sanguíneos age posteriormente ao aumento da permeabilização, sendo fundamental para a nutrição e oxigenação do tecido de granulação, ocorrendo na matriz extracelular do leito da ferida por meio da

estimulação mitogênica das células endoteliais e recrutamento de células estromais progenitoras. Fatores de crescimento PDGF, FGF, VEGF, TGF-beta, angiogenina, angiotropina, angiopoetina-1 e baixas condições de oxigênio são responsáveis pela estimulação da angiogênese (MENDONÇA & COUTINHO-NETTO, 2009; GUO & DIPIETRO, 2010).

A quarta e última fase, o remodelamento, é marcada pela maturação dos elementos e alteração da matriz extracelular. A matriz extracelular sofre reorganização e a maioria dos vasos, fibroblastos e células inflamatórias desaparece do local da lesão, devido ao processo de emigração, apoptose e/ou outros mecanismos desconhecidos. A atuação de células perivasculares e liberação dos mediadores solúveis TNF-alfa, IL-1b, PDGF e TGF- beta produzidos por fibroblastos e TGF-beta e EGF produzidos por células epiteliais, garantem a restauração da integridade do tecido epitelial (DE MENDONÇA & COUTINHO-NETTO, 2009).

2.4 Pericitos

No século 19 o pesquisador francês Charles Marie Benjamin Rouget descobriu um tipo de células contráteis que circundavam vasos sanguíneos e as nomeou como células de Rouget. Posteriormente, o alemão Karl Wilhelm Zimmermann renomeou estas mesmas células como pericitos, fazendo jus a sua localização anatômica (ROUGET, 1873; ZIMMERMANN, 1923).

Atualmente os pericitos são classificados como células perivasculares com função sobre as células endoteliais, auxiliando na maturação, desenvolvimento e remodelamento dos vasos sanguíneos (KEMP et al., 2020). Além de seu papel na estabilização da microvasculatura, os pericitos possuem alta plasticidade e capacidade de diferenciação em outros tipos celulares, características também observadas em células estromais mesenquimais. Dados recentes sugerem que os pericitos também apresentam propriedades regulatórias, sendo responsáveis por atrair leucócitos inatos, regular a ativação de linfócitos e atuar na depuração de componentes citotóxicos (BIRBRAIR et al., 2017).

A identificação dos pericitos se dá pela sua localização anatômica, seu fenótipo fusiforme e pela expressão de alguns marcadores celulares, como nestina, o antígeno neuronal glial 2 (NG2), o fator de transcrição T-box 18 (Tbx18) e o receptor beta do

fator de crescimento derivado de plaquetas (PDGFRB) (ARMULIK et al., 2011; BIRBRAIR et al., 2013a; GUIMARÃES-CAMBOA ET al., 2017). O marcador NG2 é o mais bem documentado pela literatura, sendo constitutivo de células perivasculares em vários tipos de tecido. Contudo, o NG2 não é um marcador específico de pericitos, sendo expresso em outros tipos celulares.

Birbrair e colaboradores (2013a) identificaram dois tipos de pericitos: pericitos tipo 1 (Nestina^{-} NG2+) e pericitos tipo 2 (Nestina^{+} NG2^{-}) (BIRBRAIR et al, 2013a). Entretanto, a identificação dos pericitos não é homogênea em todos os órgãos e a funcionalidade das suas subpopulações pode divergir. Os pericitos tipo 1 foram associados ao processo de formação de tecido fibroso, sintetizando colágeno tipo 1 em modelo de fibrose pulmonar, mas esta funcionalidade não foi observada em modelo de fibrose cardíaca e renal (BIRBRAIR et al, 2013b). Os pericitos tipo 2 foram associados ao processo angiogênico, participando tanto em condições fisiológicas do reparo tecidual quanto da fisiopatologia tumoral (BIRBRAIR et al., 2014; BODNAR et al., 2016; DO VALLE et al., 2020).

O processo de angiogênese é crucial para manutenção da integridade dos tecidos e envolve a ativação da migração e proliferação de células endoteliais. Estes eventos são orquestrados por um conjunto de diferentes tipos celulares que estimulam o crescimento dos novos vasos, como por exemplo as MSCs. Durante a angiogênese os pericitos se desprendem dos vasos quiescentes, permitindo uma mudança na sua estabilidade e facilitando a formação de novos brotos, através do crosstalk com o tecido endotelial e as MSCs (PAYNE L et al., 2021). Esta ação coordenada é crucial para a manutenção da integridade tecidual, colocando o direcionamento dos pericitos como alvo da terapia celular para melhora da perfusão em tecidos isquêmicos.

2.5 Células estromais mesenquimais

As células estromais mesenquimais são células imaturas, indiferenciadas e multipotentes, com alta plasticidade e capacidade de auto renovação. Em condições específicas as MSCs podem adquirir características das linhagens mesodermais adipogênica, condrogênica e osteogênica, devido ao seu potencial multilinhagem, assim como são capazes de alterar a biologia das células e tecidos ao seu redor, mediando os seus efeitos terapêuticos (DOMINICI et al., 2006). Dessa forma, desde

a sua descoberta, as MSCs vêm chamando atenção pelas suas características, representando uma promessa para o tratamento de diversas doenças (Figura 3).

In vivo as MSCs estão localizadas em nichos perivasculares, juntamente com os pericitos e as células endoteliais (CRISAN et al, 2012), podendo ser encontradas em diversos órgãos: medula óssea, tecido adiposo, pele, polpa dentária, sangue menstrual, placenta, coração, fígado etc. *In vitro*, para a caracterização das MSCs, é necessário que as células em cultivo apresentem aderência ao plástico, fenótipo fibroblastóide, capacidade de proliferação e potencial de diferenciação multinhagem. Como não possuem um marcador de superfície específico, as MSCs são identificadas pela expressão positiva de um conjunto de marcadores de superficie celular (CD29, CD73, CD90 e CD105) e expressão negativa para os marcadores CD14, CD34, CD45 e anti-HLADR (DOMINIC et al., 2006).

Nos primeiros anos de pesquisa muitos trabalhos foram baseados em estudos utilizando MSC de medula óssea, porém, como a aquisição dessas células dependia de um procedimento invasivo, foi necessário analisar o potencial de outras fontes de MSC. Diante da reclassificação do tecido adiposo como órgão endócrino, responsável pela síntese de hormônios e mediadores solúveis, este órgão se tornou uma possível fonte para isolamento de MSC (MONTERO-VILCHEZ et al., 2021). Neste contexto, pela facilidade de obtenção de material biológico, cultivo bem-sucedido e alto desempenho das células isoladas, as células estromais do tecido adiposo (ASC – do inglês Adipose derived Stromal Cells) se destacaram como uma das principais alternativas terapêuticas para a medicina regenerativa (AJIT & AMBIKA, 2021; NIADA et al., 2021).

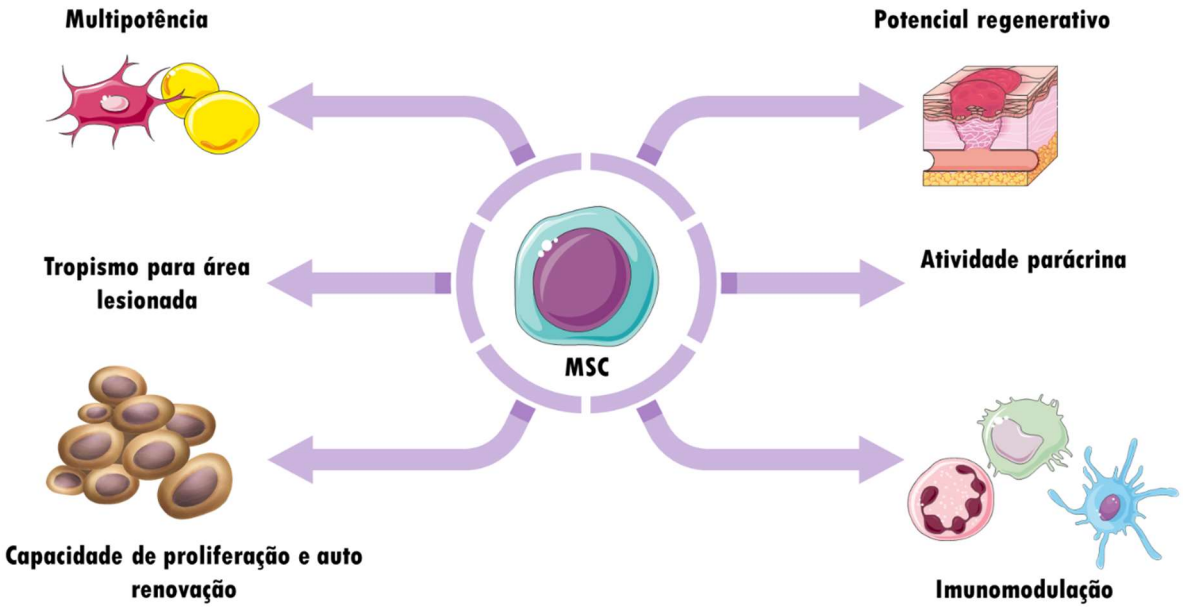


Figura 3: Propriedades das Células Estromais Mesenquimais.

2.6 Células estromais mesenquimais na medicina regenerativa

Evidências na literatura sugerem que as propriedades terapêuticas das MSCs estão relacionadas a sua interação célula-célula e a sua atividade parácrina, por meio da liberação de mediadores solúveis e de microvesículas (RATAJCZAK et al., 2012; KIM et al., 2019; TRZYNA & BANAŚ-ZĄBCZYK, 2021). O secretoma das MSCs é composto por fatores de crescimento como VEGF, bFGF, fator de crescimento de hepatócitos (HGF), fator neurotrófico derivado do cérebro (BNDF), Ang-1, proteína quimioatraente de monócitos 1 (MCP-1), fator estimulante de colônia de granulócitos-macrófagos (GM-CSF) e TGF-alfa, envolvidos na regulação da angiogênese, na quimoatração e diferenciação de células na lesão, bem como na manutenção da viabilidade celular e reparo tecidual (PARK et al., 2010; YOUSEFI et al., 2016; LIANG et al., 2016; SILVEIRA et al, 2016). Dados da literatura demonstram que a administração tópica de meios condicionados (MC) produzidos por MSC é capaz de acelerar a cicatrização de feridas e induzir a proliferação e migração de células endoteliais, não necessitando do contato célula-célula (SUN et al., 2014; RIBEIRO et al., 2019).

Por desempenhar um papel importante para a homeostase da pele, vários estudos *in vivo* evidenciam as vantagens de usar terapia com MSC para o tratamento

de úlceras cutâneas (MAXSON et al., 2012; AAMAR et al., 2021). Um trabalho recente desenvolvido pelo nosso grupo demonstrou que o pré-condicionamento em hipóxia aumentou a capacidade secretória das MSC de medula óssea e a administração local do meio condicionado induziu a angiogênese e acelerou a cicatrização de feridas em modelo murino C57BL/6 (RIBEIRO et al., 2019). Pesquisadores demonstraram que o tratamento de camundongos C57BL/6 jovens e idosos com ASC estimulou a aceleração do fechamento de úlceras de pressão, melhorando a arquitetura epidérmica/dérmica e reduzindo o infiltrado inflamatório. Isso foi observado por Hongsen et al. (2019) ao utilizar fração vascular estromal (FVS) e ASC em modelo de cicatrização com camundongos diabéticos. Os autores observaram que as duas alternativas terapêuticas foram capazes de melhorar significativamente o processo de reparo tecidual, induzindo a migração de fibroblastos, o aumento da angiogênese e por sua vez o remodelamento da matriz. Estes efeitos foram associados à regulação da expressão de 54 genes, dentre eles oncostatina M (Osm), ligante de quimiocina 2 (CCL4) e receptor de quimiocina C-C tipo 1 (CCR1) (HONGSEN et al., 2019). Estes dados comprovam que o tratamento com MSC ou seus derivados têm impacto em todas as fases do reparo tecidual, apresentando papel central na terapia celular (LU et al., 2011; MAXSON et al., 2012).

Atualmente, a utilização do meio condicionado apresenta-se como uma estratégia viável e eficaz para o tratamento de lesões cutâneas. Um dos pontos positivos para o tratamento de doenças regenerativas com o MC de MSC é que este não apresenta risco de rejeição ou de tumorigenicidade ao paciente, pois é composto apenas por moléculas bioativas e não apresenta traços celulares em sua composição. Contudo, suas limitações de uso envolvem o estabelecimento de contraindicações, padronização do método de coleta e do processamento do MC, bem como a caracterização minuciosa quanto aos seus componentes terapêuticos.

2.7 Potencial imunomodulatório das MSC

As MSCs apresentam propriedades importantes de imunossupressão e imunoregulação devido ao seu fenótipo hipoimunogênico: expressão negativa para o complexo principal de histocompatibilidade (MHC) de classe II, baixa expressão para MHC de classe I e deficiência na expressão de moléculas coestimuladoras (CD40,

CD80 e CD86) para células T e B (LEBLANC & RINGDEN, 2005). Desse modo, é sugerido que as MSCs escapam da vigilância do sistema imune por possuírem antígenos de superfície que são pobremente reconhecidos pelas células T aloreativas e células Natural Killer (células NK) (WANG et al., 2016). Além disso, as MSCs são capazes de regular a ativação e proliferação das células do sistema imune inato e adquirido, sendo o seu potencial imunomodulatório dependente de três fatores: do microambiente inflamatório, do contato célula-célula e da sinalização parácrina.

2.7.1 MSC e células da imunidade inata

Os macrófagos são células reguladoras críticas do processo inflamatório, promovendo o início e a progressão da resposta imune à lesão e remodelação tecidual. O tipo de resposta dos macrófagos é dependente do seu fenótipo, que por sua vez é determinado por estímulos externos presentes no microambiente local. Dois dos fenótipos mais bem caracterizados são o fenótipo M1 – macrófagos produtores de citocinas pró-inflamatórias (TNF-α, IL-1α e IL-6) que participam da fase inicial da inflamação, e o fenótipo M2 – macrófagos produtores de citocinas anti-inflamatórias (IL-10 e TGF-β) envolvidos no reparo tecidual (MILLS et al., 2000).

Em um processo de lesão tecidual as citocinas pró-inflamatórias clássicas como interferon gama (IFN-γ), TNF-α ou IL-1β, liberadas na fase inicial da inflamação são responsáveis por potencializar os efeitos imunomodulatórios das MSCs. Trabalho publicado por Feng e colaboradores (2019) demonstrou que MSCs foram capazes de induzir a mudança do fenótipo M1 para M2 en macrófagos estimulados por lipopolissacarídeo (LPS), com função mediada por TGF-b e ativação da via AKT/FOXO1 (FENG et al., 2019). Corroborando com estes resultados, outro estudo demonstrou que a liberação de indoleamina 2,3-dioxigenase 1 (IDO) por MSCs ativadas por IFN-γ e TNF- α esteve associada com a diferenciação de monócitos em macrófagos M2 secretores de interleucina IL-10 (CD14(+) /CD206(+)) e que esta polarização M2 implicou na supressão da proliferação de células T por meio da ação da IL-10, o que amplificou o efeito imunomodulador das MSCs (FRANÇOIS et al. 2012).

A atuação das MSCs sobre as células NK envolve a supressão de sua atividade citotóxica, inibição de sua proliferação e secreção de citocinas (Li N & Hua J, 2017).

Em um experimento de co-cultivo de MSCs com células NK previamente ativadas com IL-2, os autores perceberam que as MSCs foram capazes de reduzir a expressão de marcadores de superfície (NKp44, NKp30, NKG2D e CD132) envolvidos na ativação e proliferação de células NK. Este resultado foi associado a ação conjunta dos mediadores prostaglandina E2 (PGE2) e IDO, uma vez que a utilização de inibidores de PGE2 e IDO em co-cultivo de MSCs e células NK foi responsável por eliminar os efeitos de supressão (SPAGGIARI et al., 2008). A ação das MSCs também foi observada em estudo utilizando modelo murino de lesão hepática induzida por polyI:C. Neste trabalho, MSC de medula óssea foram capazes de atenuar a quadro pela diminuição dos níveis de alamina aminotransferase (ALT), redução da infiltração de linfócitos no fígado e supressão das células NK. A investigação, *in vitro* e *in vivo*, evidenciou ação das MSCs sobre a inibição da expressão do marcador esfingosina-1-fosfato 5 (SIPR5), responsável pela migração de células NK (QU et al., 2015).

Em relação aos efeitos sobre as células dentríticas (DCs), o potencial imunomodulador das MSCs é direcionado para a inibição da sua maturação, capacidade de secreção de IL-12 e, por sua vez, sua capacidade de ativação de células T alorreativas (Li N apud 97). Estudo *in vitro* demonstrou que as MSC foram responsáveis por inibir a maturação de monócitos CD1a+CD14+ ativados por LPS em DCs, através da supressão da expressão do marcador CD1a e redução das moléculas coestimuladoras CD86 e CD80. Além disso, as MSC alteraram o padrão secretório dos monócitos, estimulando a produção de IL-10, IL-6 e HGF e inibindo a produção de IL-12, IL-17 e TNF-alfa (DENG et al., 2016). Dados da literatura sugerem que a modulação da resposta imune e estimulação de DCs reguladoras mediada por MSC é dependente de Jagged-1/2 (ZHANG et al., 2008; CAHILL et al., 2015). Estudo evidenciou que co-cultivo de MSC com DCs maduras resultou na mudança fenotípica das DCs, que adquiriam fenótipo regulador com menor expressão de CD11c e das moléculas coestimuladoras CD80, CD86 e CD40 e expressão positiva para CD11b e Jagged/2. Assim como no estudo anterior, a capacidade secretória também foi alterada, com redução dos níveis de IL-12 e aumento dos níveis de TGF-b, IL-10. Ensaios *in vitro* e *in vivo* demonstraram que as DCs reguladoras foram capazes de inibir a proliferação de linfócitos T, sendo a imunomodulação das DCs por MSC crucial para a modulação do microambiente inflamatório (ZHANG et al., 2008).

2.7.2 MSC e células da imunidade adaptativa

As MSC apresentam potencial de imunomodulação da resposta imune adaptativa através da inibição da proliferação, diferenciação e indução da apoptose de células T. A interação das MSC com outras células da imunidade inata, como por exemplo os monócitos, é essencial para a supressão dos subconjuntos de linfócitos TCD4+ e TCD8+ e por sua vez formação de linfócitos T reguladores (Tregs). Dois importantes fatores envolvidos na formação de Tregs e inibição da proliferação celular de células T são o TGF-b e o HGF. Pesquisadores observaram que a liberação destes fatores de crescimento pelas MSC reduz a expressão de ciclina D2 e estimula a síntese de p27kipl (inibidor de ciclina-quinase) nas células T, promovendo a parada das células na fase G1 e desta forma, sua proliferação (GLENNIE S, 2005). Quanto ao processo de apoptose induzido por MSC, estudo realizado em 2012 evidenciou que a via de sinalização Fas/Faz está envolvida na ativação da morte celular em células T, através da conversão do triptofano em quinurenina (AKIYAMA et al., 2012).

