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Liliana Ivet Sous Naasani

**Análise da eficácia da membrana
amniótica humana como curativo
biológico e da sinalização purinérgica
na regeneração tecidual**

UFCSPA

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tecidual**

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Laboratório de Patologia da UFCSPA, Porto Alegre, RS, Brasil

Centre de recherche du CHU de Québec-Université Laval, Québec City, Canada

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De acordo com o estabelecido previamente pela Comissão Coordenadora do Curso de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre, realizou-se aos vinte e um dias do mês de março de 2022, das quatorze horas até as dezoito horas, na modalidade remota, via Google Meet (meet.google.com/qhg-ndsb-wbf), a defesa da Tese de Doutorado da aluna **Liliana Ivet Sousa Naasani** orientada pela professora Márcia Rosângela Wink no Programa de Pós-graduação em Ciências da Saúde da UFCSPA. O trabalho defendido intitula-se **“Análise da eficácia da membrana amniótica humana como curativo biológico e da sinalização purinérgica na regeneração tecidual”**. A Banca Examinadora foi composta pelos professores Eliane Dallegrave (UFCSPA), Tanira Alessandra Silveira Aguirre (UFCSPA), Renan Rangel Bonamigo (UFRGS) e Fabrício Figueiró (UFRGS). Após a abertura da sessão a aluna dispôs de 60 minutos para expor seu trabalho. Ao término da apresentação, foi realizada a arguição da aluna pelos membros da banca examinadora. Cada examinador teve até 30 minutos para sua arguição, tendo a aluna igual tempo para resposta. Ao término da Sessão foi proferido pela Profª. Dra. Eliane Dallegrave, o parecer conjunto da Banca que deliberou pela **aprovação da aluna com “louvor acadêmico”**. Assim sendo, após a homologação da Tese de Doutorado, a aluna receberá o título de Doutora em Ciências da Saúde: Biologia Celular e Molecular. Nada mais havendo a tratar, foi encerrada a sessão e lavrada a presente ata, que será assinada pela orientadora da aluna e pela Coordenação do Programa.

Porto Alegre, 21 de março de 2022.

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LISTA DE ABREVIATURAS

AD-MSCs: células estromais mesenquimais isoladas de tecido adiposo (*adipose derived mesenchymal stromal cells*)

ADP: adenosina difosfato

ADO: adenosina

AMP: adenosina monofosfato

ATP: adenosina trifosfato

CD: cluster de diferenciação

CD73: ecto-5'-nucleotidase

hAM: membrana amniótica humana (*human amniotic membrane*)

HE: hematoxilina-eosina

L-MSCs: células estromais mesenquimais do limbo (*limbal mesenchymal stromal cells*)

MHC: complexo principal de histocompatibilidade (*major histocompatibility complex*)

MSCs: células estromais mesenquimais (*mesenchymal stromal cells*)

NTPDase: ectonucleósido trifosfato difosfono-hidrolase

NPP: nucleotídeo pirofosfatase/fosfodiesterase

P1: receptores purinérgicos (adenosina)

P2X: receptores purinérgicos ionotrópicos

P2Y: receptores purinérgicos metabotrópicos

SASS: substitutos de pele auto-montados (*Self Assembled Skin Substitute*)

UTP: uridina trifosfato

UDP: uridina difosfato

RESUMO

O tratamento de queimaduras profundas ainda é um desafio na área da medicina regenerativa. A membrana amniótica humana (hAM) é um biomaterial que vem sendo cada vez mais explorado pelo campo da engenharia de tecidos. A fim de promover a regeneração de lesões teciduais, as células estromais mesenquimais (MSCs), podem ser transplantadas na área da lesão em um suporte (*scaffold*) que mantenha seu potencial terapêutico. Além de uma série de fatores bioativos, as MSCs liberam altas concentrações de adenosina (ADO), o que pode estar relacionado com a alta expressão de ecto-5'-nucleotidase (CD73), cuja principal função é converter ATP extracelular em adenosina imunossupressora, em conjunto com a NTPDase1 (CD39). Essas enzimas são ferramentas poderosas para controlar os efeitos mediados por purinas e pirimidinas extracelulares; no entanto, o papel dos componentes da sinalização purinérgica na regeneração tecidual é pouco compreendido. Neste trabalho avaliamos a segurança e eficácia da membrana amniótica humana como um curativo biológico, na presença ou ausência de células estromais mesenquimais através de modelos *in vitro* e *in vivo* e estudamos o papel da sinalização purinérgica como uma das vias responsáveis pela regeneração tecidual. Foi feita a caracterização física e histológica da membrana amniótica humana descelularizada (DhAM) na presença de células estromais mesenquimais derivadas de tecido adiposo (AD-MSCs), assim como a comparação do potencial da DhAM e de um substituto comercial como *scaffolds* para o cultivo de células estromais mesenquimais isoladas do limbo esclerocorneal (L-MSCs), demonstrando que o cultivo das MSCs na DhAM não alterou a potencialidade de células progenitoras *in vitro*. O efeito da DhAM na presença e ausência de AD-MSCs foi avaliado no modelo pré-clínico de queimadura de segundo grau profundo, o qual demonstrou a eficácia e segurança deste *bioscaffold* na regeneração tecidual. O papel dos componentes da sinalização purinérgica durante a cicatrização de feridas foi analisado através do modelo de substitutos de pele auto-montados (SASS), demonstrando a modulação das enzimas CD73, NTPDase1 e NPP1, assim como o potencial terapêutico do nucleotídeo UTP na regeneração tecidual. Nossos resultados confirmam a eficácia da DhAM como *scaffold* para MSCs e como uma possível terapia futura para o tratamento de queimaduras, bem como a importância de entender o envolvimento de nucleotídeos extracelulares e ectoenzimas na cicatrização de feridas, com a finalidade de oferecer novas estratégias terapêuticas.

Palavras-chaves: membrana amniótica humana descelularizada (DhAM), células estromais mesenquimais (MSCs), queimaduras, *bioscaffold*, substitutos de pele auto-montados (SASS), CD73, NTPDase1 e NPP1, UTP, adenosina.

ABSTRACT

The treatment of deep burns is still a challenge in the field of regenerative medicine. The human amniotic membrane (hAM) is a biomaterial that has been increasingly explored in the field of tissue engineering. In order to promote tissue regeneration, mesenchymal stromal cells (MSCs) can be transplanted into the injured area on a support (*scaffold*) that maintains their therapeutic potential. In addition to a series of bioactive factors, MSCs release high concentrations of adenosine (ADO), which may be related to the high expression of Ecto-5'-nucleotidase (CD73), whose main function is to convert extracellular ATP into immunosuppressive adenosine, in concert with NTPDase1 (CD39). These enzymes are powerful tools to control the effects mediated by extracellular purines and pyrimidines; however, the role of purinergic signaling components in tissue regeneration is poorly understood. In this work, we evaluated the safety and efficacy of the human amniotic membrane as a biological dressing, in the presence or absence of mesenchymal stromal cells through *in vitro* and *in vivo* models, and we studied the role of purinergic signaling as one of the pathways responsible for the tissue regeneration. The physical and histological characterization of the decellularized human amniotic membrane (DhAM) in the presence of adipose tissue-derived mesenchymal stromal cells (AD-MSCs) was performed, as well as the comparison of the potential of DhAM and a commercial substitute as *scaffolds* for the culture of mesenchymal stromal cells isolated from the sclerocorneal limbus (L-MSCs), demonstrating that the culture of MSCs in DhAM did not alter the potential of progenitor cells *in vitro*. The effect of DhAM on the presence and absence of AD-MSCs was evaluated in the preclinical model of deep second-degree burn, which demonstrated the efficacy and safety of this *bioscaffold* in tissue regeneration. The role of purinergic signaling components during wound healing was analyzed using the self-assembled skin substitutes (SASS) model, demonstrating the modulation of CD73, NTPDase1 and NPP1 enzymes, as well as the therapeutic potential of the UTP nucleotide in tissue regeneration. Our results confirm the effectiveness of DhAM as a *scaffold* for MSCs and as a possible future therapy for the treatment of burns, in addition to the importance of understanding the involvement of extracellular nucleotides and ectoenzymes in wound healing, in order to offer new therapeutic strategies.

Keywords: decellularized human amniotic membrane (DhAM), mesenchymal stromal cells (MSCs), burns, bioscaffold, self-assembled skin substitutes (SASS), CD73, NTPDase1 and NPP1, UTP, adenosine.

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1 REFERENCIAL TEÓRICO

1.1 A PELE HUMANA

A pele é o maior órgão do nosso corpo, que além de exercer múltiplas funções vitais, como a manutenção da homeostase fisiológica do corpo humano, ela constitui uma barreira que nos protege contra agressões externas.

A pele é estruturada em três camadas diferenciadas, epiderme, derme e hipoderme. Cada uma tem suas próprias características, como propriedades físicas ou composição celular (Rapini 1996).

A epiderme, a camada mais superficial, é composta por um epitélio escamoso estratificado constituído principalmente de queratinócitos e outros tipos de células epidermais como células de Langerhans, células de Merkel e melanócitos ([Kanitakis 2002](#)).

Os queratinócitos são células epiteliais derivadas do ectodermo e compreendem aproximadamente 90 a 95% das células da epiderme. Os queratinócitos passam por um processo de diferenciação terminal que inicia pelas células tronco presentes na camada basal até a camada córnea, onde são liberados da superfície da pele, completando a auto-renovação da epiderme (Watt 1989; Z. Wang et al. 2020). Essas camadas, de profunda até superficial, são a camada de células basais (estrato basal), a camada de células espinhosas ou escamosas (estrato espinhoso), a camada de células granulares (estrato granuloso) e a camada de células cornificadas ou córneas (estrato córneo). A epiderme nas palmas das mãos e plantas dos pés possui uma camada extra, denominada camada clara ou translúcida (stratum lucidum), que pode ser vista entre a camada granular e a cornificada (Kanitakis 2002; Jiang et al. 2020).

A epiderme desempenha funções vitais, incluindo limitar a perda passiva de água do corpo, impedir a absorção de produtos químicos do ambiente externo e impedir a entrada de microorganismos (Rosso et al. 2016; Wickett, Randall Wickett, and Visscher 2006). A epiderme, não contém vasos sanguíneos e é dependente da derme, onde vasos sanguíneos e linfáticos residem, para fornecer acesso a nutrientes e eliminação de resíduos (Lund et al. 2016).

A derme, abaixo da epiderme, é uma rede rica em elementos da matriz extracelular produzida pelos fibroblastos, como fibras de colágeno e elastina, que fornecem força e elasticidade à pele (Kanitakis 2002). A derme contém glândulas sudoríparas, glândulas sebáceas, folículos pilosos e terminações nervosas, além de vasos sanguíneos, que formam condutos migratórios para as células do sistema imunológico (Menon 2002; Tay et al. 2014) e desempenham papéis importantes na homeostase, inflamação e reparo da pele (Boothby, Cohen, and Rosenblum 2020). Células estromais mesenquimais (MSCs) foram identificadas em diferentes nichos desta camada (Vapniarsky et al. 2015) e têm sido isoladas da derme humana (Naasani et al. 2017).

A hipoderme é a camada mais profunda da pele, composta principalmente por lóbulos de adipócitos (Yousef, Alhajj, and Sharma 2021), onde têm sido isoladas células estromais mesenquimais do tecido adiposo (AD-MSCs) (Ritter et al. 2019) (Figura 1).

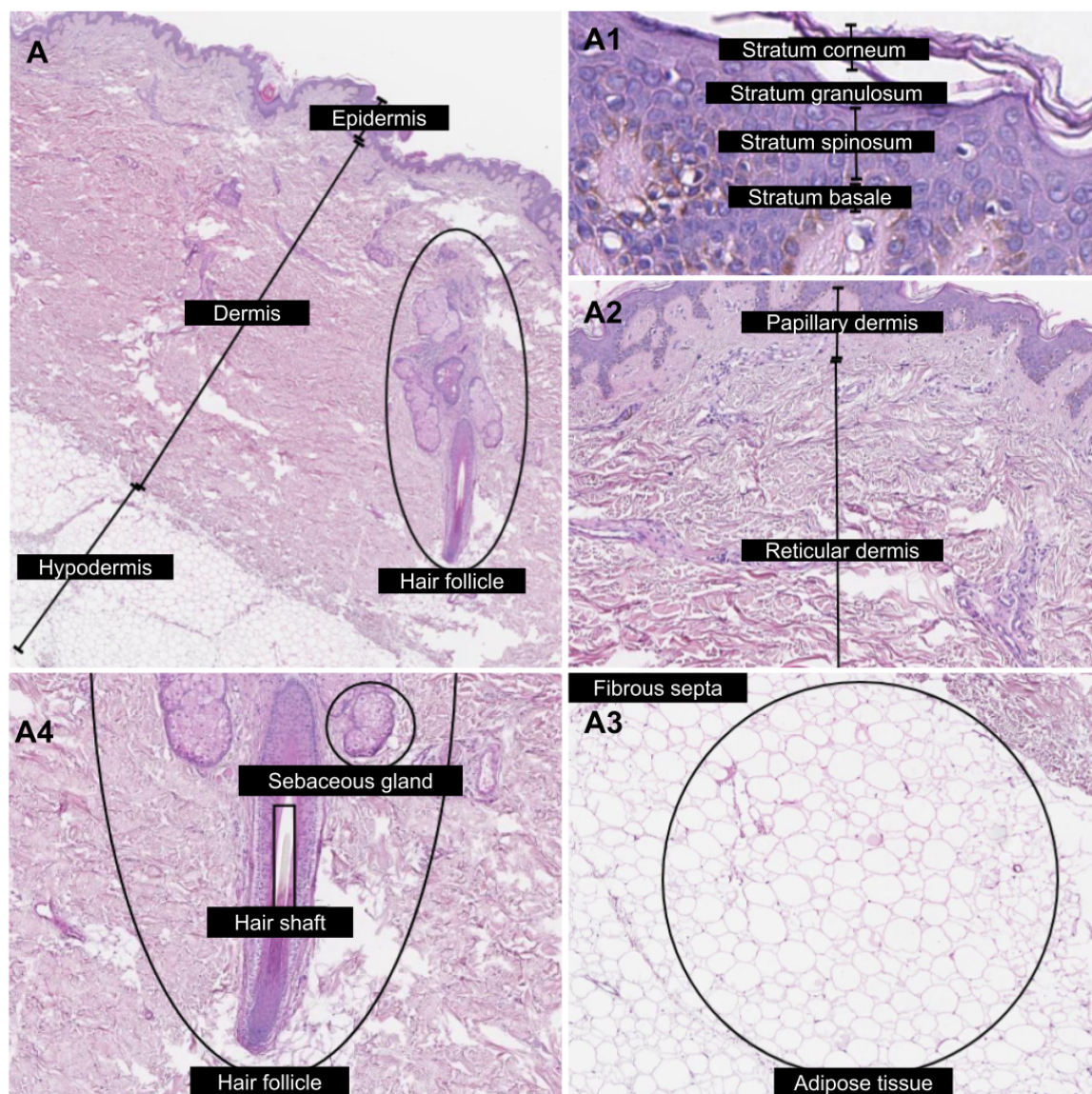


Figura 1. Histologia da pele humana. A) A pele é dividida em três estruturas principais: A1) A epiderme, formando a camada mais externa; A2) A derme, formando o tecido abaixo da epiderme; A3) A hipoderme ou tecido subcutâneo, formando a camada mais profunda e separando a pele dos órgãos subjacentes; A4) A pele também contém estruturas anexiais, como folículos pilosos e glândulas sebáceas. Coloração de hematoxilina-eosina (HE). Figura adaptada de The Human Protein Atlas (“Skin” n.d.).

1.1.1 Processo de cicatrização de feridas

Por atuar como função de barreira, a pele é exposta a diversas lesões e patologias que precisam de uma rápida regeneração. Vários tipos de células das três camadas da pele precisam se coordenar para promover o reparo tecidual. Esses estágios de hemostasia, inflamação, proliferação, reepitelização, angiogênese e remodelação ocorrem em uma

sequência temporal, mas também se sobrepõem, portanto, a reparação da pele está entre os processos mais complexos do corpo humano [Revisado em (M. Rodrigues et al. 2019)].

A hemostasia marca o primeiro estágio da cicatrização de feridas, a constrição dos vasos sanguíneos e ativação de plaquetas para formar um coágulo de fibrina, que para o sangramento após dano vascular. A fase inflamatória é anunciada pelo influxo de neutrófilos, macrófagos e linfócitos para o local da lesão. Quando a fase inflamatória termina, ocorre a angiogênese, a formação de novos vasos sanguíneos, uma das mais importantes fases da cicatrização de feridas. Enquanto novos vasos sanguíneos emergem, os fibroblastos residentes proliferam e invadem o coágulo para formar o tecido de granulação contrátil.

Simultaneamente, ocorre a reepitelização e envolve o reparo da camada epidérmica. A fase de remodelação começa quando a matriz extracelular provisória e o colágeno tipo III são substituídos por colágeno tipo I e os demais tipos de células das fases anteriores sofrem apoptose [Revisado em (M. Rodrigues et al. 2019; Gantwerker and Hom 2011)] (Figura 2).

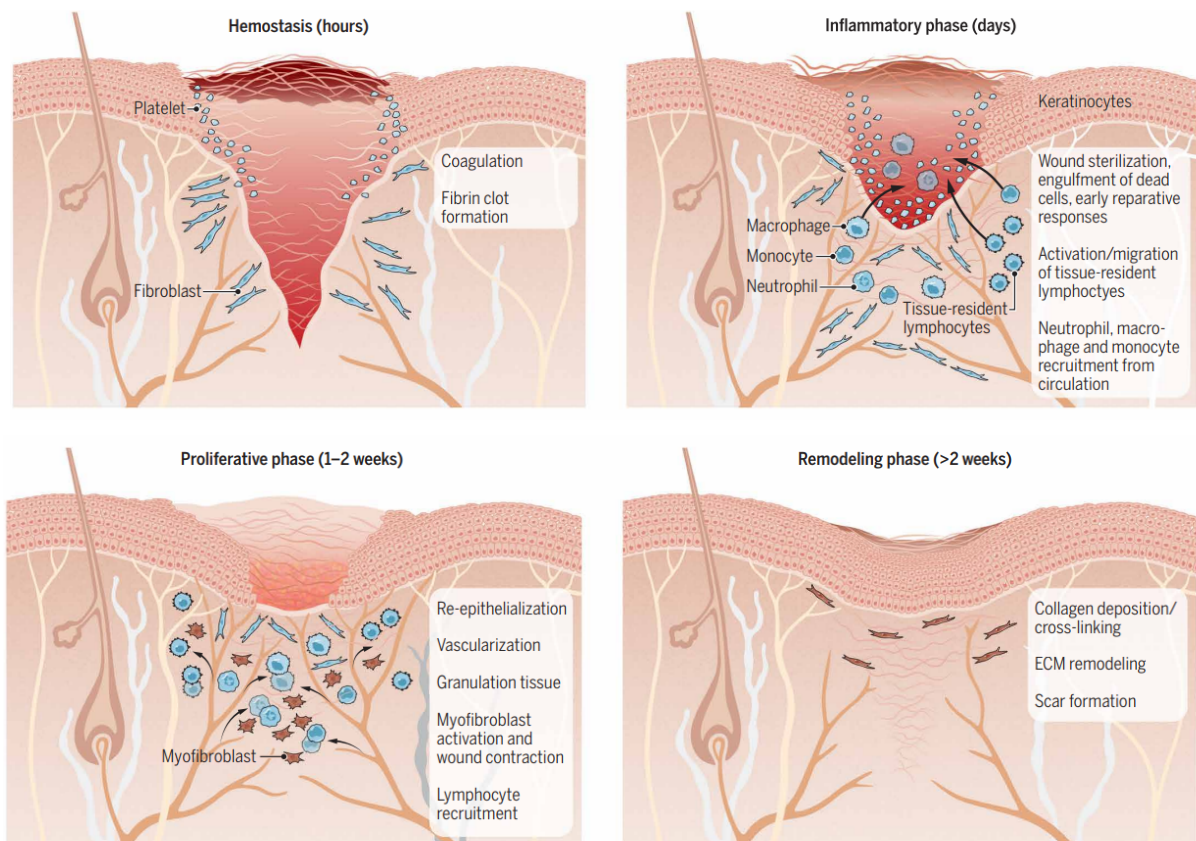


Figura 2. Progressão da cicatrização de feridas agudas na pele. (Superior, à esquerda) Imediatamente após o ferimento, um coágulo de fibrina se forma e o sangramento é interrompido. (Superior, à direita) Por vários dias após a lesão, a liberação local de sinais de dano tecidual leva ao recrutamento de neutrófilos e monócitos/macrófagos circulantes para evitar infecção e fagocitar células mortas e detritos. (Inferior, à esquerda) Conforme a inflamação é resolvida, os queratinócitos nas margens da lesão proliferam, migram e se diferenciam para reepitelizar o local da lesão e restaurar a integridade da barreira. O tecido de granulação se forma no leito da ferida e é vascularizado pela angiogênese dos vasos sanguíneos próximos. Os miofibroblastos se acumulam no tecido de granulação e se contraem para fechar a lesão. O influxo de linfócitos na ferida atinge seu pico e a polarização dos macrófagos muda de estados pró-inflamatórios para pró-reparadores. (Inferior, à direita) Depois que a lesão fecha, as células no leito da ferida diminuem e uma cicatriz se forma através de uma combinação de deposição de colágeno, reticulação e remodelação. Este processo fortalece o local da ferida às custas da regeneração da derme normal (Boothby, Cohen, and Rosenblum 2020).

Embora a regeneração descreva a substituição específica do tecido, o reparo da pele exibe uma forma inespecífica de cura em que a lesão cura por fibrose e formação de cicatriz (Reinke and Sorg 2012). Isto representa uma falha considerável da pele, a pele gravemente danificada pode cicatrizar, mas não pode regenerar. Em vez disso, forma cicatrizes. Mesmo que as cicatrizes pareçam ser mais espessas do que a pele normal, o tecido é, na verdade, mais fraco. Essas marcas não são apenas defeitos cosméticos, o tecido cicatricial pode inibir o

movimento de uma pessoa ou, por não ter glândulas sudoríparas, impedir que o corpo esfrie (Willyard 2018).

A regeneração da pele é um processo imperfeito que é impedido por uma série de fatores, por exemplo, uma resposta inflamatória excessiva ou prolongada resulta em aumento da lesão do tecido; também, os miofibroblastos no tecido de granulação, onde se contraem para fechar a ferida, tem sido associados em muitas doenças fibróticas (Boothby, Cohen, and Rosenblum 2020; Desmouliere et al. 2014). Portanto, descobrir o papel desempenhado por cada fator pode levar a novas abordagens para o tratamento de queimaduras e cicatrizes (Willyard 2018).

1.1.2 Queimaduras e tratamentos: Situação no Brasil

A perda da integridade de grandes áreas de pele pode resultar em incapacidade significativa do indivíduo devido a contrações extensas ou até mesmo em morte (Priya, Jungvid, and Kumar 2008). O trauma térmico é a causa mais comum da perda da integridade cutânea, no qual grandes áreas de pele podem ser danificadas, muitas vezes sem a possibilidade de regeneração (Shevchenko, James, and James 2010).

O tratamento padrão-ouro nestes casos é o enxerto de pele autóloga. No entanto, esta abordagem fica bastante limitada quando grandes extensões de pele foram acometidas pela queimadura, ou grandes áreas foram lesadas por traumas físicos, pela restrição de sítios doadores de pele do próprio paciente. A utilização de aloenxerto é um tratamento providencial e pode representar a diferença entre a vida e a morte desses pacientes, havendo redução da mortalidade de grandes queimados quando se dispõe dessa alternativa (Kagan, Robb, and Plessinger 2005). O Brasil atualmente tem bancos de pele nas cidades do Rio de Janeiro, São Paulo, Curitiba e Porto Alegre, que realizam a captação, processamento, armazenamento e disponibilização de lâminas de pele humana para os centros de transplante, beneficiando as vítimas de queimaduras e politraumatismos.

A terapia de pacientes queimados sempre foi um desafio global, tanto pela complexidade das lesões, quanto pela necessidade de cuidados intensivos e multidisciplinares envolvendo diversos profissionais de saúde, como clínicos, intensivistas, psicólogos, nutricionistas, fisioterapeutas e enfermeiras com experiência na área (Júnior et al. 2007).

A gravidade da queimadura é classificada de acordo com a profundidade da lesão e as camadas de pele que são afetadas: a queimadura de primeiro grau envolve apenas a epiderme, a queimadura de segundo grau superficial é uma injúria na metade superior da derme,

enquanto a de segundo grau profundo envolve quase toda a derme, e queimaduras de terceiro grau atingem a hipoderme. Quando uma queimadura é de segundo ou terceiro grau, a vasculatura é destruída e o corpo perde sua capacidade de se auto-regenerar. Procedimentos cirúrgicos combinados com terapias celulares assim, tornam-se indispensáveis (Abdel-Sayed et al. 2019).

Carboni e Colaboradores (2019) publicaram uma revisão integrativa com estudos nacionais publicados nos últimos dez anos com destaque para produtos e terapias usados em queimaduras, sendo a membrana celulósica porosa e o curativo com prata nanocristalina os mais utilizados nos estudos avaliados. Existem outros curativos comerciais sintéticos como o Mepitel®, que possui dupla camada de silicone, o Mepilex® Ag, um curativo de espuma com antimicrobiano, entre outros, mas tem poucos estudos utilizando esses curativos para o tratamento de queimaduras (Carboni et al. 2019).

Atualmente, a pele de peixe tilápia tem sido sugerida como um xenoenxerto potencial para o tratamento de queimaduras. Lima-Junior e colaboradores (2020) publicaram um relato de caso de um paciente com lesão térmica, com queimaduras superficiais e profundas de espessura parcial, que foi tratado com pele de tilápia, levando à reepitelização completa sem efeitos colaterais. A pele de tilápia carrega a promessa de um produto inovador, fácil de aplicar e altamente disponível que pode se tornar a primeira pele animal estudada nacionalmente e registrada pela Agência Nacional de Vigilância Sanitária para uso no tratamento de queimaduras. (Lima Júnior et al. 2020). No entanto, a pele de peixe tilápia encontra-se em processo de concessão de patente (BR1020150214359), o que poderia representar a sua comercialização ao preço de curativos comerciais.

Recentemente, o Conselho Federal de Medicina (CFM), no parecer CFM nº 12/2021, de 21 de outubro, liberou o uso da membrana amniótica nativa para o tratamento de queimaduras. No entanto, ainda é necessária a liberação da Comissão Nacional de Incorporação de Tecnologias no SUS (CONITEC) e da publicação de legislação por parte do ministério da saúde para uso do tecido [sistemas.cfm.org.br/pareceres (“PDF.js Viewer” n.d.)].

Embora várias terapias para a cura de feridas estejam disponíveis, elas são apenas moderadamente eficazes. Assim, há necessidade de terapias mais eficazes para a cicatrização de feridas (M. Rodrigues et al. 2019).

1.2 ENGENHARIA DE TECIDOS: EM BUSCA DA REGENERAÇÃO TECIDUAL

O estudo e tratamento de feridas remontam ao Antigo Egito, onde desenvolveram uma bandagem adesiva para unir as bordas da ferida e colocavam um pedaço de carne no primeiro dia da lesão. Produtos como mel e óleo de camomila também foram usados, e as propriedades antissépticas e antibacterianas destes compostos foram posteriormente elucidadas (Gantwerker and Hom 2011).

O déficit de material para o tratamento de lesões cutâneas tem motivado o desenvolvimento de novas estratégias na área de engenharia de tecidos em busca de processos que favoreçam a regeneração da área lesionada e, portanto, permitam devolver a função tecidual (Carrier et al. 2009) (Figura 3).

Para pacientes com queimaduras extensas, a expansão das células da pele que permitam uma função de barreira pode fazer a diferença entre a vida e a morte, e foi essa necessidade aguda que levou à iniciação da engenharia de tecidos na década de 1980 (MacNeil 2007).

O desenvolvimento de substitutos cutâneos na área de engenharia de tecidos se encontra em constante progresso. A engenharia de tecidos utiliza células, materiais biocompatíveis, fatores de crescimento e estimulação física para desenvolver um substituto vivo (Berthiaume et al, 2011).

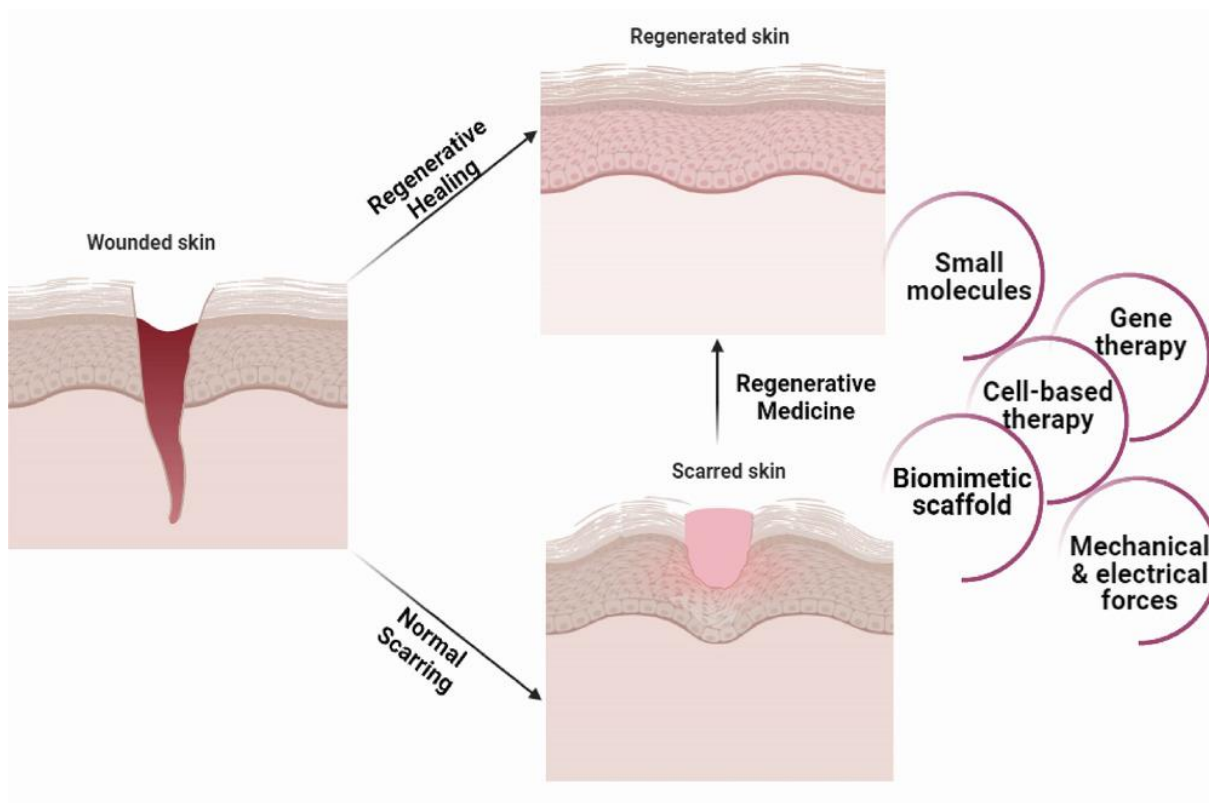


Figura 3. Abordagens potenciais para diminuir a formação de cicatrizes e melhorar a regeneração da pele (Hosseini and Shafiee 2021).

As células estromais mesenquimais (MSCs) têm sido consideradas como uma fonte de células para uso em medicina regenerativa, engenharia de tecidos e outras aplicações (Vapniarsky et al. 2015). Estas células possuem muitas propriedades que aumentam seu potencial terapêutico em dermatologia e oftalmologia, cujo mecanismo ainda está sob investigação.

Para que as células possam ser transplantadas na área da lesão, é necessário um suporte (*scaffold*) que promova o crescimento celular a fim de promover a cicatrização de lesões teciduais. Atualmente existem diversos tipos de materiais, que podem ser naturais ou sintéticos e dependendo das suas características como biocompatibilidade, bioatividade e biodegradação e das suas propriedades físico-químicas (morfologia superficial, porosidade, rigidez e resistência mecânica), pode afetar a interação com as células e o sistema vivo (Dolcimascolo et al. 2019). Portanto a escolha de células e biomateriais é fundamental no campo da medicina regenerativa.