Apesar do potencial terapêutico das MSC está associado à sua ação parácrina, os efeitos das MSC sobre as células B também são dependentes do contato célula-célula. MSC estimuladas por células T e monócitos, iniciam uma cascata de sinalização que envolve a liberação de citocinas, fatores de crescimento e vesículas extracelulares (EVs) que inibem a proliferação de células B e induzem a formação de linfócitos B reguladores (Bregs) (DI TRAPANI et al., 2016).

Esses dados sugerem que um microambiente inflamatório é capaz de ativar a capacidade imunomoduladora das MSC, que por sua vez sofrem mudanças em seu perfil secretório e atuam no controle da ativação, proliferação e diferenciação de células do sistema imune (LI et al, 2017). Desta forma, o papel das MSC visa o controle da inflamação e o restabelecimento da homeostase tecidual.

3. HIPÓTESES

Hipótese Nula 1

Não há diferença entre o pré-condicionamento em normóxia e o pré-condicionamento em hipóxia sobre a coleta de meio condicionado de ASC.

Hipótese Nula 2

O secretoma das ASC não possui efeito terapêutico sobre o reparo tecidual de lesões cutâneas

Hipótese Alternativa 2

Há diferença entre o pré-condicionamento em normóxia e o pré-condicionamento em hipóxia sobre a coleta de meio condicionado de ASC.

Hipótese Alternativa 2

O secretoma das ASC possui efeito terapêutico sobre o reparo tecidual de lesões cutâneas.

4. OBJETIVOS

OBJETIVO GERAL

Avaliar o potencial regenerativo e imunomodulatório do meio condicionado de ASC no tratamento de feridas em camundongos com anemia falciforme.

Objetivos Específicos

- Isolar e caracterizar ASC de amostras de lipoaspirado;
- Coletar e caracterizar os meios condicionados de ASC em condição de normóxia e hipóxia;
- Avaliar o potencial *in vitro* de indução da angiogênese, migração e proliferação de ASC sobre modelo de células endoteliais de cordão umbilical humano (HUVECs);
- Observar a influência do secretoma de ASC sobre a cicatrização de feridas em modelos de cicatrização de camundongos transgênicos Nestin-GFP+/NG2-DsRed+ e camundongos Towness HbSS;
- Quantificar a expressão de transcritos envolvidos na inflamação, em feridas tratadas com meio controle e feridas tratadas com meio condicionado.

5. CAPÍTULO 1

Os resultados apresentados neste primeiro capítulo da tese estão compilados e discutidos no manuscrito "**Secretome from human adipose-derived mesenchymal stem cells promotes blood vessel formation and pericyte coverage in experimental skin repair"** publicado na revista científica **PlosOne** (Silveira BM et al., 2022)

ARTIGO 1

Secretome from human adipose-derived mesenchymal stem cells promotes blood vessel formation and pericyte coverage in experimental skin repair

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Abstract

Human adipose tissue-derived stem cells (hASC) secretome display various therapeutically relevant effects in regenerative medicine, such as induction of angiogenesis and tissue repair. The benefits of hASC secretome are primarily orchestrated by trophic factors that mediate autocrine and paracrine effects in host cells. However, the composition and the innate characteristics of hASC secretome can be highly variable depending on the culture conditions. Here, we evaluated the combined effect of serum-free media and hypoxia preconditioning on the hASCs secretome composition and biological effects on angiogenesis and wound healing. The hASCs were cultured in serum-free media under normoxic (NCM) or hypoxic (HCM) preconditioning. The proteomic profile showed that pro- and anti-antiangiogenic factors were detected in NCM and HCM secretomes. *In vitro* studies demonstrated that hASCs secretomes enhanced endothelial proliferation, survival, migration, *in vitro* tube formation, and *in vivo* Matrigel plug angiogenesis. In a full-thickness skin-wound mouse model, injection of either NCM or HCM significantly accelerated the wound healing. Finally, hASC secretomes were potent in increasing endothelial density and vascular coverage of resident pericytes expressing NG2 and nestin to the lesion site, potentially contributing to blood vessel maturation. Overall, our data suggest that serum-free media or hypoxic preconditioning enhances the vascular regenerative effects of hASC secretome in a preclinical wound healing model.

Keywords: adipose derived stem cells; secretome; preconditioning; angiogenesis; wound healing.

Introduction

Human adipose tissue-derived stem cells (hASCs) are a promising therapeutic strategy in regenerative medicine explored in various models of tissue repair, ischemic injuries, immune disorders [1,2], also to improve chronic non-healing or recurrent cutaneous wounds, such as age-associated delayed healing, chronic diabetic wounds, and irradiated wounds [3,4]. Many preclinical and clinical studies have demonstrated that the hASCs secretome enhances wound healing and vascularization mainly by

stimulating cell survival, angiogenesis, cell proliferation, ECM cell migration/adhesion, and reducing inflammation [5,6]. Administration of the hASC's secretome to injured skin also increases its local metabolic activity, oxygen supply, and extracellular matrix remodeling [7], accelerating tissue healing.

The beneficial effects of *in vivo* hASCs are primarily orchestrated by their secretome enriched with trophic molecules (represented by cytokines and growth factors) and a vesicular fraction (microvesicles and exosomes) that mediate autocrine and paracrine cell communication [8,9]. Therefore, identifying critical factors secreted and characterization of their functional roles in cutaneous wound healing can be a practical approach to designing more potent secretome-based therapeutics with more predictable clinical outcomes [4]. Recently, proteomic analysis of hASCs secretome has found many players that improve wound healing *in vivo*, including epidermal growth factor (EGF), fibroblast growth factor-2 (FGF2), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), platelet-derived growth factors (PDGFs), insulin-like growth factor (IGF)-1 and 2, and stromal cell-derived factor [4,5,10]. Kim and colleagues [11] identified MCP-1, IL-8, VEGF, and angiogenin as efficient secretome biomarkers for predicting vascular regenerative efficacy in wound healing [11]. MicroRNAs that could be taken up by endothelial cells and stimulate *in vitro* and i*n vivo* sprouting angiogenesis were also described in hASC secretome [12-15].

However, the hASC secretome's composition and innate characteristics can vary depending on the cell source and culture conditions. Hypoxic preconditioning is one of the most frequent ways to improve hASC secretome, as hypoxic stress reduces oxygen, improves cellular function, and increases the concentration of paracrine factors [16,17] that promotes wound healing [18-21] with fewer scar formation [22-24] in comparison to normoxic conditions. Furthermore, hASCs secretome produced in

serum-free media has enhanced immunosuppressive and anti-fibrotic abilities because of increased vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) secretion [25,26]. These findings led us to the hypothesis that serum-free media and hypoxic preconditioning would synergistically enhance the therapeutic effects of hASCs secretome on wound healing.

In this study, first, we characterized cultivated hASCs and the soluble angiogenic-related and tissue repair factors secreted in serum-free media under 1% O_2 (hypoxic) (HCM) or normoxic conditions (NCM). Next, we assessed the secretomes' angiogenic and tissue regeneration potential using HUVECs primary culture. We demonstrated that the hASCs secretome activated the PI3K/Akt signaling cascade and enhanced endothelial proliferation, survival, migration, and *in vitro* tube formation. A Matrigel plug assay showed that NCM and HCM were potent promoters of *in vivo* angiogenesis. Next, using a pericyte labeled (or reporter) transgenic nestin-GFP/NG2-DsRed mice revealed that hASC secretomes accelerated wound healing, increased endothelial density, and vascular coverage with resident pericytes expressing NG2 and nestin to the lesion site, potentially contributing to blood vessel maturation. Overall, our data suggest that serum-free media or hypoxic preconditioning enhances hASCs secretome's vascular regenerative effects by directly recruiting NG2+nestin+ pericytes to the injury site.

Material and methods

Ethics statement

This study was approved by the institutional review board of the Health Science Institute (Federal University of Bahia, approval no 2.074.627). This study is in

compliance with the ethical principles of the revised Declaration of Helsinki. All participants read, understood and gave written consent in the form approved by the institutional review board before agree to the study activities.

Animals

All animal experiments were reviewed, approved and performed in accordance with Brazilian guidelines and regulations. The institutional review board approved animal handling and procedures for animal experimentation (CEUA, UFBA-2018-131). All animal procedures were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals and the regulation of animal protection committee to minimize the suffering and injury. The animal studies are in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

C57BL/6 mice and Nestin-GFP+/NG2-DsRed+ double-transgenic mice colonies were housed at the Health Science Institute of the Federal University of Bahia in a pathogen-free facility under a 12-hour:12-hour light/dark cycle, with *ad libitum feeding*. Nestin-GFP+/NG2-DsRed+ double-transgenic mice have been described [27]. In brief, they are transgenic mice expressing GFP under the nestin promoter and DsRed+ under the NG2 promoter. Our Nestin-GFP+/NG2-DsRed+ double-transgenic mice colony was maintained homozygous for the transgenes on the C57BL/6 genetic background. Both male and female homozygous mice were used, and their ages ranged from eight to twelve-week-old. The mice were monitored daily and euthanized humanely by overdose (three times the dose used to anesthetize) of ketamine plus xylazine (80 and 20 mg kg^{-1} i.p.) at the end of the experiment or the first sign of shortness of breath, reduced locomotion and reduced body weight.

Human cell culture

The hASC were isolated from lipoaspirate harvested as surgical waste products [28]. In order to avoid gender-related variability, only female donors were selected for this study. Following well-established isolation procedures, hASC populations were obtained from 8 healthy women (S1 Table) (aged 25 – 45 years old). A volume of 50 mL lipoaspirate was treated with collagenase I (1 mg/mL) (Sigma, C0130-1G) at 37 °C, and 30 min later, the digested material was centrifuged to obtain the stromal vascular fraction (SVF). The SVF was cultured in DMEM with low glucose (Dulbecco's Modified Eagle's Medium, Life Technologies), supplemented with 10% FBS, antibiotics (PenStrep and gentamicin) in adherent culture bottles. hASCs were expanded in a standard culture medium in a humidified atmosphere at 37°C with routine passaging at 80% confluence. The culture medium was renewed every three days. hASCs at passages three to six were used for all subsequent experimentation.

Primary human umbilical vein endothelial cells (HUVECs) cells were isolated from umbilical cords as described [29]. The HUVECs were cultured in EGM2/BulletKit medium (Lonza Group Ltd.) supplemented with 100 U/mL penicillin/streptomycin (Life Technologies) at 37 °C in 5% CO_2 and 95% air. HUVECs were seeded on 0.1% gelatin (Sigma), and EGM2/BulletKit was replaced every 2–3 days. HUVECs at passages three to six were used for this study.

Immunophenotypic and multipotency characterization of hASCs

The expression of cell-surface antigens using fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated antibodies was examined in hASCs (1

x 10^5 cells) using the following antibodies: anti-CD14 PE (clone 61D3, Lot: E026669, ebioscience), anti-CD34FITC (BD, Cat No. 348053), anti-CD45PerCP (lot: ED7029, Exbio), anti-CD146 PE (lot: 91811, BD Biosciences), anti-HLA-DR PE (clone: MEM-12, lot: 1P474T100, Exbio), antiCD29FITC (clone: TS2116, lot: E031567, ebioscience), anti-CD73 PE (clone: AD2, lot: 1P675T100, exbio), anti-CD90 (clone: ebioE10, lot: E0228253, ebioscience) PE, anti-CD105FITC (clone: SN6, lot: E029268, ebioscience) and appropriate isotype control antibodies from the same manufacturers. Samples were run on a FACScalibur flow cytometer (BD Biosciences, CA, USA) and analyzed using a BD Cell Quest pro software.

The multipotency capacity of hASC for trilineage differentiation (osteogenic, chondrogenic, and adipogenic) and the intracellular expression of mesenchymal markers were assessed as described [30,31]. Immunofluorescence staining was carried out on hASCs (2.0 x 10^4 cells/glass coverslips) with the following primary antibodies: mouse anti-SMA (Sigma, 1:500 in BSA/standard serum solution), and rabbit anti-Collagen IV (Abcam, 1:100). After PBS washing, cells were incubated with the following secondary antibodies: Alexa Fluor-555 anti-mouse; Alexa Fluor-488 anti-rabbit (Molecular Probes, 1:500 in BSA/standard serum solution).

Conditioned Medium preparation and collection

Conditioned Medium (CM) preparation was performed as described previously [30], with some modifications. The CM was obtained after 1 × 10^6 cell hASCs (passages 3 – 6) were cultured for 48h with 3 mL of serum-free EBM™-2 Basal Medium (Lonza), supplemented with 1% bovine serum albumin (Sigma-Aldrich Co) in T25 culture flasks either under standard conditions (normoxic, NCM) or combined with 0.5% of oxygen preconditioning (hypoxic, HCM) [30]. Hypoxic preconditioning was

produced in an Anaerobac Jar (Probac, São Paulo, Brazil) for 48h [32]. CMs were then collected, centrifuged at 3,000 rpm for 20 min at 4 ºC to remove cell debris and large apoptotic bodies, and maintained at -70 ºC until use. The media collected were referred to as normoxic hASC conditioned medium (NCM) or hypoxic hASC conditioned medium (HCM), respectively. In order to obtain results that were not affected by single donor variability and more representative of trophic factors released by hASCs, we pooled CMs from 2 different hASCs at the same passage, and used these pools for each specific analysis. Total protein content of conditioned medium samples was quantified using the Bradford Protein Assay according to manufacturer's protocol. Bovine serum albumin standards were used. The total protein concentration of the CM pools was normalized based on the average total protein content (2.50 +/- 0.15 mg/mL) achieved after 48h of incubation of the confluent hASC monolayer. Identical replicate doses of these normalized CMs were used in all the experiments.

Electrophoresis and Western blot analysis

HUVEC cell lysates were prepared in sodium dodecyl sulfate (SDS) buffer containing Complete Mini Proteinase Inhibitor Cocktail Tablets (Sigma-Aldrich®). Samples (100µg) of the protein lysates were loaded onto 12% polyacrylamide SDS gel, and the separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. After transfer, the membrane was blocked and incubated overnight with the following primary antibodies: β-Actin, AKT, and phospho-AKT (Ser473) (Cell Signaling Technology). The membrane was washed and incubated for one hour with a secondary antibody (Peroxidase Goat Anti-Rabbit IgG Antibody PI1000, Vector Laboratories). For development, the membrane was immersed in a

chemiluminescent solution (Immobilon Western Chemiluminescent HRP Substract, Merck Millipore), and the bands were detected using a photo-documenter.

Cell apoptosis, proliferation, and viability

Cell apoptosis was estimated with the Fluorescent terminal deoxynucleotidyl transferase nick end labeling of DNA fragments (TUNEL)-in situ cell death detection kit (Fluorescein - Roche), following the manufacturer protocol. Briefly, HUVECs were serum-starved for 4h, followed by stimulation with NCM, HCM, or vehicle control medium for 20h. Only apoptotic cells were stained by TUNEL, while propidium iodide (PI) was used for nuclear labeling**.** Five hundred cells were counted in randomly chosen fields for each sample, and the numbers of apoptotic cells were expressed as a percentage of the total cells counted.

The relative number of HUVECs incorporating 5-Bromo-2'-deoxyuridine (BrdU), indicating that these cells were going through the S phase, was performed with a cell proliferation kit (Vector). Briefly, HUVECs cultured in EGM-2 medium with 0.5% FBS for 4h were stimulated with NCM, HCM, or vehicle control medium. After 20h, HUVECs were incubated with 10 μM BrdU for 4h, before fixation. BrdU was detected by indirect immunofluorescence staining with a primary mouse anti-Bromodeoxyuridine antibody (1:200, VP-B209, Vector) and secondary anti-IgG mouse conjugate (Alexa Fluor 488, dilution: 1:2000). Images were captured with Eclipse TS100 fluorescence microscopy (Nikon Instruments Inc.). Cell viability was estimated with the fluorescent viability staining calcein-acetoxymethyl ester (calcein-AM; 1 μM; Molecular Probes, Life technologies).

Cell scratch wound healing assay

The scratch wounds were created in the confluent HUVEC monolayer using a sterile pipette tip, followed by treatment with NCM, HCM, or vehicle control medium for 18h. The wound width was determined at 0h and 18h after scratching using a light microscope equipped with a digital camera. Reference points were marked close to the scratches to evaluate the same field during image acquisition. The open wound area was quantified using the ImageJ (NIH, USA) software. The extent of wound closure was presented as the percentage by which the original scratch width had decreased at each measured time point.

In vitro 3D sprouting assay

The sprouting assay was carried out as described [33]. Briefly, HUVEC-coated microspheres were resuspended in fibrinogen solution (2.5 mg/mL fibrinogen, Sigma-Aldrich) in EGM-2 medium (without FBS), supplemented with 50 mg/mL aprotinin (Sigma-Aldrich) and plated with 0.15 U thrombin (Sigma-Aldrich) on top of a precoated fibrin layer at 37 ºC for 20 min. After the gels were allowed to polymerize, NCM, HCM, or vehicle control medium were added and replaced every two days. After four days, the number of endothelial sprouts/beads, branches, and tubule length was quantified in at least 30 microspheres per condition.

In vivo Matrigel plug assay

In vivo angiogenesis experiments were performed as described [34]. Briefly, a mixture of basement membrane matrix (ice-cold, phenol red-free, reduced growth factor, Gibco) and 10x conditioned medium (0.5 mL, 9:1 proportion) was subcutaneously injected into 8-week-old C57Bl/6 wild-type mice (n = 8, 4 per group).

Each mouse received two implants, totaling 8 plugs per group. Buffered saline was included as a negative control during the assay. After 11 days, the plugs were excised, photographed, and processed to assess the angiogenic response. The relative hemoglobin content indicating the degree of blood vessel invasion into the plug was measured as described [31].