1.2.1 Substitutos cutâneos

Nas últimas décadas, vários substitutos cutâneos acelulares, epidérmicos, dérmicos, de duas camadas ou xenogênicos, foram desenvolvidos e comercializados para o tratamento de defeitos da pele, como, Alloderm®, Integra®, Pelnac®, e Lando®. Também, a submucosa do intestino delgado porcino, OASIS®, é utilizada como substituto da pele no tratamento de queimaduras (Gentile and Garcovich 2021) (Figura 4). Embora, a relação custo-benefício desses materiais deve sempre ser considerada com cuidado, especialmente no Brasil, onde eles são comercializados a um alto custo (Ferreira et al. 2011).

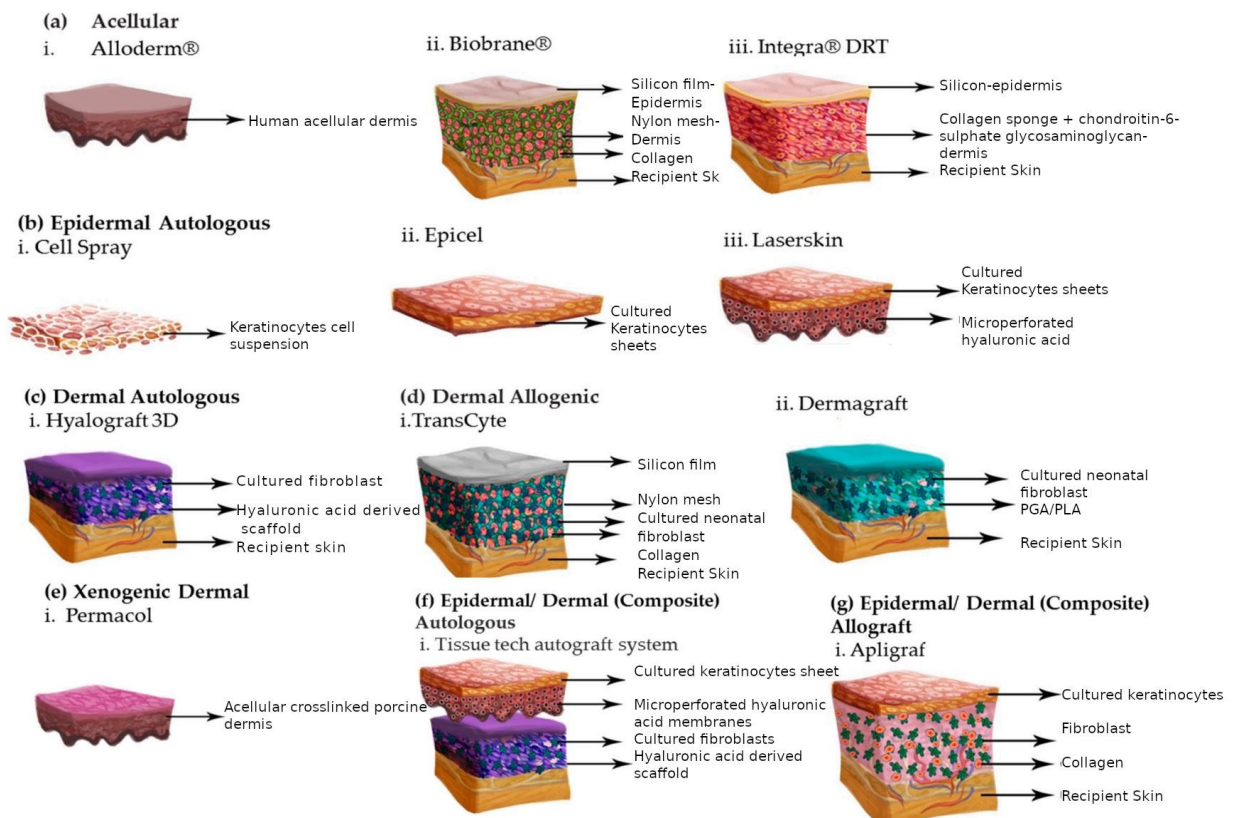


Figura 4. Substitutos dermo-epidérmicos de pele. Desenvolvidos por engenharia de tecidos com o objetivo de produzir substitutos de pele para uso clínico. Figura adaptada de (Vig et al. 2017).

Estes modelos contêm várias camadas de células epiteliais crescendo em cima de camadas de tecido com fibroblastos e matriz extracelular. Eles fornecem substitutos de tecido que podem ser manipulados experimentalmente em condições *in vitro* (Haddad et al. 2017).

Os substitutos de pele auto-montados (*Self Assembled Skin Substitute*, SASS) foram desenvolvidos pelo grupo de pesquisa do LOEX da Universidade Laval na cidade de Quebec, Canadá. Estes substitutos de pele, desenvolvidos por engenharia de tecidos, são muito semelhantes à pele humana nativa. São compostos por uma matriz extracelular de colágeno natural, secretada pelo próprio fibroblasto, sem a utilização de nenhum material sintético, subjacente a uma membrana basal funcional e uma epiderme totalmente diferenciada (D. Larouche et al. 2016; Beaudoin Cloutier et al. 2017) Figura 5.

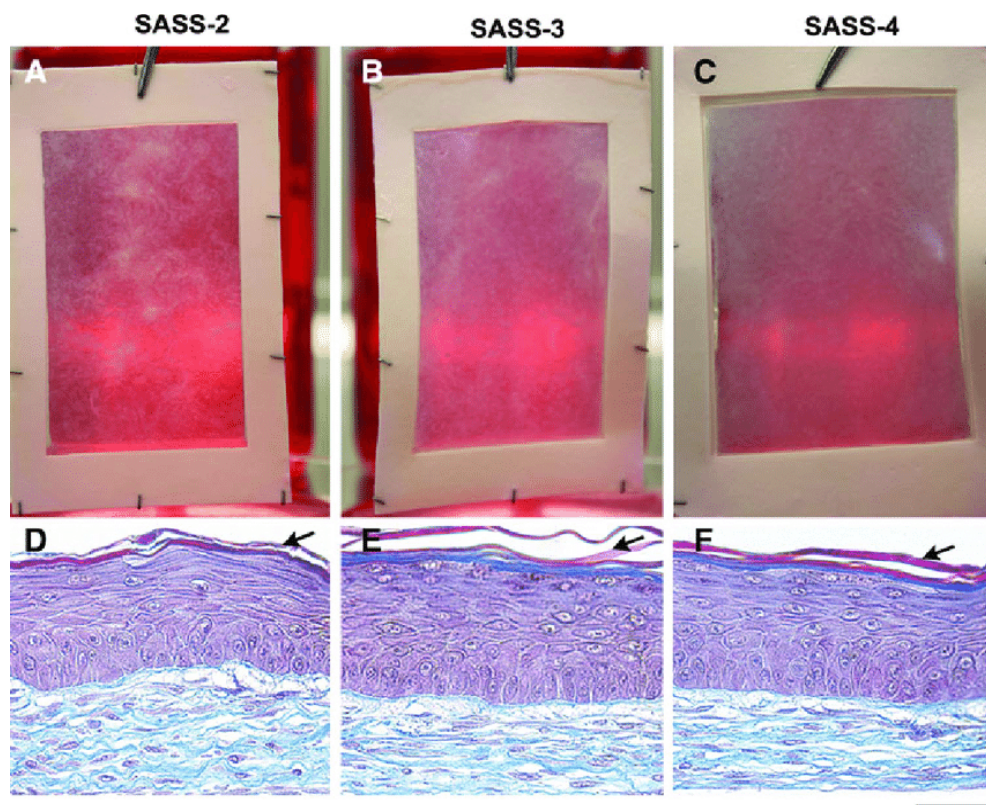


Figura 5. Análise macroscópica e histológica do substituto de pele auto-montado (SASS) produzido *in vitro*. Aspecto macroscópico (A – C) e resultados histológicos (D – F), produzidos com queratinócitos e fibroblastos do mesmo doador. Barra de escala: (A – C), 167 mm; (D – F), 50 μ m (D. Larouche et al. 2016).

Uma das maiores conquistas da tecnologia dos substitutos de pele auto-montados do LOEX é o uso de células autólogas, que permitiram o tratamento como enxerto permanente de 14 pacientes gravemente queimados, com cobertura bem sucedida e como resultado eficácia clínica (Germain et al. 2018). Dependendo do tipo de célula utilizada, esses substitutos tridimensionais podem ser desenvolvidos a fim de substituir tecidos lesados em pacientes, considerando outras abordagens que não a pele, como a córnea (Proulx et al. 2011). Além disso, eles também podem ser usados para compreender vários mecanismos fisiológicos e

patológicos, como psoríase (Jean, Soucy, and Pouliot 2011) e cicatriz hipertrófica (Moulin 2021)

Além da aplicação clínica, os substitutos cutâneos também podem ser destinados à aplicabilidade industrial e pesquisa experimental. No campo da pesquisa, são uma ferramenta valiosa para estudos *in vitro*, sendo uma excelente alternativa para estudar biologia da pele (Mieremet et al. 2019), cicatrização de feridas (Laplanche et al. 2001; Y. Xie et al. 2010), envelhecimento da pele (Diekmann et al. 2016; Weinmüllner et al. 2020), carcinoma de células escamosas cutâneo (Commandeur et al. 2009), melanoma (Hill et al. 2015) e testes de corrosão (Catarino et al. 2018), sendo as formas de pesquisa mais promissoras antes de novos avanços poderem ser traduzidos para beneficiar cirurgicamente os pacientes (Figura 6).

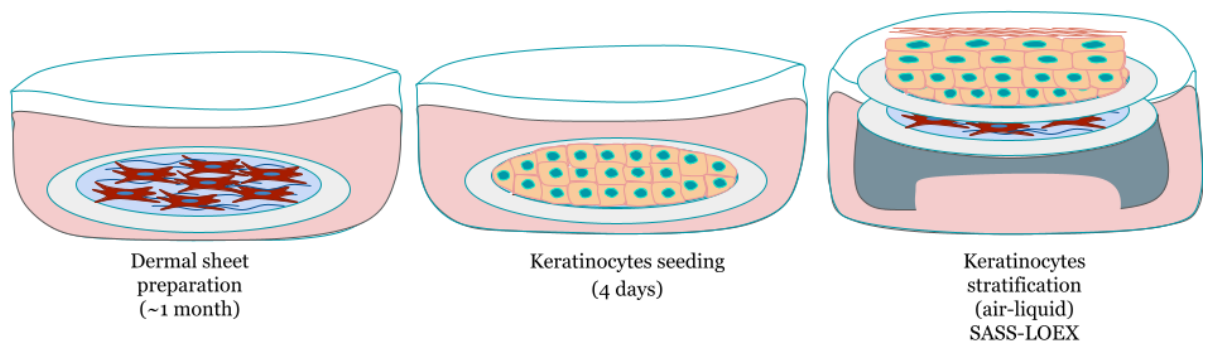


Figura 6. Elaboração do substitutos de pele auto-montado (SASS). A derme é produzida a partir da secreção de colágeno dos fibroblastos. A estratificação dos queratinócitos é produzida pelo sistema líquido-ar. Estes modelos permitem o estudo do efeito de compostos na pele *in vitro*. <http://www.crchudequebec.ulaval.ca/> (Naasani et al. não publicado).

1.2.2 Scaffold natural: Membrana amniótica humana

A membrana amniótica humana (hAM) é a camada interna da placenta e é considerada uma importante fonte de material de suporte celular. Os componentes da matriz extracelular da hAM criam um suporte quase nativo para engenharia de tecidos, a camada estromal tem uma abundância de proteoglicanos, glicoproteínas e uma rede de colágeno, principalmente tipo III (Niknejad et al. 2008).

A membrana amniótica humana é a camada interna da placenta, com uma espessura entre 20 e 50 μm e consiste em cinco camadas diferentes: uma única camada de epitélio, uma membrana

basal, uma camada compacta, uma camada de fibroblasto e uma camada esponjosa (Niknejad et al. 2008; Oyen, Calvin, and Landers 2006) (Figura 7).

A membrana placentária contém células epiteliais, fibroblastos neonatais e células-tronco mesenquimais (MSCs), promovendo efetivamente a cicatrização de feridas e já é aceita em tratamentos de oftalmologia como ulceração corneal, cobertura de lesões na conjuntiva e queimaduras químicas agudas nos olhos (Meller et al. 2011). Também, a aplicação da membrana amniótica tem sido utilizada nos campos de dermatologia e cirurgia plástica (Leal-Marín et al. 2021). No ensaio clínico em fase 1 será avaliada a utilização do âmnio humano como cobertura temporal de feridas (“Study of Donated Amnion, Fetal Placental Membrane, as Skin Substitute for Burn Patients - Full Text View - ClinicalTrials.gov” n.d.) (*ClinicalTrials.gov identifier (NCT number): NCT00592189*).

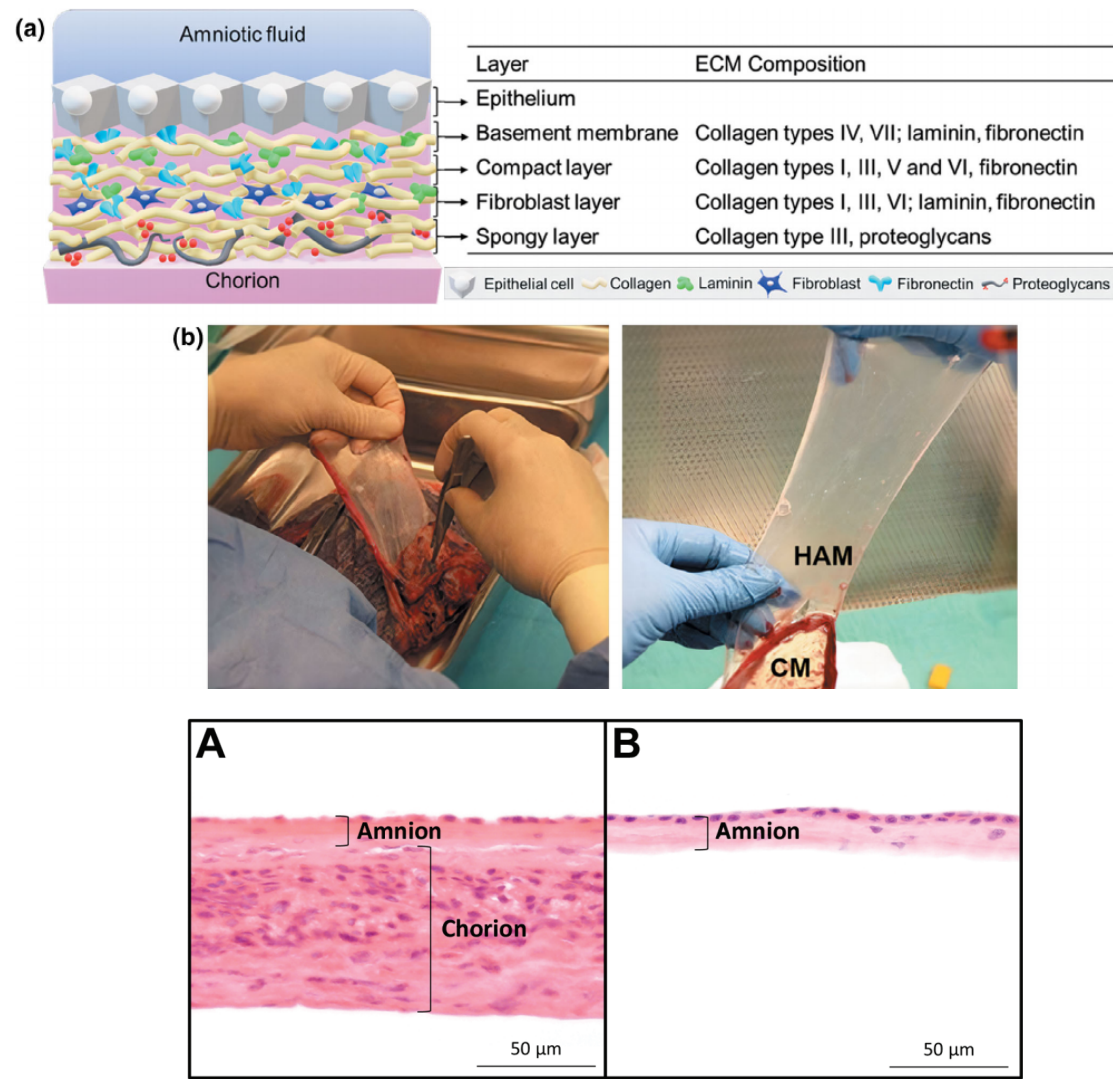


Figura 7. Membranas placentárias humanas. (a) Representação esquemática da estrutura de hAM e a composição da matriz extracelular para cada camada. (b) Aspecto geral das membranas fetais humanas (imagem à esquerda). A hAM é uma fina camada translúcida anexada ao córion (CM)

(imagem à direita). Colorações de hematoxilina e eosina de (A) enxertos de âmnio/córion e (B) enxertos somente de âmnio. O âmnio possui uma única camada de células epiteliais, sob as quais se encontra uma espessa membrana basal e uma camada acelular e avascular de mesênquima. (a-b) Figura adaptada de (Leal-Marín et al. 2021); (A-B) Figura adaptada de (Riboh et al. 2016).

Comercialmente, a membrana amniótica também já é utilizada em vários países, como por exemplo com curativo Graftix (Osiris Therapeutics Inc., Columbia, MD, EUA), um aloenxerto comercializado, criopreservado à base de placenta, que emprega a constituição nativa do tecido placentário, produzindo uma matriz extracelular que inclui várias células vivas, com estudos publicados tendo seu uso em diferentes feridas agudas e crônicas, queimaduras, incisões cirúrgicas e deiscências (Tavakoli and Klar 2021).

Na área de engenharia de tecidos, a hAM tem sido utilizada em várias formas, como fresca, nativa ou descelularizada, criopreservada, seca ao ar ou liofilizada, dependendo da acessibilidade do equipamento e dos tipos de lesões. A descelularização da hAM, sem comprometer os componentes de matriz extracelular, é muito importante para evitar a rejeição do enxerto (Milan et al. 2020). O processo de descelularização ajuda a eliminar possíveis respostas imunogênicas, removendo as células e resíduos celulares da membrana amniótica, enquanto deixa as proteínas estruturais extracelulares intactas (Milan et al. 2020; Weidinger et al. 2020).

Alguns trabalhos e patentes têm discutido a aplicabilidade de técnicas de *scaffolds* com hAM, conforme resumido na Tabela I, com o objetivo de criar compósitos utilizando biomateriais que aumentem a eficácia para diferentes aplicações, principalmente na área de reconstrução ocular.

Tabela I. Aplicação de compostos de membrana amniótica humana e produtos comerciais.

TABLE 1 Application of hAM composites and commercial products

Application	Form of hAM	Membrane material	In vitro test	In vivo test
Wound healing and tissue regeneration	hAM lyophilized and pulverized	Scaffold with a synthetic polymer and a natural polymer	Keratinocytes and fibroblast	Skin model nude mice and Yorkshire pigs
	hAM solubilized	Hyaluronic acid hydrogel		Mice
Reconstructive urology	hAM frozen	Electrospun poly-(L-lactide-co-E-caprolactone)	Mesenchymal stem cells	Wistar rats
Artificial cornea material - Keratoprosthesis	hAM decellularized	Polyvinyl alcohol (PVA)	-	Rabbit corneal epithelial cells
Aligned tissue regeneration	hAM decellularized	Electrospun fibers of PLGA	-	Skeletal muscle cells
Ocular surface reconstruction	<i>Amnioguard</i> (bio-tissue)	N/A only native hAM	Clinical test 11 patients	
Neuropathic corneal pain	<i>ProKera</i> ®(bio-tissue)	N/A only native hAM	Clinical test 9 patients	
Ocular surface disorders	<i>AmnioClip-plus</i> (DGFG)	N/A only native hAM	Clinical test 7 patients	
Premature rupture fetal membrane	hAM cell-free scaffold	Polyester urethane scaffold used as a comparison	-	Rabbit model
Vascular graft	Rolled hAM	N/A only native hAM	-	Rabbit model
Tendon regeneration	hAM pulverized	Collagen-glycosaminoglycan Hyaluronic acid	Tenocytes	-
Skin regeneration	hAM decellularized	Nanofibrous silk fibroin	Adipose tissue-derived mesenchymal stem cells	-

Fonte: (Leal-Marín et al. 2021).

Considerando os componentes da matriz extracelular da hAM, a membrana amniótica descelularizada pode ser associada com células estromais mesenquimais, como potencial alternativa no reparo de lesões cutâneas extensas como as queimaduras.

1.2.3 Células Estromais Mesenquimais

Nas últimas décadas, um grande número de estudos surgiram usando terapias baseadas em MSCs em estudos pré-clínicos para tratar muitas patologias diferentes, incluindo distúrbios neurológicos (van Velthoven et al. 2013), isquemia cardíaca (Liu et al. 2016), diabetes (Si et al. 2012) e doenças ósseas e cartilaginosas (Ozeki et al. 2016).

O potencial terapêutico das MSCs é mediado, em primeiro lugar, por sua capacidade inerente de migrar em direção aos tecidos danificados. Em seguida, as células secretam mediadores bioativos, como fatores de crescimento, citocinas e vesículas extracelulares que exercem efeitos imunossupressores, antiapoptóticos, antifibróticos, angiogênicos e antiinflamatórios (Salgado et al. 2010).

As células estromais mesenquimais são células não-hematopoiéticas com capacidade de auto-renovação, proliferação e potencial de diferenciação multilinhagem (Friedenstein, Chailakhjan, and Lalykina 1970; Pittenger et al. 1999). As MSCs têm expressão reduzida de antígenos de classe I do complexo principal de histocompatibilidade (MHC) e não expressam MHC II ou moléculas coestimulatórias como CD80, CD86 e CD40. Assim, as MSCs apresentam um fenótipo não imunogênico, possibilitando sua utilização alogênica na regeneração de tecidos e evitando a necessidade de imunossupressão após o transplante (Galindo et al. 2021) Figura 8.

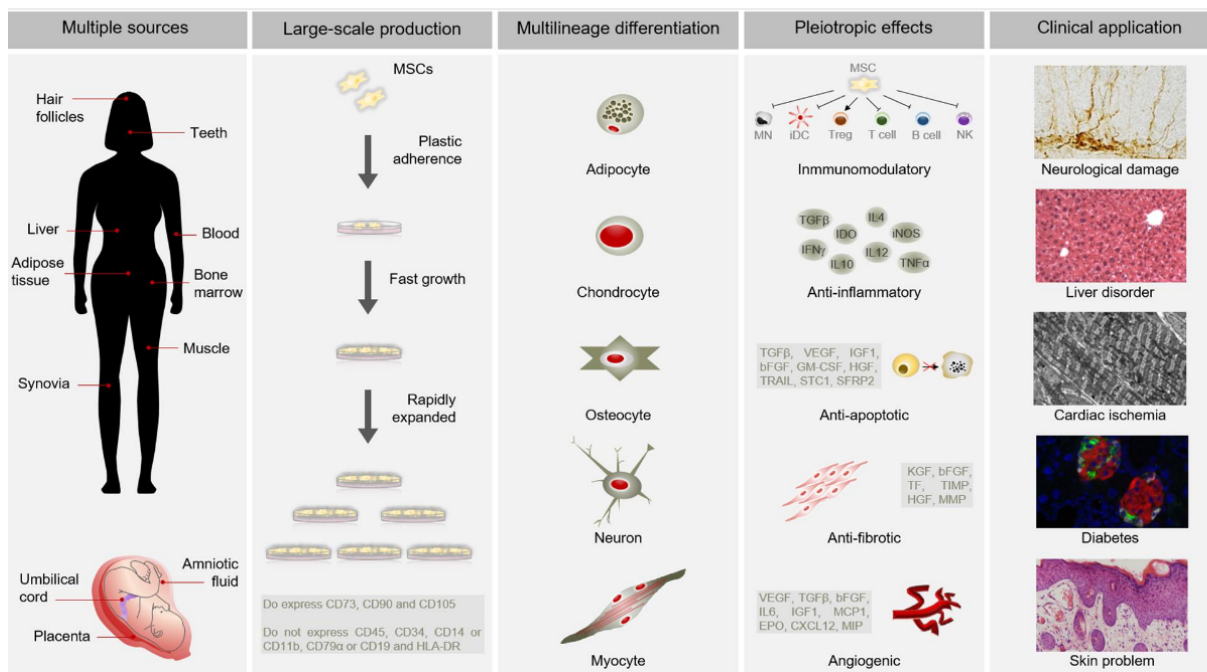


Figura 8. Vantagens das células estromais mesenquimais para uso clínico. As MSCs possuem múltiplas vantagens para aplicação clínica. Entre outros benefícios, as MSCs podem ser isoladas de várias fontes, são produzidas em grande escala, se diferenciam em vários tipos de células e têm efeitos pleiotrópicos. Todas essas vantagens tornam as MSCs adequadas para aplicação clínica em diferentes condições patológicas, como danos neurológicos, doenças hepáticas, isquemia cardíaca, diabetes ou problemas de pele (Hmadcha et al. 2020).

As MSCs têm sido isoladas de diferentes tecidos adultos, incluindo medula óssea, tecido adiposo, gelatina de Wharton do cordão umbilical, placenta, pele e córnea (Dravida et al. 2005; Vapniarsky et al. 2015). Entre os diversos materiais biológicos, o limbo esclerocorneal, a zona estreita entre a córnea e a esclera, representa uma fonte tecidual para o isolamento de células estromais mesenquimais. As células estromais mesenquimais do limbo (L-MSCs) compartilham características com as células estromais mesenquimais da medula óssea, em relação à sua morfologia, marcadores específicos, alta taxa de proliferação e baixa

imunogenicidade (Dravida et al. 2005; Du et al. 2005). Estas células fornecem um nicho e permitem a manutenção do fenótipo das células tronco limbaais epiteliais e participam na regeneração da córnea (Polisetty et al. 2008; H.-T. Xie et al. 2012).

Em 2001, Zuk e colaboradores descreveram uma nova célula estromal/tronco mesenquimal isolada de tecido adiposo após procedimento de lipoaspiração (Zuk et al. 2001). Desde então, o tecido adiposo foi amplamente estudado como uma fonte de células para engenharia de tecidos e medicina regenerativa (Patrikoski, Mannerström, and Miettinen 2019). Nosso grupo de pesquisa demonstrou que MSCs isoladas de tecido adiposo (AD-MSCs), em associação com carboximetilcelulose, são capazes de promover a regeneração da pele em um modelo pré-clínico de lesão (C. Rodrigues et al. 2014).

Recentemente, Gentile e colaboradores (2021) publicaram uma revisão sistemática onde incluíram estudos de células estromais mesenquimais derivadas do tecido adiposo, Plasma rico em plaquetas e biomateriais como novas estratégias regenerativas em feridas cutâneas crônicas e defeitos de tecidos moles. Eles concluíram que os tratamentos atuais diferem na metodologia e técnica de tratamento e mais estudos são necessários para definir protocolos padronizados, e ensaios clínicos randomizados em grande escala ainda precisam ser realizados para confirmar sua eficácia (Gentile and Garcovich 2021).

Apenas 12 estudos relacionados estão listados no banco de dados de ensaios clínicos, e apenas alguns relatos de casos com pacientes limitados foram publicados. Esses estudos comprovaram a segurança do tratamento com base em MSC e alguns mostraram boa eficácia na melhora da cicatrização de lesões por queimadura. No entanto, devido ao número limitado de pacientes, falta de regulação, grande variabilidade nos protocolos de tratamento e avaliações de resultados inconsistentes, o valor desses estudos clínicos tem sido limitado (M. Wang et al. 2021).

Um dos principais papéis das MSCs é a imunomodulação (Marigo and Dazzi 2011). Trabalhos têm sugerido que uma resposta inflamatória local intensa pode estimular e aumentar o desenvolvimento de fibrose (Marigo and Dazzi 2011; Gill and Parks 2008). Neste contexto, tem-se demonstrado uma importante participação da sinalização purinérgica na via de reparo tecidual (Geoffrey Burnstock, Knight, and Greig 2012).

Nós demonstramos que as MSCs liberam altas concentrações de adenosina (ADO) (Naasani et al. 2017), a qual vem ganhando destaque no processo de regeneração tecidual. Este aumento pode estar relacionado com a alta expressão de CD73, considerado um dos marcadores das MSCs, resultando em uma alta atividade hidrolítica do AMP em ADO (Roszek and Wujak 2018). Este acúmulo de ADO pode auxiliar no controle do recrutamento das células imunes durante o processo cicatricial (Antonioli et al. 2019). Isto é extremamente relevante, uma vez que estas células podem direcionar diferentes tipos de regeneração tecidual dependendo da sua polarização, podendo ter efeitos anti-fibróticos ou inflamatórios, o que levaria a um processo de regeneração ou formação de fibrose, respectivamente (J. Larouche et al. 2018).

1.3 PAPEL DO SISTEMA PURINÉRGICO NO REPARO TECIDUAL

Há um número crescente de estudos relacionados às aplicações terapêuticas da sinalização purinérgica na pele, incluindo crescimento de cabelo (Oura et al. 2008), cicatrização de feridas (Montesinos, Desai-Merchant, and Cronstein 2015), inflamação (Takai et al. 2011) e melanoma (White et al. 2009). O entendimento biológico dos receptores purinérgicos envolvidos pode abrir um caminho para novas modalidades de tratamento nessas importantes doenças da pele (Geoffrey Burnstock, Knight, and Greig 2012).

Os nucleotídeos extracelulares (ATP, ADP, AMP, UTP) são moléculas sinalizadoras da cascata purinérgica e influenciam uma variedade de processos fisiológicos como secreções endócrinas e exócrinas, respostas imunes, inflamação, proliferação, diferenciação, migração e morte celular (G. Burnstock 2007).

A concentração de nucleotídeos e nucleosídeos é controlada com precisão pela família das E-NTPDases (ectonucleósido trifosfato difosfono-hidrolase) a família de nucleotídeos pirofosfatase/fosfodiesterase (NPP) e ecto-5'-nucleotidase (CD73) (Zimmermann 2001; Robson, Sévigny, and Zimmermann 2006). As NTPDases e NPPs hidrolisam nucleotídeos trifosfato (ATP e UTP) e difosfato (ADP e UDP) em seu derivado monofosfato (adenosina 5'-monofosfato (AMP) e uridina 5'-monofosfato (UMP) (Kukulski et al. 2005). O AMP e UMP são posteriormente hidrolisados pela CD73 para gerar adenosina e uridina, respectivamente. Assim, essas enzimas são ferramentas poderosas para controlar os efeitos mediados por purinas e pirimidinas extracelulares (Anderson and Parkinson 1997; Robson, Sévigny, and Zimmermann 2006).

processo de cicatrização de lesões cutâneas, *in vitro* e *in vivo*, aumentando a proliferação celular, a migração celular e a expressão de proteínas da matriz extracelular (Jin et al. 2014).

O papel da ADO também vem ganhando destaque no processo de cicatrização tecidual. Shaked et al. demonstraram em pacientes queimados que existe um acúmulo de ADO no fluido de bolhas formadas em queimaduras, e que esta poderia exercer efeitos contraditórios dependendo de sua concentração, podendo ter efeitos benéficos antiinflamatórios mas também levar a disfunção sistêmica do sistema imune e síndrome da perda capilar (Shaked et al. 2007).

O progresso acelerado neste campo tem ajudado a determinar a participação desses compostos em processos bioquímicos e fisiológicos fundamentais em diferentes tecidos. O seu potencial terapêutico em uma ampla gama de enfermidades também está atraindo um interesse crescente (Geoffrey Burnstock and Knight 2018). Embora seja crescente o número de trabalhos sobre o envolvimento de nucleotídeos extracelulares na regeneração de feridas, muitos problemas ainda precisam ser resolvidos. É necessário conhecer os mecanismos pelos quais essa via modula a regeneração para que esse conhecimento possa traduzir-se em benefício clínico, para a utilização de nucleotídeos ou os seus análogos no tratamento de regeneração de feridas na pele.

2 HIPÓTESE DE TRABALHO

A elevada disponibilidade da membrana amniótica, facilidade de aceitação da doação pelas gestantes e baixo custo de manipulação, tem despertado o interesse na utilização estendida deste importante recurso biológico. Além disso, a aplicação clínica de células estromais mesenquimais alogênicas ainda não teve sua segurança biológica garantida, impedindo, desta forma, a sua utilização em humanos de maneira indiscriminada. Portanto, se a membrana amniótica descelularizada pode ser utilizada como *scaffold* para MSCs de diferentes tecidos, *in vitro*, e como curativo biológico para tratamento de queimaduras, *in vivo*, a potencialidade das células será mantida assim como a segurança biológica deste biomaterial, o que pode representar um avanço no uso da membrana amniótica na prática clínica do Brasil, além do interesse e participação no setor produtivo.

A compreensão dos mecanismos que modulam a regeneração da pele humana, bem como a necessidade de terapias alternativas que minimizem o processo de formação de cicatrizes ainda precisa de muita atenção na comunidade científica. Se os nucleotídeos como o ATP, UTP e seus produtos de degradação, exemplo, adenosina, estão envolvidos no processo de regeneração da pele, será observada a modulação dos componentes da sinalização purinérgica, no modelo de substituto cutâneo desenvolvido por Germain e colaboradores no Centro de Regeneração da Universidade Laval, Quebec, Canadá, o que terá uma importante contribuição para o país e futuramente na melhora das terapias para o tratamento de lesões cutâneas.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a segurança e eficácia da membrana amniótica humana como um curativo biológico na regeneração de lesões, na ausência ou presença de células estromais mesenquimais através de modelos *in vitro* e *in vivo*, bem como, avaliar o papel da sinalização purinérgica como uma das vias responsáveis pela regeneração tecidual.

3.1.1 Objetivos Específicos

1. Padronização e caracterização protocolos de descellularização e preservação da membrana amniótica humana.
2. Padronização da extração de células estromais mesenquimais derivadas de materiais biológicos de descarte (tecido adiposo e limbo esclerocorneal) e de tecido adiposo de ratos, bem como a caracterização fenotípica e o potencial de diferenciação multilinhagem.
3. Desenvolver um *bioscaffold*, associando-se a membrana amniótica humana descellularizada, células estromais mesenquimais, derivadas de tecido adiposo humano de descarte hospitalar e analisar as características físicas, histológicas e moleculares.
4. Comparar a membrana amniótica descellularizada com uma membrana biológica comercial, a submucosa de intestino delgado porcino, como *scaffold* para células estromais mesenquimais derivadas do limbo esclerocorneal humano.
5. Padronizar um modelo murino de lesão cutânea por queimaduras de segundo grau profundo para avaliar a eficácia e segurança da membrana amniótica humana.
6. Avaliar o efeito da membrana amniótica descellularizada na presença ou ausência de células estromais mesenquimais derivadas de tecido adiposo de ratos na regeneração tecidual em modelo pré-clínico de queimadura de segundo grau profundo, através de análises histológicas e de imunofluorescência.
7. Reproduzir um modelo de substituto cutâneo com células humanas para estudar os mecanismos envolvidos na regeneração da pele (realizado como estágio no exterior na Universidade de Laval, Canadá).

8. Avaliar o papel da sinalização purinérgica, como um dos mecanismos envolvidos na regeneração tecidual *in vitro*, através de um substituto cutâneo e *in vivo*, no modelo pré-clínico de queimadura.

4 ORGANIZAÇÃO DA TESE

Os resultados produzidos nesta Tese serão apresentados na forma de capítulos organizados da seguinte forma:

- Capítulo I: Artigo publicado, intitulado: **“Decellularized human amniotic membrane associated with adipose derived mesenchymal stromal cells as a bioscaffold: Physical, histological and molecular analysis”** (objetivos 1, 2 e 3).
- Capítulo II: Artigo publicado, intitulado: **“Comparison of Human Denuded Amniotic Membrane and Porcine Small Intestine Submucosa as Scaffolds for Limbal Mesenchymal Stem Cells”** (objetivos 1, 2 e 4).
- Capítulo III: Artigo publicado, intitulado: **“Bioscaffold developed with decellularized human amniotic membrane seeded with mesenchymal stromal cells: assessment of efficacy and safety profiles in a second-degree burn preclinical model”** (objetivos 1, 2, 5, 6 e 8).
- Capítulo IV: Artigo publicado, intitulado: **“UTP increases wound healing in the Self Assembled Skin Substitute (SASS)”** (objetivos 7 e 8).
- Capítulo V: Artigo em preparação, intitulado: **“CD73 is overexpressed during the wound healing process in Self Assembled Skin Substitute (SASS) model”** (objetivos 7 e 8).

5 CAPÍTULO I - Artigo 1

Decellularized human amniotic membrane associated with adipose derived mesenchymal stromal cells as a bioscaffold: Physical, histological and molecular analysis

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A membrana amniótica humana tem um grande potencial como curativo e sua combinação com células estromais mesenquimais representam uma terapia alternativa para a regeneração de lesões. Para alcançar o benefício do potencial terapêutico das células estromais mesenquimais na regeneração de feridas, é muito importante utilizar um *scaffold* biocompatível que mantenha a potencialidade de células progenitoras, sem alterar a viabilidade celular, para que estas possam apresentar suas propriedades imunomoduladoras e quimioatraentes quando transplantadas na área ferida. A membrana amniótica é histologicamente semelhante ao tecido cutâneo, no entanto, para a elaboração do *bioscaffold*, em combinação com células estromais mesenquimais, a membrana é exposta a processos de descélularização. Estes processos devem garantir uma descélularização eficaz sem a alteração da histoarquitetura do biomaterial. Neste trabalho analisamos as características físicas, histológicas e moleculares da membrana amniótica descélularizada na presença e ausência de células estromais mesenquimais, isoladas de tecido adiposo. Essa etapa é fundamental para que se possa utilizar com segurança esse *bioscaffold* em modelos pré-clínicos.



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Regular article

Decellularized human amniotic membrane associated with adipose derived mesenchymal stromal cells as a bioscaffold: Physical, histological and molecular analysis



Liliana Ivet Sous Naasani^{a,1}, Aline Francielle Damo Souza^{b,1}, Cristiano Rodrigues^a, Samlai Vedovatto^a, Jéssica Gonçalves Azevedo^a, Ana Paula Santin Bertoni^a, Marilda Da Cruz Fernandes^c, Silvio Buchner^d, Márcia Rosângela Wink^{a,*}

^a Departamento de Ciências Básicas da Saúde, Laboratório de Biologia Celular, Universidade Federal de Ciências da Saúde de Porto Alegre - UFCSPA, Porto Alegre, RS, Brazil

^b Banco de Tecidos Humanos - Pele Dr. Roberto Corrêa Chem, Hospital Irmandade da Santa Casa de Misericórdia de Porto Alegre - ISCMPA, Porto Alegre, RS, Brazil

^c Departamento de Ciências Básicas da Saúde, Laboratório de Patologia, Universidade Federal de Ciências da Saúde de Porto Alegre - UFCSPA, Porto Alegre, RS, Brazil

^d Instituto de Física, Universidade Federal do Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil

HIGHLIGHTS

- The hAM is thermally stable at high temperatures.
- The hAM decellularization does not compromise their collagen types I and III content.
- Analysis of NMA shows that decellularized human amniotic membrane (D-hAM) does not cause nuclear alterations in AD-MSCs.
- The D-hAM allowed the AD-MSCs to grow without compromising their stemness.
- The D-hAM has the potential to be used as a bioscaffold associated with MSCs.

ARTICLE INFO

Keywords:

Human decellularized amniotic membrane
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ABSTRACT

The human amniotic membrane (hAM) has a great potential as a wound dressing and its combination with mesenchymal stromal cells (MSCs) represents an alternative therapy for wound healing. The scaffold should maintain the stemness of cells, so that they can present their immunomodulatory and chemoattractant properties when transplanted into the injured area. In this work we analyzed the histological and physical characteristics of amniotic membrane preserved in glycerol, in the presence and absence of human adipose derived mesenchymal stromal cells (AD-MSCs). No statistical difference was observed when comparing collagen fibers types I and III in intact (I-hAM) and amniotic membrane (D-hAM). We compared the degradation temperature with the thermogravimetric analysis in I-hAM, D-hAM, and D-hAM with AD-MSCs and characterized the structure and composition of the biological tissue material with Fourier Transform Infrared (FTIR). The intact hAM showed to be more thermally stable; however, it was observed that the hAM, intact and decellularized, is stable at high temperatures. The AD-MSCs were able to grow and expand on D-hAM without showing alterations on cell biology parameters as adhesion, viability, nuclei morphology and actin cytoskeleton. Importantly, the AD-MSCs did not lose their differentiation potential and their mesenchymal cell markers expression after growth on D-hAM. These results constitute a main step for the use of this bioscaffold in pre-clinical models.

1. Introduction

Tissue engineering and stem cell therapy are very promising strategies for wound healing. There are many human diseases depending on

the development of successful cell-based therapies and the treatment of large cutaneous losses is one of the main injuries that could benefit from them [1,2].

The skin, as a protective layer of the human body, is a vulnerable

* Corresponding author at: Federal University of Health Sciences of Porto Alegre – UFCSPA, Sarmento Leite, 245, CEP 90050-170, Porto Alegre, RS, Brazil.

E-mail address: marciawink@yahoo.com.br (M.R. Wink).

¹ These two authors contributed equally to this work.

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system in the organism and its physiologically impaired healing response to chronic wounds is still poorly understood and thus a matter of debate [1,3].

Normal wound healing implies a chain of harmonized cellular and molecular events. In cases of severe skin burns a series of local and systemic responses to the lesion are triggered as intense dehydration, high levels of inflammatory cytokines secretion, micro-organisms colonization, loss of vascularization and extensive cell destruction that interrupts the healing process [4].

There are many strategies based on new technologies as bioengineering and 3D bioprinting to develop skin substitutes to regenerative medicine (revised by Chocarro-Wrona et al.). Examples of available skin replacement products are Integra™, made up of bovine collagen and chondroitin 6-sulfate, and MatriDerm® that has bovine collagen and elastin hydrolysate. Both products have been demonstrated to improve the healing process; however, they are still expensive therapies to developing countries insert in their public health systems [5].

Stem cells applications and tissue engineering have become a novel therapeutic modality for damaged skin regeneration. These strategies involve the combination of cells, growth factors and scaffolds that can supply cellular biological components allowing to restore the tissue function [6].

Human adipose derived mesenchymal stromal cells (AD-MSCs) are often the choice of cell source for transplantation in cell-based therapy, such as burn wound healing, due to easy access to adipose tissue, which yields high cellular quantities [7,8]. These cells can undergo multi-lineage differentiation and secrete growth factors that can enhance wound-healing processes by promoting angiogenesis, hence increasing local blood supply [8]. Although AD-MSCs have been widely studied *in vitro* and *in vivo*, demonstrating their potential and safety, randomized clinical trials on humans are still in process [1,9].

To reach the benefit from the therapeutic potential of these cells in wound healing, it is very important to find a biocompatible scaffold. It should maintain cell viability without toxicity and allow cells transplant to the injured area, where they will migrate to the site requiring repair [10].

The human amniotic membrane (hAM) has anti-inflammatory, angiogenic, and anti-bacterial nature leading to its successful clinical use [11]. The hAM is an avascular tissue that makes up the inner part of the fetal membrane [12]. It is composed of a single cubic epithelium monolayer, a thick basement membrane and an avascular stromal matrix, also called the spongy layer. The basement membrane consists of a network of reticular fibers, such as laminin, fibronectin and collagen type I, III, IV, VI and XVII [13], and some fibroblastic cells, such as mesenchymal stromal cells in the connective tissue [14]. The spongy layer is constituted by proteoglycans and glycoproteins, producing a spongy appearance in histologic preparations. It contains a nonfibrillar meshwork of mostly type III collagen [15].

The study of this important biological resource over the years has led to a better understanding of its action in the regeneration of lesions. When in contact with the wound bed, the hAM forms a physical barrier against bacterial invasion, reduces loss of body fluids and proteins, reduces pain, and stimulates the release of growth factors and healing modulators that stimulate proliferation and migration of keratinocytes, favoring the healing process [16,17].

The hAM has the advantages of being highly available and of low handling cost, being a strong competitor to the products already in the market. However, considering it has different processing methods, it is important to determine whether the way of preservation, storage and decellularization is adequate for therapy. Also it is necessary to investigate whether the steps employed during preparation of the tissue can alter cell biology.

Therefore, in this work we analyzed the histological and physical characteristics of the decellularized amniotic membrane preserved in glycerol, in the presence and absence of AD-MSCs and determine the cell biology parameters and stemness maintenance of AD-MSCs after

contact with D-hAM.

2. Materials and methods

2.1. Isolation of AD-MSCs

AD-MSCs were extracted from the abdominal adipose tissue of female healthy donors (ages between 31–45 years) with normal body mass indexes (BMI). The patient consent was obtained before the liposuction and all protocols were approved by the Research Ethics Committee (REC-ISCMPA no 3.029.141) from the Irmandade Santa Casa de Misericórdia de Porto Alegre (RS, Brazil). The AD-MSCs were obtained as already described [18]. Briefly, the fat tissues were transferred to a separatory funnel and were washed with a phosphate-buffered saline (PBS) to remove the blood tissue. The fat fragments were digested with collagenase solution (Type I, 1 mg/mL solubilized in DMEM) and incubated in a water bath (37 °C) for 30 min with vortex agitation every 10 min. The collagenase solution was inactivated by DMEM 10% FBS. The cells were then centrifuged at $600 \times g$ for 10 min and the supernatant was discarded. Next, they were resuspended in an erythrocyte lysis solution (150 mM NH₄Cl, 10 mM NaHCO₃ and 1 mM EDTA) diluted in ultrapure water 1:1 (v/v), undergoing simultaneous vortex agitation and then 15 min incubation at room temperature. After new centrifugation ($600 \times g$, 10 min), the cells were resuspended in a known volume of DMEM 10% FBS and were counted using 0.4% trypan blue (Bio-Rad, Hercules, CA, USA) exclusion method. The cells were seeded in 25 cm² cell culture flasks with DMEM 10% FBS (1000 mg/L glucose) at 37 °C in a humidified incubator filled with 5% CO₂. The first exchange of the medium was realized 48 h later, when adherent cells appeared, and it was repeated each 4 days.

2.2. Differentiation of AD-MSCs

For AD-MSCs differentiation we used the StemPro® Adipocyte Differentiation Basal Medium, StemPro® Osteogenesis Differentiation Basal Medium and StemPro® Chondrocyte Differentiation Basal Medium (Gibco) for adipogenic, osteogenic and chondrogenic differentiation, respectively, has already been published by Naasani et al. 2017 [19]. Control cells were maintained with standard medium that was changed every three days, until the specific periods of cultivation. After 14 days of adipogenic differentiation conditions, the culture was fixed with 4% formaldehyde solution for 30 min and stained with Oil Red O in deionized water (1:3). The pre and post-treatment was made with 60% isopropanol (v/v) in water. Then, after 23 days in culture with chondrogenic medium, the synthesis of proteoglycans by chondrocytes was seen with 1% Alcian Blue staining, prepared in 0.1 N HCl. For osteogenic cultures, it was used 2% Alizarin Red S solution (pH 4.2) after 25 days, to see the calcium phosphate deposited by osteoblasts. Stained induced cultures were compared with stained controls. Photomicrographs were performed using a BX-50 Olympus microscope with optical lens (10X/0.30 Ph1-UplanFI), coupled to a camera Motican 2500 (Olympus, Hamburg, Germany).

2.3. Cell surface antigen expression

We detected the AD-MSCs surface antigen expression by flow cytometry. Cultures were dissociated with trypsin-EDTA, centrifuged and incubated for 30 min at 4 °C using the antibodies: FITC-anti-CD44, PE-anti-CD105, PE-anti-CD45, PE-anti-CD14, FITC-anti-CD34 (Invitrogen™, Waltham, MA, USA), PE-mouse-Anti-Human-CD73 (BD Pharmingen™) and PE anti-human-CD90 (Thy1) Clone: 5E10 (BioLegend) [20]. The cytometer was equipped with 488 nm argon laser (Becton-Dickinson, San Diego, CA, USA), and CellQuest software. At least 10.000 events were collected and data were evaluated using the Flowing Software 2 (Perttu Terho, Finland).

2.4. Processing of hAM

For amniotic membrane processing, the protocol used by Paggiaro and colleagues was followed [21]. Briefly, the pregnant women were screened serologically for HIV, hepatitis B and C, HTLV, syphilis, Chagas disease, toxoplasmosis (IgM antibodies) and cytomegalovirus (IgM antibodies). Only patients with negatives results were selected. In all phases of processing fragments were collected for microbiological analysis. Under sterile conditions, the hAM was separated from the other fetal membranes and it was washed once with sterile saline solution. Membrane was treated with antibiotics (penicillin 1.000.000 U/L and streptomycin 1 g/L) and it was preserved in glycerol 85% solution under refrigeration and up to this point the samples were used as an intact amniotic membrane (I-hAM). To obtain decellularized hAM (D-hAM), the membrane was submitted to decellularization protocol by enzymatic method using 0.05% trypsin / 0.02% EDTA during 20 min at 37 °C and, finally, it was washed with PBS and hydrated overnight at 37 °C with culture medium before seeding cells.

2.5. Collagen fibers analysis of I-hAM and D-hAM

A histomorphometric method was used to determine quantitatively and qualitatively the collagen content in the intact and decellularized hAM. Firstly, intact and decellularized AM were included in paraffin after being sectioned into slices of 4 µm and deparaffinized. Collagen fibers were assessed by 2 h incubation of the slides in a Sirius Red solution, consisting of saturated picric acid containing both 0.1% Direct Red 80 and 0.1% Fast Green (Sigma-Aldrich, St. Louis, MO, USA). After that, the sections were shortly dipped in distilled water, and were then washed once with 100% ethanol. The color hues were assessed to determine the collagen distribution, using polarized light microscopy (Olympus BX-53, Shinjuku, Tokyo, Japan) at $\times 200$ magnification. Each image was splitted into three color channels, using the RGB stack by ImageJ software (National Institutes of Health, Bethesda, MD, USA). Adjusting the threshold tool, the color channels monochromatically revealed the collagen content, according to their type: I (red) and III (green). The relative area occupied by each type of collagen was then calculated in proportion to the total area of the membranes. Data were analyzed by one-way ANOVA, following Tukey's multiple comparison tests using the Graph Pad Prism 6 software (La Jolla, CA, USA). Statistically significant differences were accepted when $p < 0.05$, being expressed as mean $\pm$ standard deviation (SD).

2.6. Expression of fibronectin in I-hAM and D-hAM by immunohistochemistry

Deparaffinized sections (4 µm) of I-hAM and D-hAM were treated with Tris (pH 9,0) for 20 min and blocked with 5% H₂O₂/methanol 10 min followed by incubation with 1% BSA. Sections were then sequentially incubated with polyclonal rabbit anti-human fibronectin (DakoCytomation) for 1 h at 37 °C. After was used the EnVision™ + Dual Link System-HRP (Dako, K4061) according to the manufacturer's protocol. The images were obtained using the EVOS FL Auto 2 Cell Imaging System (Invitrogen) at $\times 400$ magnification.

Quantification of fibronectin was performed using the ImageJ Software, calculating the proportion of area occupied by the strong brown staining in samples. We adjusted the color threshold to measure the stained area and then, calculate the proportion to the total area of the membranes. Statistically significant differences were accepted when $p < 0.05$, being expressed as mean $\pm$ standard deviation (SD).

2.7. Histological analysis of hAM

The histological sections of intact hAM, decellularized hAM and decellularized hAM + AD-MSCs were stained with hematoxylin-eosin (HE). The samples were fixed with xylene and hydrated with decreasing

concentrations of ethyl alcohol (100, 70 and 60%) and water for 5 min and then stained with hematoxylin solution (Merk, Germany) for 4 min. After washing with running water, it was stained with eosin for 2 min and then dehydrated with increasing concentrations of ethyl alcohol, ending with xylene for 5 min. Histological micrographs were performed with a Leica DM6 B microscope.

2.8. Infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA)

The intact hAM, decellularized hAM and decellularized hAM + AD-MSCs were analyzed with Fourier transform infrared (FTIR) spectra (4 cm^{-1} resolution, 512 scans) and were collected in reflectance mode using a Perkin-Elmer Spectrum 400 MIR/FIR and a Bomem MB 100 spectrometers.

The Thermogravimetric analysis (TGA) scans of the samples were carried out using Shimadzu TGA-50 to detect the mass variation. TGA experiments were performed in a Pt-crucible in a temperature range between 20 and 650 °C under flowing nitrogen (50 cm^3/min) in rate of 20 °C/min.

2.9. MTT tetrazolium assay

The relative viability of the AD-MSCs seeded on D-hAM were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. The hAM was cut in fragments of approximately 6 mm in diameter and it was placed in 96-well plate conical bottom without adherence. Cells were seeded at a density of 10×10^3 cells/well. Additionally, D-hAM control was analyzed to determine if the MTT reacted with the membrane. After 72 h, they were incubated with MTT for 4 h. The crystals were dissolved in dimethyl sulfoxide (DMSO), and the amount of formazan (presumably directly proportional to the number of viable cells) was measured by SpectraMax Microplate Reader (Molecular Devices Corporation, USA) at 570 nm. Average absorbance corresponded to 100% viability in the control.

2.10. Actin filaments staining

Actin cytoskeleton organization of AD-MSCs seeded on D-hAM was assessed via F-actin staining. Briefly, AD-MSCs were seeded at 10×10^3 cells/ cm^2 on glass coverslips (control) and on D-hAM and then cultured for 72 h with standard medium under appropriate atmosphere. The culture was washed once with PBS and it was fixed with 4% paraformaldehyde solution in PBS for 30 min at 4 °C. Cells were washed with PBS followed by incubation with permeabilization solution (0.1% Triton X-100 in PBS) for 1 h. Actin filaments were stained with FITC-Phalloidin (Sigma) and cells were also stained with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for the identification of nuclei (40 min, 4 °C, dark room). Images were obtained with a fluorescence microscope (Olympus IX51/Olympus U-RFL-T) coupled with 460–490, and 372 nm band-pass excitation filters, respectively.

2.11. Nuclear morphometric assay (NMA)

Nuclear morphometry was analyzed using an NMA program [22]. AD-MSCs were seeded at a density of 1.5×10^3 cells/ cm^2 on D-hAM and the control was seeded on glass coverslips for 72 h. After this time, the cells were fixed with 4% paraformaldehyde (v/v in PBS) for 30 min at room temperature. Nuclei were marked with 300 nM DAPI for 30 min, at room temperature and images of the DAPI-labeled nuclei were captured using a fluorescence microscope (Olympus BX-50) with optical lens ($\times 10/0.30$ Ph1-UplanFI) coupled with 372 nm band-pass excitation filter.

Quantification of nuclei types were performed using Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, USA). The results were analyzed by one-way ANOVA, following Tukey's post-hoc test. Data

were presented as a plot of Area versus Nuclear Irregularity Index (NII) and all samples were compared with control.

2.12. Senescence-associated- β -galactosidase staining

Senescence-associated (SA) β -galactosidase expression of AD-MSCs seeded on D-hAM was analyzed using Senescence Cells Histochemical staining kit (Sigma), according to the manufacturer's protocol. Briefly, the AD-MSCs in sixth passage were cultured on D-hAM and tissue culture plate (control) for 72 h with standard medium under appropriate atmosphere. Subsequently, the cells were fixed with 1X fixation buffer, washed with PBS and incubated with Staining mixture (X-gal solution) overnight at 37 °C without CO₂. Finally, the SA- β -gal-positive cells exhibited a blue color. Cells were also stained with DAPI (30 min, 4 °C, dark room) to identify the number of nuclei/cells on the D-hAM. Images were obtained with a light/fluorescence microscope (Olympus IX51/Olympus U-RFL-T). The number of positive cells was counted for every 300 cells using ImageJ software.

2.13. RT-qPCR analysis

To better evaluate the decellularization process, we evaluated the presence of mRNA in hAM before and after the decellularization. Samples of ~4.0 cm² of intact and decellularized membrane were disrupted in a rotor-stator tissue homogenizer in presence of TRIZOL LS reagent (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions and stored at -80 °C.

The complementary DNA (cDNA) was synthesized with M-MLV reverse transcriptase enzyme (Promega, Madison, WI, USA) and 1 μ g total RNA in a final volume of 20 μ L with a random hexamer primer in accordance with the manufacturer's instructions. Real-time PCRs were carried out in an Applied Biosystems StepOnePlus™ Real-Time PCR cycler and done in duplicate. Reaction settings were composed of an initial enzymatic activation step at 50 °C and 95 °C cDNA denaturation step, both for 2 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 60 s, followed by analysis of the melting curves from 60 °C to 95 °C at 0.1 °C/s to produce the denaturation curve of the amplified products. The reaction was performed in a final volume of 12.5 μ L composed of 6.25 μ L of SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 μ L of primer pairs 10 μ M, 4.75 μ L of DEPC-treated water and 1 μ L of cDNA. A list of primers used in this study is presented in Table 1. At the end of the PCR cycles, a melting curve was generated to identify the specificity of the products. For RT-qPCR normalization we evaluated glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TATA-Binding Protein (TBP) e β -actin

Table 2

Dispersion data for each reference gene. Analysis of their results indicating less stability for GAPDH.

	ACTB	TBP	GAPDH
CV%	9.89	6.51	12.60
SD	2.97	2.02	3.22
MFC	1.27	1.18	1.36

*CV (coefficient of variation), SD (standard deviation) and MFC (maximum fold change).

(ACTB) housekeeping genes and combination of ACTB and TBP primers were selected because of its higher stability (Table 2; Supplementary Fig. 1). The data were presented as the ratio of target gene expression/(ACTB-TBP mean). The relative quantification was obtained by the comparative Ct method (2- $\Delta\Delta$ Ct).

2.14. Scanning electron microscopy (SEM) examination

Morphological aspects of AD-MSCs on D-hAM scaffold were observed from scanning electron microscopy (SEM). Cells were seeded on membrane and after 72 h of culture they were fixed with 2.5% glutaraldehyde in PBS for 96 h at 4–8 °C. After it the samples were washed with PBS for 12 h at the same temperature. Then, the cells were incubated with 1% osmium tetroxide in PBS for 20 min at room temperature, followed by washing it three times with PBS. In the sequence, successive dehydration with ascending concentrations of ethyl alcohol (50, 60, 70, 80, 90, and 100%) was performed for 10 min each, and then more 20 min for the last two percentages. The critical point was used as the last drying step of the samples and then they were mounted onto aluminum stubs and coated with gold. Samples were kept in the desiccator until reading in the SEM. Imaging was conducted using a scanning electron microscope (Carl Zeiss EVO).

2.15. Characterization of AD-MSCs after seeded on D-hAM

In order to analyze whether the contact with D-hAM could change the characteristics of AD-MSCs after their culture, we seeded the cells on D-hAM and we started the treatment with StemPro® Adipocyte, Osteogenesis and Chondrocyte Differentiation Basal Medium (Gibco). The controls were maintained with standard medium as described above.

For adipogenic differentiation, AD-MSCs were grown during 24 h on D-hAM and then treated for two weeks with the adipocyte differentiation medium. Considering that the stain reagents used to the

Table 1

Characteristics and sequences of the candidate genes for normalization of the RT-qPCR and genes of interest for the analysis of the interaction of AD-MSCs with the D-hAM.

SEQUENCE NAME	SEQUENCE (5' - 3')	FUNCTION	AMPLICON (bp)
GAPDH_F_human	GTCAGGCTGAGAACGGGAA	Carbohydrate metabolism	158
GAPDH_R_human	AAATGAGCCCCAGCCTTCTC		
TBP_F_human	GCATCACTGTTTCTTGCGGT	Initiation of transcription of the RNA polymerase II promoter	112
TBP_R_human	CGCTGGAACCTCGTCTACTA		
ACTB_F_human	AAGACAGTGTGTGGGTGAGG	Structural protein of the cytoskeleton	126
ACTB_R_human	TGGGATGGGGAGTCTGTTC		
CDH2_F_human	AGGCTTCTGGTGAATCGCAT	Cellular adhesion molecule	90
CDH2_R_human	GCTGTCCTTCATGCATCCT		
FN1_F_human	AATGGTCGTGGGGAATGGAC	Cell matrix organization glycoprotein	114
FN1_R_human	CCCTCTTCATGAGCCTTGT		
VIM_F_human	CGGAGAAATTGCAGGAGGA	Intermediate filament protein	105
VIM_R_human	AAGGTCAAGACGTGCCAGAG		
KI67_F_human	AGAAGACAGTACCGCAGATGA	Marker of cell proliferation	
KI67_R_human	AGTGCATTCTGCCATTACG		

Abbreviations in Table 1: ACTB: beta-actin; TBP: TATA box binding protein; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; CDH2: N-caderin; FN1: Fibronectin; VIM: Vimentin; bp: base pairs.

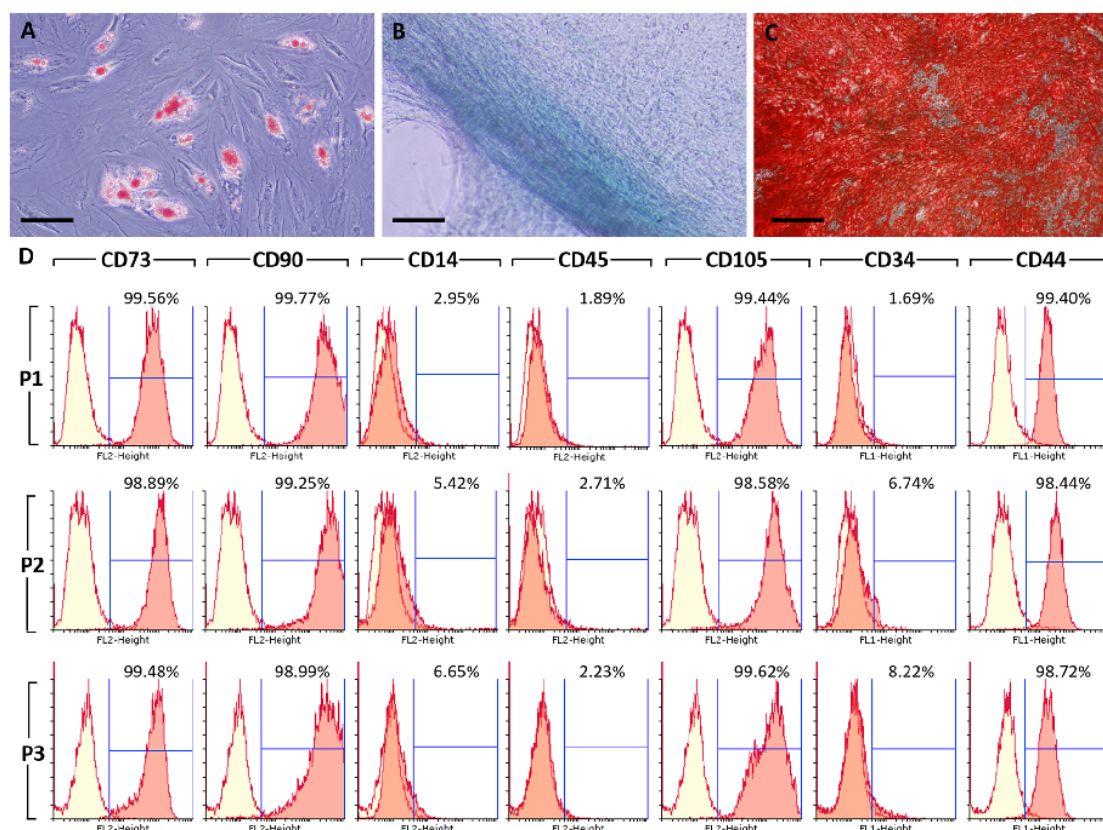


Fig. 1. Characterization of AD-MSCs. Cells differentiated into adipocytes (A), chondrocytes (B) and osteocytes (C). Flow cytometry histograms (D) show the expression (yellow curves, left) of selected molecules (CD73⁺, CD90⁺, CD14⁻, CD45⁻, CD105⁺, CD34⁻ and CD44⁺) by AD-MSCs populations from three tissue donors, compared with controls (yellow curves). Phase contrast micrograph. Scale bar: 100 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identification of osteogenic and chondrogenic differentiation impregnates the amniotic membrane, avoiding the analysis, we had to adapt the protocol. First, AD-MSCs were seeded and expanded on D-hAM for 7 days. After that, we washed it with PBS and dissociated with trypsin-EDTA (0.05%) trypsin - (0.02%) EDTA during 15 min at 37 °C and seeded them on tissue culture plates. Finally, we treated them with Osteogenesis and Chondrocyte Differentiation Basal Medium for two weeks

Staining for the identification of the three lineages was done as already described above (section 2.2 Differentiation of AD-MSCs).