In vivo wound healing assay and Immunofluorescent labeling

Two cohorts of mice were used in 2 independent experiments (n = 12, 6 per group). Nestin-GFP+/NG2-DsRed+ double-transgenic mice (on the C57BL/6 genetic background) were anesthetized with a combination of ketamine plus xylazine (80 and 20 mg kg^{-1} i.p.) and shaved. Four full-thickness (including the panniculus carnosus) were performed on the back of the mouse, two on each side of the animal midline, by using a 4.0 mm biopsy punch. Wounding day was coded as day 0. Immediately after excision, the wounds on the right side were treated with vehicle control medium and those on the left side were treated with the conditioned medium. One group was treated with normoxic hASC conditioned medium (NCM) and the other group with hypoxic hASC conditioned medium (HCM). A total of 50 ⌈L of conditioned medium (2.5 ± 0.15 mg/mL) or vehicle control medium were subcutaneously injected into four diametrically opposed points at the wound site (1 mm from the wound edges) on days 0 and 2. Wounds were then left uncovered. Digital pictures of wounds were taken at indicated days after wounding. After seven days, mice were euthanized, and the skin sample was harvested, processed, embedded in OCT medium, and stored at - 80°C for further immunofluorescence staining, as described [30]. Quantification of blood

vessels and perivascular cells was achieved using immunofluorescent visualization of blood vessels on frozen sections. Frozen skin sections were stained with goat anti-CD31 antibody (R&D System, 1:100 in BSA/standard serum solution), rabbit anti-RFP (Abcam, 1:100), chicken anti-GFP (Abcam, 1:100), followed by incubation with the following secondary antibodies: Donkey anti-rabbit 555 (Molecular Probes, 1:500 in BSA/ standard serum solution), Donkey anti-goat 647 (Molecular Probes, 1:500) and Donkey anti-chicken 488 (Jackson Immuno Research, 1:500). Images were captured using fluorescence microscopy analysis (Leika SP8). Quantitative analysis was carried out with the ImageJ (IJ2.3.0/1.53f; Bethesda, MD) software. Colocalizations were assessed with the Leica Application Suite Advanced Fluorescence software. Colocalization was quantified with the RG2B colocalization, Colocalization_Finder, and JACoP plugins of the ImageJ program [35,36]

Antibody-Based Protein Array Analysis

Analysis of different trophic factors in CM was performed using a Proteome Profiler Human Angiogenesis Array kit (R&D Systems) according to the manufacturer's instructions. All analyses were performed starting from the same amount of proteins (3mg) for each preparation. Briefly, either 1 mL of NCM or HCM were incubated with the membrane arrays, positive spots were identified by chemiluminescence, and data quantification was performed by densitometry using the ImageJ (NIH, USA) software.

Functional enrichment of genes associated with regulation of angiogenesis

We used the STRING database (http://www.string-db.org/) to assess the protein-protein interactions (PPIs) of the most abundant proteins identified in our array analysis. The functional enrichment of these PPI by Gene Ontology (GO), including biological process, cellular component, and molecular function, was assessed and visualized with the Cytoscape 3.9.1 software [37]. The hypergeometric test computed the p-values, the minimum required interaction score was 0.7 for high confidence, and the Benjamini & Hochberg false discovery rate (FDR) correction was also defined at a significance level of 0.05.

Statistical analysis

Statistical analyses were performed with Statistical Package for the Social Sciences (SPSS) version 25.0 software (IBM, Armonk, New York, USA) and GraphPad Prism v6.0 (Graphpad Software, San Diego, California, USA). The significance level (p values) was below 0.05. Quantitative variables distribution was carried out using the Shapiro-Wilk test. Unpaired t-test or Mann–Whitney U tests were used to compare two groups according to the variable distribution, or one-way analysis of variance (ANOVA), followed by the Bonferroni *post-ho*c test for three-group comparisons.

Results

Adult hASCs displayed multilineage potential and maintained viability under serum-free medium and hypoxic preconditioning

First, adult hASCs were characterized according to their ability to adhere to plastic, the surface antigen markers expression and the multilineage differentiation potential. In culture, the hASCs were constitutively positive for typical mesenchymal markers, with high (>95%) expression of CD29, CD73, CD90 and CD105, and low (< 2%) expression of hematopoietic markers (Fig 1A). The isolated hASC were *in vitro* differentiated in adipogenic, osteogenic and chondrogenic lineages, as showed in Fig 1B. The hASCs expressed low but detectable amounts of α-SMA and collagen-IV as shown in Fig 1C. These data indicated that the isolated hASC consistently meet all basic criteria required for human mesenchymal stem cells derived from lipoaspirate samples, and can be used for subsequent studies. Characterized hASCs cultured in serum-free medium under standard conditions (normoxic) or combined with low oxygen level (hypoxic) preconditioning displayed elongated spindle shapes. Upon hypoxic preconditioning, the viability of hASCs was maintained compared to normoxic condition, as indicated by calcein cell-permeant staining (Fig 1D). These results suggest that 48h serum-free media combined with hypoxic preconditioning did not affect hASCs viability.

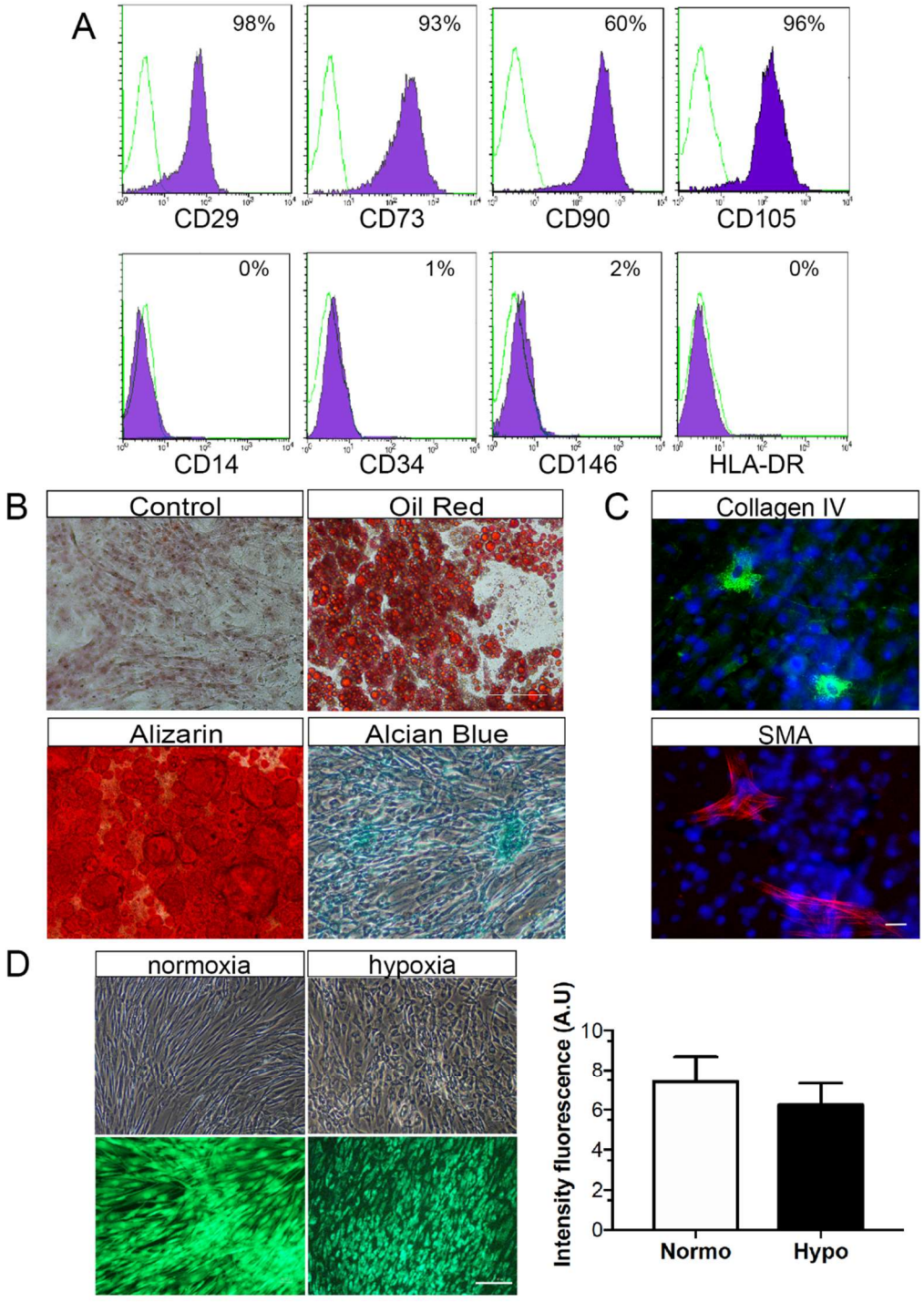

Fig 1. Characterization of hASCs. (A) Flow cytometry histograms shows that hASC were positive for CD29, CD73, CD90 and CD105, while negative for CD14, CD34, CD146 and HDLA-DR. The percentage of cells staining for each marker (purple area) and the respective isotype-control (green line) are provided. **(B)** Phase-contrast microscopy imaging of hASCs exposed to adipogenic, osteogenic, or chondrogenic differentiation media. Accumulation of intracellular lipid vacuoles shown by oil red-O

staining, calcium-rich extracellular matrix as evidenced by Alizarin red S, and glycosaminoglycans stained with Alcian Blue. **(C)** Immunocytochemistry detection shows α -SMA and collagen-IV-positive hASCs. Nuclei were stained with Hoechst dye (blue). **(D)** After normoxic and hypoxic preconditioning, hASC displayed spindle-shaped fibroblast-like morphology. Staining with Calcein-AM indicated that hASC viability remained unaffected by hypoxic preconditioning. Scale bars: 25μm in C and 75μm in D. Values are expressed as means ± SD of at least three independent experiments (n = 8).

The secretome profile of hASCs is enriched with trophic factors

Analysis of the conditioned medium revealed that of 55 angiogenic-related mediators analyzed, 27 were consistently detected on the membrane in at least three samples of at least one group (Fig 2A). A total of 12 growth factors/cytokines with relevant expression (OD Abs > 0.5) were expressed in both CMs, and eight trophic factors were differentially expressed between NCM and HCM (Fig 2B). Since this result indicated the presence of both pro-and antiangiogenic factors, we identified enriched pathways and biological processes in the whole secretome. The list of consistently more abundant proteins in NCM and HCM samples was analyzed to predict a protein-protein interaction network (PPI) with the String-online database (Fig 2C). GO enrichment analysis showed that the more abundant proteins played an essential role in angiogenesis, vasculature development, cell migration, and tissue repair (Fig 2D and S1A Fig). The KEGG pathway enrichment analysis results showed that the differentially expressed proteins were mainly involved in the pathways related to response to hypoxia, cell proliferation, and migration, including PI3K-Akt, HIF-1, Rap-1 signaling cytokine-cytokine receptor interaction pathways (S1B Fig).

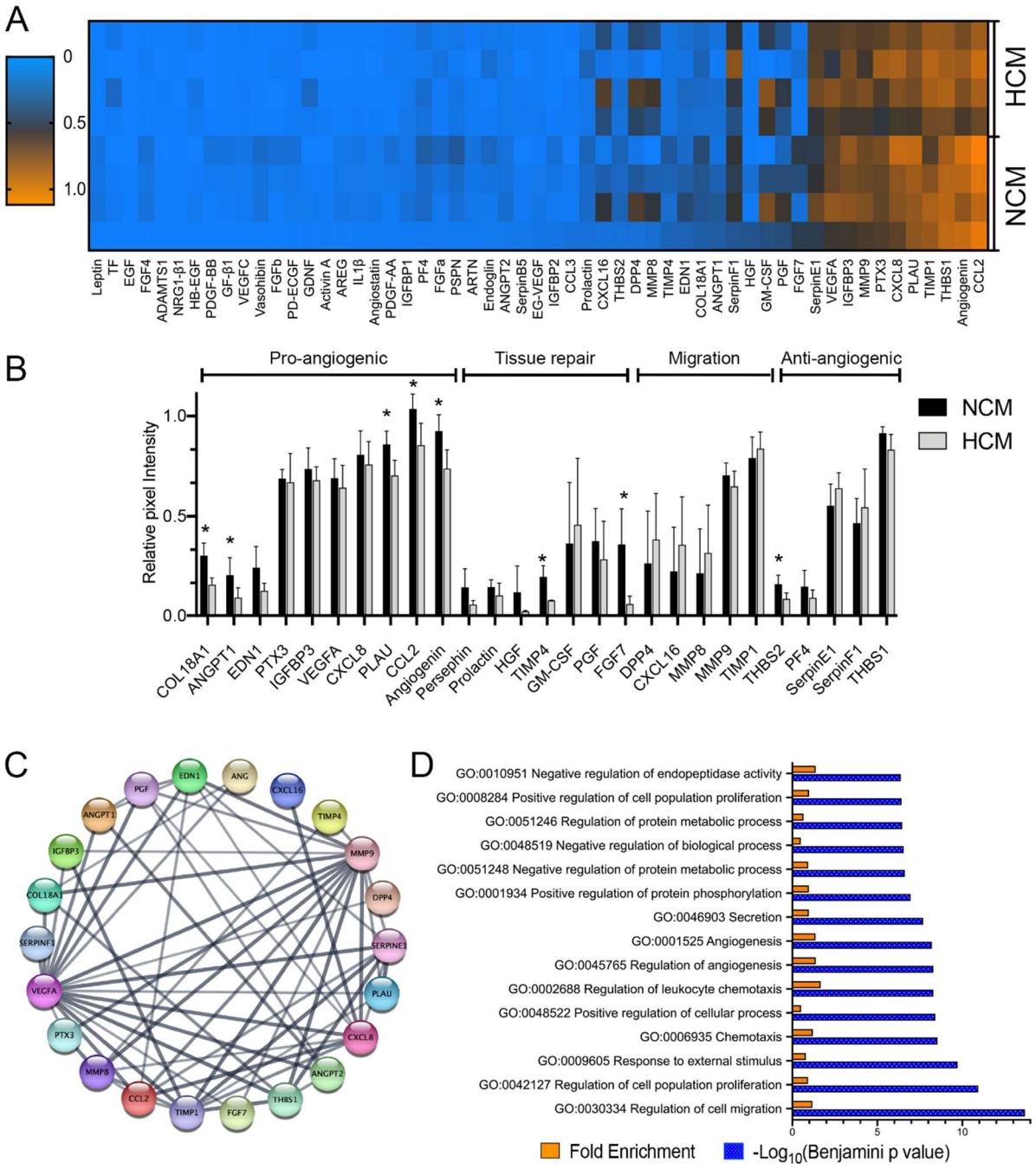

Fig 2. Characterization of HCM and NCM secretomes of human adipose mesenchymal stem cells (hASCs). (A) Heatmap depicts the overall profile of 55 trophic factors, cytokines, and chemokines relative to protein abundance (mean pixel intensity). Each column represents a different protein, while each row represents a sample. **(B)** NCM and HCM were analyzed by antibody-based protein array kit. Values are normalized to positive reference spots. Data are representative of three independent experiments, and values are expressed in mean ± SD. **(C)** STRING analysis uncovering protein-protein interaction (PPI) network of most abundant trophic factors expressed in NCM and HCM visualized by

Cytoscape. **(D)** David Gene Ontology analyses of proteins more abundant in hASC secretome. GO enrichment analysis showed the biological process of most representative trophic factors ranked by p-value. The top 15 processes were selected based on the Benjamini p-value ($-\text{Log}_{10}$ Benjamini p-value are reported as blue bars). Fold enrichment is also reported as orange bars. * $p<0.05$. N= 8.

hASCs-conditioned medium maintained viability and induced proliferation of HUVEC.

To verify the *in vitro* effects of NCM and HCM on the viability of endothelial cells, we performed the TUNEL assay. The experiment indicated a decreased number of late apoptotic cells in HUVECs treated with NCM or HCM when compared to the control group (0.6 ± 0.7, 0.8 ± 1.0 vs 5.2 ± 2.6, $p<0.05$), indicating that both conditioned media exhibited antiapoptotic characteristics and had a positive effect on cell survival (Fig 3A). Next, we evaluated the incorporation of BrdU in HUVECs treated with NCM or HCM. After 24h, a significant number of BrdU-positive HUVECs was observed in the presence of NCM and HCM when compared with the control group ($p<0.05$). These results indicate that NCM and HCM were able to induce proliferation in HUVECs (Fig 3B). Various signaling pathways, including PI3K-AKT, have been implicated in the pro-angiogenic function of endothelial cells. Accordingly, we tested if NCM and HCM conditioned media induced intracellular signaling in HUVECs. Serum and growth factor starved HUVECs were stimulated in a time-dependent manner. We found that serum-starved HUVECs stimulated with NCM or HCM activated the PI3K-AKT pathway. Both conditioned media induced activation of the AKT pathway after 5min ($p<0.05$) (Fig 3C, Fig 3D and S1 Fig). These data showed that both CMs were able to activate the PI3K-

AKT pathway, which is involved in cell survival mechanisms, corroborating the predicted PPI network and previous results about the effect of NCM and HCM on endothelial cell survival and proliferation.