Cell surface antigens expression of AD-MSCs after culture on D-hAM was detected by flow cytometry. For this, the cultures in contact with this membrane were dissociated with trypsin-EDTA, centrifuged and incubated for 30 min at 4 °C using the antibodies: PE-anti-CD14, FITC-anti-CD34, FITC-anti-CD44, PE-anti-CD45, PE-anti-CD105 (Invitrogen™, Waltham, MA, USA), PE-mouse-Anti-Human-CD73 (BD Pharmingen™) and PE anti-human-CD90 (Thy1) Clone: 5E10 (BioLegend), in the same conditions mentioned before.

As a control, we also analyzed the amniotic membrane after decellularization process in order to evaluate the presence of remaining cells.

2.16. Statistical analysis

Data were analyzed statistically by one-way ANOVA followed by Tukey's post-hoc test and T-Test. using the Graph Pad Prism 7 software (La Jolla, CA, USA). Statistically significant differences were accepted when $p > 0.053$.

3. Results

3.1. Isolation and characterization of AD-MSCs

We isolated a population of AD-MSCs from discarded human abdominal adipose tissue that presented a fusiform morphology, plastic adherence, self-renewal and trilineage differentiation potential. We observed the intracellular lipid vacuoles with oil red, the synthesis of proteoglycans with alcian blue and a calcium-rich mineralized matrix with alizarin red S staining, which shows that they differentiated into adipocytes, chondrocytes and osteoblasts, respectively, when in contact with differentiation factors (Fig. 1A–C). Also, we analyzed the AD-MSCs surface antigen markers from three tissue donors by flow cytometry. Cells showed the expression of selected molecules as expected (CD73⁺, CD90⁺, CD14⁻, CD45⁻, CD105⁺, CD34⁻ and CD44⁺) (Fig. 1D).

3.2. Analysis of hAM histoarchitecture after the decellularization process

The hAM was successfully separated from other fetal membranes and preserved in glycerol at 4 °C before its decellularization. We evaluated, histologically, the impact of decellularization process of hAM by picro-sirius red staining and fibronectin expression (Fig. 2). After hAM processing, the concentration of both types of collagen fibers did not present statistical difference, suggesting that the decellularization by trypsin-EDTA method does not cause loss of collagen. The relative area occupied by collagen increased ($27.4 \pm 10.2\%$ to $42.3 \pm 3.7\%$) when calculated in proportion to the total area of membrane (Fig. 2A). Complementarily, it appears that the amount of type III collagen

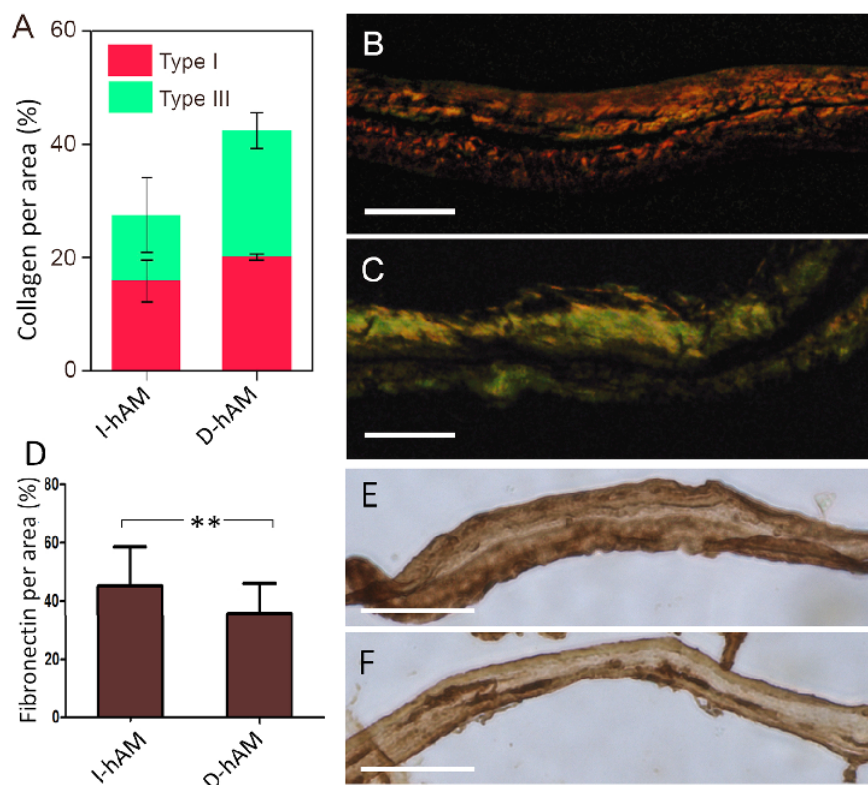


Fig. 2. Picro-sirius red staining and fibronectin expression of I-hAM and D-hAM. (A) Stacked graph shows the relative hAM area (%) occupied for collagen fibers types I (bottom) and III (top) before and after decellularization process (mean $\pm$ SD). No statistical difference was observed when comparing intact and decellularized membranes. Longitudinal micrographs demonstrate both (B) intact and (C) decellularized membrane sections under polarized light microscopy, revealing differences in the birefringence of collagen fibers. (D) Relative area (%) of fibronectin expression for in I-hAM and D-hAM showed a significant decrease of 21% after decellularization process (mean $\pm$ SD) ($p < 0.05$). Immunohistochemical staining for fibronectin expression in I-hAM (E) and D-hAM (F). Scale bar: 50 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(green) was more visible in the D-hAM, changing from 42 to 52% of the total, pointing to an increase in small fibers (Fig. 2B, C). The immunohistochemistry for fibronectin expression showed a decreased of 21% in the protein content after the decellularization process ($43 \pm 10.22\%$ for I-hAM and $34.33 \pm 7.88\%$ for D-hAM) (Fig. 2D, E, F).

3.3. Histological analysis of D-hAM associated with AD-MSCs

In order to analyze the integration of AD-MSCs seeded on D-hAM, after 72 h of culture the membrane was fixed for histological sections analysis. In the intact amniotic membrane, we observed a monolayer of epithelial cells which was successfully separated after decellularization. The AD-MSCs seeded on D-hAM formed a monolayer without altering their fusiform morphology (Fig. 3A–F).

3.4. Thermogravimetric analysis (TGA)

Thermogravimetric analysis for D-hAM + AD-MSCs, D-hAM and I-hAM samples is shown in Fig. 3G. In this figure is shown also the TGA measurement for liquid glycerol used to preserve the samples. The TGA curve of Glycerol showed that there is a gradual loss in weight until complete decomposition. This occurred from room temperature to 150 $^{\circ}$ C, suggesting that Glycerol was not impregnated in the samples at superior temperature.

The degradation of the D-hAM + AD-MSCs, D-hAM, I-hAM samples occurred in the same temperature range, but with different rates. The TGA measurement showed a characteristic weight loss that was smaller around the temperature of 100 $^{\circ}$ C when compared with the total mass. A significant decomposition was observed in the range of 250–390 $^{\circ}$ C for all samples. However, the D-hAM + AD-MSCs sample followed with a high composition rate up to 415 $^{\circ}$ C. It can be observed that at temperatures above 650 $^{\circ}$ C occurred D-hAM + AD-MSCs sample complete

degradation (662 $^{\circ}$ C), while D-hAM was completely degraded at an even higher temperature (692 $^{\circ}$ C). Meanwhile, at 700 $^{\circ}$ C, the I-hAM remained with 22% of its mass in relation to its initial value, showing to be more thermally stable.

3.5. Infrared spectroscopy

To characterize the structure and composition of the biological tissue material we analyzed the samples with Fourier Transform Infrared (FTIR). The absorption bands 1652 cm^{-1} , 1550 cm^{-1} and 1238 cm^{-1} were observed when the hAM in the presence of AD-MSCs was analyzed (Fig. 3H). In the case of D-hAM samples the absorption bands were slightly shifted. They were observed in 1675 cm^{-1} , 1560 cm^{-1} and 1240 cm^{-1} . It should be noted that in the region of 1652 cm^{-1} there is a small inclination, this band can be identified with a range of 1675–1653 cm^{-1} . In the I-hAM sample the amide I is shown in range 1700–1685 cm^{-1} , the second absorption band was identified in 1550 cm^{-1} and amide III was not shown.

3.6. Morphology and viability of AD-MSCs on D-hAM

The use of a simple phase-contrast microscopy allowed us to observe AD-MSCs on the D-hAM due to membrane transparency (Fig. 4A–C). However, to improve this observation and to analyze the morphology, a Phalloidin/DAPI staining and scanning electron microscopy examination were made. By actin filaments analysis, there were no alterations in its cytoskeleton and nuclei (Fig. 4D–G). This is in agreement with the results of the MTT analysis, in which there was no statistically significant loss of cell viability when AD-MSCs were in contact with the membrane (Fig. 4H).

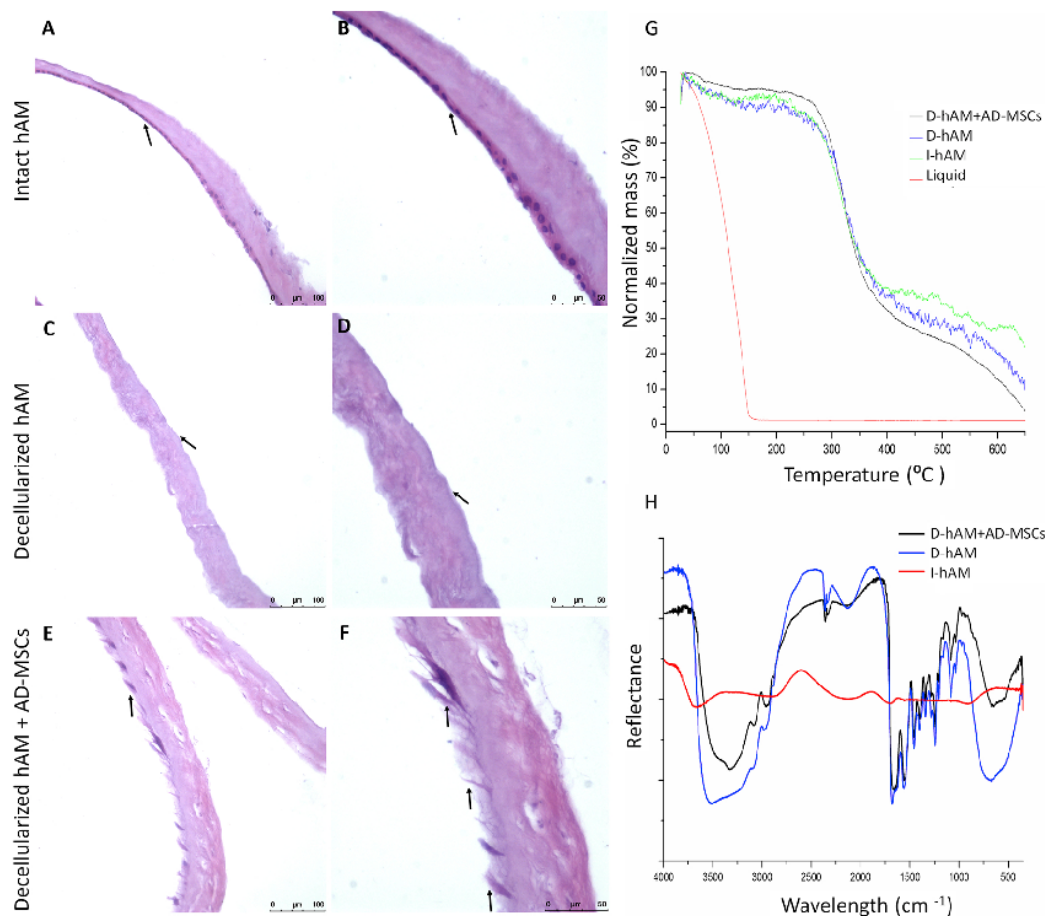


Fig. 3. Hematoxylin and eosin (HE) staining, thermogravimetric analysis and FTIR spectra of human amniotic membranes. (A–B) Intact amniotic membrane shows its epithelial cells monolayer in HE staining. (C–D) Decellularized amniotic membrane shows it was effectively decellularized. (E–F) The AD-MSCs adhered in the D-hAM. G) Thermogravimetric analysis of D-hAM + AD-MSCs, D-hAM, I-hAM and glycerol. H) FTIR spectra of D-hAM + AD-MSCs, D-hAM and I-hAM. Scale bar: (A, C, E) 100 μ m; (B, D, F) 50 μ m.

3.7. Nuclear morphometric analysis assay and senescence-associated β -galactosidase expression of AD-MSCs on D-hAM

The Nuclear Morphometric Analysis Assay (NMA) allows to identify seven different nuclear morphologies that have different biological means: normal, no damage; irregular and large irregular, damaging event; small regular, apoptosis in an early or intermediary stage; small, mitosis; small irregular, apoptosis with damage or nuclear fragments; and large regular, senescence. In this study, the nuclear morphology of AD-MSCs seeded in both, hAM and control on glass coverslips, presented three different nuclear morphologies (normal, large regular and irregular) and there was no significant difference between the two scaffolds analyzed (Fig. 5A–B). Analysis of NMA showed that D-hAM scaffold did not cause nuclear alterations as, for example, apoptotic nuclei. It means that this structure, besides being a suitable scaffold, can maintain cells viability. We confirm these results with the senescence-associated β -galactosidase staining. The SA- β -gal-positive cells exhibited a blue color in senescent cells and no significant difference was observed between the D-hAM and control (Fig. 5C–G).

3.8. Gene expression

We performed qPCR for a normalizing gene (TBP) after a disintegration of decellularized membrane. We did not consider only the amounts of total RNA measurement in absorbance at 260 nm, because

degraded RNA and single nucleotides also would contribute to the 260 nm reading. qPCR analysis showed that only about 3% of mRNA remains intact (Supplementary Fig. 2A). This data is in accordance with other study in the literature that showed a reduction in the DNA content from $341 \pm 29.60 \mu\text{g/ml}$ to $39.38 \pm 4.04 \mu\text{g/ml}$, after the decellularization procedure of h-AM [23].

The results obtained from real time RT-qPCR for adhesion and proliferation genes presented an important variability, avoiding statistically analysis. Besides the diversity among patients, it was possible to conclude that the did not lose its expression of vimentin, fibronectin, N-cadherin and Ki67 genes after grown on D-hAM (Fig. 6A–C).

3.9. Characterization of AD-MSCs grown on D-hAM and evaluation of stemness maintenance

By SEM it was possible to obtain a general and magnified view of the interaction between cells and membrane. Cells formed a monolayer on the membrane and at higher magnification it was possible to identify the cytoplasmic prolongations, added to the substrate and the fibers that give structure to membrane (Fig. 7A–C). In order to determine the stemness maintenance of AD-MSCs after contact with hAM, their potential for multilineage differentiation and the expression of characteristic markers were evaluated. It was possible to identify the formation of lipid vesicles in the cells that were grown on membranes. However, technically, it was not possible to characterize the other

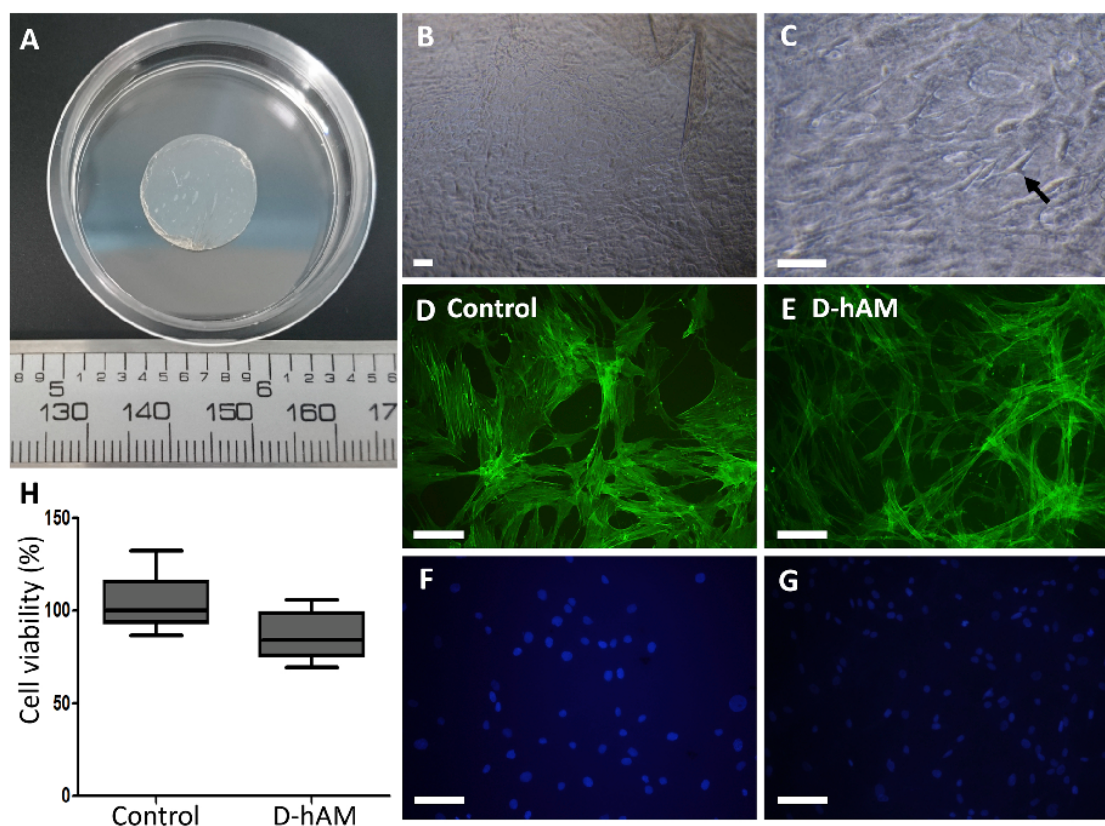


Fig. 4. Morphology and viability of AD-MSCs cultured on hAM scaffold. (A) The hAM scaffold for cell culture is shown. (B–C) AD-MSCs seeded on hAM. Their fusiform morphology is indicated. Phalloidin and DAPI staining for AD-MSCs on the control (D,F) and hAM (E,G) showing cytoskeleton and nuclei of these cells. (H) The MTT assay shows no significant differences in viability (%) of AD-MSCs seeded on the D-hAM in comparison with control cells (plastic culture plates surface). Scale bar: (B) 200 μ m; (C–G) 100 μ m.

differentiations on D-hAM, because the reagents of staining impregnated the membrane (Fig. 7D, E). For that, we seeded the AD-MSCs on D-hAM and then expanded them on the membrane. We dissociated the cells with trypsin-EDTA and seeded them on tissue culture plates to perform osteogenic and chondrogenic differentiations. It was possible to identify the synthesis of proteoglycans (Fig. 7F, G) and a calcium-rich mineralized matrix (Fig. 7H, I), confirming the maintaining of tri-lineage differentiation capacity.

Regarding to cell surface antigen expression pattern, there was no alteration in molecules expression in comparison to control (Fig. 7J).

In addition, as a control, to be sure that MSCs present in the intact amniotic membrane were removed by the decellularization process, we incubated the D-hAM with trypsin and analyzed the presence of cells positive to CD44 and CD105 markers. We observed the absence of a representative population of cells after decellularizing the human amniotic membrane (Supplementary Fig. 2B).

4. Discussion

Human AM has been widely used in many medical procedures, including skin transplantation to treat burned and ulcerated skin, and in critical ophthalmologic surgeries [14,24,25]. In addition to associated growth factors, the composition of the hAM extracellular matrix by proteins such as collagen, laminins and fibronectin, has been identified as the main cause of its elevated potential for tissue regeneration [26,27].

The mechanical integrity of hAM is guaranteed by interstitial collagens types I and III, while collagens type V and VI form filamentous

connections between interstitial collagens and the epithelial basement membrane. [15]. In this work, the collagen content of hAMs was accessed using picrosirius red staining, a specific method for collagen detection in tissue sections. It is well performed with different approaches, e.g. to detect fibrosis, to estimate tumor progression, wound healing, biomaterial performances and homo and xenografts evaluation after decellularization process. The total amount of collagen fibers were quantified in decellularized hAM and compared with intact hAM. Under polarized light microscopy, large collagen fibers are bright red or orange, and the thinner ones, including reticular fibers, are green. According to Junqueira and colleagues, the birefringence is highly specific for collagen types I and III [28]. Our results showed that there was no difference between collagen content before and after processing. Moreover, by the application of color threshold filters, it is apparent that there is an increase in the proportion of type III fibers, signaling an augmentation in spatial distribution of small fibers after decellularization processing. According to our findings, Wilshaw and colleagues reported that hAMs can increase the total collagen amounts after treatment. This increase might be due to a loss of soluble proteins and cells from the matrix after decellularization with EDTA solution [29]. The abundance of glycosaminoglycans, together with collagen and elastin, gives to human AM a rich complex of structural elements to act in wound healing, or as a scaffold for cells aiming bioengineering applications. In addition, Taghiabadi et al. 2014 showed similar results measuring the hydroxyproline content of intact hAM in comparison to hAM treated with ethylenediaminetetraacetic acid (EDTA, 0.1% w/v) and 0.03% (w/v) solution sodium dodecyl sulphate (SDS) [23]. The levels increased from 361 μ g/mg to 479 μ g/mg after the

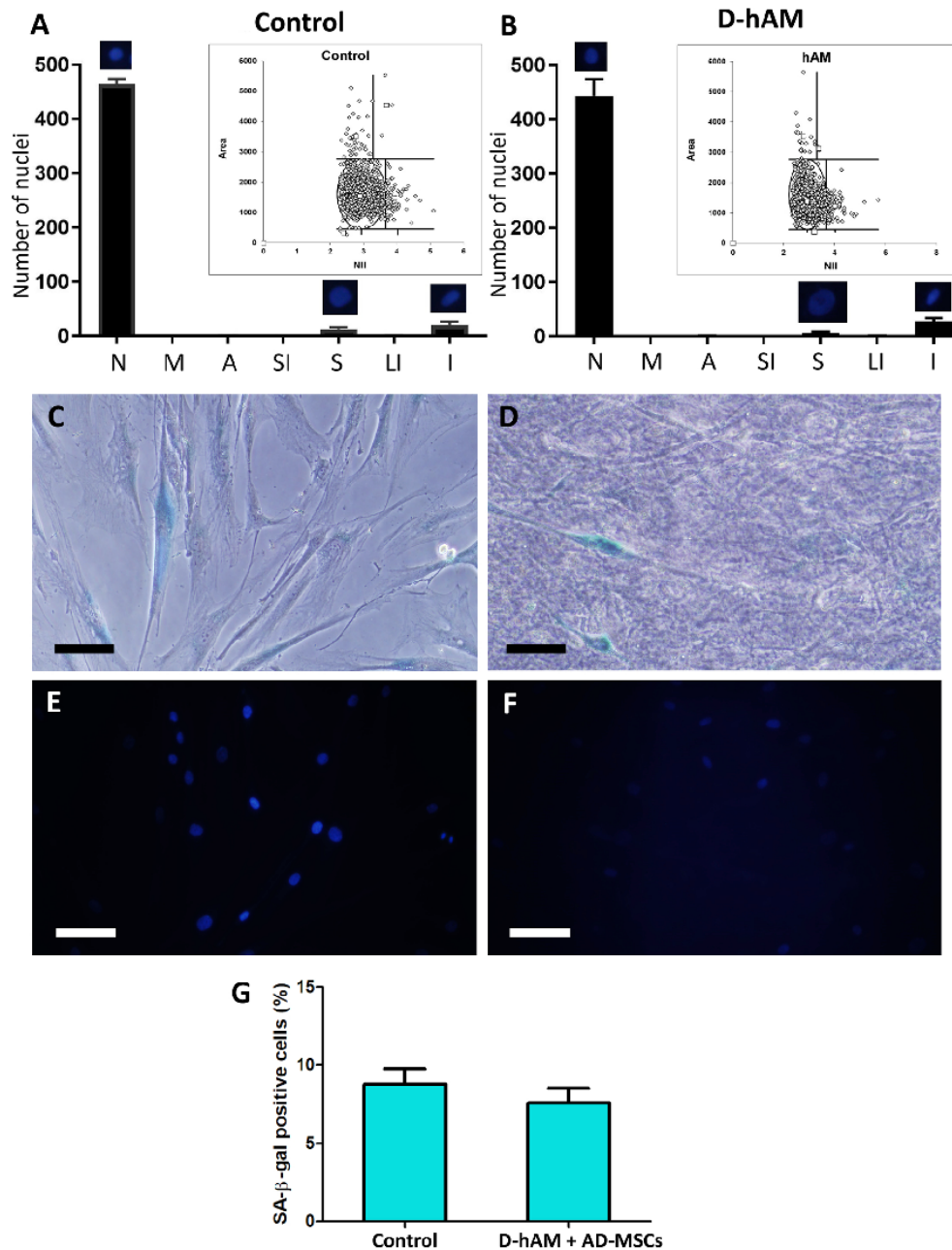


Fig. 5. Nuclei morphometric analysis (NMA) and senescence-associated β -galactosidase analysis of AD-MSCs on D-hAM. AD-MSCs control (A) and D-hAM (B) showing the number of nuclei of normal (N), mitotic (M), apoptotic (A), small and irregular (SI), senescent (S), large and irregular (LI) or irregular (I) nuclei after 72 h of culture. Images show an example of each nuclei types analyzed. The SA- β -gal-positive cells (blue color) and DAPI staining for control (C, E) and AD-MSCs on D-hAM. (G) The graph shows the percent of senescent AD-MSCs. No significant difference was observed between cells grown on D-hAM and control (plastic culture plate). Statistically significant differences were accepted when $p > 0.05$, being expressed as mean $\pm$ standard deviation (SD). Scale bar: 100 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decellularization. In another study conducted by Lim et al. (2010), the authors analyzed the collagen (types I, II, IV, VI and VII) in decellularized hAM by immunofluorescence, however the type III was not investigated, thus avoiding us to compare our results of collagen type III quantification [30].

Considering the importance of structural elements in order to hAM

act in wound healing, we also analyzed the fibronectin expression, in intact hAM and after decellularization. We observed that the decellularization process caused a loss of around 21% in the expression of the protein. The fibronectin content was already analyzed from micronized hAM [31]. The authors found an amount of 1.84×10^3 ng/mg. However, they did not evaluate the fibronectin content after the

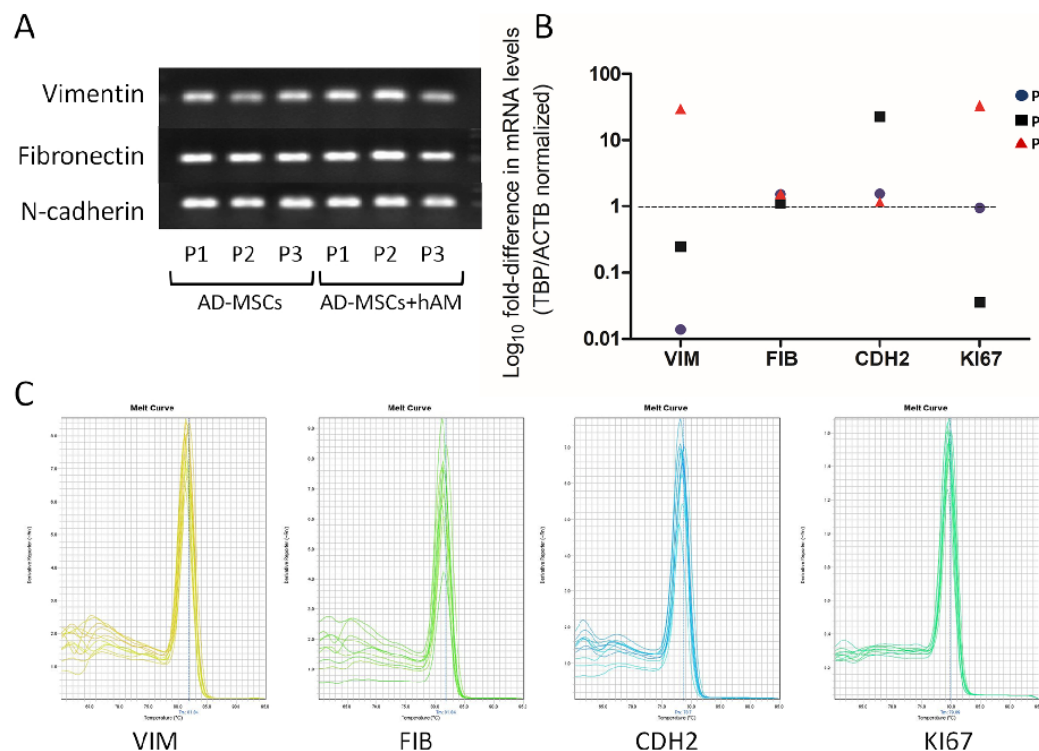


Fig. 6. Relative mRNA expression for vimentin (VIM), fibronectin (FIB), N-cadherin (CDH2) and Ki67 in AD-MSCs adhered to decellularized amniotic membrane (AD-MSCs + D-hAM) by real-time RT-qPCR. (A) Agarose gel electrophoresis of amplified fragments. (B) The AD-MSCs used in the qPCR experiments were isolated from three different donors (P1, P2 and P3). We extracted mRNA from each sample in three independent experiments and cDNA was analyzed in duplicate in the qPCR, as default. Data is represented as fold-differences relative to the AD-MSCs + D-hAM (gray line; downregulated (< 1) or upregulated (> 1)). (C) Melting curves for target genes indicating its specificity.

decellularization. In another study the fibronectin content was measured by immunohistochemical staining in decellularized and dehydrated human amniotic membrane (DHAM), in comparison with cryopreserved human amniotic membrane (CHAM). In accordance with our data, the authors found that the decellularization process caused a loss in the fibronectin expression [30].

The tissue collection and preservation are essential steps which determines the success of any transplantation [21,32]. There are several ways to decellularize and preserve the amniotic membrane and it is important that the way in which it is preserved does not alter the composition of its matrix so that it can be used as a scaffold for cell culture. For that, we followed a protocol of preservation with a high concentration of glycerol, without cryopreservation. The use of glycerol is a disadvantage because of its high cellular toxicity, however, for our purpose, it is not essential since we decellularized the membrane without preserving its cells, while maintaining the structural integrity of the tissue [21]. In addition, this method represents simple storage, long-term preservation as well as a low cost procedure [33].

The intact amniotic membrane showed to be more thermally stable. However, it was observed that human amniotic membrane is stable at high temperatures in all analyzed conditions: decellularized and decellularized covered with AD-MSCs. The collagen begins to slowly denature above 37–43 °C [25] and we observed a significant decomposition in the range of 250–390 °C for all samples. According to Ma and colleagues, due to collagenous constitution, the hAM reveals three principal characteristic absorption bands: amide I (C=O stretching), amide II (N–H bending) and amide III (N–H bending) [34]. In this work the absorption bands reported above were observed in hAM with

AD-MSCs. The peaks at 3320 cm⁻¹ were assigned to the stretching vibrations of N–H. The absorption band observed in 2929–2958 cm⁻¹ for D-hAM + AD-MSCs and 2981 cm⁻¹ for D-hAM samples were also observed at 2929 cm⁻¹ by Ma and colleagues. Kumar and colleagues cited that peaks at 2922 cm⁻¹ (cross-linked membrane) and 2936 cm⁻¹ (non-cross-linked membrane), meaning the presence of aliphatic group [35].

Mesenchymal stromal cells express a large number of bioactive molecules such as adhesion molecules, extracellular matrix proteins, cytokines and receptors for growth factors. They act by modulating the inflammatory response, angiogenesis and mitosis of cells involved in the tissue repair process. These characteristics allow them to survive host immune rejection, which justifies their use for allograft applications. For that purpose is necessary a suitable biocompatible scaffold that does not alter the cellular properties when transporting these cells to the injured area to promote regeneration [36–39]. According, we observed that the AD-MSCs were able to grow and expand on D-hAM without showing significant alteration on adhesion, viability, senescence, nuclei morphology and actin cytoskeleton. Similar results were also observed in a previous study from our group, analyzing the growth of limbal mesenchymal stem cells on hAM in comparison to Porcine small intestinal submucosa (SIS) [40]. The property of the D-hAM to promote an suitable environment to preserve the AD-MSCs signaling to external stimuli may be related to its structure rich in collagen, elastin, laminin and fibronectin [7].

The evaluation of the expression of vimentin, fibronectin, N-cadherin and Ki67 made it possible to indirectly verify the cellular response of the AD-MSCs to D-hAM, regarding cytoplasmic integrity, cell adhesion and migration, sequestration of nutrients and growth factors, and

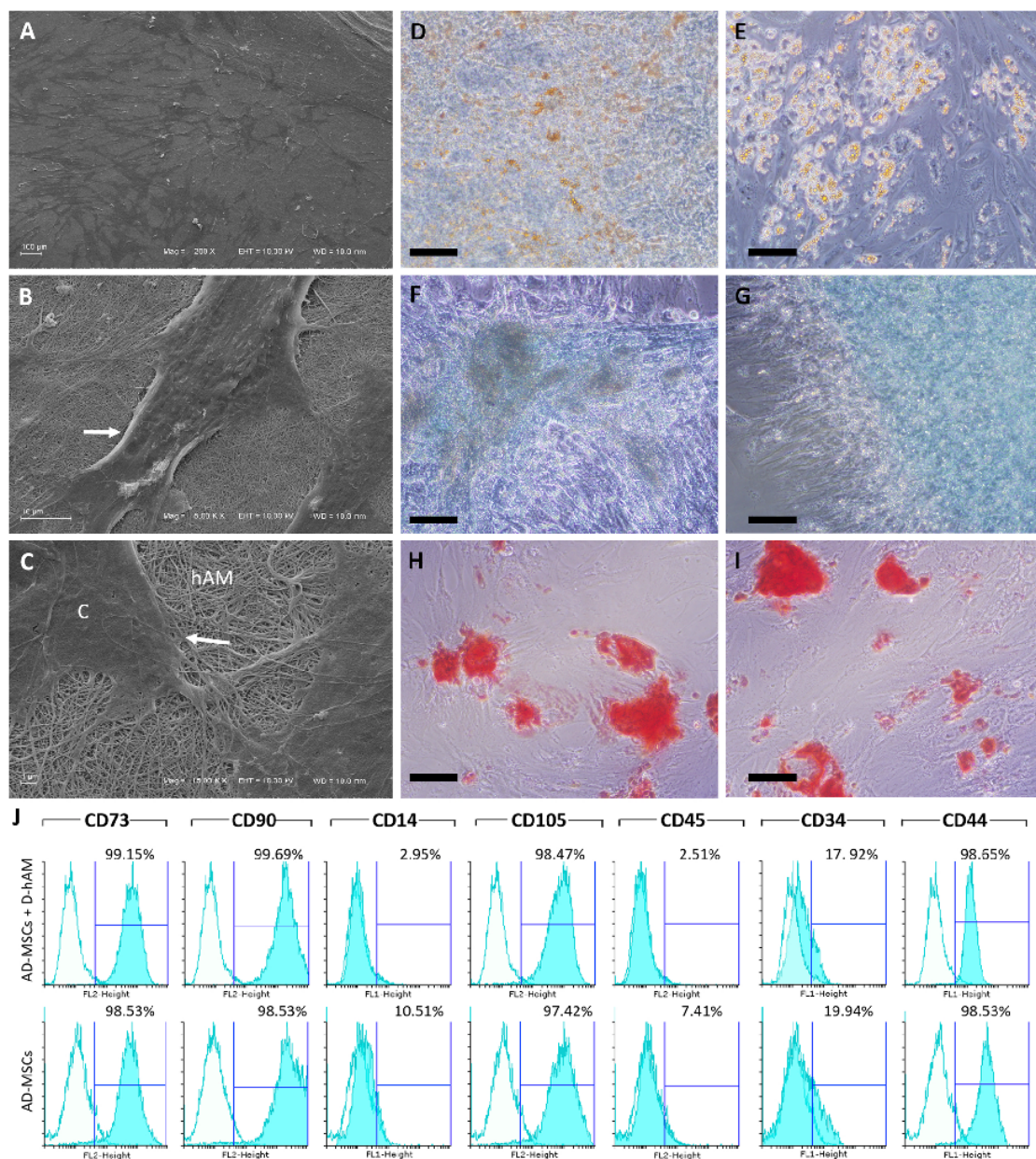


Fig. 7. Characterization of AD-MSCs after seeding on D-hAM. (A–C) Micrographs of scanning electron microscopy of AD-MSCs seeded on D-hAM. Cells and cytoplasmic extensions are indicated. C: Cells; hAM: human amniotic membrane. Adipocytes on hAM (D) and culture plate (E) showing the lipid vacuoles stained with Oil Red. AD-MSCs cultured on D-hAM showed potential for chondrogenic (F) and osteogenic differentiation (H) similar to AD-MSCs cultured on tissue plate (G, I). (J) Flow cytometry histograms of AD-MSCs cultured on D-hAM show the expression of selected molecules (CD73⁺, CD90⁺, CD14[−], CD105⁺, CD45[−], CD34[−], and CD44⁺) in comparison to controls AD-MSCs grown on tissue plate. Negative control analyses with no antibody (left). Scale bar: (B) 10 μ m; (C) 1 μ m; 100 μ m (A, D, F, E, G, H, I). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

formation of granulation tissue [41–44]. Considering the scaffolds properties that can affect the efficiency for application in regenerative medicine, cell adhesion is among the most critical characteristics [7]. The mRNA expression of N-cadherin, vimentin and fibronectin were preserved by AD-MSCs after their growth on D-hAM. This result together with the preserved histoarchitecture of the hAM, after the decellularization process, could be responsible to the stemness maintenance of the AD-MSCs. Taken together, these results indicate that the amniotic membrane is a scaffold with potential to become stem cell

therapy more suitable for clinical application, since other forms of cell administration imply complex questioning about the capacity to reach the target tissue [45].

5. Conclusions

In this study, we analyzed the physical and histological characteristics of decellularized amniotic membrane in the presence and absence of human adipose derived mesenchymal stromal cells. We found that

cells isolated from adipose tissue did not lose their differentiation potential and expression markers when in contact with human amniotic membrane, thus, showing the maintenance of their therapeutic potential, when adhered to this biological matrix. Therefore, these results constitute a main step for the use of this bioscaffold in pre-clinical models.

Founding

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Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.bej.2019.107366>.

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6 CAPÍTULO II - Artigo 2

Comparison of Human Denuded Amniotic Membrane and Porcine Small Intestine Submucosa as Scaffolds for Limbal Mesenchymal Stem Cells

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A córnea é um tecido que, assim como a pele, se encontra exposta a diversas lesões que podem levar à deficiência de células estromais do limbo esclerocorneal, as células responsáveis pela regeneração da córnea. Isso implica que a superfície da córnea não possa ser regenerada, causando a uma perda da acuidade visual. As células estromais mesenquimais do limbo esclerocorneal têm sido consideradas como uma fonte de células para o seu uso em medicina regenerativa, em aplicações dermatológicas e oftalmológicas, cujo mecanismo ainda está sob investigação. Assim como a membrana amniótica humana, outros biomateriais como a submucosa de intestino delgado porcino têm sido utilizados no desenvolvimento de matrizes biológicas. Este capítulo tem como objetivo comparar a membrana amniótica descelularizada, com a submucosa de intestino delgado porcino, como *scaffolds* para o crescimento e manutenção de células mesenquimais do limbo esclerocorneal.

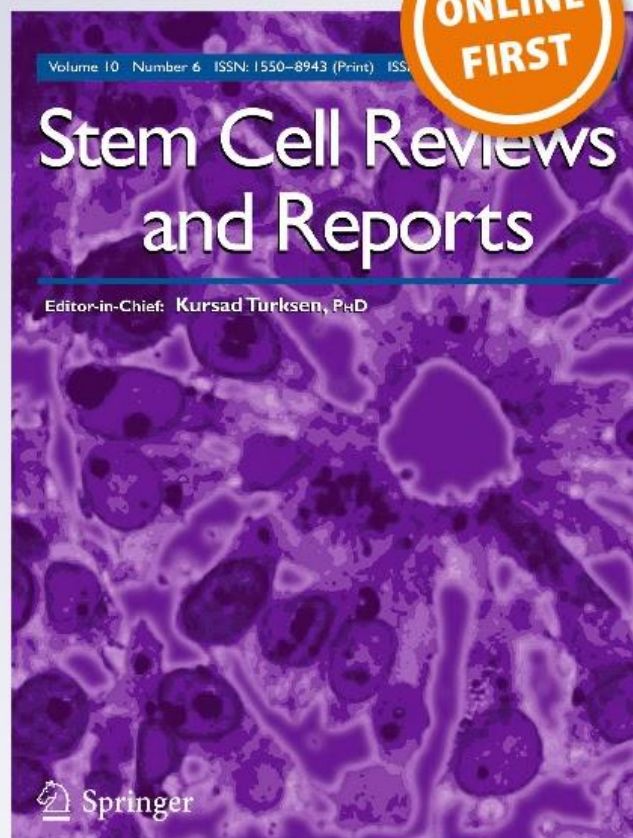
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Comparison of Human Denuded Amniotic Membrane and Porcine Small Intestine Submucosa as Scaffolds for Limbal Mesenchymal Stem Cells

Liliana I. Sous Naasani¹ · Cristiano Rodrigues¹ · Jéssica Gonçalves Azevedo¹ · Aline F. Damo Souza^{1,2} · Silvio Buchner³ · Márcia R. Wink¹

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Abstract

Blinding corneal scarring is usually treated with allogeneic graft tissue. Nevertheless, the global shortage of donors leaves millions of patients in need of therapy. Traditional tissue engineering strategies involves the combination of cells, growth factors, and scaffolds that can supply cellular biological components allowing to restore the tissue function. The mesenchymal stem cells found in the limbal stroma (L-MSCs) have a self-renewal potential for multilineage differentiation. Thus, in this work we compared the potential of human amniotic membrane (hAM) and porcine small intestine submucosa (SIS) as scaffolds for L-MSCs, aiming at potential applications in corneal regeneration. For that, L-MSCs were seeded on hAM and SIS and we analyzed their viability, actin cytoskeleton, nuclei morphology, cell density, adhesion and surface markers. Our results showed that cells adhered and integrated into both membranes with a high cell density, an important characteristic for cell therapy. However, due to its transparency, the hAM allowed a better observation of L-MSCs. In addition, the analysis of surface markers expression on L-MSCs after two weeks showed a slight increase in the percentages of negative markers for MSCs grown on SIS membrane. Thus, considering a long-term culture, the hAM was considered better in maintaining the MSCs phenotype. Regarding the function as scaffolds, SIS was as efficient as the amniotic membrane, considering that these two types of biological matrices maintained the cell viability, actin cytoskeleton, nuclei morphology and mesenchymal phenotype, without causing cell death. Therefore, our data in vitro provides evidence for future pre-clinical studies were these membranes can be used as a support to transport mesenchymal stem cells to the injured area, creating a kind of temporary curative, allowing the release of bioactive molecules, such as cytokines and growth factors and then promoting the tissue regeneration, both in human and veterinary medicine.

Keywords Amniotic membrane · Porcine small intestine submucosa · Limbal mesenchymal stem cells · Scaffolds · Sclerocorneal rims

Introduction

The external ocular surface extending from the conjunctiva into the cornea plays a critical role in visual perception. The cornea is a transparent avascular tissue that provides protection to the internal structure of the eye. It serves as an adequate surface of refraction, allowing to send the incident light to the retina, which

is essential for visual acuity [1]. Corneal tissue is composed of three layers with different types of cells. Beneath the corneal epithelium is the corneal stroma, which encompasses about 90% of the corneal volume, presenting as components mesenchymal stromal cells, collagen type I, and proteoglycans [2].

The high regenerative capacity of the corneal surface occurs through continued division, differentiation, and migration of the population of limbal epithelial stem cells present at the periphery of the cornea [3, 4]. This transition zone, between the cornea and sclera is known as the sclerocorneal limbus and represents the niche of the stem cells responsible for corneal regeneration. This area was recognized by the experiments carried out by Dr. Tung-Tien Sun in the '80s [5]. In addition, it was shown that mesenchymal stem cells (MSC) found in the stroma of sclerocorneal limbus, have the capacity for self-renewal potential for multilineage differentiation [6, 7]. These cells share characteristics with MSCs from bone marrow, in relation to its morphology, specific markers, high proliferation rate and low immunogenicity. In addition, the limbal

✉ Márcia R. Wink
mwink@ufcspa.edu.br; marciawink@yahoo.com.br

¹ Departamento de Ciências Básicas da Saúde e Laboratório de Biologia Celular, Universidade Federal de Ciências da Saúde de Porto Alegre – UFCSPA, Sarmiento Leite, 245, CEP, Porto Alegre, RS 90050-170, Brazil

² Hospital Irmandade Santa Casa de Misericórdia de Porto Alegre – ISCMPA, Porto Alegre, RS, Brazil

³ Departamento de Física, Instituto de Física, Universidade Federal do Rio Grande do Sul – UFRGS, Porto Alegre, RS, Brazil

mesenchymal stem cells (L-MSCs) provide a niche, allowing the maintenance of the limbal epithelial stem cells phenotype and participate in angiogenesis and regeneration of stromal tissue, during corneal wound healing [8, 9].

Damage to the stem cells or disruption of the niches, may lead to limbal stem cells deficiency. This implies that the corneal surface cannot be regenerated, then it is replaced by conjunctival cells causing conjunctivalization and vascularization of the cornea, thereby leading to a loss of visual acuity [10]. Additionally, others conditions as several pathologies, poor responses to surgery, injuries, and infections can result in scarring of the corneal stroma, which disrupts the vision of millions of people around the world [11]. These corneal scars can remain for decades in humans causing the loss of regular structure of the collagen fibers, also low transparency of the cornea [12]. Currently, surgical stromal replacement is the only successful approach to restoring vision in healed corneas. However, one of the main limitations for this treatment is that the amount of patients needing this tissue exceeds the donor availability [2]. These limitations have motivated the development of strategies using the new technological advances in tissue engineering.

Traditional tissue engineering strategies involves the combination of cells, growth factors, and scaffolds that can supply cellular biological components, allowing to restore the tissue function [13]. Among cells used in these combinations are MSCs, a population of non-hematopoietic stem cells with a high proliferative, and self-renewal potential [14, 15]. It is well established the differentiation capabilities and immunosuppressive properties that aid them to surviving from host immune rejection, which justifies its use for allograft applications [16]. One of their potential is the therapy in ophthalmology, due to their properties to increase the therapeutic potential of regeneration [17].

For tissue engineering applications, the biomaterials often serve as scaffold for a specific cell type. Most scaffold materials with great compatibility are naturally derived from mammalian tissues. The human amniotic membrane (hAM) is derived from the inner layer of the placenta. Histologically, it consists of a simple epithelium, a basement membrane, and an avascular stroma [18, 19]. This membrane has a low immunogenicity, and a low anti-inflammatory, anti-microbial, anti-fibrosis, and anti-scarring properties. The hAM has been tested as a treatment in ophthalmology for corneal ulceration, conjunctival lesions coverage, and acute chemical burns of the eye, demonstrating promising results [20].

Other potential source of scaffolding is the porcine small intestinal submucosa (SIS) that is composed mainly of extracellular connective tissue. This xenograft scaffold is a non-immunogenic biomaterial that has been widely used for tissue reconstruction of different organs, including the eye, almost in veterinary ophthalmology [21, 22].

Therefore, the main objective of this study was the isolation of L-MSCs from human sclerocorneal rims, and its culture on

hAM and SIS scaffolds. To this, we assessed parameters as the maintenance of viability, cell adhesion, density, compliance of actin cytoskeleton, nuclei morphology and phenotype of the cells on these membranes.

Materials and Methods

Ethical Aspects

The sclerocorneal rims (from cadaveric donors) were donated by Cornea Bank of Irmandade Santa Casa de Misericórdia de Porto Alegre hospital (ISCMPA) with the acceptance of the internal Research Ethics Committee on Human Beings (REC-ISCMPA n°070925/2015). Human amniotic membranes (hAM) were acquired from Obstetrical Center at ISCMPA hospital. Informed consent was applied before cesarean sections and all protocols were also approved by REC-ISCMPA (n°882,968/2014). All procedures were conducted according to the ethical principles expressed in the Declaration of Helsinki.

Obtainment, Culture and Characterization of L-MSCs

The isolation of mesenchymal stem cells from sclerocorneal rims was consistent with what we have reported [23]. In short, we cut the corneal tissue in fragments of 4 mm² and washed once with isopropyl alcohol/iodine 1:1 (v:v), followed of phosphate buffer saline (PBS) with 2% penicillin/streptomycin (Sigma Chemicals, Co. St. Louis, MO, USA). Tissue fragments were incubated with 2 mg/mL dispase II (Sigma) with 1% penicillin/streptomycin overnight at 37 °C to separate the epithelial membrane of the stroma. After removing the epithelial layer, the fragments of the stroma were placed on 6-well microplates (Nest Biotech) to isolate the cells by migration. The growth medium used was a Dulbecco's Modified Eagle's Medium (DMEM) with low glucose (Sigma), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, N.Y., USA) and 1% penicillin/streptomycin. Medium was changed every three days. L-MSCs cultures were maintained at 37 °C, 5% CO₂, and 95% humidity to reach confluence. After confluence, cells were trypsinized and expanded in 75 cm² culture flasks (Kasvi, Curitiba, Brazil).

For cell differentiation, L-MSCs at 5 passage were seeded at density 1×10^4 cells/cm² into a 12-well plate (Nest Biotech, New Jersey, USA). The growth medium was changed after three days in culture by the StemPro® Adipocyte Differentiation Basal Medium, StemPro® Osteogenesis Differentiation Basal Medium and StemPro® Chondrocyte Differentiation, Basal Medium (Gibco) for adipogenic, osteogenic and chondrogenic differentiation, respectively. Control cells were maintained with standard medium that was changed every three days, until the specific periods of cultivation. After two weeks of adipogenic differentiation conditions, the culture was fixed with 4%

formaldehyde solution for 30 min and staining with Oil Red O in deionized water (1:3). The pre and post-treatment was made with 60% isopropanol (v/v) in water. Then, after 17 days in culture with chondrogenic medium, the synthesis of proteoglycans by chondrocytes was seen with 1% Alcian Blue staining, prepared in 0.1 N HCl. For osteogenic cultures, was used 2% Alizarin Red S solution (pH 4.2) after 25 days, to see the calcium phosphate deposited by osteoblasts. Stained induced cultures were compared to dye impregnation between controls. Photomicroscopes performed using a BX-50 Olympus microscope with optical lens (10X/0.30 Ph1-UplanFI), coupled to a camera Motican 2500 (Olympus, Hamburg, Germany).

Cell surface antigen expression by L-MSCs was detected using flow cytometry. Cultures were dissociated with trypsin, centrifuged and incubated for 30 min at 4 °C with direct immunofluorescence staining using the antibodies: FITC-anti-CD44, PE-anti-CD105, PE-anti-CD45, PE-anti-CD14, FITC-anti-CD34 (Invitrogen™, Waltham, MA, USA) and PE-anti-CD73 (BD Pharmingen™, San Diego, USA). The cytometer was equipped with 488 nm argon laser (Becton-Dickinson, San Diego, CA, USA), and CellQuest software. At least 10,000 events were collected and data were evaluated using the Flowing Software 2 (Perttu Terho, Finland).

Scaffolds Preparation

Processing of hAM

Previously, the pregnant women were screened serologically for HIV, hepatitis B and C, HTLV, syphilis, Chagas disease, toxoplasmosis (IgM antibodies) and cytomegalovirus (IgM antibodies). Only patients with negatives results were selected. Under sterile conditions, the hAM was separated from the other fetal membranes and it was washed once with sterile saline solution. Membrane was treated with antibiotics (penicillin 1,000,000 U/L and streptomycin - 1 g/L) and it was preserved in glycerol 85% solution under refrigeration. In all phases of processing fragments were collected for microbiological analysis. For the tests, the hAM was submitted to decellularization protocol by enzymatic method using 0.05% trypsin / 0.02% EDTA during 20 min at 37 °C and finally, it was hydrated overnight at 37 °C with culture medium before seeding cells.

Preparation of SIS

The membrane of porcine intestinal submucosa was obtained from OASIS Wound Matrix, Smith & Nephew Inc., London, UK. The decellularized protocol is detailed by Luo et al. (2011) [24]. Briefly, the membranes were cut into 6-mm-diameter disks, and sterilized by UV-C radiation (254 nm) for 10 min. Then, the SIS disks were rehydrated overnight using standard culture medium for MSCs prior seeding cells.

MTT Tetrazolium Assay

The relative viability of the L-MSCs seeded on scaffolds were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) tetrazolium reduction assay. The hAM and SIS were cut in fragments of approximately 6 mm in diameter and it was placed in 96-well plate conical bottom without adherence. We seeded at density of 1.5×10^3 cells/well to provided sufficient cell-cell contact on the hAM, SIS and a control of the cells on the flat surface of the 96-well plate, additionally, cell-free membrane control was performed to determine if the MTT reacts with the membranes. After 48 h, the scaffolds were changed from well to avoid the cells that were adhered on well plate, and they were incubated with MTT for 4 h. The crystals were dissolved in dimethyl sulfoxide (DMSO), and the amount of formazan (presumably directly proportional to the number of viable cells) was measured by SpectraMax Microplate Reader (Molecular Devices Corporation, USA) at 570 nm. Average absorbance corresponded to 100% viability in the control.

Actin Filaments Staining

Actin cytoskeleton organization was assessed via F-actin staining. Briefly, L-MSCs were seeded at 5×10^3 cells/cm² on each scaffolds and glass coverslips (control) and were cultured for 48 h and two weeks with standard medium over appropriate atmosphere. Then, the culture was washed once with PBS and it was fixed with 4% paraformaldehyde solution in PBS for 30 min at 4 °C. Cells were washed with PBS followed by incubation with permeabilization solution (0.1% Triton X-100 in PBS) for 1 h. Actin filaments were stained with FITC-Phalloidin (Sigma) and cells were also stained with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for the identification of nuclei (40 min, 4 °C, dark room). Images were obtained with a fluorescence microscope (Olympus IX51/ Olympus U-RFL-T) coupled with 460–490, and 372 nm band-pass excitation filters, respectively.

Nuclear Morphometric Assay (NMA) and Density Analysis

Nuclear morphometry was analyzed using a NMA program developed by Filippi-Chiela et al. 2012 [25]. MSCs were seeded at density 1.5×10^3 cells/well on the hAM, SIS and a control on glass coverslips for 48 h. After this time, the cells were fixed with 4% paraformaldehyde (v/v in PBS) for 30 min at room temperature. Nuclei were marked with 300 nM DAPI for 30 min, at room temperature and images of the DAPI-labeled nuclei were captured using a fluorescence microscope (Olympus BX-50) with optical lens ($\times 10/0.30$ Ph1-UplanFI) coupled with 372 nm band-pass excitation filter. Quantification of nuclei types were performed using Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring,

Fig. 1 Isolation and characterization of L-MSCs from sclerocorneal rims. **a** The esclerocorneal rims. **b** The stromal cells migrating from limbus are indicated. **c** Confluent primary culture of L-MSCs. **d** Isolated cells show fusiform morphology. **e** Alcian blue staining demonstrates glycosaminoglycan production. **f** The cells differentiated into osteoblasts showing the bone matrix stained by Alizarin red. **g** Adipocytes showing the lipid vacuoles stained with Oil Red. **h** Flow cytometry dot plot of isolated L-MSCs shows the expression of selected molecules (CD105⁺, CD44⁺, CD73⁺, CD14⁺, CD34⁺, and CD45⁺), in comparison to each control. Phase contrast micrograph. Scale bars: 200 μ m (**a-d**); 100 μ m (**e-h**)

USA). Data are presented as a plot of Area versus Nuclear Irregularity Index (NII) and all samples were compared between control. In the same way, we quantify the number of nuclei per area, to determine the cell density per mm² in each substrate using ImageJ k 1.45 software.

Scanning Electron Microscopy Examination

Morphological aspects of L-MSCs on hAM, and SIS scaffolds were observed from scanning electron microscopy (SEM). Cells were seeded in each substrates, and before 48 h of culture were fixed with 2.5% glutaraldehyde in PBS for 96 h at 4–8 °C. After this, the samples were washed with PBS for 12 h at same temperature. Then, the cells were incubated with 1% osmium tetroxide in PBS for 20 min at room temperature, followed by washing three times with PBS. In the sequence, successive dehydration with ascending concentrations of ethyl alcohol (50, 60, 70, 80, 90, and 100%) for 10 min at each, and more 20 min for two last percentages. The critical point was used as the last drying step of the samples and then they were mounted onto aluminum stubs, and coated with gold. Samples were kept

in the desiccator until reading in the SEM. Imaging was conducted using a scanning electron microscope (Carl Zeiss EVO).

Confirmation of Phenotype Maintenance

The Cell surface antigens expression of L-MSCs after two weeks in culture on hAM and SIS, was detected by flow cytometry. For this, the cultures were dissociated with trypsin, centrifuged and incubated for 30 min at 4 °C with direct immunofluorescence staining, using the antibodies: FITC-anti-CD44, PE-anti-CD105, PE-anti-CD45, PE-anti-CD14, FITC-anti-CD34 (Invitrogen™, Waltham, MA, USA), in the same conditions mentioned above.

Statistical Analysis

Data were analyzed statistically by one-way ANOVA for MTT analysis and a two-way ANOVA for NMA and density analysis, followed by Tukey's post-hoc test. Differences between mean values were considered significant when $p < 0.05$.

Results

Isolation and Culture of L-MSCs

In this study, we isolated MSCs from fragments of human sclerocorneal rims (Fig. 1a) that migrated from the tissue before two weeks of culture, and reached the confluence in approximately 26 days. These cells adhered to plastic and showed a typical fusiform morphology (Fig. 1b-d).

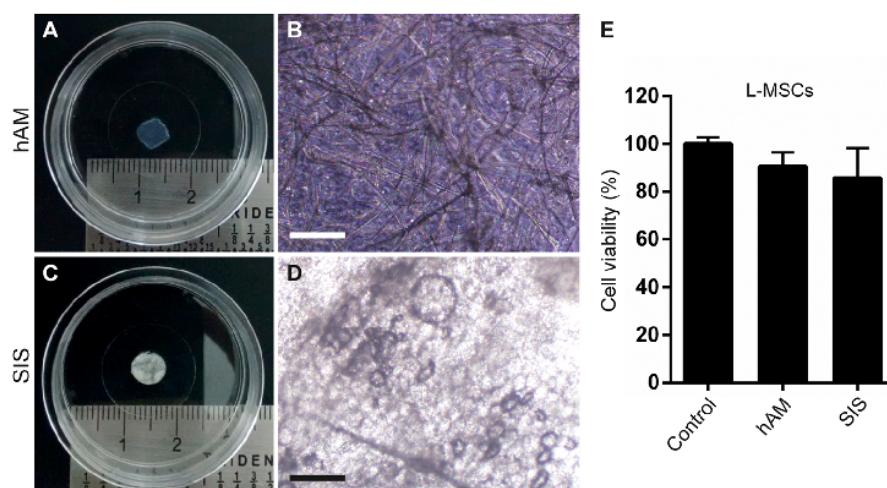


Fig. 2 L-MSCs viability on SIS and hAM scaffolds. The size and the transparency of the (a) hAM and (c) SIS fragments are shown. The cells were seeded on (b) hAM and (d) SIS and it was cultured for 48 h to MTT assay. (e) Graph shows no significant differences in the viability (%) of the L-MSCs seeded in the hAM and SIS in comparison with the control

cells (plates surface). Bars represent means of percentage compared to the control $\pm$ standard error of the mean of three independent experiments performed in triplicate. Data were analyzed statistically by one-way ANOVA followed by Tukey post-hoc test. Phase contrast micrograph. Scale bars: 100 μ m

L-MSCs Characterization

To demonstrate the differentiation potential of isolated cells, they were exposed with osteogenic, adipogenic and chondrogenic medium. In the differentiation assay, the Alcian blue staining demonstrated the glycosaminoglycan production after 17 days in culture, confirming the presence of chondrocytes after induction; while Alizarin Red S staining showed a calcium-rich mineralized matrix produced by osteoblasts, after 25 days under osteogenic differentiating conditions. Completely, adipocytes showed the formation of intracellular lipid vacuoles, which were confirmed with Oil Red staining after two weeks under adipogenic differentiation conditions (Fig. 1e-g). Continuing the confirmation of cellular identity, the immunophenotyping assay was performed. The analysis of cell surface antigen expression showed a great part

of L-MSCs population was positive for mesenchymal markers (CD73, CD105 and CD44), and negative for hematopoietic markers (CD14, CD34, and CD45) (Fig. 1h).

Cell Viability of L-MSCs on SIS and hAM Scaffolds

To test the viability of cells on these scaffolds, we performed the MTT assay. Using phase contrast microscopy it was not possible to observe the adhered cells in each biomaterial, mainly in the SIS that is less transparent than the hAM, preventing the passage of light (Fig. 2a-d). MTT assay showed that the viability of cells plated on hAM and SIS was not affected by both biomaterials when compared to the control cells, plated on culture plastic plates. Nevertheless, a great deviation was obtained on the membranes, mainly in the SIS. This may be due to the fact that some cells descended

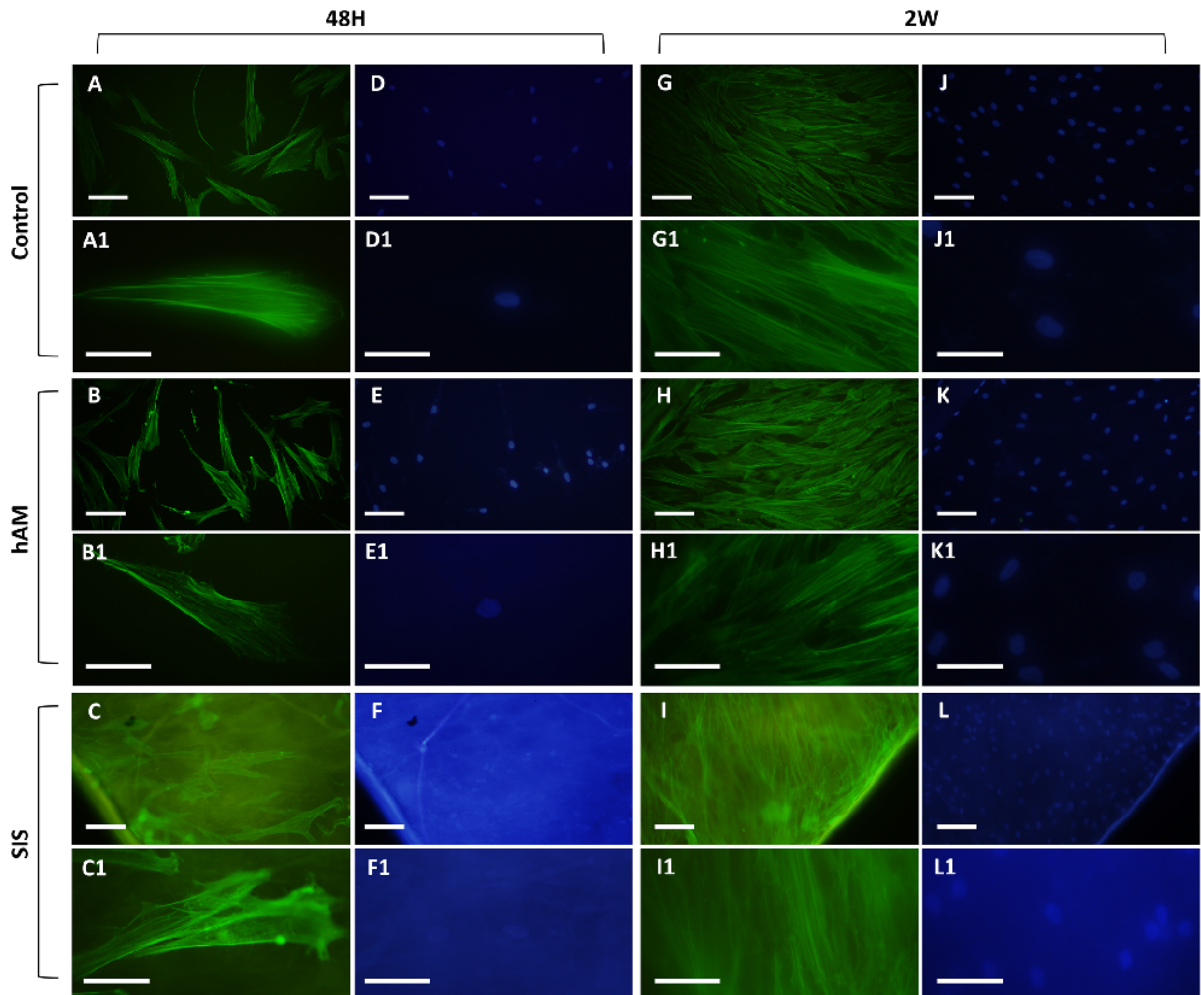


Fig. 3 Fluorescence micrograph of actin filaments and nuclei staining of L-MSCs cultured on hAM and SIS scaffolds. Phalloidin and DAPI staining for L-MSCs on the **a** control, **b** hAM and **c** SIS showing the

cytoskeleton and nuclei of these cells on the surface of each biomaterials after 48 h of culture. Scale bars: 50 μ m

to the conic bottom of the plate, thus decreasing the number of cells adhered in the substrates. However, no statistically significant difference was observed between the substrates in comparison to the control (Fig. 2e).