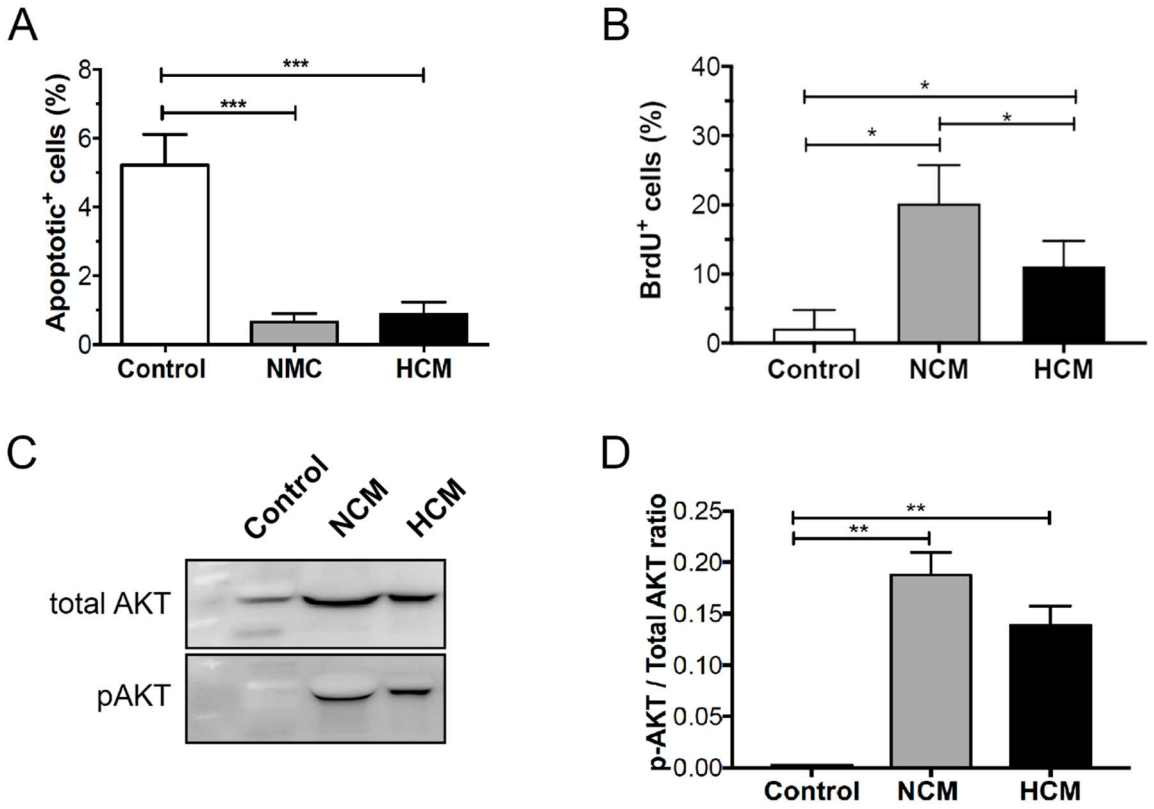

Fig 3. Normoxic- and hypoxic- hASCs secretomes increase cell proliferation and attenuate apoptosis in HUVECs. (A) HUVECs were cultured for 24h in medium alone (control) or supplemented with either NCM or HCM. TUNEL-positive cells were counted, and the apoptotic index was calculated as the average number of positive cells compared to the total number of cells in at least six visual fields. Values shown are the mean ± SD of at least three independent experiments. **(B)** HUVECs were pulse-labeled with 10 μM BrdU, cultured for up to 24 hours in the presence of NCM or HCM, and BrdU was visualized by immunocytochemistry and quantified by cell counting. **(C)** Representative western blots of pAKT and total AKT expression in HUVECs exposed to NCM or HCM. **(D)** Quantitative data representing the average values of three independent experiments. The Fig 3C was cropped to improve the clarity and conciseness of the presentation, and the full-length blots are presented in Supplementary file. Results indicate mean normalized expression relative to control ± SD. Cell experiment was repeated three times independently. *$p<0.05$; ** $p<0.01$ ($n = 4$).

hASCs-conditioned medium stimulates migration and sprouting of endothelial cells

Angiogenesis is a multistep process that requires cell migration, proliferation, survival, and tube formation. We evaluated whether the hASCs-conditioned medium stimulates angiogenesis. First, the chemotactic potential of the conditioned medium was examined with the *in vitro* scratch wound assay. HUVEC migration was significantly enhanced (~2.0-fold) in the presence of NCM and HCM, in comparison to the vehicle control medium (99.5 ± 0.5; 99.2 ± 0.9 vs 55.5 ± 8.5 % of original scratched area, *** $p<0.0005$), with monolayer scratching 100% recovered after 18h (Fig 4A). Next, *in vitro* angiogenesis was examined in HUVECs seeded on collagen-coated Cytodex beads embedded in a fibrin gel. Under control conditions, no sprouts were formed, and this group was removed from the analyzed graphs. In the presence of either NCM or HCM, endothelial sprouts appeared around day 2, and capillary-like structures were formed around day 6. Figure 4B exhibits the formation of a more significant number of endothelial sprouts/beads (6.5 ± 1.6 vs 5.0 ± 2.8 sprouts/beads, $p<0.05$) with greater lengths (229.4 ± 49.6 vs 172.8 ± 73.7 μm, $p<0.0001$) in the presence of NCM, as compared to HCM. A similar response was observed in the Matrigel plug assay to evaluate *in vivo* angiogenesis. Both NCM or HCM conditioned media were able to induce invasion of blood vessels into the Matrigel plug when compared to control ($p<0.05$) (Fig 4C). These results indicate that NCM and HCM showed pro-angiogenic capacity, and hypoxia preconditioning did not alter the effects of hASCs.

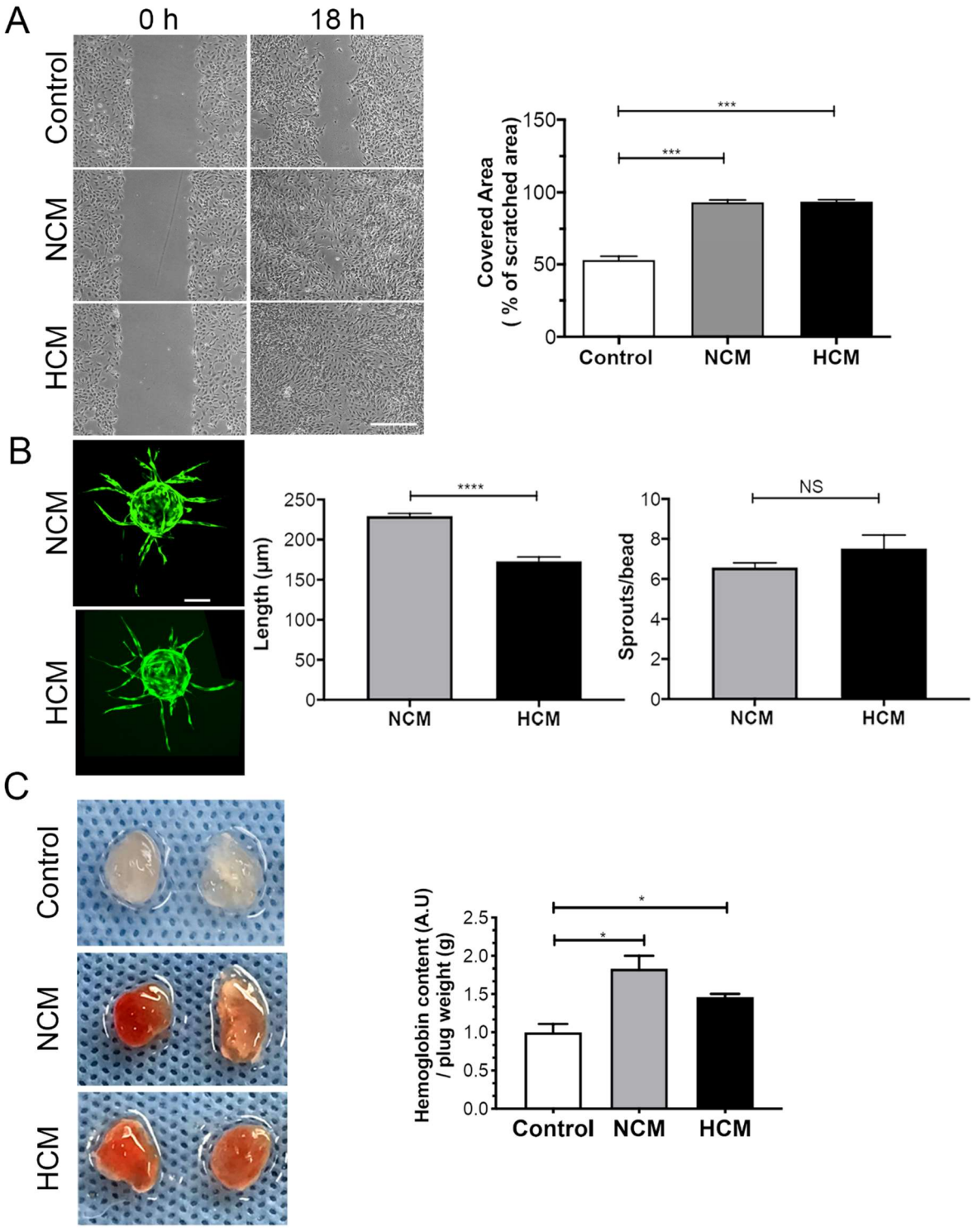

Fig 4. Hypoxic- (HCM) and normoxic- (NCM) hASCs secretomes accelerate *in vitro* cell migration and show *in vitro* and *in vivo* pro-angiogenic potential. (A) Representative image of the migration of HUVEC cells after 0 or 18 h of incubation in HCM, NCM or control. Quantification of the percentage of variation in the wound area (% migration area). Scale bars = 250 µm **(B)** Representative images of EC-coated beads with NCM or HCM on fibrin gel and stained with calcein. Quantitation of EC sprouting

formation and sprout length in arbitrary units formed after 4 days. Scale bars = 100μm **(C)** Mixture of matrigel-filled plugs containing indicated conditioned medium (NCM or HCM) or PBS (control) were injected subcutaneously in mice (n = 8). After 11 days, hemoglobin content representative of invading vessels was measured. The two-way ANOVA test and the Bonferroni post-test were used to analyze the differences among the groups. Values are expressed as mean ± SD of at least three independent experiments. (C) **p<0.01; ***p<0.001 (n = 12, 6 per group).

The hASC-derived conditioned medium enhances skin repair in a wound healing mouse model.

The reparative potential of the conditioned medium was evaluated in a murine full-thickness excisional wound healing model. The wounds were treated with a control medium, NCM, or HCM, and observed on days 0, 3, 5, 7, 9, and 11. On day 7, the wound healing process was accelerated in wounds exposed to NCM and HCM, when compared with the vehicle control group, with a reduction of the injured area on days 9 and 11 ($p<0.05$) (Fig 5A). The relative abundance of CD31+ (endothelial cell marker) and Nestin+NG2+ perivascular (pericyte cell markers) populations were analyzed in wounded skin (Fig 5B). Upon injury, our immunofluorescence staining demonstrated that NCM or HCM treatment led to a significant increase in CD31+ capillaries. These CD31+ capillaries exhibited a higher percentage of endothelial vessels associated with pericytes when compared to the control medium ($p<0.05$) (Fig 5C). These results suggest that human hASCs secretome enhances endothelial blood vessel formation and pericyte coverage during full-thickness excisional skin repair.

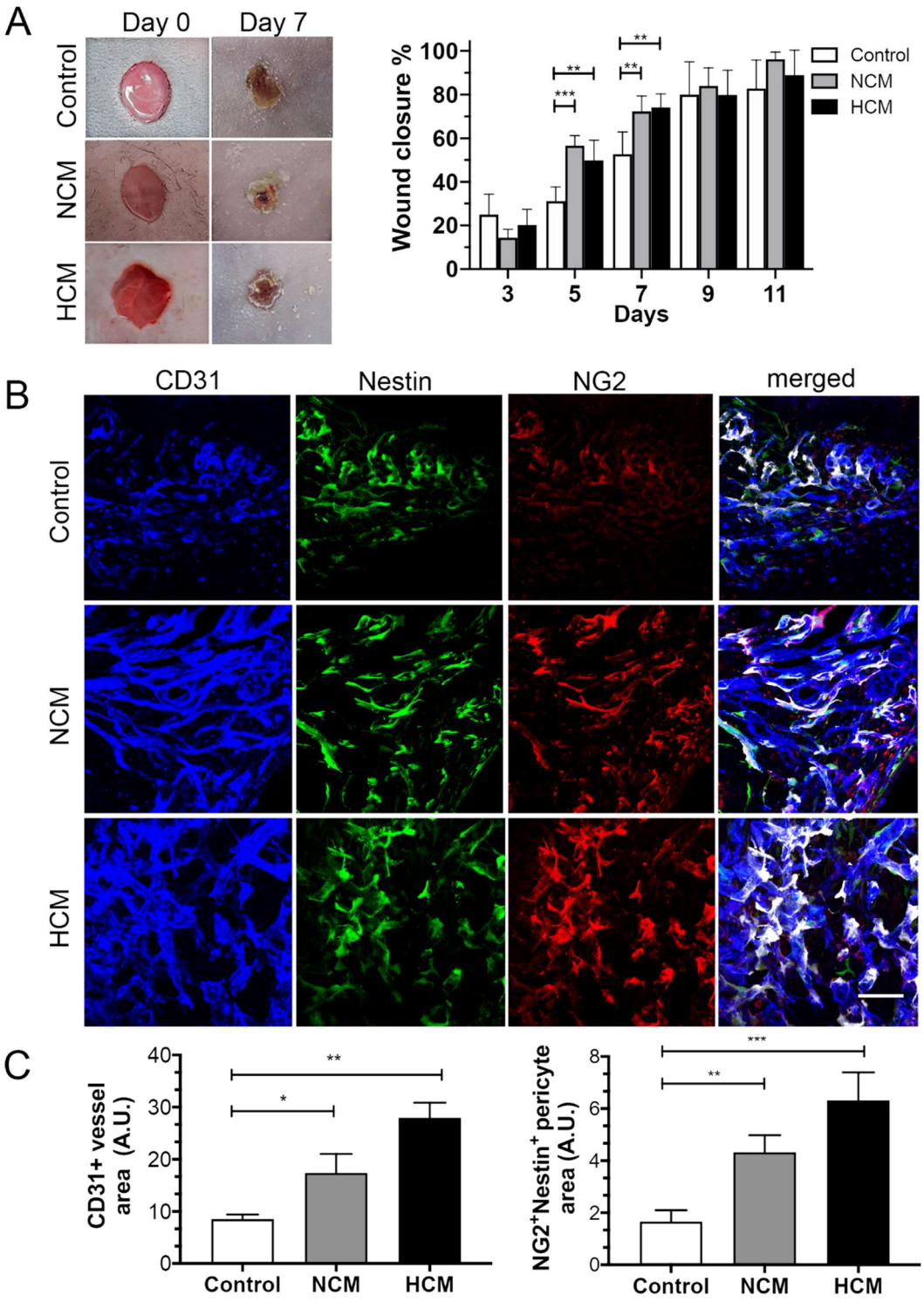

Fig 5. NG2+Nestin+ pericytes surrounding the blood vessels and in close contact with the vascular wall in hASCs secretomes treated animals. (A) Representative macroscopic images showing cutaneous wounds on days 0 and 7 after injection of control, hypoxic- (HCM) or normoxic- (NCM) secretomes. **(B)** HCM and NCM accelerated wound closure and microvessel density. Confocal images of the skin wound sections labeled NG2+(red)/Nestin+(green) pericytes and CD31+ blood

vessels (blue). Use of pseudocolor (white) to display colocalization of NG2+(red) and nestin+(green) pericytes around CD31+microvessels (blue). **(C)** The extent of microvessel density was determined by assessing the CD31+ vessel area or NG2+nestin+ area in each of 4 randomly chosen high-power fields within the injury site. Scale bar, 100 μm for images in (B). Results are given as the means ± the SD. *p<0.05, **p<0.01; ***p<0.001 (n = 12, 6 per group).

Discussion

Emerging evidence shows that the therapeutic effects of hASCs result from their intense paracrine activity mediated by their secretome. However, hASCs represent a heterogeneous cell population with varied secretory behavior. hASCs preconditioning is a valuable alternative to improve secretome function and reduce inter-individual cell variability [38, 39]. The present study used serum-free medium alone or combined with hypoxia as preconditioning strategies to yield normoxic (NCM) or hypoxic (HCM) secretomes, respectively. We showed that both secretomes enhanced endothelial cell proliferation and migration and reduced endothelial cell apoptosis. Additionally, in vitro endothelial sprouting and *in vivo* Matrigel invasion were almost equivalent for both normoxic and hypoxic secretomes. Moreover, we demonstrated that both normoxic and hypoxic hASCs secretomes accelerated wound healing, blood vessel formation, and resident pericyte coverage in the murine full-thickness excisional wound healing model. These findings suggest that serum-free media or hypoxia preconditioning promoted the angiogenic and skin repair potential of hASCs secretomes.

Several studies indicate that serum-free media may benefit mesenchymal stem cells proliferation, differentiation, and paracrine activity while maintaining their stemness, immunosuppressive and antifibrotic abilities [40-42]. In general, secretomes

collected under serum-free conditions are much more appropriate for clinical use because of reduced contamination and safety concerns related to xenogeneic infectious agents [43-45]. Serum-free conditions facilitate the proteomic analysis of secretomes [5,46]. In addition, hypoxic preconditioning has been widely studied to improve hASCs paracrine activity in models in which proangiogenic and skin-repairing effects of secretomes are expected [4,47]. Therefore, we investigate whether serum-free media alone or combined with hypoxic preconditioning constituted an effective strategy to increase the number and function of secreted molecules. The present study found that serum-free condition induces paracrine activity in hASCs to produce a relatively enriched secretome. In addition, the combination of serum-free condition and hypoxia preconditioning has a limited effect on the secretome composition. Our results further indicated that serum-free media and hypoxia preconditioning for 48h did not affect hASC's spindle-shaped cell morphology and viability, consistent with previous studies [40, 47]. The predicted PPI and the protein array profiling of NCM and HCM secretomes showed that the synergism employed here caused no substantial alteration of the paracrine factor landscape, with only minimal significant differences between these two types of secretomes. Similarly, Peltzer et al. [39] showed that hypoxia preconditioning has no additional effect on mesenchymal paracrine activity primed with Interferon-g, and only a limited number of secreted molecules was affected by hypoxia. Moreover, Ferreira et al. [20] described that hypoxia preconditioning for short periods significantly impacted cell proliferation and increased ASCs cell survival and paracrine ability. These discrepancies are likely due to experimental conditions or donor inter-variability [49]. Therefore, our results suggest that hypoxic preconditioning has no additional effect on the serum-free media condition to improve the secretome composition.

hASCs secretomes enriched with angiogenic and other paracrine growth factors display a notable function to accelerate healing and promote wound angiogenesis in various preclinical animal models. In our study, both NCM and HCM secretomes showed strong angiogenic and healing potential, as evidenced by HUVEC proliferation, sprouting formation, and *in vivo* angiogenesis. In this context, we analyzed hASCs secretomes to identify possible paracrine factors that are functionally involved in the acceleration of wound healing processes. Predominantly, we identified regulators of tissue repair, including pro- (VEGF-A, angiogenin, IGFBP3) and anti-angiogenic (Serpins F1 and E1, THBS1) factors, inflammatory response mediators (CCL2, CXCL8, PTX3), migration (MMP-9 and TIMP-1) and coagulation (PLAU) factors. Recently, Cases-Perera et al., [50] identified 8 proteins enriched in hASCs secretome, including VEGF, TIMP-1, THBS1, Serpin F1/E1, IGFBP-3, and PTX3. Remarkably, our results also indicated high expression of CCL2, Angiogenin, CXCL8, that are critical players with proangiogenic effect, as previously described by Kim et al., [11]. Although the composition of hASCs secretomes have differed slightly with culture preconditioning, the presence of these proteins in similar amount suggests that their potential role are equivalents [50]. These results are in agreement with previous reports suggesting that hASCs secretome are an abundant source of trophic factors, with marked growth and angiogenic properties—factors, that could be relevant to accelerate wound healing and enhance in vivo skin repair [3, 11, 50].