Actin Cytoskeleton and Nuclei Staining of L-MSCs Cultured on the SIS and hAM

When L-MSCs were seeded in the hAM and SIS, we had difficulty to observe them by phase contrast microscopy, then in order to improve the visualization of L-MSCs on membranes, we performed an actin filaments and nuclei staining. Stained cells showed the actin cytoskeleton and a fusiform morphology in both substrates, and as expected, they showed the actin filaments distributed throughout cell shape (Fig. 3a-f). We observed that there was no alteration in the distribution of actin filaments. The nuclear staining with DAPI showed that cells formed a monolayer on the substrates, coating the entire surface (Fig. 3g-l).

Nuclear Morphology and Density Analysis

The nuclear morphology of L-MSCs cultivated on hAM and SIS was analyzed through the Nuclear Morphometric Analysis (NMA) [25]. This program allows to identify seven different nuclear morphologies that have different biological means: normal, no damage; irregular and large irregular, damaging event; small regular, apoptosis in an early or intermediary stage; small, mitosis; small irregular, mitosis with damage or nuclear fragments; and large regular, senescence. In this study, we identified four different nuclear morphologies in all groups analyzed (normal, small regular, large regular and irregular). However, cells growing on hAM and SIS did not undergo significant changes in their nuclear morphology,

when compared to control cells in culture plastic plates (Fig. 4a-c). Also, Nuclear density analysis showed that cultures of L-MSCs in hAM and SIS scaffolds have a higher cell density per area (mm^2), when compared to control on the culture plate (Fig. 4d).

Scanning Electron Microscopy of L-MSCs Cultivated on Scaffolds

Figure 5 shows the SEM images of hAM (Fig. 5a, b and c) and SIS (Fig. 5d, e, and f) populated with L-MSCs. The SEM images showed a cell monolayer in the surface of both biomaterials. It was possible to observe the cytoplasmic extensions of the cells adhered to each substrate.

Confirmation of Phenotype Maintenance

In order to analyze whether the long-term culture of L-MSCs over the membranes could alter the expression of mesenchymal markers on cell surface, we grown cells during two weeks on hAM and SIS and analyzed their phenotype, in comparison with cells grown on tissue culture plastic for the same time (control). As can be observed in Fig. 6, the flow cytometry histograms of L-MSCs show that the largest population of the cells maintained their phenotype (CD44^+ , CD105^+ , CD34^- , CD45^- and CD14^-). However it is possible to observe that there is a slight increase in the percentages of negative markers for mesenchymal cells grown on SIS membrane.

Discussion

Stem cell-based therapy holds great promise for the treatment of severe injuries as well as a number of incurable diseases.

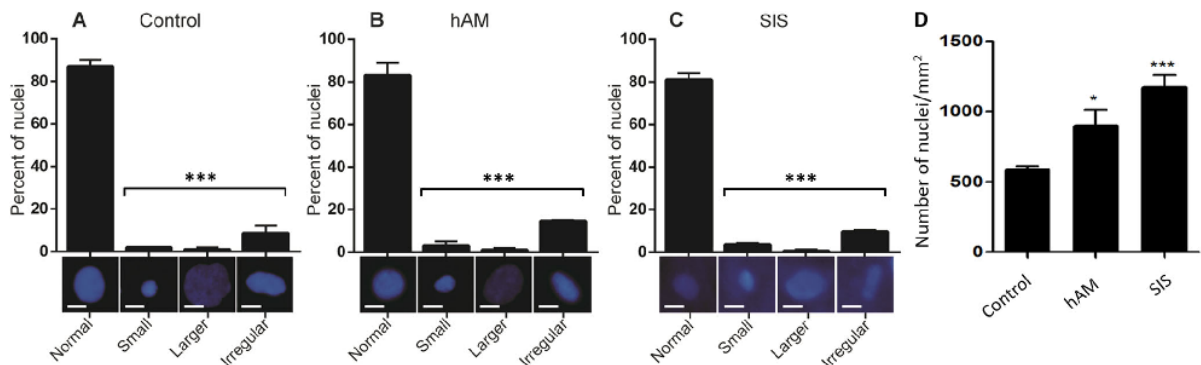


Fig. 4 Nuclei morphometric and cell density analysis of L-MSCs on substrates. Comparison of nuclei types in **a** control (glass coverslips), **b** hAM and **c** SIS. The graphic shows the percent of normal, small (apoptotic), larger (senescent) or irregular nuclei after 48 h of culture. The images show an example of each nuclei types analyzed. **d** Analysis of the nuclear density of cells in the different substrates, showing the

number of nuclei per mm^2 . Results for NMA are expressed in percentage $\pm$ standard error of the mean; results for cell density are expressed in number of nuclei per $\text{mm}^2 \pm$ standard error of the mean and data were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test. * different from control. Scale bars: 100 μm

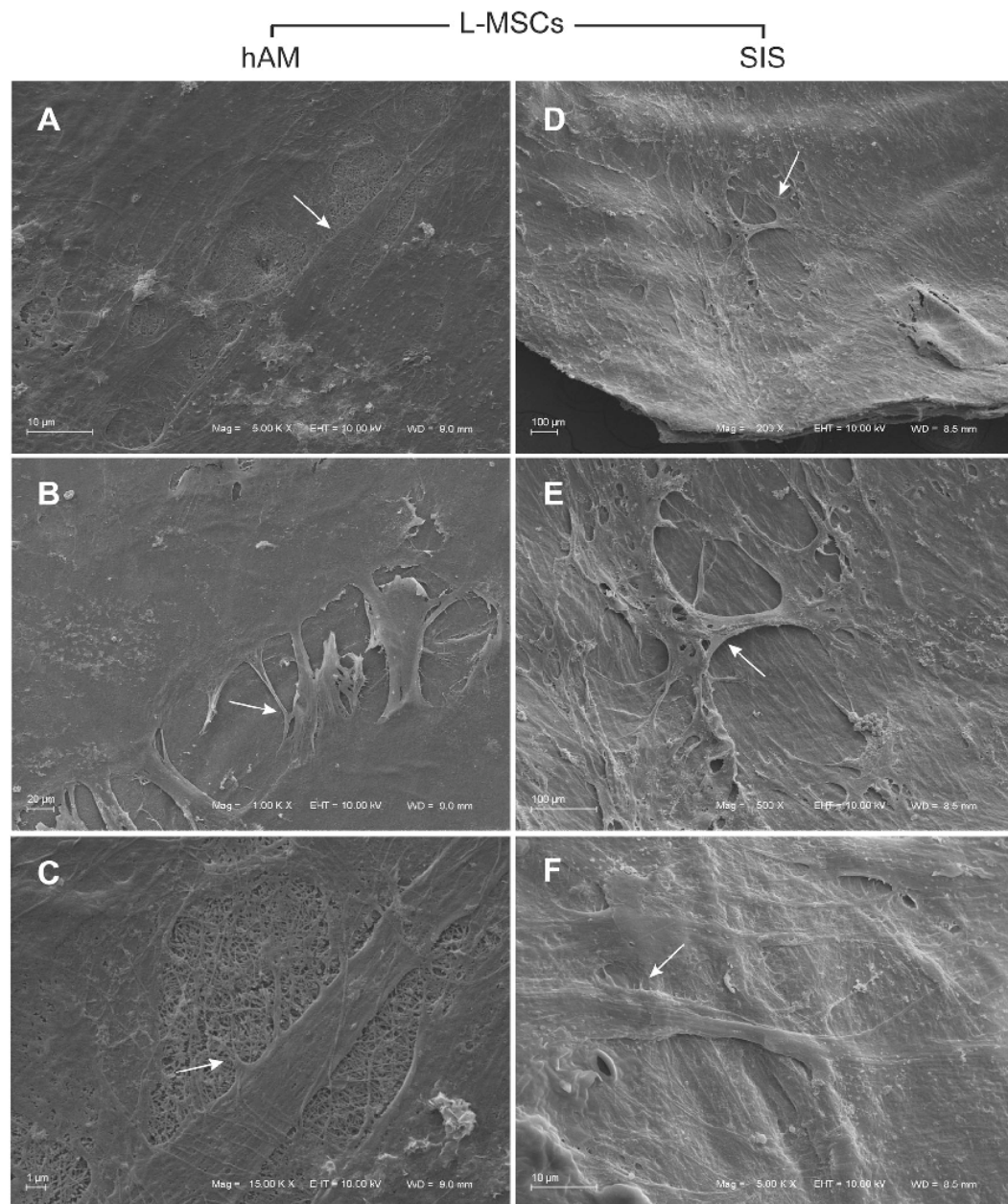


Fig. 5 Micrographs of scanning electron microscopy of L-MSCs seeded on scaffolds. The cells formed a monolayer coating the surface of the (a-c) hAM and (d-f) SIS. The L-MSCs are indicated on the surface of (a, b)

hAM and (d, e) SIS. Cytoplasmic extensions of L-MSCs adhered to the (c) hAM and (f) SIS are indicated. Scale bars: 10 μ m (a, f); 100 μ m (d, e); 20 μ m (b); 1 μ m (c)

[26]. The use of MSCs is based on their ability to differentiate into various cell types, besides those of mesodermal origin, including its potential for proliferation and immunomodulatory properties [15, 27, 28]. In our study, the isolated cells by migration from corneal sclerotic tissue, demonstrated a potential for multilineage differentiation, and the expression of cell surface antigens, associated with human mesenchymal stem cells. Also, they have no/low expression of negative

hematopoietic lineage markers, which coincides with previous published results about L-MSCs [29, 30]. In addition, these cells showed a filamentous actin stress fibers distributed throughout cell shape, characteristic of mesenchymal-migrating cells [31].

Tissue engineering requires three key factors: 1) a unique niche with a defined medium, 2) a biocompatible scaffold with structural characteristics that favors cell adhesion, and

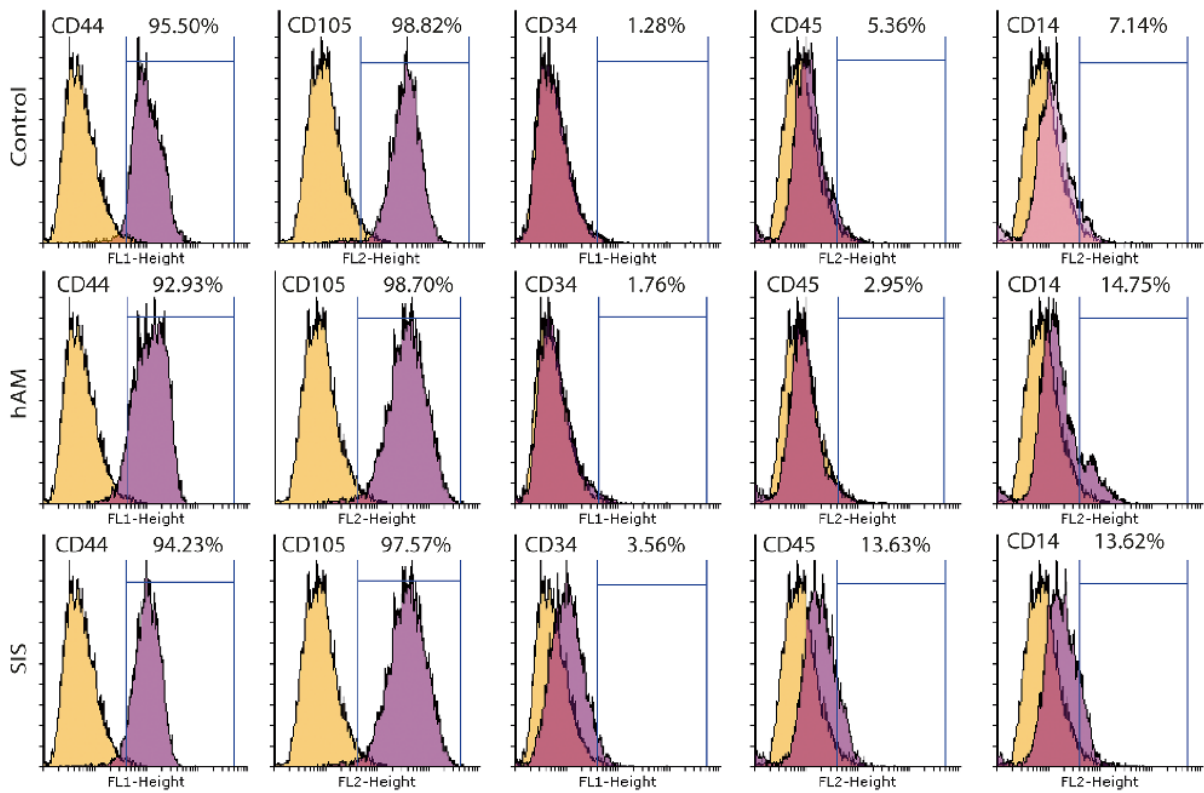


Fig. 6 Flow cytometry histograms of L-MSCs after growing for two weeks on control (plastic tissue culture), hAM and SIS scaffolds. Cell markers expression of selected molecules (CD44⁺, CD105⁺, CD34⁺, CD45⁻ and CD14⁻) were evaluated in a population of at least 10,000

events of each condition. The results showed that L-MSCs maintained the expression of positive markers for mesenchymal stem cells and the negative markers for hematopoietic markers

3) the ability to promote the metabolic activities of cells [13]. In order to evaluate whether the hAM and SIS can be a suitable scaffolds for L-MSCs, we seeded cells on membranes and analyzed their viability, morphology, adhesion, density, and maintenance of the mesenchymal phenotype.

The viability and actin filaments distribution of cells that were expanded on both membranes were not changed. In addition, there was no alteration in the distribution of actin filaments and in the mesenchymal phenotype, after a period of two weeks of cell culture on both membranes, which demonstrates the maintenance of L-MSCs on both scaffolds. Likewise, no nuclear morphology changes were observed in L-MSCs cultivated on hAM and SIS. This is important to ensure that these scaffolds are not able to induce an injury process in L-MSCs, considering that alteration in nuclear morphology occurs in several processes associated to cell death [32] and epithelial-mesenchymal transition-like (EMT-like) process, as already shown by our group [33].

SEM image analysis showed a cell monolayer with cytoplasmic extensions in the surface of both membranes, confirming the adhesion capacity. In addition, both membranes maintained a high cell density, which is an important

factor, due to the need of a large number of cells for cellular therapy in regenerative medicine [34]. Our results are in accordance with data in literature, confirming the properties of these matrices as biological scaffolds [35, 36].

Cells originated from several sources in combination with biomaterials has been used in different studies for corneal bioengineering [37, 38]. The integration of appropriate cells with the most appropriate biomaterials is necessary to repair each cellular layer of the cornea, either epithelium, stroma, endothelium or full-thickness. A successful clinical outcome, defined as corneal epithelialization without central conjunctivalization.

The first successful clinical outcome of cultivated corneal epithelial transplantation in two patients was published by Pellegrini and colleagues in 1997. After this initial report, various researchers have reporting transplantation of in vitro cultivated corneal epithelial cells on scaffolds, such as amniotic membrane, fibrin, and collagen hydrogel, with a great impact for clinic corneal transplantation [39, 40]. Tsai et al. 2000 in their review concluded that transplantation of autologous limbal epithelial cells cultured on amniotic membrane is a simple and effective method of reconstructing the corneal

surface and restoring useful vision in patients with unilateral deficiency of limbal epithelial cells (LSCD) [41]. However, the success is restricted to the availability of healthy limbal tissue taken from contralateral eye in autologous cases or tissues from living related donors or cadaveric eyes for allogeneic transplantation. Despite the benefits, it remains difficult to estimate the appropriate amount of limbal tissue from a donor eye for autologous transplantation. Inadequate volume may lead to the failure of eye surface-reconstruction and excess extraction tends to impair the healthy donor eye [38].

Growth factors play an important role in corneal epithelial wound healing and repair. Thus, various experimental treatments that aim to promote wound healing and minimize inflammation are being evaluated, such as human mesenchymal and adipose stem cells [42].

The L-MSCs are a cell population, reaching the minimum criteria that defining them as human mesenchymal cells [6, 7]. Therefore, they could be used as much for the understanding the stem cells biology, as a potential tool for developing better limbal epithelial stem cell constructs, for the treatment of ocular surface disorders.

Amniotic membrane is a rich source of growth factors and is increasingly being used in the treatment of acute ocular burns. It has anti-inflammatory and antifibrotic properties that promote the restoration of a stable ocular surface [43, 44]. Ma et al. 2006 reported that bone marrow- derived human MSCs have also been transplanted into rat corneas, using the amniotic membrane as a carrier after chemical burns. This lead to the successful reconstruction of the damaged corneal surface. The therapeutic effect of transplantation may be associated with the inhibition of inflammation and angiogenesis rather than epithelial differentiation from MSCs [45].

The SIS has been used in veterinary ophthalmology. Bussieres et al. 2004 support that SIS is suitable for the repair of full-thickness corneal wounds in dogs, cats and horses. Similarly, a preliminary evaluation of the biocompatibility of SIS biomaterial with the rabbit cornea, suggests that remodeling of SIS in rabbit cornea resulted in a relatively clear corneal stroma leaving only moderate amounts of scar tissue [46, 47].

In our study, from the point of view of scaffold for L- MSCs, SIS membrane was as efficient as the hAM. However, it is possible to observe that there is a slight increase in the percentages of negative markers (CD34⁺, CD45⁺ and CD14⁺) for L- MSCs grown on SIS membrane for two weeks. Thus, at long term culture, the hAM was considered better in maintaining the MSCs phenotype. Indeed, due to its transparency, it was possible to observe better the cells on the amniotic membrane. Thus, the amniotic membrane would be more suitable to treat corneal injury.

Our main proposal at this stage of the study was to investigate whether these membranes were able to maintain the cell viability, actin cytoskeleton, nuclei morphology and phenotype of L- MSCs. Therefore, our data in vitro provides

evidence that both membranes can be used as a support to transport mesenchymal stem cells to an injured area, creating a kind of “temporary curative”, not only in corneas, but also in other tissues, as skin.

Future pre-clinical studies are necessary to demonstrate that these combinations of cells and biomaterials will allow the release the bioactive molecules, such as cytokines and growth factors and then promote the tissue regeneration.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no potential conflicts of interest.

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7 CAPÍTULO III -Artigo 3

Bioscaffold developed with decellularized human amniotic membrane seeded with mesenchymal stromal cells: assessment of efficacy and safety profiles in a second-degree burn preclinical model

Artigo publicado na revista **Biofabrication**

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Após a elaboração e análise do *bioscaffold* de membrana amniótica humana e células estromais mesenquimais, onde a membrana amniótica foi efetivamente descelularizada e as células estromais mesenquimais mantiveram seu potencial de células progenitoras, o seguinte passo é avaliar como esse *bioscaffold* pode ser utilizado em modelos pre-clínicos. Neste capítulo foi desenvolvido um modelo murino de lesão cutânea por queimadura, para avaliar o efeito da membrana amniótica descelularizada na presença ou ausência de células estromais mesenquimais derivadas de tecido adiposo. Foi padronizada a realização de queimaduras de segundo grau profundo, assim como os curativos externos para evitar a remoção dos tratamentos. Os resultados derivados deste capítulo poderão confirmar a eficácia e segurança deste curativo de baixo custo e elevada disponibilidade para a futura aplicação clínica como terapia para o tratamento de queimaduras.

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Bioscaffold developed with decellularized human amniotic membrane seeded with mesenchymal stromal cells: assessment of efficacy and safety profiles in a second-degree burn preclinical model

Liliana Ivet Sous Naasani¹, Luiza Pretto¹, Carla Zanatelli¹, Thaís Casagrande Paim¹, Aline Francielle Damo Souza², Pablo Fagundes Pase³, Marilda Da Cruz Fernandes⁴, Jean Sévigny⁵ and Márcia Rosângela Wink^{1,*}

¹ Departamento de Ciências Básicas da Saúde, Laboratório de Biologia Celular, Universidade Federal de Ciências da Saúde de Porto Alegre—UFCSPA, Porto Alegre, RS, Brazil

² Banco de Tecidos Humanos—Pele Dr. Roberto Corrêa Chem, Hospital Irmandade da Santa Casa de Misericórdia de Porto Alegre—ISCOMPA, Porto Alegre, RS, Brazil

³ Cirurgia Plástica—Hospital de Pronto Socorro e Moinhos de Vento, Porto Alegre, RS, Brazil

⁴ Departamento de Ciências Básicas da Saúde, Laboratório de Patologia, Universidade Federal de Ciências da Saúde de Porto Alegre—UFCSPA, Porto Alegre, RS, Brazil

⁵ Département de Microbiologie-Infectiologie et d'Immunologie, Faculté de Médecine, Université Laval, Québec, QC G1V 0A6, Canada

* Author to whom any correspondence should be addressed.

E-mail: mwink@ufcspa.edu.br and marciawink@yahoo.com.br

Keywords: decellularized human amniotic membrane (DhAM), adipose derived mesenchymal stromal cells (AD-MSCs), deep second degree burn, murine preclinical model, CD73, CD11b

Supplementary material for this article is available [online](#)

Abstract

Therapies to deep burn injuries remain a global challenge. Human amniotic membrane (hAM) is a biomaterial that has been increasingly explored by the field of regenerative medicine. A decellularized hAM (DhAM) can be used as scaffold for mesenchymal stromal cells (MSCs) to grow without the loss of their stemness potential, allowing its application as cell therapy for wound healing. In this work, we associated DhAM with adipose-derived MSCs (DhAM + AD-MSCs), as a therapy strategy for second-degree burns in a preclinical model. Animals with induced second-degree burns were divided into four groups: control, which consists of a non-adherent gauze; a synthetic commercial dressing as the positive control (Control+); DhAM; and DhAM plus rat AD-MSCs (DhAM + AD-MSCs), followed by detailed and long term analysis (5 weeks). The macroscopical analysis showed the healing improvement in the wound area after the DhAM + AD-MSC treatment. Histological analysis also showed no alteration in the animal organs and a regular epithelial progression in comparison to the control. This observation was also confirmed by the analysis of suprabasal layers in the neoepidermis with CK10, showing a stratified and differentiated epithelium, when compared to Control and Control+. A strong CD73 (ecto-5'-nucleotidase) labeling was observed in the first 2 weeks postburn in dermis and epidermis. The expression in dermis was stronger in the second week in the middle of the wound, when comparing the Control+ with DhAM + AD-MSCs ($p = 0.0238$). In the epidermis the expression of CD73 was increased in all regions when compared to the control. This data suggests the involvement of this protein on wound healing. A low CD11b labeling was observed in DhAM + AD-MSCs treatment group mainly in the last treatment week, in comparison to Control and Control+ ($p < 0.0001$), which indicates a reduction in the inflammatory process. MSCs

through CD73 can release high concentrations of adenosine, an immunosuppressive molecule, suggesting that this could be the mechanism by which the inflammation was better modulated in the DhAM + AD-MSCs group. The results obtained with this preclinical model confirm the effectiveness and safety of this low-cost and highly available dressing for future clinical application as a therapy for burn treatments.

1. Introduction

Burns are traumatic injuries that mainly affect the skin and its appendices. This damage—that can be caused by thermal, physical, chemical or biological conditions—can lead to a loss of skin integrity (Jeschke *et al* 2020). For this reason, the way to cure damaged skins requires critical care management, followed by long-term care and rehabilitation. Nowadays, the treatments available are basically allogeneic skin transplants and commercial skin substitutes. The first one bumps into the disproportion between the number of donors and the number of burned individuals, in addition to the large burned areas versus the skin bandages available. The second is implicated in high costs per patient treated, often making its use unfeasible in public health systems care.

In this complex scenario, the regenerative medicine field has become prominent as the study of new approaches urges. Through it, the development of new therapies based on tissue engineering becomes possible and its potential should be explored to overcome the barrier found in cases of large burns (Daar and Greenwood 2007).

The human amniotic membrane (hAM) is a biomaterial that has been increasingly explored by the regenerative medicine field. The hAM is the inner part of fetal membranes and histologically resembles the skin tissue (Bourne 1960). Besides, it has peculiar biological properties. It counts with the presence of important cells of the point of view of regenerative medicine—such as mesenchymal stromal cells (MSCs), and fibroblasts—a wide array of cell growth factors, proteins, proteoglycans and cytokines (Farhadhosseinabadi *et al* 2018) and secretes peptides with antimicrobial character (Tehrani *et al* 2017). In the last years, it has been studied for the repair of peripheral nerve defects (Bourgeois *et al* 2019, Zhang *et al* 2019), full-thickness skin (Song *et al* 2017), wound diabetic ulcerations (Paggiaro *et al* 2018), ophthalmological conditions (Navas *et al* 2018), burns (Puyana *et al* 2020), intrauterine adhesion due to endometrial fibrosis (Chen *et al* 2019), osteoinductive (Tang *et al* 2018), cartilage grafts (Motamed *et al* 2019), soft tissue filling (Buday and Ozturk 2019), associated with colistin and silver nanoparticles to treat infected burns in animal model (Wali *et al* 2022), among others. In addition, its application as a scaffold

for cells (Dragúňová *et al* 2019), as drug-delivery systems (Mencucci *et al* 2011) and organ-on-chip technology (Richardson *et al* 2020) are promising for clinical therapies.

MSCs are multipotent cell progenitors capable of differentiating into different lineages and mesodermal phenotypes, as first proposed by Caplan (1991). They play many roles in the body and participate in many signaling systems and pathways. In addition, MSCs can be isolated from various sources, both embryonic and adult tissues, such as adipose tissue, bone marrow, dermis, esclerocorneal limbus, umbilical cord, endometrium, dental pulp, blood peripheral, synovial fluid, and muscles (Iser *et al* 2014, Naasani *et al* 2017, Berebichez-Fridman and Montero-Olvera 2018). Among these tissues, adipose tissue is a good strategy for tissue engineering applications due to its abundance (Naasani *et al* 2019).

MSCs associated with decellularized hAM (DhAM) have been studied in several *in vitro* and *in vivo* models. *In vitro* studies showed that DhAM can be used to grow MSCs derived from human tissues as from dental pulp, iliac crest bone-marrow (Echarte *et al* 2022) human adipose tissue-derived stem cells (Gholipourmalekabadi *et al* 2016, Naasani *et al* 2019, Hadipour *et al* 2021), sclerocorneal limbus (Naasani *et al* 2018) and epithelial cells (Dragúňová *et al* 2019). The DhAM was combined with bone marrow MSCs to investigate the wound healing effects in full thickness skin defects in rabbits (Kim *et al* 2009). Recently, the DhAM associated with MSCs derived from adipose tissue and placenta was tested *in vivo* in an excisional wound model showing superior regenerative effects when compared to control groups (Aghayan *et al* 2022).

A great advantage of MCS for cell therapy is their immunosuppressive property. Indeed, MSCs are hypoimmunogenic and unable to express costimulatory molecules (Naji *et al* 2019, Casagrande Paim and Wink 2022). Our group observed that dermal MSCs release high concentrations of adenosine, due to the CD73 (ecto-5'-nucleotidase) expression, one of the MSCs markers. Its main function is to convert extracellular adenosine triphosphate (ATP) to the immunosuppressive adenosine in normal tissues, limiting the excessive immune response (Jin *et al* 2010, Naasani *et al* 2017, Roszek and Wujak 2018, Roh *et al* 2020). Although the role of extracellular adenosine generated by CD73 on tumor cells is sufficient to mediate immune evasion, facilitating tumor growth and

metastasis (Jin et al 2010, Stagg et al 2010, Naasani et al 2017, Roszek and Wujak 2018, Roh et al 2020), the involvement of the CD73 enzyme on the wound healing process remains unclear.

Therefore, the objective of this study was to associate the DhAM with adipose derived MSCs (AD-MSCs), as a therapy strategy to treat second-degree burns on a preclinical model with detailed and long term follow up of wound healing process. As a novelty we also investigated the expression of the CD73 in the unburned and burned skin regions.

2. Materials and methods

2.1. Human amniotic membrane (hAM)

2.1.1. Isolation

For the hAM isolation, we used fresh placentas from cesarean surgery of patients between 18 and 40 years old, after the application of the Informed consent form, approved by the Human Research Ethics Committee (REC-ISCMPA N° 3.029.141) from the Irmandade Santa Casa de Misericórdia de Porto Alegre (RS, Brazil). Patients who were donated the amniotic membrane had no history of obstetric complications, an undergone prenatal care, and obtained non-reactive results for serological tests for HIV, Hepatitis B and C, Syphilis, Chagas disease, Toxoplasmosis (IgM antibodies) and cytomegalovirus (IgM antibodies), with no symptoms in the neonate, maternal fever above 38 °C, gestation less than 34 weeks, rupture of fetal membranes for more than 12 h before delivery and duration of delivery longer than 24 h. The procedures were conducted according to the ethical principles expressed in the Declaration of Helsinki.

2.1.2. Preservation

Under sterile aseptic conditions, the hAM was mechanically separated from the chorion and it was washed with sterile saline solution with antibiotics (penicillin 1.000 000 U l⁻¹ and streptomycin 1 g l⁻¹). The hAM fragments were preserved in 85% glycerol solution at 4 °C until the decellularization process.

2.1.3. Decellularization

The hAM was decellularized as we described previously. Briefly, an enzymatic method using 0.05% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA) during 20 min at 37 °C was added on hAM fragments and the solution was inactivated with Dulbecco's Modified Eagle's Medium (DMEM) low glucose (Sigma), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, N.Y., USA) and 1% penicillin/streptomycin.

2.2. Animals

2.2.1. Ethical aspects

All procedures rigorously followed the guidelines for animal experimentation proposed by the National

Council for the Control of Animal Experimentation (CONCEA), mainly the 'Procedures—Rodents and Lagomorphs maintained in facilities of teaching or scientific research institutions' (Resolução Normativa N° 33) and the Practice of Euthanasia of the CONCEA (Resolução Normativa N° 13). Eight weeks old female and male Wistar Rats (*Rattus norvegicus*), weighing between 275 and 300 g, were used. The procedures were approved by the Animal Use Ethic Committee of the Federal University of Health Sciences of Porto Alegre (CEUA-UFCSPA N° 539/17) and developed at the bioterium of this institution.

2.3. Cell isolation and culture of AD-MSCs

The rat AD-MSCs were isolated from bilateral inguinal regions of Wistar rats. Three animals were used for cell isolation. The animals were euthanized with a dose three times greater than the dose required for general anesthesia of Ketamine and Xylazine, 90 mg kg⁻¹ and 10 mg kg⁻¹ respectively, intraperitoneally. At this dose, the drug acts quickly and is an acceptable form of euthanasia. The adipose tissue was washed with phosphate-buffered saline (PBS) and the fat fragments were digested with collagenase solution (Type I, 1 mg ml⁻¹ solubilized in DMEM) and incubated in a water bath (37 °C) for 30 min with agitation every 10 min. The collagenase solution was inactivated by DMEM 10% FBS. The cells suspension was centrifuged at 600 × g for 10 min and resuspended in an erythrocyte lysis solution (150 mM NH₄Cl, 10 mM NaHCO₃, and 1 mM EDTA) diluted in ultrapure water 1:1 (v:v). The cell viability was assessed by 0.4% trypan blue (Bio-Rad, Hercules, CA, USA) exclusion method. Then, the cells were seeded in 25 cm² cell culture flasks with DMEM supplemented with 10% FBS (1000 mg l⁻¹ glucose) and 1% penicillin/streptomycin and maintained at 37 °C, 5% CO₂, and 95% humidity to reach confluence. Medium was changed every second day and AD-MSCs were passed five times before experimental use.

2.4. Characterization of AD-MSCs

For multilineage differentiation, 1 × 10⁴ cells cm⁻² AD-MSCs were seeded on 12-well plates. For adipogenic, osteogenic, and chondrogenic differentiation we used the StemPro[®] Adipocyte, Osteogenesis, and Chondrocyte Differentiation Basal Medium (Gibco), respectively, following the manufacturer instructions. The culture was fixed with 4% formaldehyde solution for 30 min and stained with Oil Red O in deionized water (1:3) for adipocytes, 2% Alizarin Red S solution (pH 4.2) for osteocytes, and 1% Alcian Blue staining, prepared in 0.1 N HCl for chondrocytes. Stained induced cultures were compared between the respective controls without the differentiation medium. Micrographs were performed using a BX-50 Olympus microscope with optical lens (10X/0.30

Ph1-UplanFI), coupled to a camera Motican 2500 (Olympus, Hamburg, Germany).

2.4.1. Flow cytometry

The AD-MSCs surface antigen expressions were detected by flow cytometry. The cells were incubated with phycoerythrin (PE) anti-CD29 (Molecular Probes), PE anti-CD45 (Invitrogen), PerCP-Cy5.5 anti-CD90 (Molecular Probes) and CD73 (Ectonucleotidases-ab, Canada). The cytometer was equipped with a 488 nm argon laser, with the filters 527/32 nm (FITC), 585/42 nm (PE) and 700/54 nm (Cy5.5) (Becton-Dickinson, San Diego, CA, USA), and CellQuest software. At least 10,000 events were collected and data were evaluated using Flowing Software 2 (Perttu Terho, Finland).

2.4.2. Actin filaments staining

In order to observe the cytoskeleton organization of AD-MSCs, phalloidin staining was performed. 10×10^3 cells cm^{-2} AD-MSCs were seeded on glass coverslips and maintained for 48 h with a standard medium under an appropriate atmosphere. The culture was fixed with a 4% paraformaldehyde solution in PBS for 30 min at 4 °C and permeabilized with 0.1% Triton X-100 in PBS for 1 h. Actin filaments were stained with fluorescein isothiocyanate (FITC)-Phalloidin (Sigma) and cells were also stained with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for the identification of nuclei (40 min, 4 °C, dark room). Images were obtained with a fluorescence microscope (Olympus IX51/Olympus U-RFL-T) coupled with 460–490, and 372 nm band-pass excitation filters, respectively.

2.5. Bioscaffold

The 4 cm^2 approximately fragments of DhAM were placed on nitrocellulose filter paper on 60 mm Petri dishes and hydrated overnight at 37 °C with culture medium before seeding cells. 5×10^4 cells cm^{-2} were seeded on each DhAM in order to form a monolayer on the membrane and were cultured with a standard culture medium for MSCs for 24 h prior to the surgical procedure.

2.6. Burn surgery model

The apparatus used to perform the burn model was kindly provided by the plastic surgeon and researcher Dr Neil Grant Venter (Venter *et al* 2015). The aluminum cylindrical head (ACH) of 23 mm diameter allows a deep second-degree burn when heated to 70 °C and in contact with the animal skin for 10 s.

Initially, the animals were anesthetized with 90 mg kg^{-1} Ketamine and 10 mg kg^{-1} Xylazine and the anesthesia was confirmed with a negative test for muscle responsiveness. For depilation, a hair remover machine was used in association with a depilatory cream commercially available (VEET®). Then, a new test for muscle responsiveness was performed before

the dorsal side burn was made. To perform the burn, the animal back was laterally supported on a rigid surface, to allow a homogeneous burn, and the ACH was put in contact with the skin for 10 s. Running water was applied just in the lesion area for 3 min before the respective treatments and standardized photographs were taken as time 0.

2.7. Treatments and wound area measure

The animals were divided into four groups: control, which consists of a non-adherent gauze; a polyurethane synthetic commercial dressing as a positive control (Control+); DhAM; and DhAM plus rat AD-MSCs (DhAM + AD-MSCs), which were maintained for 1, 2 and 5 weeks. We used five animals per group, for four treatments and three times, a total of 60 animals ($N = 60$, $n = 5$). For the DhAM and DhAM + AD-MSCs, the membrane was grafted directly to the injured area to allow the cells to be in contact with the burn, and the nitrocellulose paper, which helped to carry the membrane in a uniform way, was removed at the treatment time.

After the burn, the animal received the treatment assigned to its group and a non-adherent gauze was placed just above. Above this, another bandage with non-adherent gauze was made around the entire animal back and covered with medical adhesive tape, to avoid the removal of the treatment still allowing air exchange and humidity maintenance. During the postoperative period, the animals received 5 mg kg^{-1} Tramadol hydrochloride, an analgesic drug, every 12 h for 48 h. The animals were allocated two by two to reduce stress in appropriate cages, with free access to standard laboratory chow and water *ad libitum*. The rooms where the animals were allocated had programmed light and dark cycles (12 h light and 12 h dark cycle) and controlled conditions of temperature and humidity. The Rat Grimace Scale was used to monitor pain in animals throughout the treatment time.

The treatments were maintained for 1, 2 and 5 weeks and in these times the injuries were photographed. Each group was euthanized at their respective week with cardiac exsanguination preceded by general anesthesia (90 mg kg^{-1} Ketamine and 10 mg kg^{-1} Xylazine).

A skin fragment containing unburned and burned skin regions from approximately 2.25 cm^2 was removed for histological analysis. The unburned skin, the burn margin (transition region between the unburned skin and the burn) and the middle region of the burned skin were analyzed microscopically.

The wound area was analyzed in ImageJ software and reported as the percentage of initial wound area in day zero (D0) for each animal and treatment.

2.8. Biochemical parameters

Blood samples collected with cardiac puncture were centrifuged and the enzymes alanine transaminase

(ALT) and aspartate transaminase (AST) were measured according to the manufacturer's protocol (Bioclin), in the serum.

2.9. Histological analysis

The histological skin and organ sections were stained with hematoxylin-eosin (HE). The skin sections were also stained with Azan trichrome stain. Briefly, the samples were deparaffinized in xylene and hydrated with decreasing concentrations of ethyl alcohol (100%, 70%, and 60%) and water for 5 min. For the HE coloration, they were stained with hematoxylin (Merk, Germany) followed by eosin solutions. Ending with increasing concentrations of ethyl alcohol, and xylene for 5 min. For the Azan trichrome stain, the samples were stained with Azocarmine followed by phosphotungstic acid. Then, Aniline Blue-Orange G was used, finishing with dehydration in 100% ethyl alcohol and clarifying with xylol. Histological micrographs were performed using the EVOS FL Auto 2 Cell Imaging System (Invitrogen). In the skins, the unburned skin, the burn margin (transition region between the unburned skin and the burn) and the middle region of the burned skin were identified, and at least four images were taken from each region. Regarding the organs, at least four images of each tissue were taken at random, with magnifications of 10 $\times$, 20 $\times$ and 40 $\times$.

2.10. Immunofluorescence analysis

The skins with the unburned and burned regions were embedded in Tissue-Tek[®] Optimal Cutting Temperature Compound (Sakura Finetek USA), frozen in liquid nitrogen and maintained at -80°C for cryostat sectioning. Indirect immunofluorescence assays were performed on 6 μm -thick cryosections of skins following permeabilization with acetone (10 min at -20°C). The following primary antibodies were used: rabbit monoclonal anti-Cytokeratin 10 (CK10) (NOVUS); mouse monoclonal anti-CD11b (Invitrogen) and Polyclonal rabbit anti-human ecto-5'-Nucleotidase/CD73 antibodies (Ectonucleotidases-ab, Canada). The tissue sections were incubated with the primary antibody for 1.5 h at room temperature. After PBS washes the respective secondary antibody was added and incubated for 30 min in a humid chamber. The following secondary antibodies were used: goat anti-rabbit IgG (H + L) Alexa Fluor 488 (Molecular probes) and goat anti-mouse IgG (H + L) Alexa Fluor 488 (Sigma). The cell nuclei were counterstained with DAPI (Molecular Probes), ending with Fluoromount-G mounting medium (SouthernBiotech). Fluorescent micrographs were observed and photographed under an EVOS FL Auto 2 Cell Imaging System (Invitrogen). The CD73, CK10 and CD11b relative fluorescence intensity was quantified as described by Jensen (2013). We adjusted the threshold to select the fluorescence regions and the intensity measurements were performed within

regions of interest (ROIs). The relative fluorescence intensity was calculated as the integrated density (IntDen) by each total ROI area in μm^2 .

2.11. Statistical analysis

Data were analyzed statistically by Two-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Statistically significant differences were accepted when $p < 0.05$.

3. Results

3.1. Isolation and characterization of rat AD-MSCs

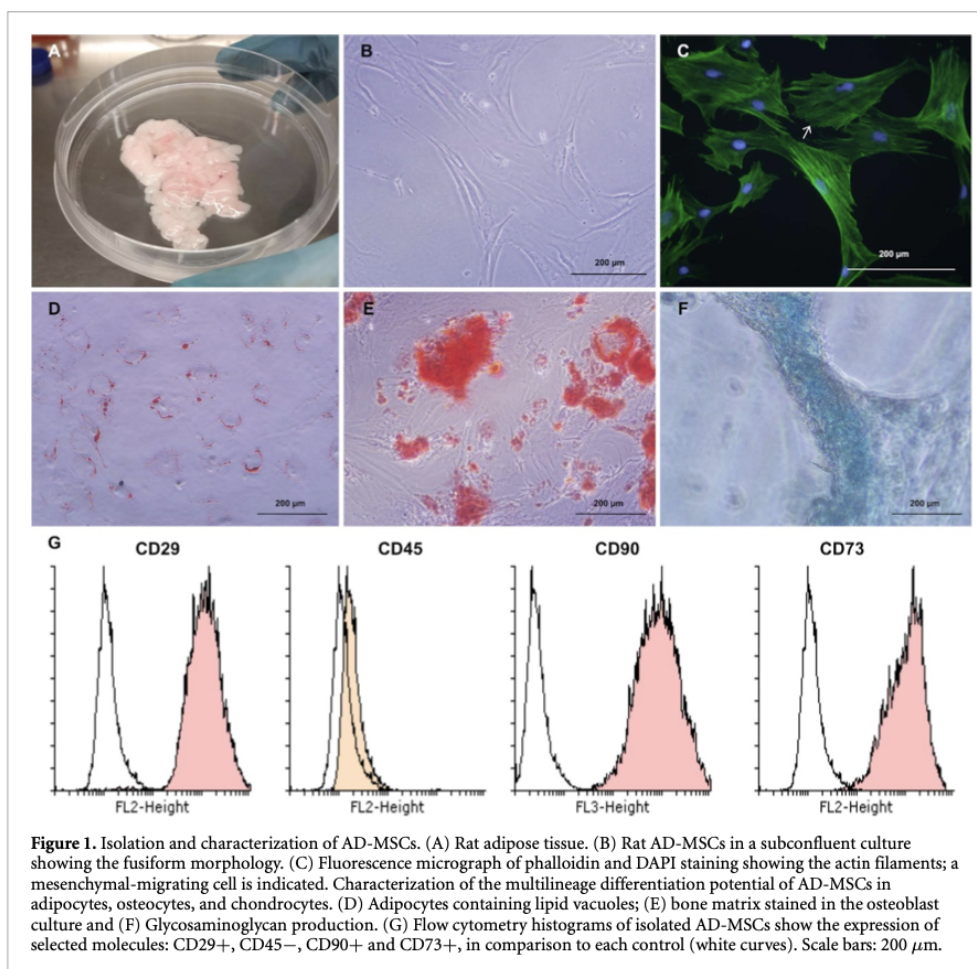
We isolated a population of AD-MSCs from rat adipose tissue (figure 1(A)) with fusiform morphology, plastic adherence, self-renewal, and trilineage differentiation potential. Cells showed the filamentous actin stress fibers, typical of mesenchymal-migrating cells (figure 1(B)). After the treatment with the differentiation media, cells differentiated into adipocytes, osteoblasts, and chondrocytes showing intracellular lipid vacuoles stained with oil red, a calcium-rich mineralized matrix stained with alizarin red S and the synthesis of proteoglycans stained with alcian blue, respectively (figures 1(D)–(F)). The analysis of surface markers indicated that the cell population isolated from rat adipose tissue was positive for mesenchymal markers (CD29, CD90, CD73) and negative for a hematopoietic marker (CD45).

3.2. Isolation of hAM and bioscaffold preparation

A large amount of hAM was successfully isolated from fresh human placenta. The amnion, a transparent membrane, was effectively separated manually from the chorion, which is highly vascularized (figures 2(A)–(D)). The hAM can be preserved until the decellularization process to be used as a scaffold (figures 2(E)–(H)). To avoid membrane contraction when performing the graft, we prepare the DhAM as a scaffold on a nitrocellulose filter. To perform the bioscaffold, previously characterized AD-MSCs were seeded on the DhAM in order to form a monolayer (figures 2(I) and (J)). Therefore, it is important to keep the side to be transplanted and allow cells to make contact with the lesion.

3.3. Burn model and treatments

Deep second-degree burns on the dorsal part of Wistar rats were performed with the burn apparatus. At day zero of burn, it was possible to identify the homogeneous burned area with visible margins (figures 3(A) and (B)). To study the effect of the bioscaffold as treatment for deep second-degree burns, we used a non-adherent gauze as Control; a synthetic commercial dressing as positive control (Control+); DhAM; and the bioscaffold: DhAM associated with rat AD-MSCs (DhAM + AD-MSCs). The DhAM and the DhAM + AD-MSCs were effectively transplanted



to the area of the injury, without contractions, allowing the cells from the bioscaffold to be in contact with the burn (figures 3(C)–(F)).

3.4. Macroscopic evaluation of wound healing and safety of the treatment

To evaluate the effect of the different treatments macroscopically and histologically during 1, 2, and 5 weeks, each animal was analyzed independently at the end of their respective treatment week. The burn at the first week showed a white color, with a pink border. In the Control group with non-adherent gauze, was observed, from the first week, the process of formation of a layer of dry necrosis. In the Control+ group, this drying does not occur, we observed a more advanced autolytic removal of necrotic tissue with centripetal scar reduction. In the DhAM group, a smaller amount of non-viable tissue (pearly white) was observed, since the first week. The greater speed of debridement of both intervention groups (compared to the control groups)

and the consequent final scar of smaller dimensions was notorious. Specifically, in relation to the DhAM + AD-MSCs group, there is an apparent greater capillary density and a better visual aspect of the scar at the fifth week (figures 4(A)–(D3)). The wound closure was measured at each time point (1, 2, and 5 weeks) and the main differences, both in granulation tissue and in wound closure, were observed with DhAM + AD-MSCs treatment at the first week in comparison with the control ($p = 0.0216$) and positive control ($p = 0.0387$) and at two and five weeks vs the control treatment ($p = 0.0459$; $p = 0.0403$) (figure 4 (E)).

The wound closure was measured at each time point (1, 2, and 5 weeks). The differences in granulation tissue and wound closure were observed with DhAM + AD-MSCs treatment at the first week in comparison with the control and positive control and at 2 and 5 weeks with the control treatment (figure 4 (E)). To evaluate the effect of the different treatments macroscopically and histologically during

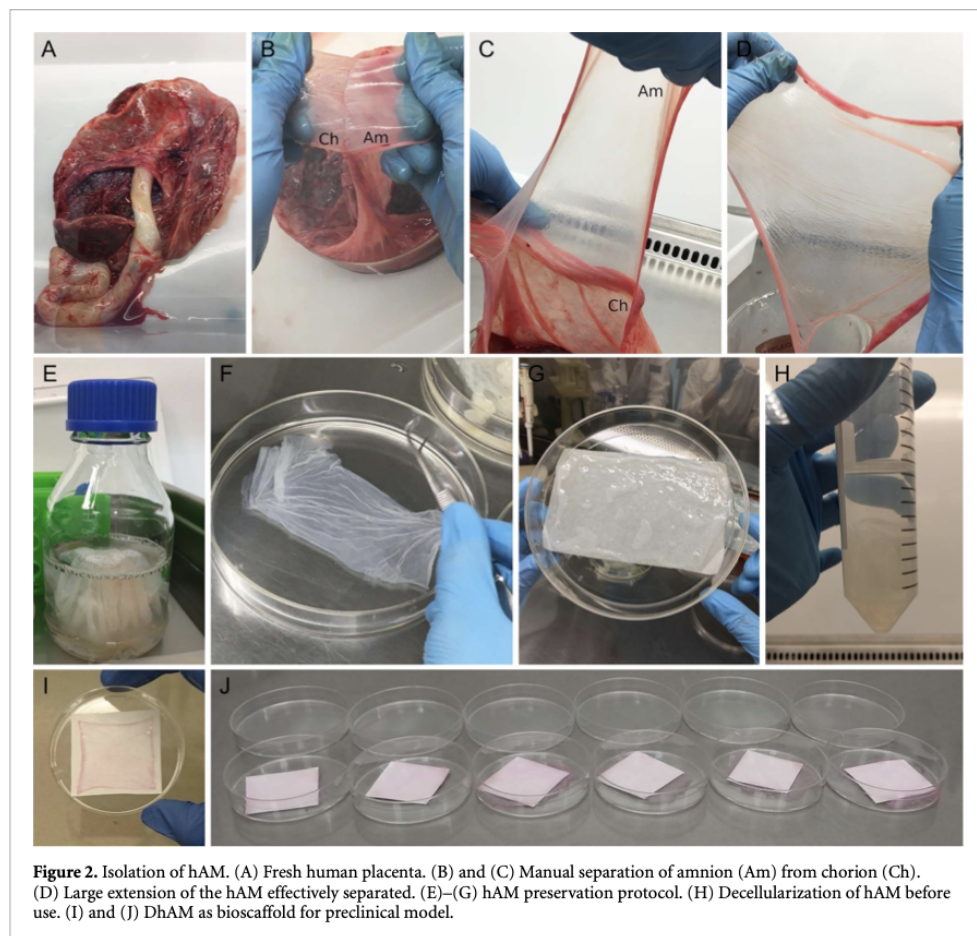


Figure 2. Isolation of hAM. (A) Fresh human placenta. (B) and (C) Manual separation of amnion (Am) from chorion (Ch). (D) Large extension of the hAM effectively separated. (E)–(G) hAM preservation protocol. (H) Decellularization of hAM before use. (I) and (J) DhAM as bioscaffold for preclinical model.

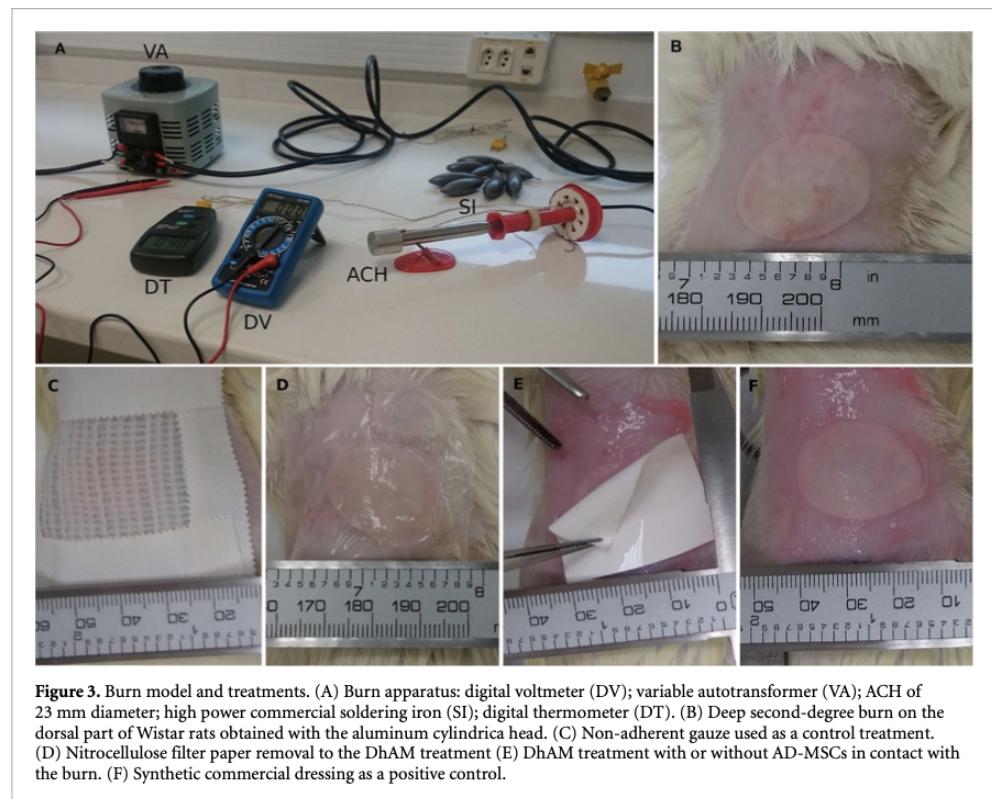
1, 2, and 5 weeks, each animal was analyzed independently at the end of their respective treatment week. The granulation tissue at the first week shows a white color, with a pink border, with more pink and red regions at the second week (figures 4(A)–(D2)). At 5 weeks a burn scar tissue was observed (figures 4(A3)–(D3)). Additionally, no inflammatory infiltrates were observed in the organs of all groups and there was no significant difference in the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (figure 5).

3.5. Histological analysis

Animals exposed to 70 °C contact temperature created a deep second-degree burn, where not only the epidermis, but also the deep layer of the dermis were affected. The histological section showed the difference between unburned skin and burned skin, by the presence of burned hair follicles and burned sebaceous glands on a deep burn (supplementary figure 1). The granulation tissues are shown in detail, where the differences between the unburned region, the burn margin, and the middle were

identified. At first week total necrosis of the epithelium was observed in the middle region, and the neoepidermis was observed in the burn margin. The burned hair follicles and sebaceous glands burned were noted in the burn margin and middle region (figures 6(A)–(H1)). An irregular epithelial progression was observed in the control treatment. However, a complete stratified epithelium until the stratum corneum was observed in the burn margin of the DhAM + AD-MSCs treatment at the second week (figures 6(I)–(L1)). The re-epithealization in the middle region was observed at 5 weeks postburn for the Control+, DhAM and DhAM + AD-MSCs treatments (figures 6(M)–(P1)).

Histologic changes occurring at 1, 2 and 5 weeks were also observed with Azan trichrome staining, which stains cytoplasm in pink, muscle, and nucleus in red, collagen and fibrin in blue. On unburned skins, the collagen bundles distributed along the skin surface and the collagen fibers were well organized and aligned in each bundle. In the granulation tissue (burn margin and middle region), at one week postburn, the stained image was more blurred



in comparison with the unburned skins, however, the individual bundles were still clearly visualized. Inflammatory cells were observed mainly in the control treatment. The granulation tissue with burned hair follicles and blood vessel formation were also observed (figures 7(A)–(D2)).

At 2 weeks, more fibroblast and inflammatory cells were identified, mainly in the middle region of the papillary dermis (near the epidermis) from the control treatment and the burn margin from the Control+ treatment (figures 7(E)–(H2)). At 5 weeks, compact collagen bundles and loosely packed collagen fibers with irregular arrangement were observed in the Control, Control+ and DhAM groups, while mainly collagen bundles with regular arrangement were observed with DhAM + AD-MSCs treatment (figures 7(I)–(L2)).

3.6. Cytokeratin 10 and CD11b expressions in burned skins

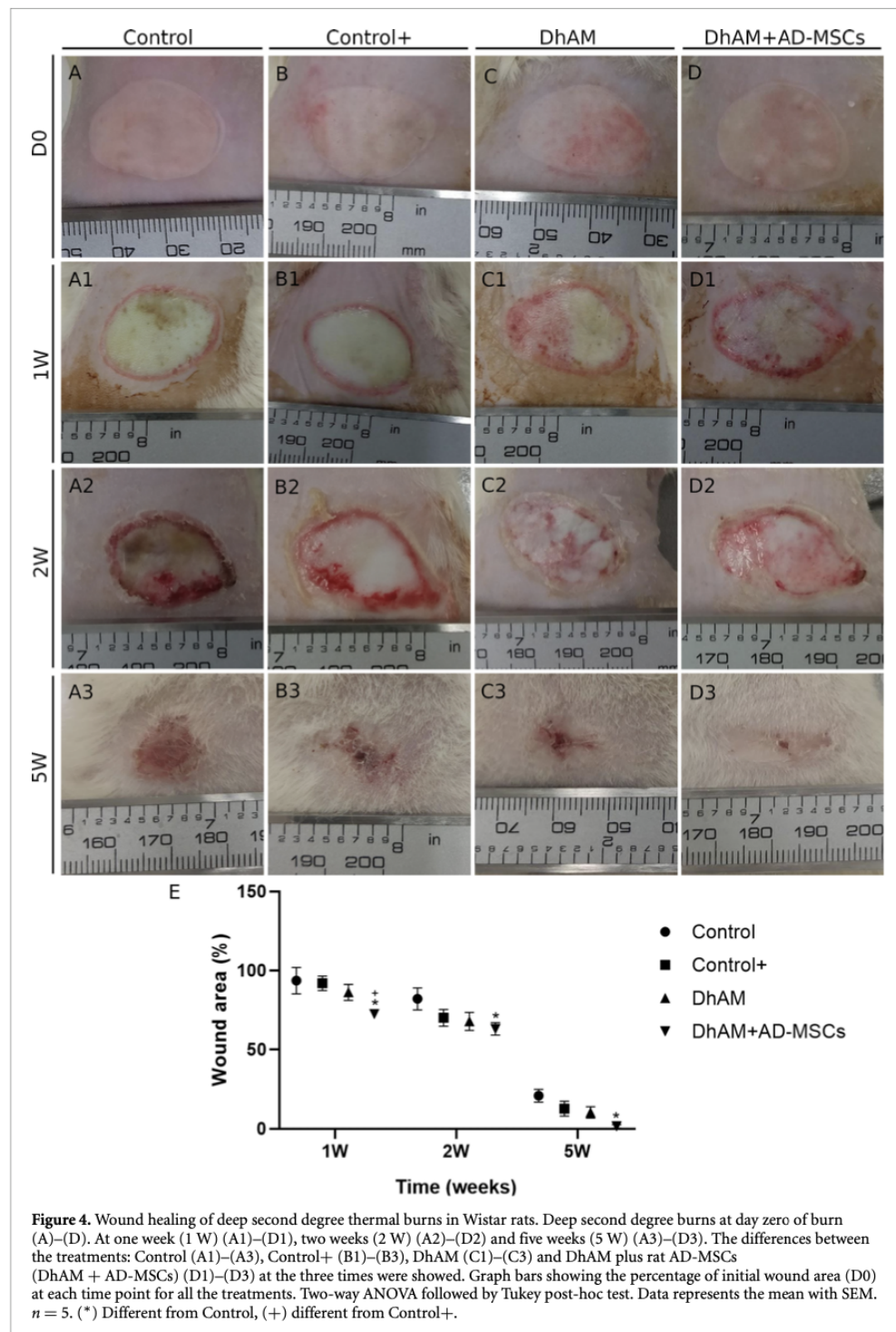
The expression of cytokeratin 10 and CD11b expressions were evaluated during wound healing in burned skins. The CK10 labeling was observed on suprabasal layers in the epidermis, as expected. In the middle region, the CK10 expression was observed in the DhAM and DhAM + AD-MSCs treatments (figures 8(A)–(H2)), showing strong and regular labeling during all weeks in DhAM + AD-MSCs treatment, where it was possible to observe a stratified

and differentiated epithelium, compared to Control and Control+ (figures 8(A)–(L2)) (table 1; Cytokeratin 10).

The inflammatory response in the granulation tissue was evaluated with CD11b labeling. A strong expression was observed in the first weeks in the Control and Control+ treatments accompanied by abundant positive cells in the papillary dermis, near the lesion, which was reduced in the treatment with DhAM + AD-MSCs (figures 9(A)–(H2)). At the fifth week we still observed a strong labeling in the granulation tissue from the Control and Control+ treatment (figures 9(I)–(L2)) (table 1; CD11b).

3.7. CD73 expression in burned skins during wound healing

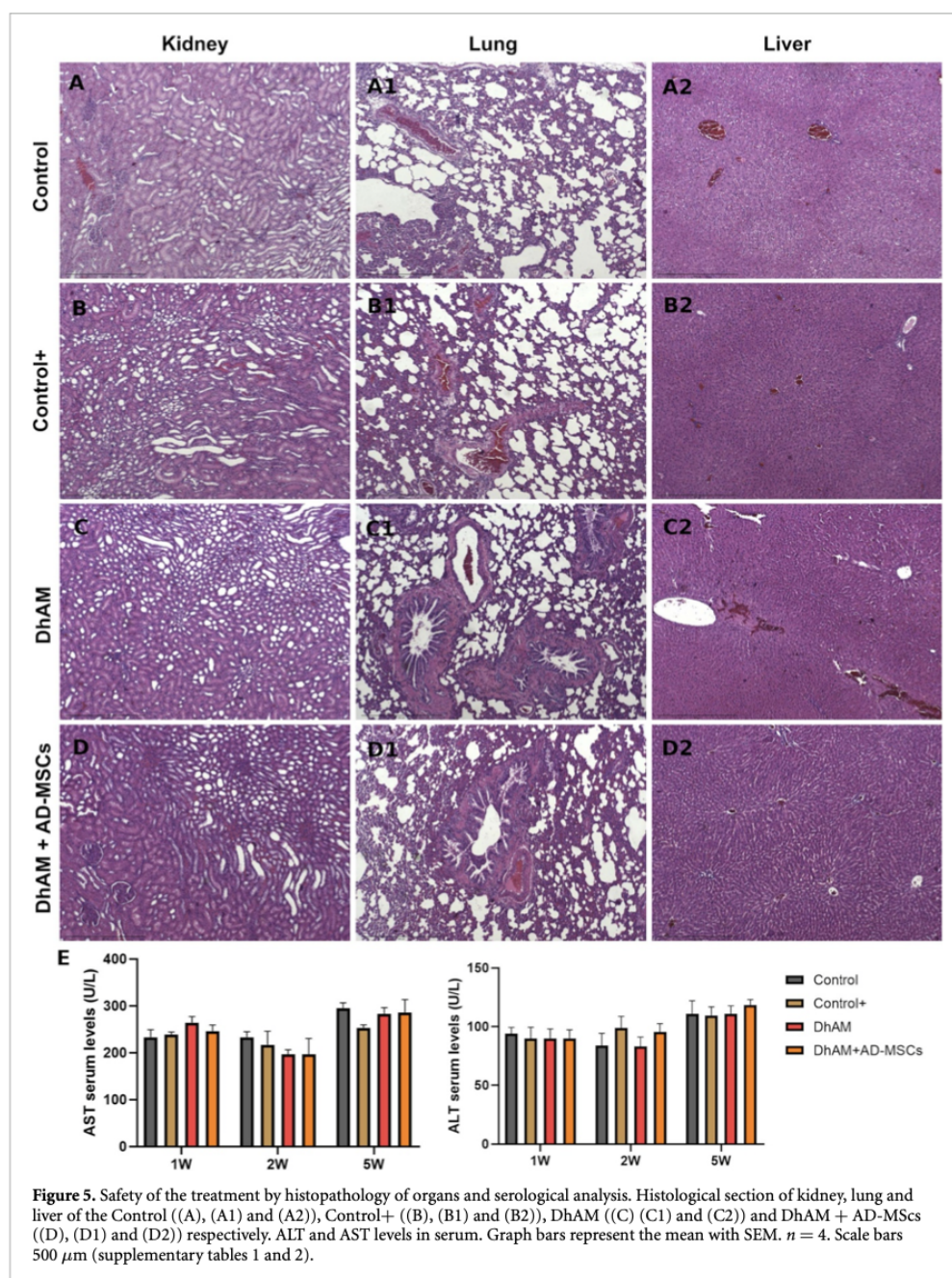
In unburned skin, the CD73 labeling was observed in keratinocytes, fibroblast, hair follicle cells, sebaceous gland epithelial cells and endothelial cells. In burn margin and middle regions, CD73 positive cells were identified mainly in epidermis, fibroblast and endothelial cells, due to the burned hair follicles and sebaceous glands. Interestingly, we observed a strong CD73 labeling in the epidermis and dermis at the first and second week postburn, which was reduced at the fifth week postburn (table 1; CD73). This strong expression was observed mainly in the papillary dermis, close to the burned epidermis, and in the neoepidermis (figures 10(A)–(L5)).