Even though available reports state that under standard culture conditions, hASCs secrete high levels of VEGF-A and other proangiogenic factors [51, 52], which can be even higher under low oxygen conditions [53, 54], we found equivalent expression levels of VEGF-A in both normoxic and hypoxic secretomes. The serum-free condition has been shown to induce a stress response that might be sufficient to

stimulate a paracrine activity in hASCs, rendering uncertain the effect of the low oxygen tension [55, 56]. Similarly, a recent report has shown that normoxic or hypoxic hASCs secretomes contain equivalent levels of angiogenin, VEGFA, CCL2, and IGFBP3 and accelerate the healing process by increasing fibroblast migration and granulation tissue formation [57]. Additionally, after mass spectrometry analysis of hASCs secretome, Riis and colleagues [24] could not detect a significant effect of hypoxic preconditioning. Only a relatively small fraction (9.6%) of the proteome was affected by low oxygen compared to normoxia and serum-free conditions [24]. From this perspective, An et al. [52] analyzed the proteomic profile of serum-free hASCs secretome. They revealed a remarkably high amount of protein molecules for wound healing, i.e., TGF-β1 and VEGF, besides more than 700 proteins highly involved in the extracellular matrix organization, angiogenesis, and cell migration. In accordance, a comprehensive quantitative proteomics approach recently explored the protein composition of serum-free hASC secretome and identified more than 1977 proteins involved in ECM organization (hyaluronan and glycosaminoglycan metabolism) and immunological regulation (e.g., macrophage and IkB/NFkB signaling regulation) [5].

Among the in-silico hASCs-enriched processes, platelet degranulation, extracellular matrix organization, and regulation of smooth muscle cell-matrix adhesion pathways emerged. Our *in vivo* results demonstrated that normoxic or hypoxic hASCs secretome enhanced blood vessel density and pericyte coverage during wound healing. These results are consistent with other preclinical studies of skin repair, demonstrating that paracrine factors increase vessels density and accelerate healing in full-thickness excisional wounds [58, 59], diabetic wounds [60-62], hypertrophic and keloid scars [63], skin flaps [64] and hair loss [65]. However, it is noteworthy that most studies do not assess the presence of perivascular cells (i.e., pericytes) surrounding

the newly formed blood vessels mediated by the hASCs secretomes. Pericytes play a pivotal role in maintaining vascular integrity and restoring skin function after acute injury [66]. In this context, our results completely align with previously described results for wound angiogenesis and skin repair [67].

In our study, we found that nestin+/NG2+ pericytes were surrounding blood vessels seven days post-wound. We also quantified perivascular cells, and found the highest number of nestin+/NG2+ pericytes in wounds treated with hASC secretome and accelerated healing. It is suggestive that these perivascular cells may help vessel stabilization, maturation, or vessel remodeling. These results were consistent with previous observations that nestin+/NG2+ pericytes generate blood vessel tissues [27,68], and NG2+ perivascular cells are co-localized with blood vessels for up to 10 days post-wound [69]. Recently, do Valle et al. [67] demonstrated that an increased number of nestin+/NG2+ pericytes and undifferentiated cells were mobilized to wound edges in the initial experimental periods and accumulated in the dermal regions of the wound afterward. Further work is needed to explore the role of nestin+/NG2+ pericytes in stabilizing blood vessels, contributing to vascular maturation, remodeling, and regulating the permeability and blood flow in wound healing. The healing effect accomplished by hASCs secretome presumably was because it provided essential trophic and mediating factors that helped create a proangiogenic response at different stages of the wound healing process. However, it remains unclear whether hASCs secretome contributes to the direct activation of pericytes and endothelial cells or is indirectly responsible for the recruitment of these and other cells (tissue-resident progenitors, inflammatory cells) due to metalloproteinases and chemokines detected in secretomes.

hASCs secretomes are currently in phase I and II clinical trials for diverse applications [70 - 73], even though the road toward an exhaustive characterization of this new generation of cell-free therapeutic is still a very long one. Compared to cell therapy, the use of secretomes has lower biological risks, less tumorigenicity and reduced cost [3]. However, the risk of disease transmission as well as xenogeneic immune reactions in the recipient is a clear limitation. In addition, the commercial use of secretome still requires challenges to be addressed, such as the optimization and reproducibility of production methods, better characterization of its constituents (content of cytokines, growth factors, microRNAs, lipids and microvesicles), better preservation and dose-effect evaluation to achieve the best efficacy profile for clinical applications. The use of filter sterilization to remove biological contaminants during the preparation of commercially intended hASCs secretomes should be carefully evaluated, as it could considerably affect the contents of microvesicles and extravelllar vesicles with therapeutic potential [3,4].

In conclusion, our results suggest that serum-free media or hypoxic preconditioning hASCs secretomes display a very similar composition of specific bioactive factors. Both NCM and HCM were potent in promoting *in vitro* and *in vivo* angiogenesis, tissue vascularization, and vascular coverage of resident pericytes expressing NG2 and nestin to the lesion site, potentially contributing to blood vessel maturation. Overall, normoxic and hypoxic preconditioning enhanced the vascular reparative effects of hASCs secretome on the preclinical wound healing model. Therefore, from the clinical translation perspective, hASCs secretome represents a potential therapeutic product in a practical and minimal manipulation procedure, affording a more feasible scale-up approach.

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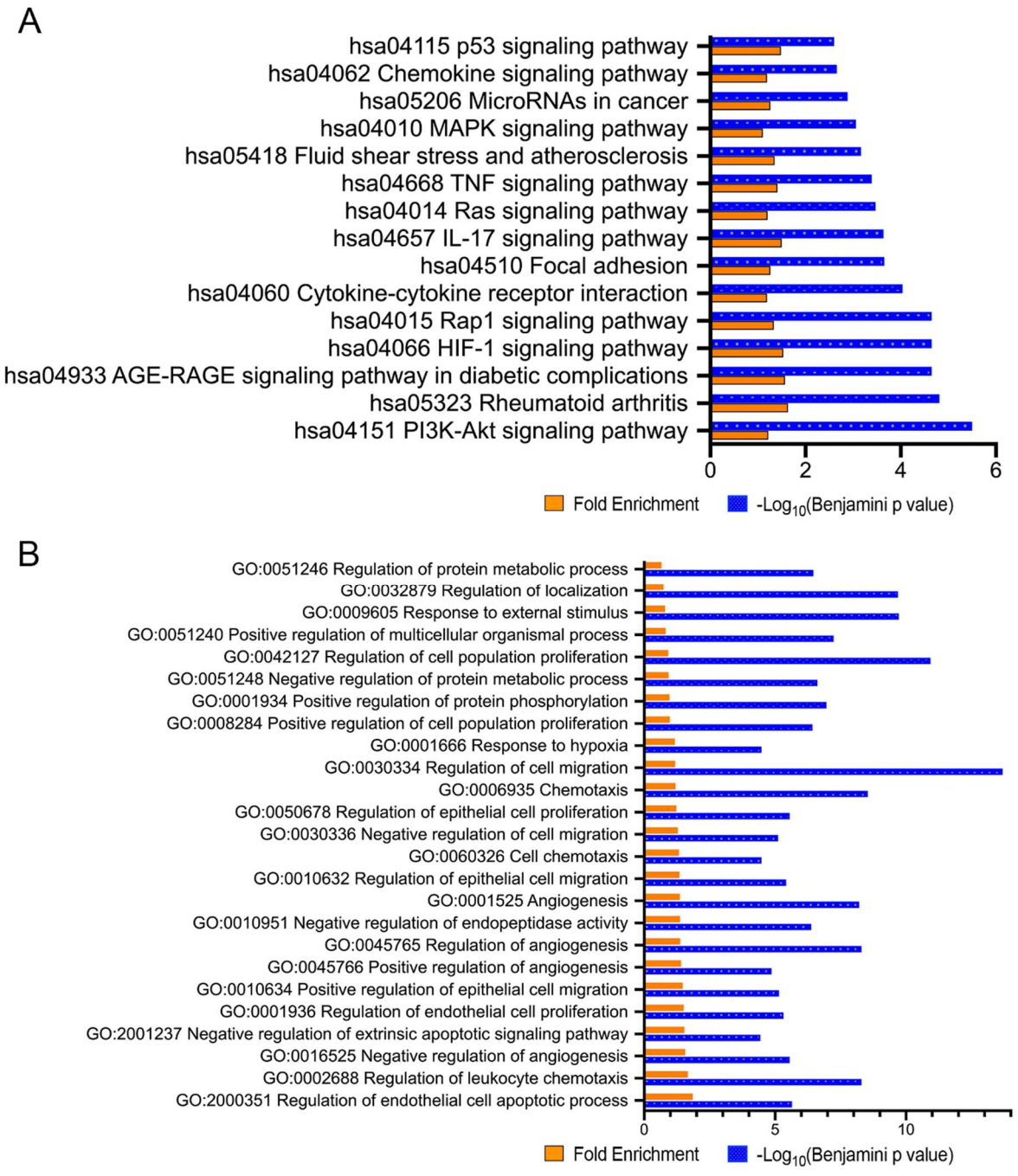
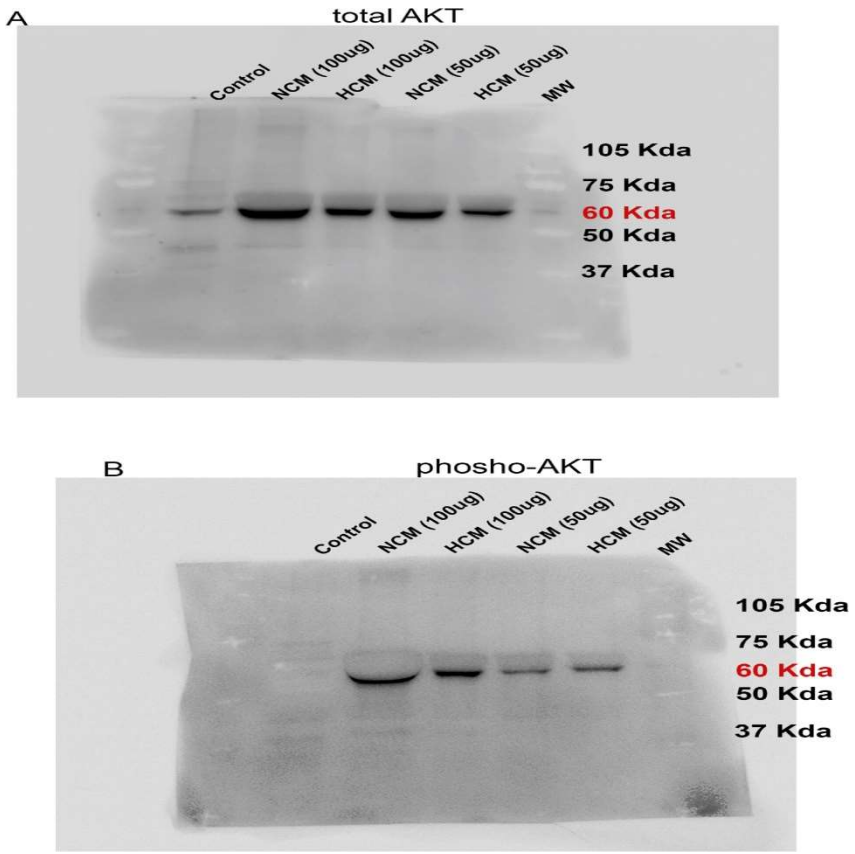
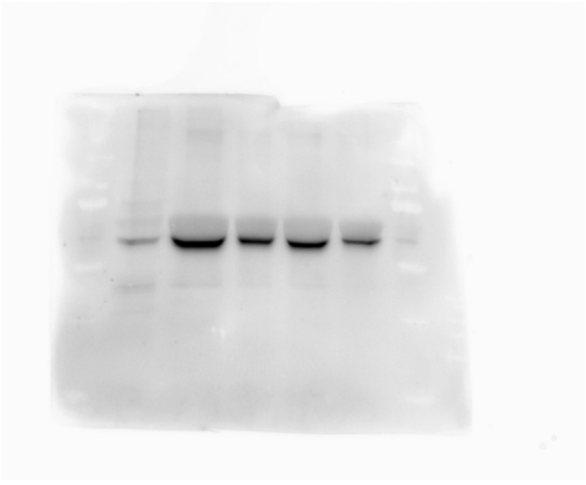
Supporting information



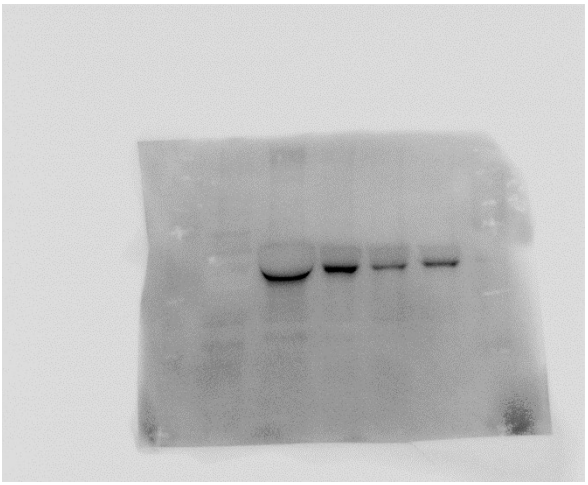
S1 Fig. (A) KEGG enriched pathways of most representative trophic factors in hASC secretome ranked by p-value. − Log_{10} Benjamini p-value are reported as blue bar and Fold enrichment as orange bars. **(B)** Gene Ontology analyses of proteins more abundant in hASC conditioned medium ranked by Fold enrichment. The top 25 processes are shown.



S2 Fig. Total AKT and phospho-AKT(Ser473) were detected in HUVECs after incubations with hASC secretomes. Representative Western blots for total-Akt (A) and P-Akt$_{Ser473}$ (B) from HUVECs that were starved in 0.1%BSA EBM-2 and stimulated with control medium, NCM or HCM for 5 min.



S2A_raw_images. Total AKT.



S2B_raw_images. phospho-AKT(Ser473)

S1 Table. Patient and tissue harvesting information

Identification	Ethnic group	Gender	Age	Source
Patient 1	Black	Female	30	abdominal liposuction
Patient 2	White	Female	45	abdominal liposuction
Patient 3	White	Female	35	abdominal liposuction
Patient 4	White	Female	27	abdominal liposuction
Patient 5	Black	Female	38	abdominal liposuction
Patient 6	White	Female	40	abdominal liposuction
Patient 7	White	Female	25	abdominal liposuction
Patient 8	White	Female	30	abdominal liposuction

6. CAPÍTULO 2

Os resultados apresentados neste segundo capítulo da tese estão compilados e discutidos no manuscrito **"Adipose derived stromal cell-conditioned accelerated wound healing in a murine model of sickle cell anemia"** a ser submetido na revista científica **PloSOne** (Silveira BM et al., 2023).

ARTIGO 2

Adipose derived stromal cell-conditioned accelerated wound healing in a murine model of sickle cell anemia

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Abstract

Sickle cell disease is a hemoglobinopathy, characterized by the substitution mutation of glutamic acid for valine, generating hemoglobin S. Erythrocytes with hemoglobin S assume a sickle-like phenotype, resulting from the polymerization of defective hemoglobin. This phenotype hinders its flow through the capillaries, causing secondary complications, such as chronic leg ulcers (UCL). Adipose derived stromal cells (ASC) represent a therapeutic alternative for regenerative medicine. Due to their ability to secrete

bioactive molecules involved in angiogenesis and tissue repair, ASC secretome is a therapeutic alternative for the treatment of non-healing wounds. The present study evaluated the effects of ASC secretome on wound healing in Towness mice HbSS. The secretome collected from ASC culture was administered to dorsal full-thickness wounds on sickle cell anemia mice. The secretome's therapeutic effect was analyzed in a wound healing model through wound reduction rate, gene expression analysis by RT-PCR, histological and immunofluorescence assay. Wounds treated with ASC secretome showed improved healing over 7 days (n=08). Histological sections showed a reduction in the inflammatory infiltrate, an increase in the number of fibroblasts and thickening of the epidermis in the wound lesions treated with the ASC secretome. Immunofluorescence analysis revealed positive expression for CD31, α-SMA and SM22 markers in both treated groups. Differentiated expression of SM22 in the group control on the 7th day suggests the delay of the remodeling phase in untreated wounds. Finally, analysis of expression of transcripts by RT-PCR revealed a reduction in the levels of soluble pro- and anti-inflammatory mediators. In sum, the ASC secretome promoted the acceleration of wound healing in sickle cell mice, reducing local inflammation and restoring the structural morphology of the epidermis. However, it may be necessary to stimulate ASC with the aim of optimizing the immunomodulatory capacity of the secretome.

Keywords: adipose derived stem cells; sickle cell anemia; secretome; chronic leg ulcers;

Introduction

Sickle cell anemia (SCA) is a genetic hemoglobinopathy caused by the substitution mutation of glutamic acid for valine, in position 6 of the N-terminus of the ß globin chain, with production of hemoglobin S (HbS). Defective red blood cells assume a sickle-like phenotype, resulting from the polymerization of abnormal hemoglobin [1,2]. The clinical manifestations of SCA are consequences of pathophysiological alterations such as vaso-occlusion events, oxidative stress and inflammation. Sickle cell patients may develop some clinical conditions such as recurrent painful crises of vaso-occlusion, pulmonary hypertension, ischemic stroke,

priapism, osteonecrosis and chronic ulcers of the lower limbs [3-6]. The treatment of chronic leg ulcers in sickle cell patients is an important public health problem due to the recurrence of lesions, requiring specific care for pain control, debridement of the area and infection control, resulting in high costs for health services and social problems [7-9].

The use of Mesenchymal Stromal Cells (MSC) in regenerative medicine has generated great interest due to its capacity for self-renewal, tropism for the injured area and paracrine activity, through the secretion of molecular mediators that help the reduction of the inflammatory microenvironment, promoting the migration and proliferation of endothelial cells and tissue repair [10-12]. Data from the literature demonstrate that MSC treatment has an impact on all phases of skin ulcer repair, acting on inflammation, proliferation, angiogenesis and tissue remodeling [13-15]. However, the use of MSCs has some limitations: (1) low survival rate after administration, (2) selection of the best cell population and (3) risk of tumorigenicity, thus making the use of its secretome a safer and more effective alternative [16,17].

Our previous study demonstrated that the secretome produced by Human Adipose derived Stromal Cells (hASC) is composed of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), interleukin (IL-8), monocyte chemoattractant protein-1 (MCP-1), angiogenin (ANG) and hepatocyte growth factor (HGF), that directly influence the migration and proliferation of endothelial cells *in vitro*, acting on the tissue regeneration process in wild-type C57BL/6 mice [18]. Similarly, a study by Se-Ra Park and collaborators demonstrated that the ASC secretome, composed of bFGF, hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin like growth factor binding protein 6 (IGFBP-6) and transforming growth factor beta (TGF-b), accelerated wound healing in Nude mice. The authors reported the

effects of the secretome in inducing migration and proliferation of fibroblasts, as well as the mitogenic and anti-apoptotic action of HGF on endothelial and epithelial cells, through activation of the PI3K/AKT and FAK/ERK1/2 pathway [19]. Furthermore, the regenerative role of MSCs is not only associated with the secretion of bioactive factors, MSCs secrete microvesicles that are able to act in the remodeling and healing of cutaneous wounds [20-22].

Thus, the objective of this work was to investigate the therapeutic potential of the conditioned medium (CM) from hASC in reducing the inflammatory process and stimulating tissue repair of wounds in mice with sickle cell anemia. In the first stage of this study, we produced the conditioned medium from hASC and evaluated its effects in a wound healing murine model over 7 days. Then, we observed the immunomodulatory and regenerative potential of the CM through the processing of the lesions by histological and immunofluorescence assay and analyzed the expression of transcripts by RT-PCR. In summary, the treatment of wounds with CM accelerated the wound healing, inducing the reduction of inflammatory cells, increasing the number of fibroblasts and stimulating the angiogenic process at the site of injury. Therefore, we suggest that CM from hASC may represent an important therapeutic alternative for the treatment of chronic leg ulcers in patients with sickle cell anemia.

Methodology

Ethical statement

This work was approved by the ethics committee of the Institute of Health Sciences (Federal University of Bahia, opinion nº 2,074,627). Liposuction was collected after signing the Informed Consent Form (TCLE). A total volume of 50 ml of

biological material was collected and transported in RPMI medium withi 10% of Fetal Bovine Serum (FBS) and ciprofloxacin (100U/mL), under refrigeration at 4^0C, for isolation of the Stromal Vascular Fraction (SVF) within 24 hours.

Animals

The use and management of animals was reviewed and approved by the institutional review board (CEUA, Fiocruz-Ba-021/2019). All procedures were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals and the regulation of the animal protection committee, aiming to minimize the pain and suffering of the animals. The HbSS-Townes mouse model (Jackson Lab), used in this study, was created on a mixed genetic background in which the adult murine α-globin genes were replaced by the human α-globin gene (genotype: Hbahα / hα) and the murine adult β-globin genes were replaced by fragments of the linked human βS and fetal αγ-globin gene (genotype: Hbb hAγβS / hAγβS).

The Towness B6:129 subtype HbSS mice were kept in the Experimental Animal Facility of the Instituto Gonçalo Muniz, Fiocruz/Bahia (Fiocruz-Ba), in a controlled environment, free of pathogens and other stressors. The animals were housed in mini isolators attached to a "rack" type shelf produced by Alesco (www.alesco.ind.br), under a light/dark cycle every 12 hours and controlled feeding.

ASC Isolation and cultivation

The cells used in this study were from liposuction samples from 08 healthy female patients (age range 25-45 years). The processing of adipose tissue was performed by mechanical digestion (magnetic shaker SL-91/A solab) and enzymatic digestion with collagenase I (1 mg/mL) (Sigma, C0130-1G), at a temperature of 37 °C. After 30 min of enzymatic digestion, the lipoaspirate was centrifuged to collect and

culture the vascular stromal harvest (SVF). The SVF was maintained in T25 adherent bottles in low glucose EM medium (Dulbecco's Modified Eagle's Medium, Life Technologies) with 10% FBS, PenStrep and gentamicin.

ASC characterization

The isolated cells were characterized by analyzing the expression of cell surface antigens and analyzing the potential for multilineage differentiation. Cellular immunophenotyping analyzes were performed using antibodies conjugated with fluorescein isothiocyanate (FITC) and conjugated with phycoerythrin (PE), with analysis in a FACScalibur flow cytometer (BD Biosciences, CA, USA). Cell preparation (1 x 105 cells) consisted of resuspension in 0.9% saline solution and incubation for 30 min, in cytometry tubes, with anti-CD14 PE antibodies (clone 61D3, Batch: E026669, ebioscience), anti-CD34FITC (BD, Cat No. 348053), anti-CD45PerCP (batch: ED7029, Exbio), anti- CD146 PE (batch: 91811, BD Biosciences), anti-HLA-DR PE (clone: MEM-12, batch: 1P474T100, Exbio), anti-CD29FITC (clone: TS2116, batch: E031567, ebioscience), anti-CD73 PE (clone: AD2, batch: 1P675T100, exbio), anti-CD90 (clone: ebioE10, lot: E0228253, ebioscience) PE, anti-CD105FITC (clone: SN6, lot: E029268, ebioscience) (27) or isotype control antibodies (BD Biosciences), diluted 1:100. At the end of the incubation, the tubes were centrifuged at 1500rpm/5min and the cells were washed twice with PBS. Approximately 5×10^4 events were acquired and analyzed using BD Cell Quest pro software.

Conditioned Medium collection

For the production and collection of conditioned medium (CM), a total of 1×10^6 ASC (passages 2 - 6) were cultured for 48h in T25 culture bottles with EBMTM-2

medium (Lonza), with fetal bovine serum deprivation (FBS) and 1% albumin (Sigma-Aldrich Co). After cultivation, the CM was collected, centrifuged at 3,000 rpm for 20 min at 4ºC to remove cell debris and stored at -70ºCThe protein quantification of the conditioned media was performed using the modified Lowry methodology [23]. Bovine serum albumin standards were used in a range of 0.1 to 2 mg/mL following standard procedures.

In vivo wound healing model

Male and female Towness B6:129 subtype HbSS mice, between 2 and 3 months of age, were used for analysis of the healing model (n=12). To carry out the experiment, the animals were anesthetized using isoflurane (2% for induction and 1-2% for maintenance), being kept at a temperature of 37°C, using an adjustable heating plate and subsequent removal of hair from the dorsal region with the aid of a shaving machine and depilatory cream. Using a 4mm diameter biopsy needle, four excisions were performed on the back of the mice to remove the skin (including the panniculus carnosus muscle). In the same animal, on days 0 (wound day) and 2, different compounds were administered according to the delimitation of each study group:

1. Control group wounds: wounds in the upper and lower left regions, treated with saline solution;

2. NCM Group wounds: wounds in the upper and lower right regions, treated with conditioned medium;

In the postoperative period, the animals were kept separately (one animal per box) for recovery and observation of tissue healing, with euthanasia (ketamine hydrochloride 100 mg/kg and xylazine hydrochloride 14 mg/kg intraperitoneally) at three different times: T03 (after 03 days), T05 (after 05 days) and T07 (after 07 days). To carry out

the photometric analysis, photographic recording and measurement of the width, area and closure of the wound were made on days 0, 3, 5 and 7, using the Fiji software (NIH).

Immunohistochemical analysis

To perform the immunohistochemical analysis, the mice were euthanized after 3, 5 and 7 days. Wound samples were collected and fixed in 4% paraformaldehyde (PFA) for 48h, then washed in water, dehydrated in an alcohol and xylene battery, and embedded in paraffin at 60°C. The paraffin blocks were sectioned using a microtome (Leica Biosystems) and placed on slides, stained with Hematoxylin-eosin (HE) and Masson's Trichome. The quantification of inflammatory cells, fibroblasts, delimitation of the granulation tissue area and the thickness of the epithelium were performed using the Fiji software (NIH). All slides were analyzed using an optical microscope (Platform Leica DMi8).

Immunofluorescence Analysis

To evaluate the arrangement of blood vessels, fibroblasts and smooth muscle cells in the wound bed, immunofluorescence assay was performed. Wound samples collected at times T03, T05 and T07 were fixed in 4% paraformaldehyde (PFA) for 48 hours and embedded in OCT tissue Tek medium (Tissue-Tek O.C.T. Compound Medium – Sakura). The OCT blocks were sectioned into 60um sections for subsequent immunofluorescence staining. Skin sections were stained with goat anti-CD31 IgG antibody (R&D System, Lot YZUO119021), rabbit IgG α-SMA antibody (abcam Lot: GR3183259-30), rabbit IgG SM22 antibody (abcam, Lot GR 3206537-1), donkey anti Goat IgG Alexa Fluor 555 secondary antibody (ThermoFisher A-214 32) and donkey

anti-Rabbit IgG secondary antibody 488 (ThermoFisher A-21206), diluted 1:100 in BSA/standard serum solution. The capture of images was obtained through visualization in the confocal microscope (Leica TCS SP5 X) from Fiocruz-BA and Quantitative analysis was performed with the software Fiji (NIH).

RT-qPCR

In this assay, the following transcripts were evaluated: TNF, TGF, IFN -γ, IL-1b, IL-4, IL-12, IL-10, NOS-2, LY6C, LTBR4, STAT-1, MCP-1, ARG-1 and CCR2 (S1 Table1). The collection of primers was performed using the Primer blast databases (available at: http://www.ncbi.nlm.nih.gov/tools/primer-blast/). RNA was isolated using silica columns (RNeasy Mini Kit – Qiagen, lot 160054560) and methodology provided by (www.qiagen.com). The determination of the quality of the isolated RNA was made by the ratio Abs260ηm/Abs280ηm, having, as a satisfactory purity, a ratio close to 2.0. Conversion to cDNA was performed using SuperScript VILO Master Mix (Invitrogen, Catalog Number: 11755-050, 11755-250 and 11755-500). All qRT-PCR reactions were performed in duplicate, using the dye SYBR® Green Master Mix (Thermo PowerUp™ SYBR™ Green Master Mix Cat No. /ID A25777), in the QuantStudio 12k Flex Real Time (Applied Biosystems). Results were calculated using the modified ΔΔCt method, using the b-Actin gene as a reference gene.

Statistical analysis

Statistical analyzes were performed using GraphPad Prism v6.0 (Graphpad Software, San Diego, California, USA), with p values below 0.05 being considered statistically significant. The distribution of quantitative variables was performed using the Shapiro-Wilk test. Paired t test or Mann-Whitney U test were used to compare two

groups according to the distribution of variables, or one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc test for comparisons of three groups

Results

ASC characterization and conditioned medium production

The cells isolated from liposuction samples exhibited a characteristic pattern of MSCs, showing proliferation capacity, adherence to plastic and fibroblastoid morphology. Flow cytometry analysis indicated that MSCs positively expressed the typical mesenchymal surface markers CD29, CD73, CD90 and CD105 and showed negative expression for hematopoietic markers CD14, CD34, CD45, CD146 and HLADR (Fig 1A). After 21 days in a multilineage differentiation assay, MSCs acquired a characteristic phenotype of adipogenic, osteogenic, and chondrogenic mesodermal lineages, with the presence of lipid droplets, bone mineralization matrix, and expression of cartilage-specific proteoglycans, respectively (Fig 1B).

To use the secretome of the MSCs as a therapeutic alternative, MSCs were submitted to culture in a medium with serum deprivation, for 48 hours, to collect the conditioned medium. After pre-conditioning, using the inverted microscope, we observed that the MSCs maintained their viability, preserving their fibroblastoid phenotype and their ability to adhere to plastic, evidenced by positive staining for calcein-AM (Fig 1C). In summary, the evaluated cell populations presented all the minimum criteria required for the identification of ASCs, as previously described by Dominici [24], preserving their integrity and morphological characteristics before and after collection of the conditioned medium.

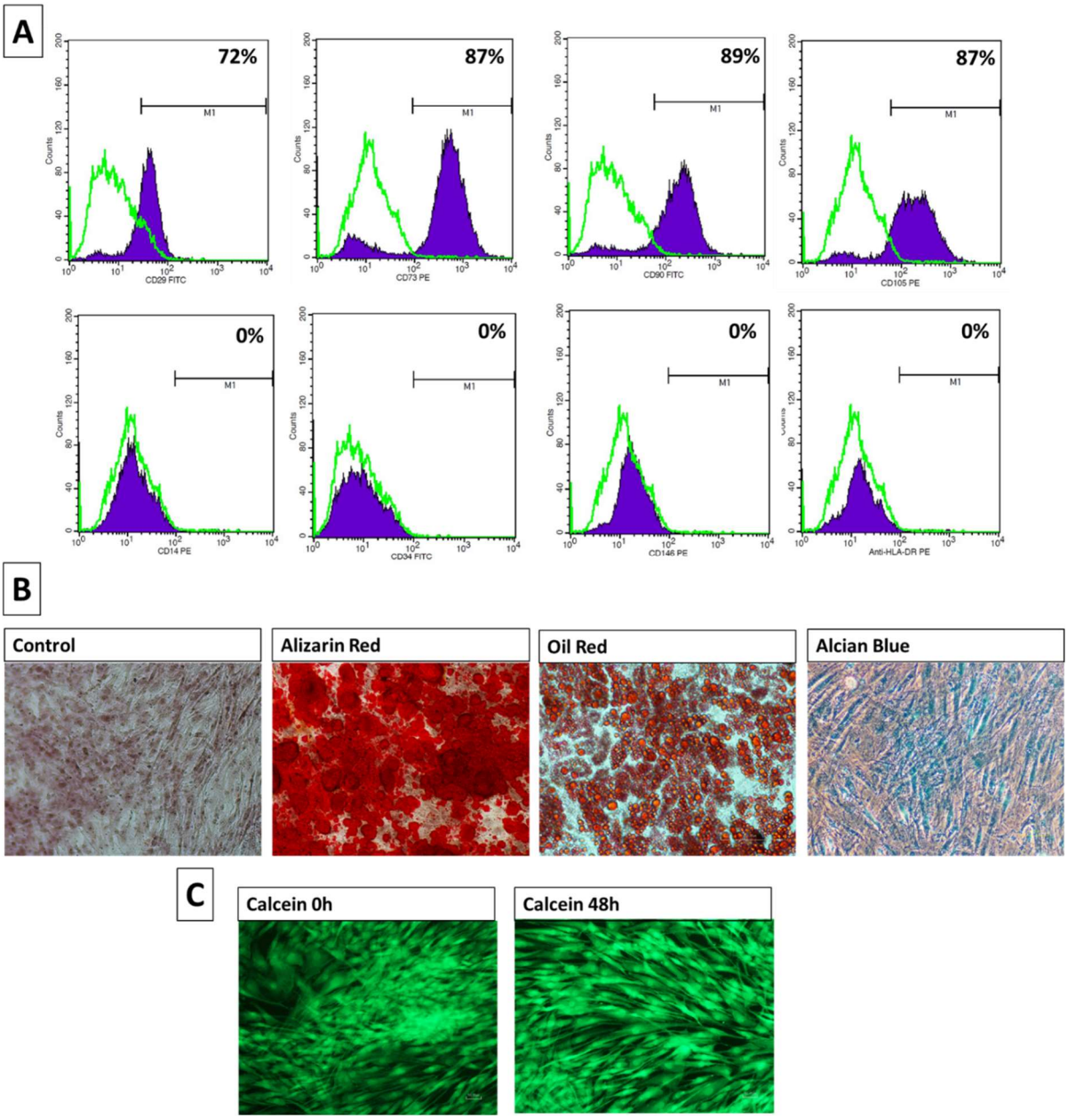

Fig 1: Isolated cells showed expression of specific MSC markers and multilineage differentiation pontential. (A) Cells cultured in passage 03 showed positive expression for surface markers CD29, CD73, CD90 and CD105 and negative expression for markers CD14, CD34, CD146 and HLA-DR; **(B)** After 21 days of culture in cell differentiation medium, ASC showed multilineage potential with osteogenic (staining by Alizarin Red), adipogenic (staining by Oil Red) and chondrogenic (staining by Alcian Blue) characteristics; **(C)** Assay with calcein-AM staining demonstrated that ASC maintained their viability in cultures with and without FBS deprivation (n=08).

ASC derived conditioned medium improved wound healing in a murine model of sickle cell anemia

The regenerative potential of NCM was evaluated in a murine sickle cell model of full-thickness excisional wound healing. The administration of the conditioned medium produced by ASCs was able to accelerate the tissue repair process in a wound healing model in Towness HbSS mice. To evaluate the regenerative properties of the therapeutic product, we administered NCM in wounds located on the dorsal region of the mice, with observation over days 3, 5 and 7. The *in vivo* analysis demonstrated that the wound healing process was accelerated in the group treated with NCM, showing wound closure on day 7. The group of wounds treated with control medium showed delayed healing, showing no restoration of epithelial tissue on the last day of analysis.7 ($p<0.001$) (Fig 2).

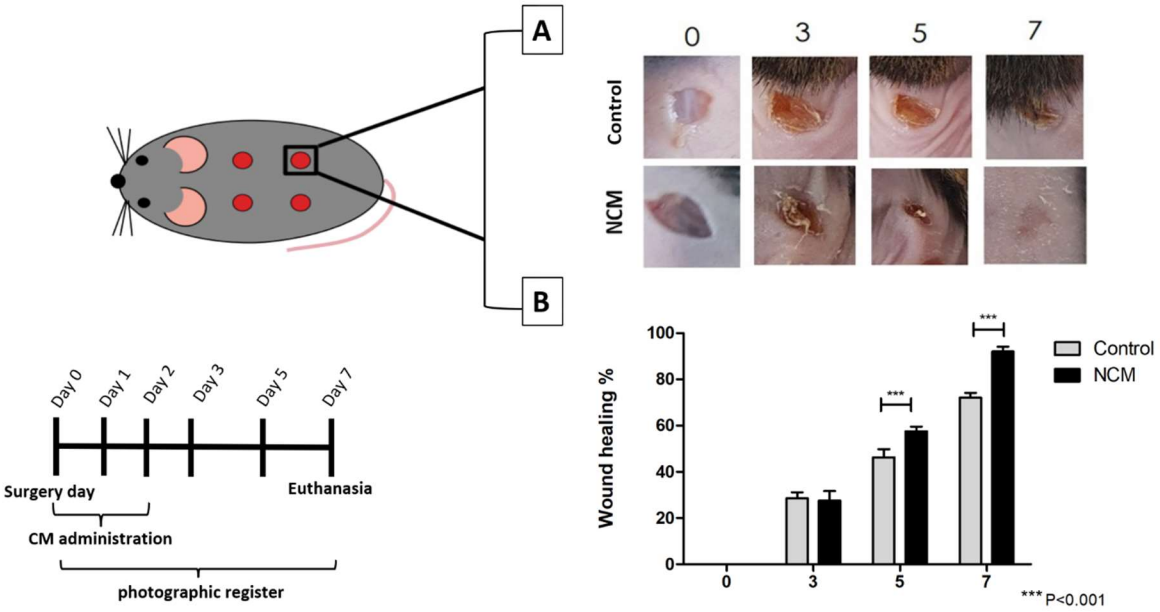

Fig 2: NCM treatment accelerated wound healing in HbSS mice. (A) Representative images of dorsal wound healing in townes HbSS mice (age: 03 months) over 7 days. The experiment involved two treatment groups (group 01: treated with control medium and group 02: treated with NCM) and wound

measurement was performed using Fiji software. **(B)** Data referring to daily measurements between the control and NCM groups (***p<0.001, n=07 per group).

ASC conditioned medium was able to reduce local inflammation and promote tissue regeneration

Skin sections stained with hematoxylin-eosin (HE) showed reduced inflammation and increased tissue regeneration in groups of wounds treated with NCM (Fig. 3). Wounds treated with our therapeutic product showed an increase in the number of fibroblastoid cells at the injury site on days 3 and 7, with greater significance on day 7 (*p=0.0128; ***p<0.001) (Fig. 3A and 3D). The opposite was observed when analyzing the population of inflammatory cells at the injury site. We observed a reduction in the number of inflammatory cells in wounds treated with NCM when compared to wounds treated with control medium (***p<0.001). The number of inflammatory cells decreased with healing time, recording lower counts in 7-day wounds treated with NCM (***p<0.001) (Fig. 3B and 3E). In addition, there was an increase in the thickness of the epidermis in wounds treated with NCM, Improved performance was evidenced in 7-day wounds treated with NCM (p=0.0021). This result that wasn't observed in control group wounds. (Fig 3C and 3F).

Wound treatment with NCM was also able to increase collagen deposition at the wound site. Skin sections stained with Masson's Trichrome were used to measure the presence of collagen fibers in groups of wounds treated with control medium and NCM (Fig 4A). Comparing the groups of treated and untreated wounds on days 3, 5 and 7, greater Masson's Trichrome staining (collagen stained in blue) was detected in NCM group, with reduced levels of collagen in wounds treated with the control group on all analyzed days (Fig 4B, 4C and 4D).

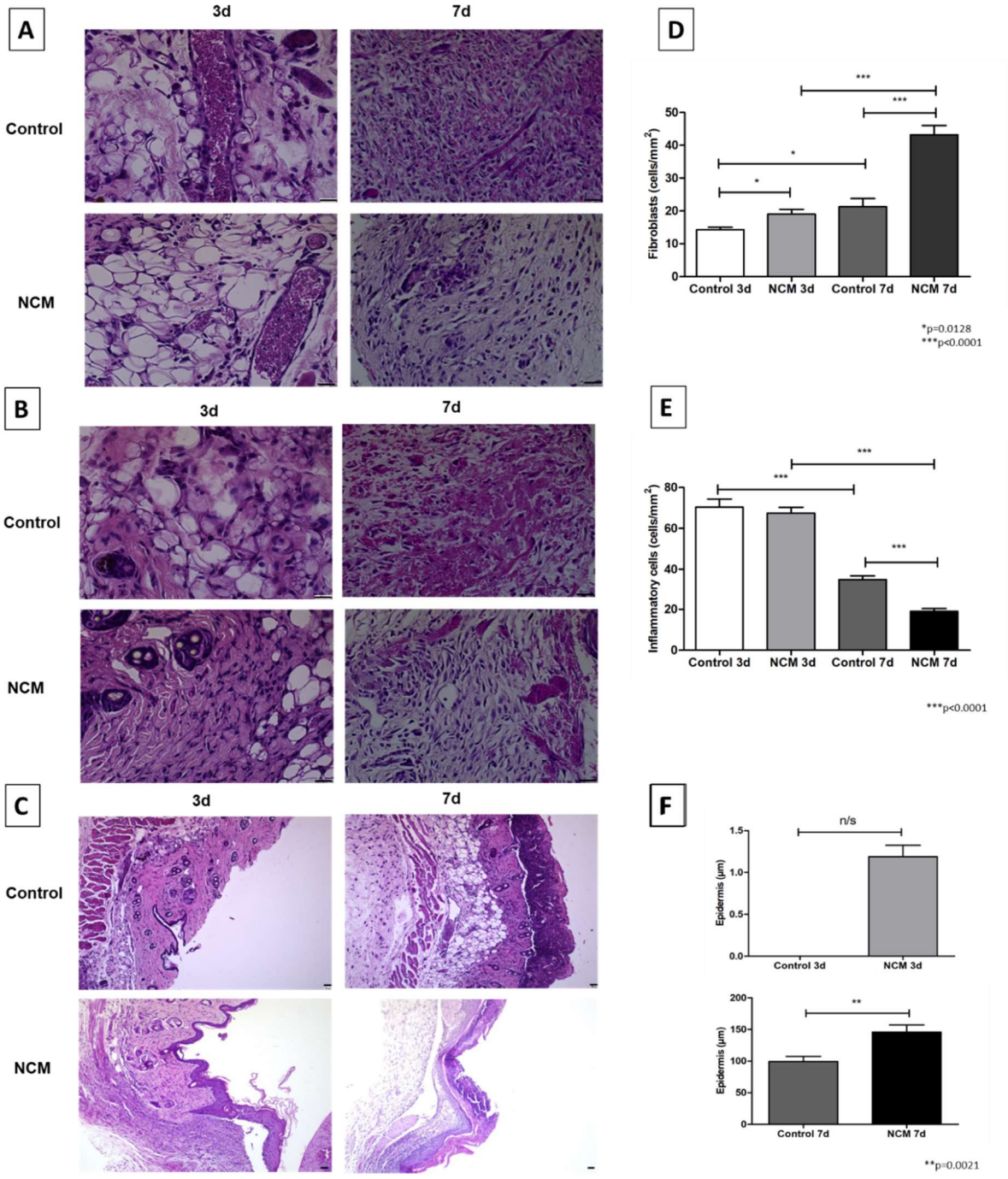

Fig 3: The conditioned medium reduced the inflammation and stimulated wound healing. (A-C) Representative images of skin sections stained with hematoxylin and eosin (scale: 20um); (D) Counting the number of fibroblasts at the site of lesions at 3 and 7 days (*p=0.0128, ***p<0.0001). (E) Number of inflammatory cells between control and NCM-treated groups. (F) Thickness of the epidermis in skin wounds treated with control medium and NCM, on days 3 and 7 (**p=0.0021). The quantification of the number of fibroblasts, inflammatory cells and the measurement of the thickness of the epidermis were performed using the Fiji software (n=03 per group).

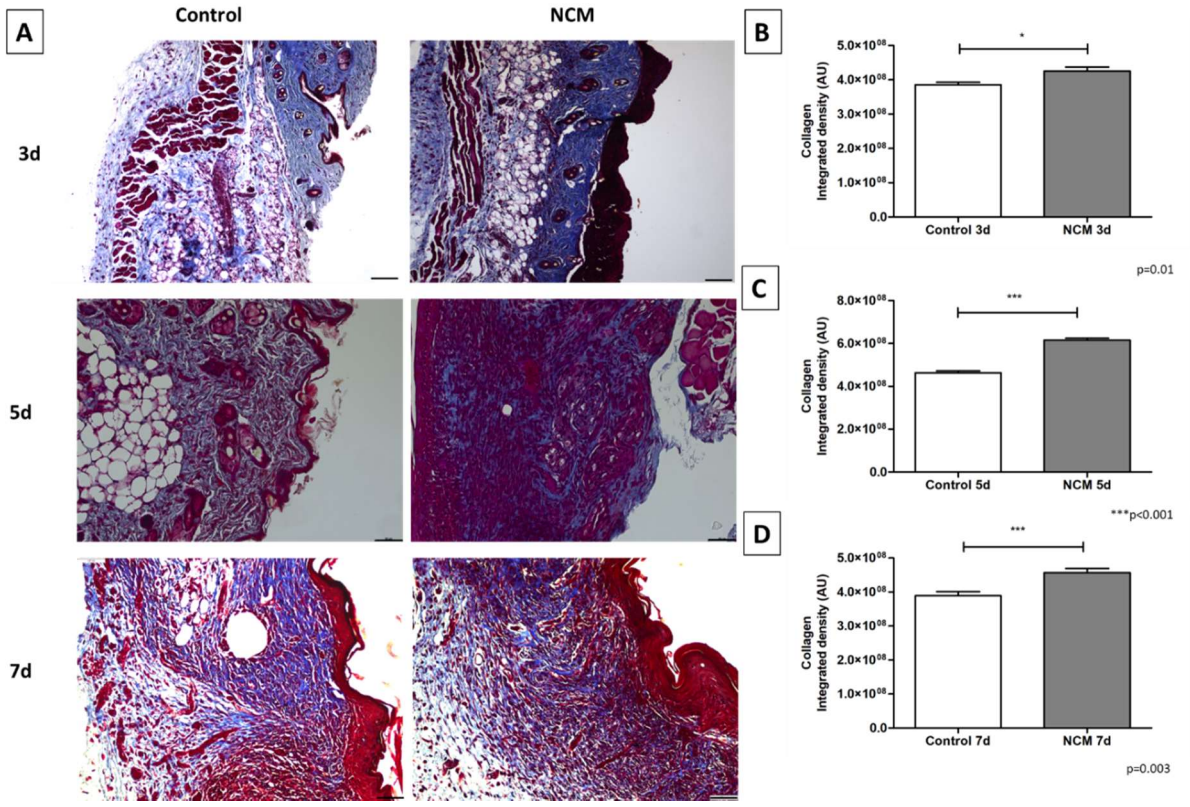

Fig 4: Wounds treated with NCM showed greater collagen deposition. (A) Representative images of skin sections at 3, 5, and 7 days stained with Masson's Trichomic (Scale: 50 um). (B,C,D) Quantification of collagen deposition after treatment with control medium and NCM, in periods of 3, 5 and 7 days respectively. (*p=0.01, *** p=0.003 and p<0.001). Quantification of collagen fibers was performed using the Fiji software (n=03 per group).

Wounds treated with control and conditioned medium showed positive expression of CD31, α - SMA and SM22

To evaluate the expression of CD31, α-SMA and SM22 at the lesion site, skin samples from days 3, 5 and 7 were processed and analyzed by immunofluorescence (Fig 5). All biological samples analyzed showed positive staining for CD31, α-SMA and SM22 antibodies in the control and NCM groups. Interestingly, at time 3 there was a higher expression of the CD31 antibody in the group of wounds treated with control medium, when compared with the wounds treated with NCM (*p=0.0189) on the same

day (Fig 5C). There were no statistical differences for the expression of CD31+ cells in the groups treated with control medium and NCM medium on days 5 and 7 (Fig 5E and 5G). The skins collected on day 3 also showed an increase in positive α-SMA cells in wounds treated with control medium, with a lower expression of positivity in wounds treated with NCM (**p=0.001) (Fig 5B).

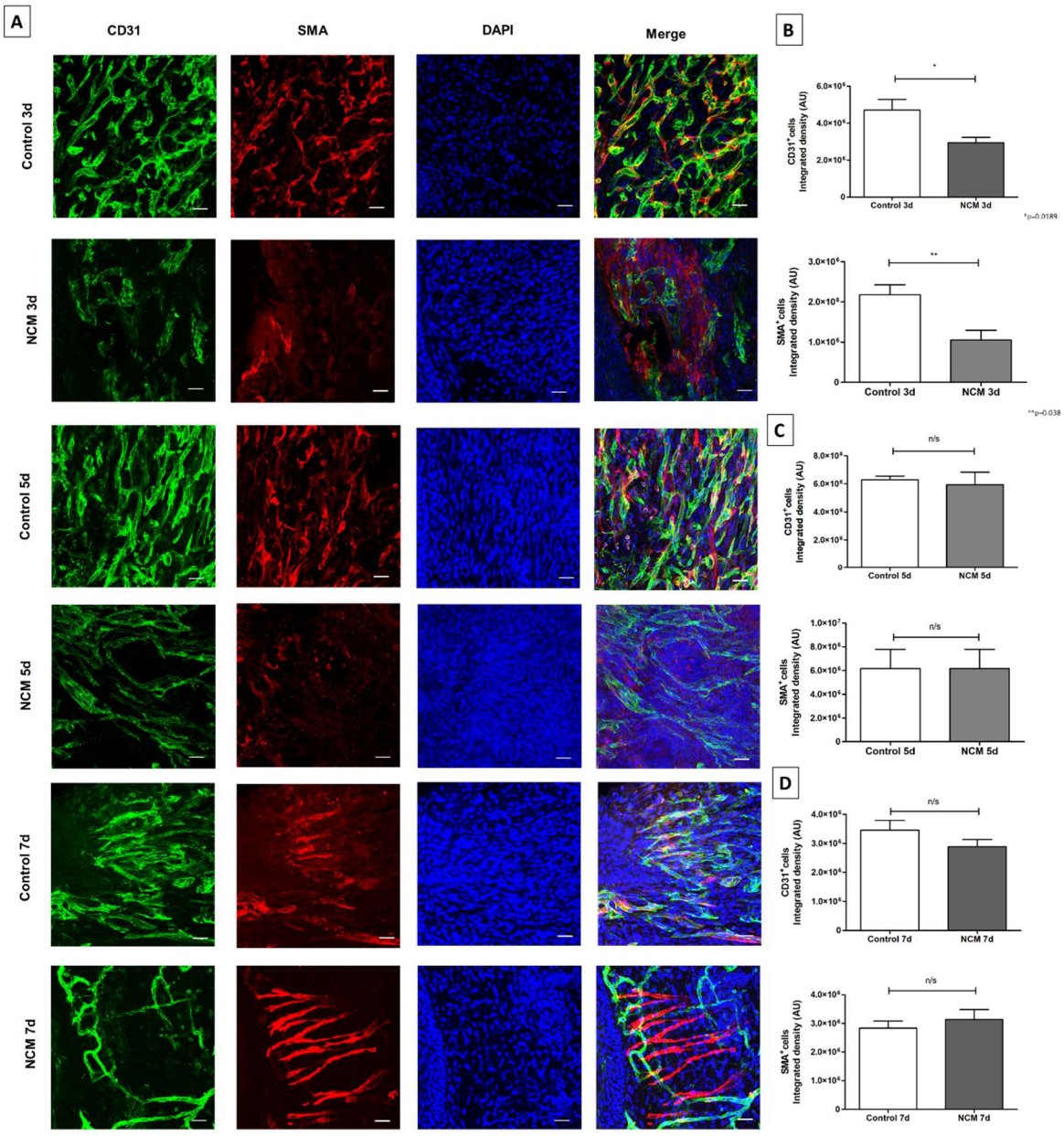

Fig 5: Immunofluorescence assay showed positive expression for CD31 and α-SMA markers. (A) Representative images for the positive staining of anti-CD31 and anti-SMA antibodies in wounds processed on days 03, 05 and 07; **(B)** Wounds from the group control on day 03 showed higher expression of CD31 and α-SMA when compared to the group treated with NCM; **(C)** There was no

statistical difference between the wound groups treated with control medium and NCM medium on days 05 and 07 (n= 03 per group) Scale: 50um.

Corroborating with previous results, the skins from days 05 and 07 did not show statistical differences between the control and NCM groups for marking positive α-SMA cells (Fig 5D and 5F). Finally, there was no difference in the expression of SM22+ cells in wounds treated with control and NCM media on days 3 and 5. However, on day 7, marking with the SM22 antibody was only evidenced in the control group (Fig 6).

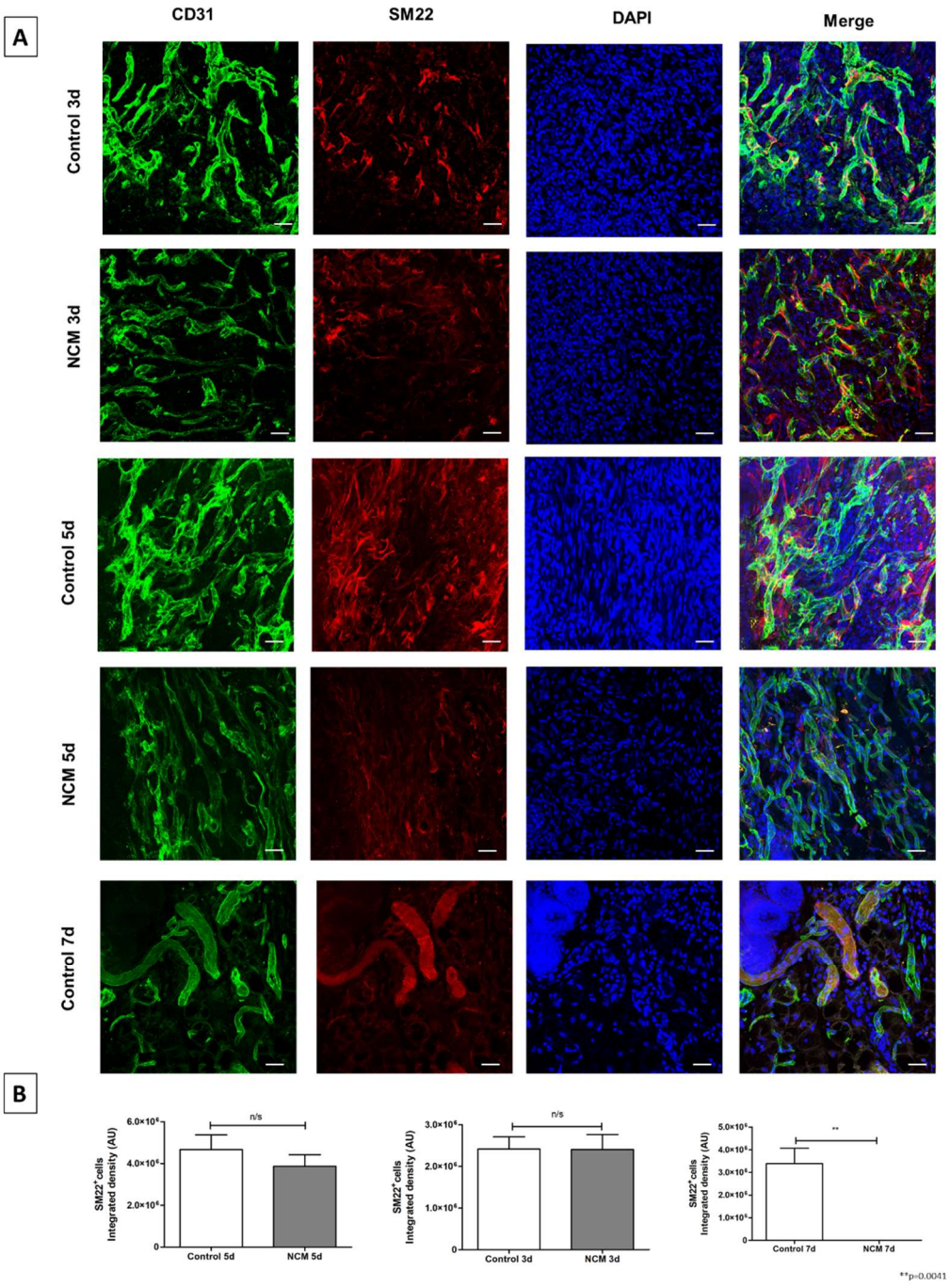

Fig 6: Immunofluorescence assay showed differential expression for SM22 marker in wound treated with NCM. (A) Representative images of the positive staining for anti-CD31 and anti-SM22 antibodies in wounds processed on days 03, 05 and 07; **(B)** There was no statistical difference for the marking of SM22 on days 03 and 05**; (C)** Expression of SM22 on day 07 was observed only in the group control, with no marker in the NCM group (n= 03 per group) Scale: 50um.

NCM treatment reduced the expression of genes involved in the inflammatory phase of wound healing

To analyze the expression of gene transcripts involved in the inflammation process, we used the qRT-PCR technique (quantitative real-time PCR). The genes analyzed by the in wounds after 3 days of healing were: IL-4, CCR2, TGF, LY6C, IL-10, IL-12, IL-1b, NOS2, ARG-1, LTBR4, TNF, STAT-1, MCP1 and IFN.

We compared gene expression levels between the group of wounds treated with control medium and the group of wounds treated with NCM. The results were normalized using the endogenous genes: HPRT and B-actin. Due to its greater stability in both conditions, the endogenous B-actin gene was used to show the results.

The genes TNF (*p=0.04), TGF (**p=0.0034), IL-1b (**p=0.0015), IL-4 (*p=0.018), IL-12 (*p=0.0043), LY6C (**p=0.0003), NOS2 (***p<0.0001), LTBR4 (*p=0.0114), ARG-1 (***p<0.0001) and CCR2 (*p=0.0014) showed reduced expression in 3-day wounds treated with NCM compared to wounds treated with control medium (Fig 7). There was no statistical difference for the expression of IFN, IL-10, STA-1 and MCP1 transcripts.

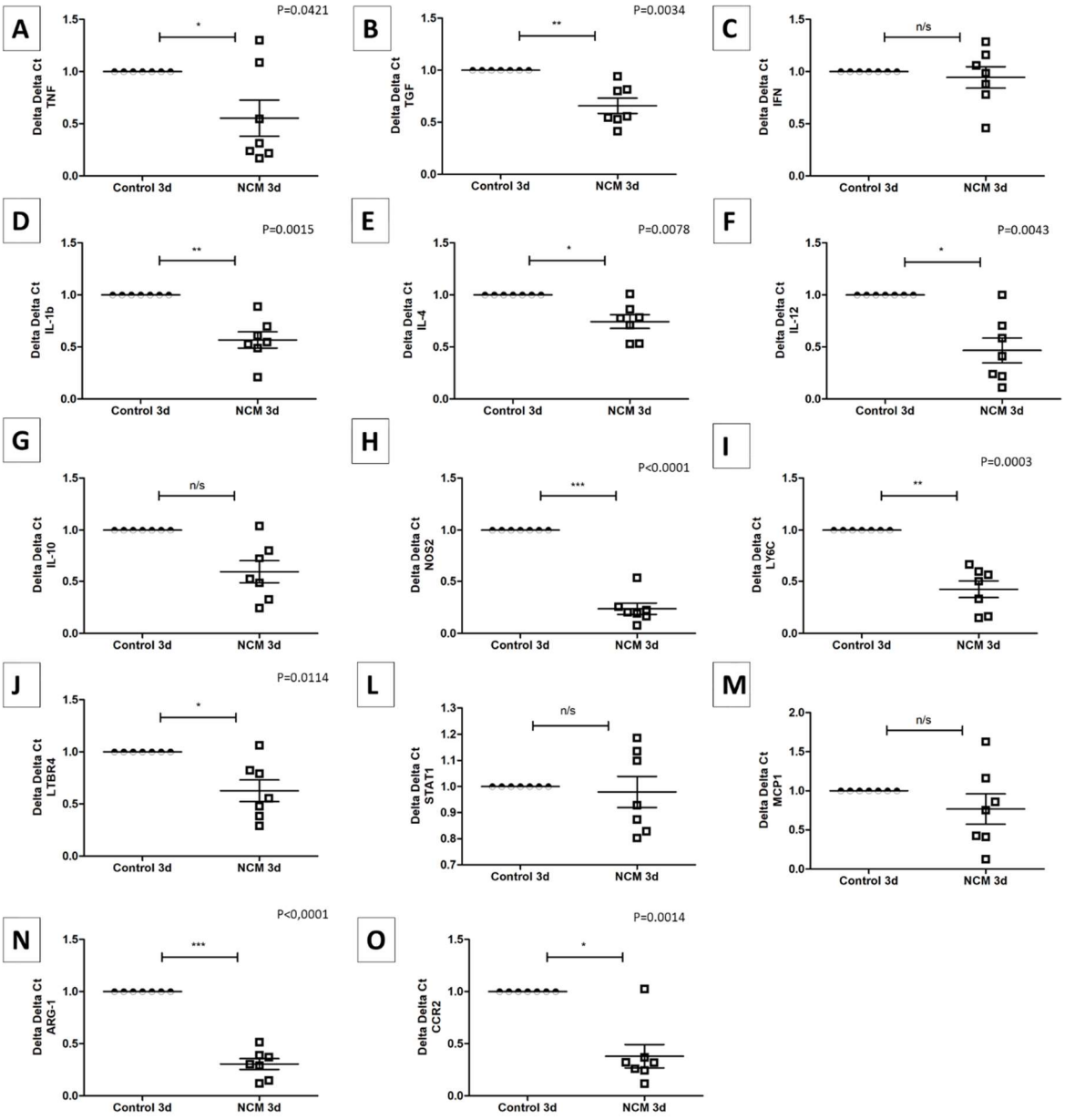

Fig 7: NCM treatment reduced the expression of transcripts involved in inflammation. Wounds treated with NCM showed reduced expression of transcripts **(A)** TNF (*p=0.04), **(B)** TGF (**p=0.0034), **(D)** IL-1b (**p=0.0015), **(E)** IL-4 (*p=0.018), **(F)** IL-12 (*p=0.0043), **(H)** NOS2 (***p<0.0001), **(I)** LY6C (**p=0.0003), **(J)** LTBR4 (*p=0.0114), **(N)** ARG-1 (***p<0.0001) and **(O)** CCR2 (*p=0.0014). The expression of IFN, IL-10, STA-1 and MCP1 did not show statistical significance when compared to the control group.Each symbol represents a wound sample. The horizontal lines represent the mean and standard deviation (n=07).

Discussion

Clinical conditions that affect wound healing, such as the development of skin ulcers in sickle cell patients, require advanced therapeutic approaches that help restore skin integrity. So far, the management of these cutaneous ulcers is based on conservative treatments that are not effective for all patients [25,26]. In this context, cell-based therapy has stood out in recent decades as an attractive and promising option for the treatment of CLUs. We have recently demonstrated that the secretome from mesenchymal stromal cells of adipose tissue is composed of bioactive molecules capable of promoting biological effects responsible for stimulating the migration of fibroblasts, endothelial and perivascular cells to the injured site, promoting tissue regeneration [27, 28, 29]. Considering these findings, we decided to investigate the therapeutic potential of the ASC secretome in a murine model of sickle cell anemia. The present study reported the potential effects of secretome collected from ASC on wound healing in Towness mice. We identified structural and cellular differences at the wounds sites treated and not treated with NCM, observing the decrease in local inflammation and acceleration of wound healing when exposed to NCM treatment.

The use of transgenic mice carrying human sickle cell hemoglobin [30] provided data on how our therapeutic product may help in the management and regeneration of sickle cell cutaneous ulcers. Data from the literature show that the quality of the MSC secretome depends on some factors: donor source, type of biological sample, culture conditions and stimulus administered on the MSC [31]. Studies report that the MSC secretome is primarily responsible for their therapeutic capacity, playing a central role in local homeostasis, promoting cell recruitment and stimulating the formation of new blood vessels in the target organ [32, 33]. However, some authors point out that cell-cell contact is important for optimizing the immunomodulatory activity of MSCs [34].

The present study was dedicated to demonstrating the effects of all cellular secretome, without fractionation of its components or specific treatment, using a cell-free treatment. Our findings demonstrate that NCM was able to accelerate wound closure in sickle cell mice, restoring epithelial integrity in 7 days. Similar to data found in the literature, NCM stimulated re-epithelialization by increasing the proliferation of fibroblastoid cells, reducing the number of inflammatory cells and thickening the epidermis [18,19].

In the wound healing process, the transition from the inflammatory to the proliferative phase is essential for increasing blood supply and improving oxygen delivery near the injured area. The formation of new blood vessels and increased collagen deposition are critical to the entire healing process. In this phase, there is action of growth factors that promote neovascularization and influence the proliferation of dermal fibroblasts, also activating the migration of endothelial cells that act directly in the reconstruction of the cutaneous structure [35]. Our data report an increase in collagen deposition in wounds treated with NCM compared to wounds in the control group, which may be associated with an increase in the number of collagen-producing cells at the injury site.

Immunohistochemical analysis showed that there were significant differences between the groups of wounds treated with control medium and NCM medium, which corroborated the data shown in the wound healing curve. As we expected, there was intense expression of CD31 and α-SMA markers in both groups. However, on day 03, the control group showed greater expression of CD31 and α-SMA when compared to the NCM group, suggesting that there was greater vascularization and myofibroblastic transformation in the group of untreated wounds in this period. This event was not observed over days 05 and 07, with similar expression of CD31 and α-SMA markers

in both analyzed groups. Differing from our results found in C57BL/6 Dsred^{+}/Nestin+ mice wound healing model, CM administration did not induce an increase in the number of blood vessels at the injury site (29).

One of the factors that impair the healing process is accentuated inflammation, the continuous action of inflammatory cells at the injury site affects the reconstruction of the extracellular matrix (ECM), favoring the development of cutaneous ulcers and the formation of scars. In an inflammatory microenvironment, MSCs are capable of inhibiting activation, proliferation and inducing a change in the phenotype of cells of the immune system (36). Therefore, considering the influence of some inflammatory mediators for the maintenance of cutaneous ulcers, we decided to evaluate the expression of some transcripts in wounds treated and not treated with NCM in the inflammatory phase of healing. Of the 14 genes analyzed, we evidenced a decrease in relevant mediators for the inflammatory process: TNF-alpha and IL-1beta, directly involved in activation, cell recruitment and maintenance of the inflammatory microenvironment (37). We also observed reduced expression of the NOS2 transcript, marker of pro-inflammatory macrophages (M1) and reduced expression of Interleukin-12, a soluble mediator synthesized by M1 macrophages and DC cells during the inflammatory phase (38). Furthermore, our work identified the reduction of classic monocyte markers: Ly6c, CCR2 and MCP-1, in wounds treated with NCM. However, we did not register an increase in the expression of the anti-inflammatory transcripts TGF-beta, IL-4, IL-10 and ARG-1, which may be related to the time the sample was analyzed or the need for cell-cell stimulation to enhance immunomodulation.

In conclusion, this study reports the therapeutic potential of the ASC secretome in the treatment of sickle cell wounds mice. Our results demonstrate a strong effect of

NCM on accelerating the healing process, by changing the extracellular microenvironment with reducing local inflammation and stimulating the formation of well-structured and functional new blood vessels. These findings suggest that the use conditioned medium of ASC has relevant regenerative effects for the treatment of sickle cell cutaneous ulcers.

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Supporting information

S1 Primers Table

Gene	Forward 5'->3'	Reverse 5'->3'
IL-1β	CCTTGTGCAAGTGTCTGAAGC	CAAAGGTTTGGAAGCAGCCC
TNF-α	TCTTCTCATTCCTGCTTGTGG	GATCTGAGTGTGAGGGTCTGG
NOS2	CCTGCTTTGTGCGAAGTGTC	CCCAAACACCAAGCTCATGC
IFN-γ	GCTACACACTGCATCTTGGC	CATGTCACCATCCTTTTGCCAG
CCL2 (MCP-1)	GGTCCCTGTCATGCTTCTGG	GGCGTTAACTGCATCTGGC
IL-10	ACCTGGTAGAAGTGATGCCC	GGAGAAATCGATGACAGCGCC
IL-12	TGGCCAGCTTCGAGAAAGAG	TGGCAAGACCTTACGGACATC
Arg1	TGAGGAAAGCTGGTCTGCTG	AACTGCCAGACTGTGGTCTC
TGF-β1	CCCGAAGCGGACTACTATGC	CATAGATGGCGTTGTTGCGG
STAT-1	CTGAAGACCTGAACACCGCT	GGCCACGTGCATTGCTTT
Ly6c	AAGCTCTCCAGAAGCAATCCTG	GAAGAATTGCCAGACCTGTGAC
CCR2	GGAGCCATACCTGTAAATGCC	ATGCCGTGGATGAACTGAGG
IL-4	GCACAGGAGCCAAGAGTGAA	TTCACATCACAGCTCCCCAC
Ltb4r1	GGACCCTGGCACTAAGACAG	CAGCCATCAAAAGGACAGGG
GAPDH	GGAGAGTGTTTCCTCGTCCC	ACTGTGCCGTTGAATTTGCC
β-actin	TAGGCACCAGGGTGTGATGG	AGGTGTGGTGCCAGATCTTC
HPRT	CTCCTCAGACCGCTTTTTGC	CATCGCTAATCACGACGCTG

7. CONCLUSÃO GERAL

Embora a fisiopatologia das úlceras cutâneas falciformes ainda não esteja totalmente elucidada, sabe-se que a disfunção eritrocitária, associada ao processo de falcização e hemólise das hemácias corroboram para a promoção de um microambiente inflamatório propício para o estabelecimento de uma ferida não cicatrizante (SUNDD et al., 2019). O processo de cicatrização de feridas é complexo, formado por quatro etapas que se apresentam de forma dinâmica e integrada a fim de restaurar a barreira cutânea. Neste processo, a atuação de fatores de crescimento, quimiocinas, citocinas e microvesículas na formação de novos vasos sanguíneos é crucial para o fechamento da ferida.

Na primeira parte deste trabalho, a composição do secretoma de ASC foi influenciada pela variação de oxigênio no meio. Nossos resultados demonstraram que tanto em condições normóxicas quanto hipóxicas as ASCs secretaram mediadores envolvidos no processo angiogênico e de reparo tecidual. Usando um modelo murino Nestin-GFP+/NG2-DsRed+ para avaliação da cicatrização de feridas, o presente estudo demonstrou que NCM e HCM foram capazes de acelerar o processo de reparo tecidual estimulando a proliferação de células CD31 positivas (células endoteliais) e células Nestin positivas (células regenerativas) no local da lesão, não necessitando de pré-condicionamento em hipóxia para potencialização dos efeitos.

A segunda parte do nosso trabalho corroborou com os dados encontrados no capítulo 1, demonstrando que assim como nos camundongos C57BL/6, o NCM manteve seu potencial terapêutico e foi capaz de acelerar a cicatrização de feridas em camundongos towness HbSS. Contudo, os efeitos imunomodulatórios encontrados no presente estudo possibilitou uma análise crítica da necessidade de otimização do processamento do meio condicionado, visando a produção de um produto que seja capaz de atingir seu máximo de potencial.

Por fim, concluímos que a utilização do meio condicionado para o tratamento de úlceras cutâneas falciformes é uma ferramenta promissora que apresentará benefícios diretos para a qualidade de vida do paciente, contribuindo para a sua recuperação e reinserção social.

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APÊNDICES

PLOS ONE

RESEARCH ARTICLE

Investigating the potential of the secretome of mesenchymal stem cells derived from sickle cell disease patients

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Abstract

Sickle cell disease (SCD) is a monogenic red cell disorder associated with multiple vascular complications, microvessel injury and wound-healing deficiency. Although stem cell transplantation with bone marrow-derived mesenchymal stem cells (BMSC) can promote wound healing and tissue repair in SCD patients, therapeutic efficacy is largely dependent on the paracrine activity of the implanted BM stromal cells. Since *in vitro* expansion and culture conditions are known to modulate the innate characteristics of BMSCs, the present study investigated the effects of normoxic and hypoxic cell-culture preconditioning on the BMSC secretome, in addition to the expression of paracrine molecules that induce angiogenesis and skin regeneration. BMSCs derived from SCD patients were submitted to culturing under normoxic (norCM) and hypoxic (hypoCM) conditions. We found that hypoxically conditioned cells presented increased expression and secretion of several well-characterized trophic growth factors (VEGF, IL8, MCP-1, ANG) directly linked to angiogenesis and tissue repair. The hypoCM secretome presented stronger angiogenic potential than norCM, both *in vitro* and *in vivo*, as evidenced by HUVEC proliferation, survival, migration, sprouting formation and *in vivo* angiogenesis. After local application in a murine wound-healing model, HypoCM showed significantly improved wound closure, as well as enhanced neovascularization in comparison to untreated controls. In sum, the secretome of hypoxia-preconditioned BMSC has increased expression of trophic factors involved in angiogenesis and skin regeneration. Considering that these preconditioned media are easily obtainable, this strategy represents an alternative to stem cell transplantation and could form the basis of novel therapies for vascular regeneration and wound healing in individuals with sickle cell disease.

Introduction

Sickle cell disease (SCD), the most common inherited hemoglobinopathy worldwide, is characterized by repeated vaso-occlusion crises secondary to sickled red blood cells [1]. It is associated with significant microvessel injury, as well as impairments in neovascularization, wound

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ORIGINAL RESEARCH
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Leukemia Inhibitory Factor (LIF) Overexpression Increases the Angiogenic Potential of Bone Marrow Mesenchymal Stem/Stromal Cells

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Mesenchymal stem/stromal cells (MSCs) have the ability to secrete bioactive molecules, exerting multiple biological effects, such as tissue regeneration, reduction of inflammation, and neovascularization. The therapeutic potential of MSCs can be increased by genetic modification to overexpress cytokines and growth factors. Here we produced mouse MSCs overexpressing human leukemia inhibitory factor (LIF) to assess their proangiogenic potential *in vitro* and *in vivo*. Mouse bone marrow-derived MSCs were transduced by using a second-generation lentiviral system to express human LIF. Leukemia inhibitory factor expression was confirmed by RT-qPCR and by ELISA, allowing the quantification of the transcript and secreted protein, respectively. Flow cytometry analysis and trilineage differentiation assay showed that the MSC_LIF cell line maintained the immunophenotype and a multipotency characteristic of MSCs. The immunosuppressive activity of MSC_LIF was confirmed using a lymphoproliferation assay. Moreover, gene expression analysis demonstrated upregulation of genes coding for strategic factors in the neovascularization process, such as angiogenin, IL-8, MCP-1, and VEGF, and for the perivascular cell markers αSMA, Col4a1, SM22, and NG2. To evaluate the pro-angiogenic potential of MSC_LIF, we first tested its effects on endothelial cells obtained from umbilical vein in a scratch wound healing assay. Conditioned medium (CM) from MSC_LIF promoted a significant increase in cell migration compared to CM from control MSC. Additionally, *in vitro* tube formation of endothelial cells was increased by the presence of MSC_LIF, as shown in microvessel sprouting in aortic ring cultures. Finally, an *in vivo* Matrigel plug assay was performed, showing that MSC_LIF were more potent in promoting *in vivo* angiogenesis and tissue vascularization than control MSCs. In conclusion, LIF overexpression is a promising strategy to increase the proangiogenic potential of MSCs and sets precedents for future investigations of their potential applications for the treatment of ischemic diseases and tissue repair.

Keywords: mesenchymal stem/stromal cells, genetic modification, LIF, proangiogenico factors, angiogenesis