4. Discussion

Previously our research group developed a bioscaffold composed of DhAM and MSCs. The data showed that the histoarchitecture of the DhAM did not alter

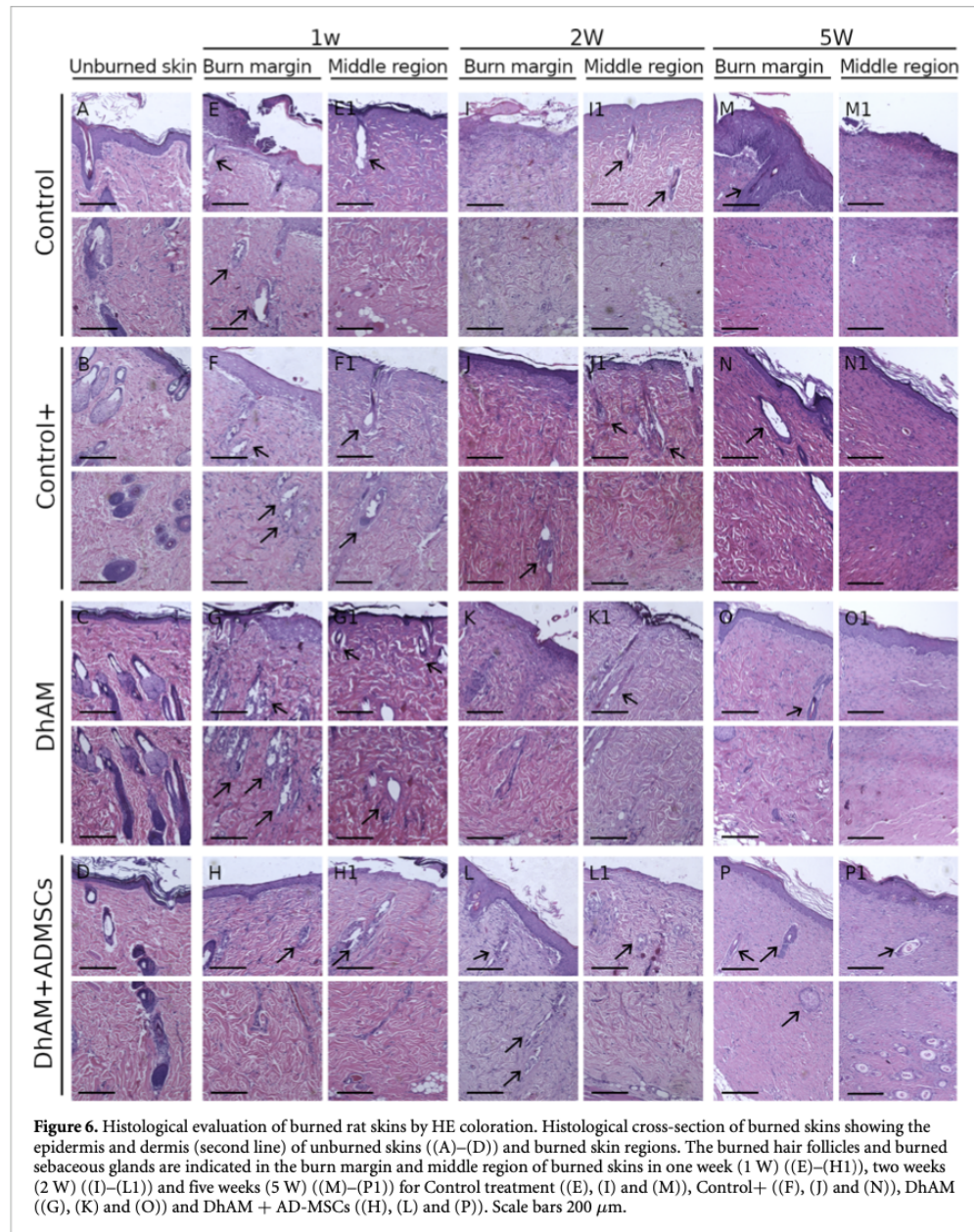
the cell adhesion, viability, nuclei morphology, actin cytoskeleton, stromal cell potential and trilineage differentiation of the MSCs (Naasani *et al* 2018, 2019). Considering these previous positive results, and the multiple benefits of MSCs and their secretome, in



the present study we evaluated the safety and efficacy of this bioscaffold in a preclinical model, aiming its future application as a treatment for burns.

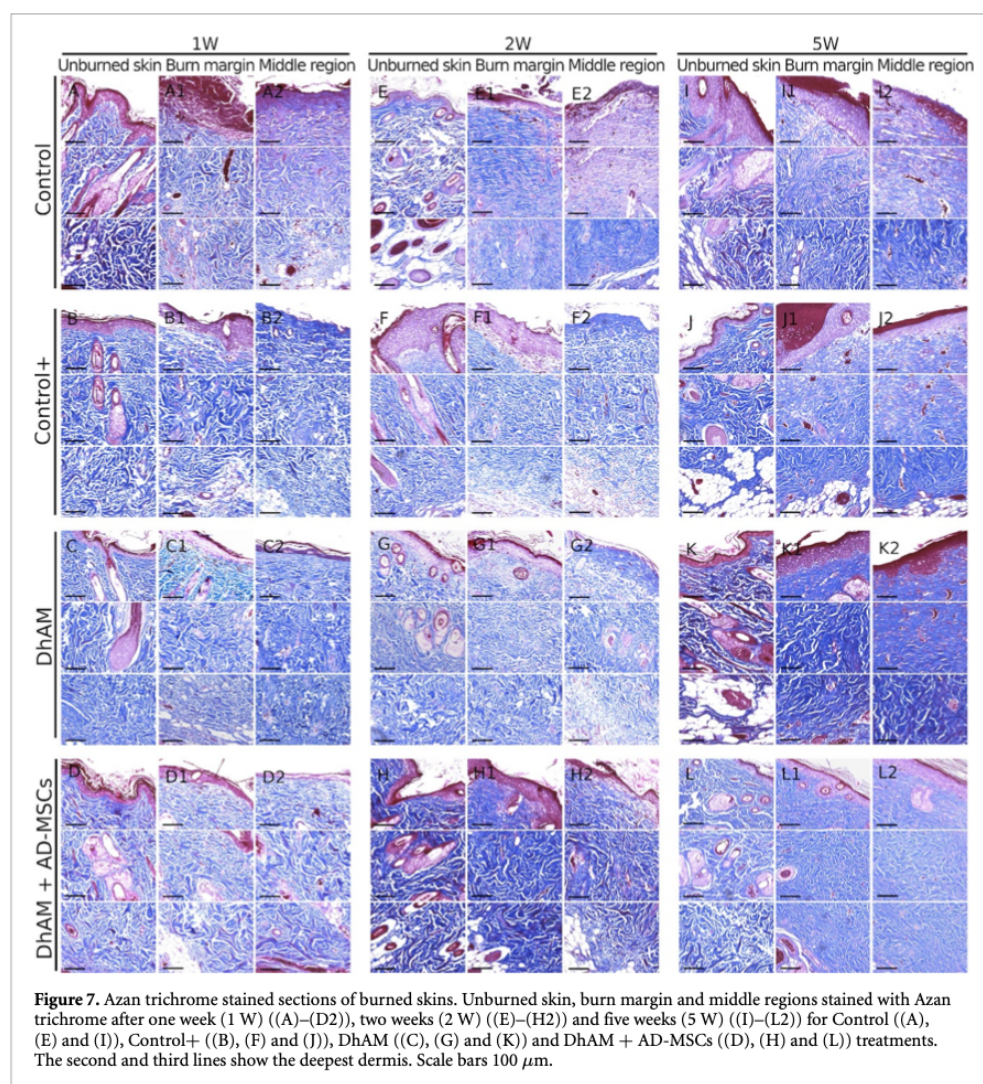
Macroscopical analysis showed that the DhAM allowed the maintenance of humidity and gas exchange. In the Control group, we observed, from the first week, the formation of a layer of dry necrosis, that culminated in a lesion with a larger diameter at the end of the fifth week (a probable but not exclusive effect of mechanical obstruction to contraction

of the edges caused due dry necrosis layer). By difficult evaporation with the addition of only a film over the lesion (Control+ group), this drying does not occur; thus, we observed a more advanced autolytic removal of necrotic tissue with centripetal scar reduction. Corroborating the hypothesis of the mechanical effect of the necrosis layer, it was observed that, from the Control+ group, the scar begins to conform to the lines of force of the skin, adopting a less circular and more longitudinal appearance. From the



intervention group with only amniotic membrane, a smaller amount of non-viable tissue (pearly white) since the first week. Even crediting this effect to a possible discrepancy in initial depth at the time of production of the burn, the greater speed of debridement of both intervention groups (compared to the control groups) and the consequent final scar of smaller dimensions is notorious. Specifically, in relation to the DhAM + AD-MSCs group, there is an apparent greater capillary density and a better visual aspect of the scar. Because they are difficult to quantify, such findings are only an inference and not an absolute conclusion.

Histologically, the main differences between the unburned and burned regions of the skin were observed by the presence of burned hair follicles and sebaceous glands, which also reached the epidermis. After the re-epithelialization, an irregular epithelial progression was observed in the control treatment, different from the treatment with the bio-scaffold containing the AD-MSCs. This observation was also confirmed by the analysis of suprabasal layers in the neoepidermis, using CK10, which indicated strong and regular labeling in DhAM + AD-MSCs treatment, showing a stratified and differentiated epithelium, compared to Control and Control+



(commercial dressing). Additionally, with the Azan trichrome stain, it was also possible to observe the differences in the collagen bundles between the unburned and burned skin regions. In the fifth week, mainly collagen bundles with regular arrangement were observed with DhAM + AD-MSCs treatment. A similar result was reported by Guo *et al* (2016) that used bone marrow MSCs seeded on small intestinal submucosa (SIS) to repair the deep partial-thickness burns. They showed that MSCs were able to improve the re-epithelialization and revascularization significantly. Also, they observed regenerated collagen bundles with a regular arrangement and extensive distribution in SIS alone and MSC-seeded SIS group, while loosely packed collagen fibers with irregular arrangement were seen in the control group (without any treatments) (Guo *et al* 2016).

Similarly with our results, Song *et al* (2017) treated full-thickness skin of 25 rats with dhAM,

demonstrating reduced fluid secretion, formation of a thinner scab and smaller wound area, resulting in a better recovery than the control (Song *et al* 2017). Milan *et al* (2020) reported the efficacy of dhAM in an *in vivo* model with balb/c mice and a clinical trial with 12 patients suffering from second-degree burn. According to the authors, the hAM enhanced re-epithelialization, granulation tissue formation, angiogenesis and healing compared to the control group (without any treatment), the repair successfully occurred 15 d post-wounding. The findings also demonstrated the clinical usefulness of hAM for the healing of partial thickness burn wounds, as no immunologic reaction or dermatitis was found during the study (Milan *et al* 2020). Recently, Wali *et al* (2022) evaluated the antibacterial activity of the DhAM combined with silver nanoparticles, as a dressing for infected burn wounds, showing an antimicrobial activity (Wali *et al* 2022).

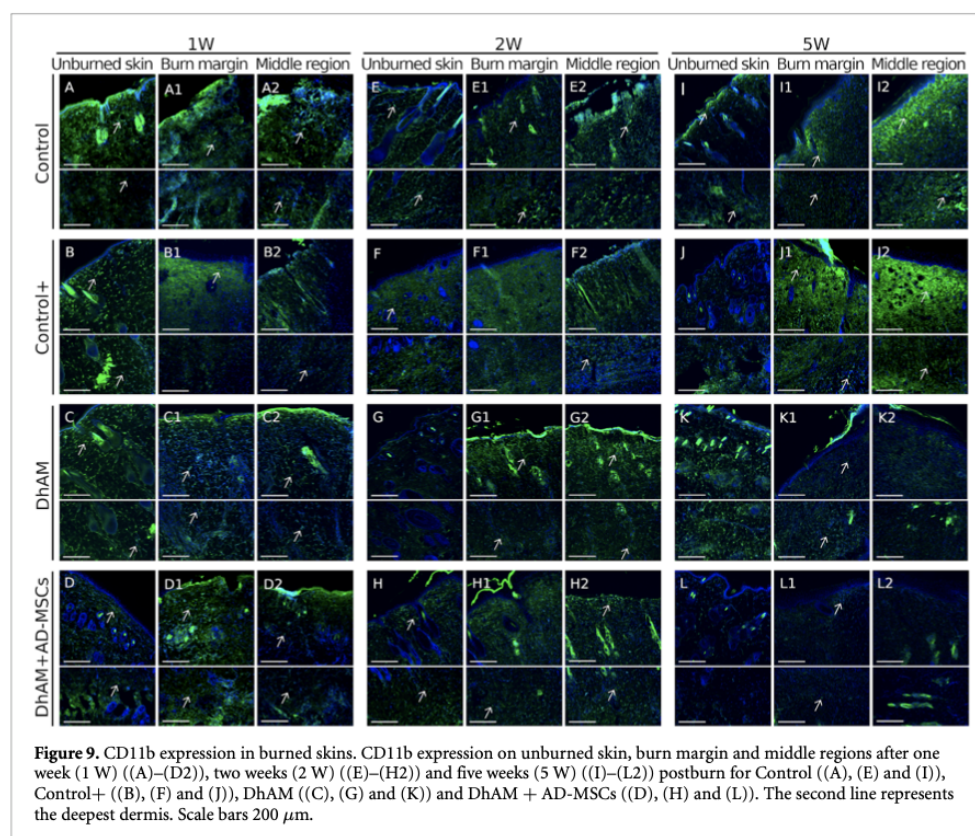
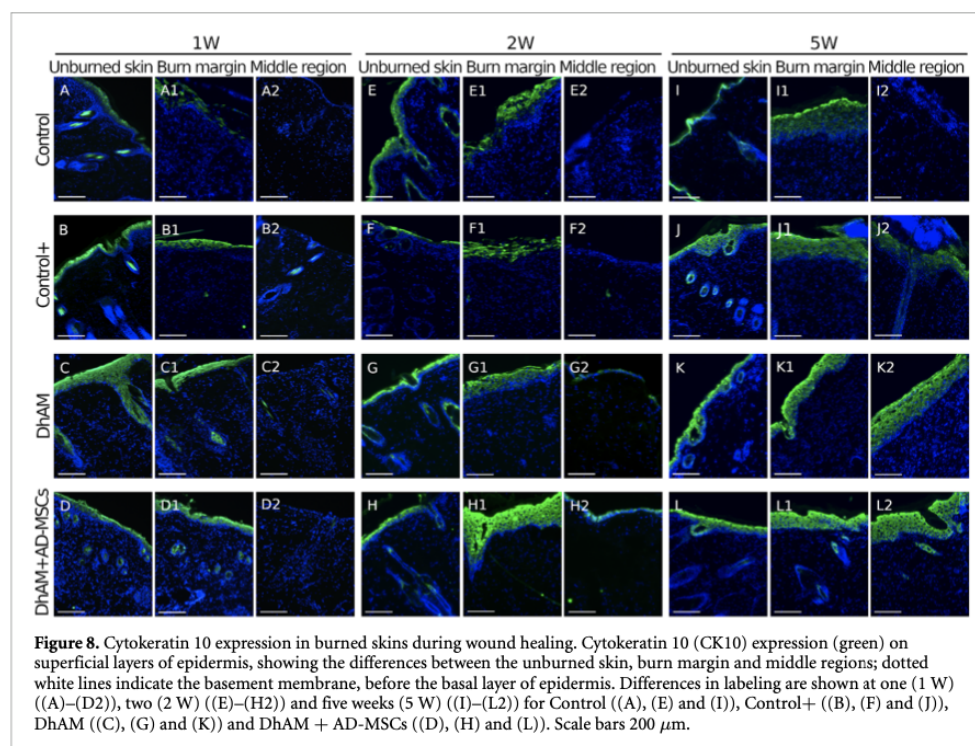


Table 1. Expression levels of CD73, Cytokeratin 10 and CD11b in burned skins during wound healing.

Antibody	Treatment	1 W			2 W			5 W		
		Unburned Skin	Burn Margin	Middle Region	Unburned Skin	Burn Margin	Middle Region	Unburned Skin	Burn Margin	Middle Region
CD73 (Epidermis)	Control	+	++	nd	++	+-	nd	+-	+-	+-
	Control+	+	+	nd	+	+-	nd	+-	+-	+-
			$*p = 0.0009$			$*p < 0.0001$				
	DhAM	+	+	nd	+	+	nd	+-	+	+-
		$*p = 0.0095$	$*p = 0.001$		$*p = 0.0007$			$*p < 0.0001$	$*p < 0.0001$	
		$\#p = 0.0074$						$\#p = 0.0016$	$\#p < 0.0001$	
CD73 (Dermis)	DhAM + AD-MSCs	++	++	+	++	+	+	+	+	+
		$*p < 0.0001$	$*p = 0.0009$	$*p < 0.0001$	$*p < 0.0001$	$*p = 0.0089$	$*p < 0.0001$	$*p < 0.0001$	$ap < 0.0001$	$*p < 0.0001$
		$\#p < 0.0001$	$\#p < 0.0001$	$\#p < 0.0001$	$\#p < 0.0001$	$\#p = 0.0363$	$\#p < 0.0001$	$\#p < 0.0001$	$\#p < 0.0001$	$\#p < 0.0001$
		$ap = 0.0014$	$ap < 0.0001$	$a < 0.0001$			$ap < 0.0001$			$ap < 0.0001$
	Control	++	+	++	+-	++	++	+-	+-	+-
	Control+	+-	+	+	+	+	+-	+-	+-	+-
Cytokeratin 10		$*p = 0.0024$				$*p = 0.0044$	$*p = 0.0112$			
	DhAM	+	+	+	+	++	+-	+-	+-	+-
							$*p = 0.0023$			
	DhAM + AD-MSCs	++	++	+-	+-	+	++	+-	+-	++
		$\#p < 0.0001$					$\#p = 0.0238$			$*p = 0.0163$
		$ap = 0.0005$					$ap = 0.0054$			$\#p = 0.0243$
CD11b										$ap = 0.0452$
	Control	+	+-	nd	+	+-	nd	+	+-	+-
	Control+	+	++	nd	+	+	nd	+	+	+
									$*p = 0.0019$	$*p = 0.0099$
	DhAM	+	++	nd	++	+	+	+	++	++
								$*p = 0.0112$	$*p < 0.0001$	$*p < 0.0001$
CD11b										$\#0.0024$
	DhAM + AD-MSCs	+	++	nd	+	++	+	+	++	++
								$*p = 0.0117$	$*p < 0.0001$	$*p < 0.0001$
										$\#p < 0.0001$
	Control	++	++	++	+-	+	+	+	+	++
	Control+	++	++	++	+	+	++	+	++	++
CD11b		$*p = 0.026$							$*p = 0.0066$	
	DhAM	+	+	++	+-	+	+	+	+-	+-
		$*p = 0.0017$	$\#p = 0.0253$	$*p = 0.0342$					$\#p = 0.0009$	$*p < 0.0001$
										$\#p < 0.0001$
	DhAM + AD-MSCs	+-	+	+	+-	+	+	+-	+-	+-
		$*p < 0.0001$	$*p = 0.0071$	$*p = 0.0012$				$\#p < 0.0001$		$*p < 0.0001$
CD11b			$\#p = 0.0029$	$\#p = 0.0113$						$\#p < 0.0001$

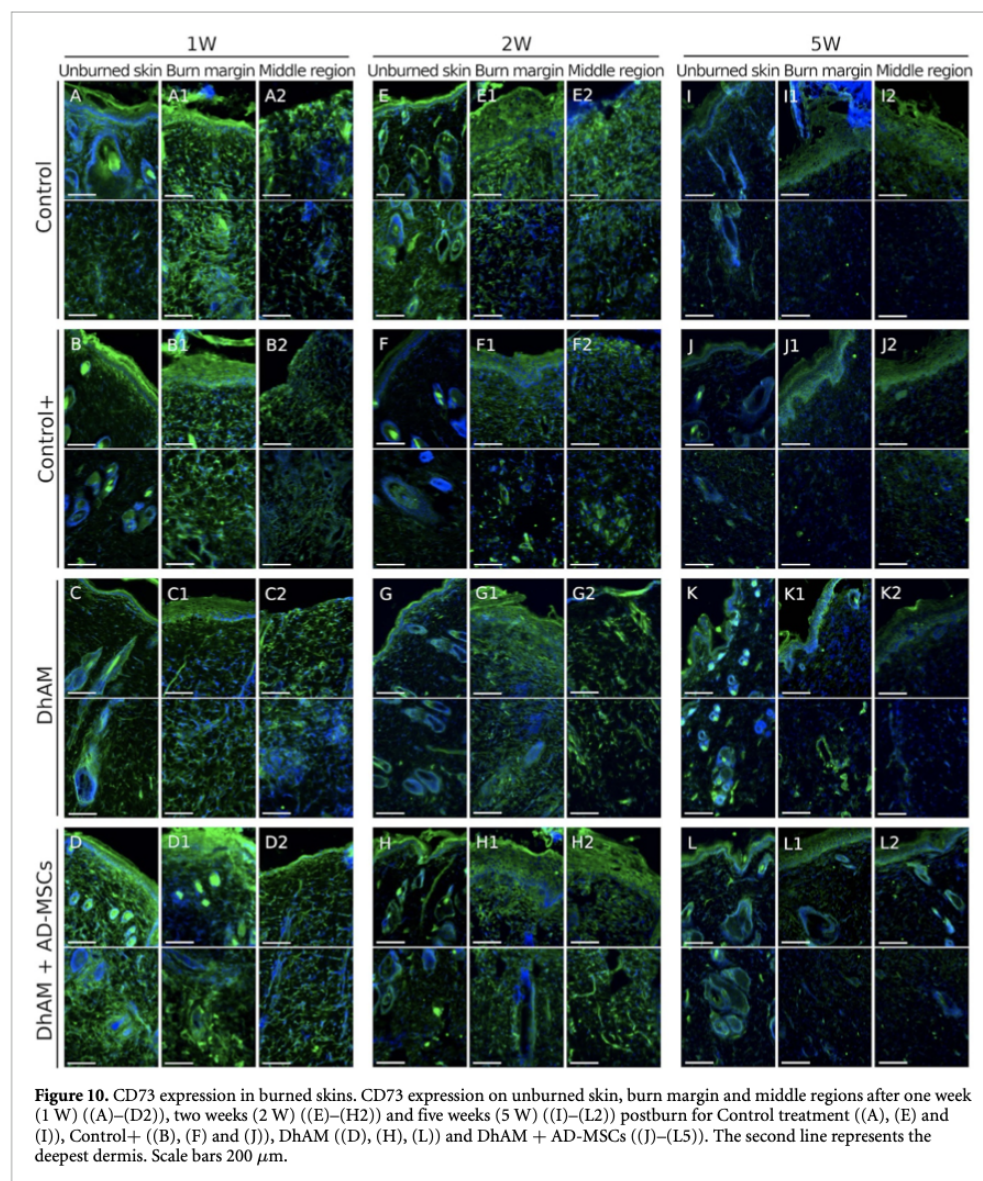
++: high expression; +: moderate expression; +-: weak expression; nd not detected; *: different from Control; #: different from Control+; a: different from DhAM.

There are few studies published in the literature that tested *in vivo* hAM in the presence of AD-MSCs. Recently, Aghayan *et al* (2022) demonstrated the feasibility, safety, and efficacy of tissue-engineered dressings composed of hAM and two different types of MSCs, human adipose-derived MSCs and placenta-derived MSCs, in the excisional wound model in rats. The results of wound closure rate, re-epithelialization, angiogenesis, and collagen remodeling demonstrated that MSC-seeded acellular AMs had superior regenerative effects in excisional wound animal model, in comparison with the control groups, showing better healing effects with the placenta-derived MSCs group (Aghayan *et al* 2022).

MSCs have immunomodulatory properties (Meisel *et al* 2004, Dabrowska *et al* 2020, Müller *et al* 2021) and reveal their immunomodulatory potential via functional changes of monocytes/macrophages (Németh *et al* 2009), T cells (Ge *et al* 2010, Obermajer *et al* 2014), B cells (Luz-Crawford *et al* 2016, Luk *et al* 2017), dendritic cells (Jiang *et al* 2005) and natural killer cells (Spaggiari *et al* 2008). This may be related to the fact that MSCs release high concentrations of

the immunosuppressive adenosine, due to the CD73 expression, limiting the excessive immune response (de Oliveira Bravo *et al* 2016, Naasani *et al* 2017, Roszek and Wujak 2018, Roh *et al* 2020, Galgaro *et al* 2021). In addition to adenosine, MSCs contribute to granulation tissue through their trophic, paracrine and immunomodulatory functions by secretion of growth factors, proangiogenic factors, cytokines and chemokines, such as transforming growth factor beta (TGF- β), insulin-like growth factor-1, prostaglandin E2, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor, basic fibroblast growth factor-2, vascular endothelial growth factor (VEGF), angiopoietin-1, stromal cell-derived factor-1, erythropoietin, monocyte chemoattractant protein-1 and exosomes, reviewed in (Jiang and Scharffetter-Kochanek 2020). Wang *et al* (2019) were the first to report that MSCs coated by the extracellular matrix could promote wound healing in diabetic rats and to enhance the expression of growth factors VEGF, PDGF, and EGF (Wang *et al* 2019).

In order to investigate the mechanisms underlying the effect of MSCs in wound healing, as a novelty,



we analyzed the expression of the CD73 during the wound healing process. We observed a high expression of this protein in the first 2 weeks postburn, mainly in the granulation tissue. We observed a high expression, with the treatment with DhAM + AD-MSCs. In the fifth week, a reduction in CD73 labeling was observed in the burned regions. Further, more inflammatory cells were identified in the Control and Control+ treatments. The inflammation was assessed using the CD11b-antibody, expressed on the surface of monocytes, macrophages, and granulocytes. This was demonstrated with CD11b labeling. A strong expression was observed in the first weeks in the Control and Control+ treatments accompanied by abundant CD11b+ immune cells in the papillary

dermis, near the lesion, which was reduced in the treatment with DhAM + AD-MSCs.

Shaked *et al* (2007) analyzed the burn blister fluid from 11 patients who suffered a second degree burn injury, and reported for the first time an increased concentration of adenosine in burn blister fluid. The initial host response to burns is an inflammatory reaction, whose main purpose is to protect the body against microbial invasion through damaged tissues. The presence of adenosine in the blister fluid could explain some of the important features of the pathophysiology response to the burn, since adenosine is a potent anti-inflammatory agent (Shaked *et al* 2007). Adenosine can trigger different cellular responses, aimed to restore tissue homeostasis, such as, limit

inflammatory and immune responses, to prevent tissue damage and promote the healing process. In fact, adenosine acts as an immunosuppressive molecule, capable of inhibiting the functions of different cell populations and subsets of the immune system, including T and B lymphocytes, NK cells, dendritic cells, monocytes and macrophages, reviewed in (Morandi *et al* 2018).

The extracellular adenosine is produced from the adenosine monophosphate (AMP) hydrolysis by the enzymatic activity of CD73 purinergic enzyme (ecto-5'-nucleotidase). At physiological conditions, CD73 is expressed by stromal cells, follicular dendritic cells, and endothelial cells (Antonioli *et al* 2016), also by variable proportions of adaptive immune cells (B cells and some T-cell subsets) (Thomson *et al* 1990, Bono *et al* 2015). At hypoxic conditions and several inflammatory mediators, CD73 expression and function is increased (Antonioli *et al* 2016). After a tissue damage, as a burn, ATP is released to the extracellular environment and can be degraded by ectoenzymes chain, to adenosine or act on different purinergic receptors (Burnstock 2007, Abbracchio *et al* 2009). The balance between ATP and adenosine is crucial to prevent uncontrolled tissue damage due to excessive inflammatory responses (Roh *et al* 2020). While ATP mediates inflammatory responses through their P2 purinergic receptors, it is rapidly hydrolyzed by the enzymatic cascade via CD39 (NTPDase1) and CD73, to generate adenosine that acts as an anti-inflammatory mediator to downregulate the immune cell function. Therefore, CD73, by degrading extracellular AMP to adenosine, has a critical role for this pathway (Roh *et al* 2020).

5. Conclusion

We have demonstrated the safety and efficacy of the use of DhAM associated with AD-MSCs as a treatment to deep second degree burns. The engineered bioscaffold did not cause any alteration in the animal organs, once there were no inflammatory infiltrates among treatments. In addition, the biochemical parameters of blood did not change once no significant differences were found between control and treated groups analysis. The DhAM was able to heal the wounds as efficiently as the positive control (commercial dressing), improving the healing, when in the presence of AD-MSCs. The presence of CD73+ cells suggest the involvement of the adenosinergic signaling on wound healing, as the enzyme produces the adenosine that is an immunosuppressive molecule. This idea is reinforced by the lower expression of the marker CD11b in the DhAM + AD-MSCs treatment group. This preclinical study presents an efficient strategy to treat burn wounds that is also economically viable, as it can be engineered with human tissues that are usually discarded in the hospitals.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Conflict of interest

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

ORCID iD

Márcia Rosângela Wink  <https://orcid.org/0000-0001-5987-3509>

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8 CAPÍTULO IV - Artigo 4

UTP increases wound healing in the Self Assembled Skin Substitute (SASS)

Artigo publicado na revista **Journal of Cell Communication and Signaling**
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O papel dos receptores purinérgicos P1 e P2 tem sido analisado em vários tipos de células da pele. Entretanto, os mecanismos envolvidos na sinalização extracelular do ATP são pouco compreendidos, particularmente na derme humana. Neste capítulo usamos os substitutos de pele auto-montados (SASS), para estudar o impacto de agonistas e antagonistas de sinalização purinérgicos, como ATP, UTP e um antagonista do receptor P2Y, Reactive Blue 2 (RB2) durante a cicatrização de feridas. Após o tratamento das peles feridas, avaliamos a área da lesão, reepitelização e qualidade das peles através da estrutura da membrana basal e a diferenciação epidérmica. Além disso, a expressão das principais ectoenzimas capazes de hidrolisar nucleotídeos (NTPDase1 e NPP1) foram investigadas nas regiões da ferida. Os resultados obtidos neste capítulo poderão demonstrar o envolvimento de nucleotídeos extracelulares e principais ectoenzimas, na cicatrização da pele humana, o que poderia contribuir nas futuras aplicações clínicas utilizando o potencial de nucleotídeos para regeneração de tecidos.



UTP increases wound healing in the self assembled skin substitute (SASS)

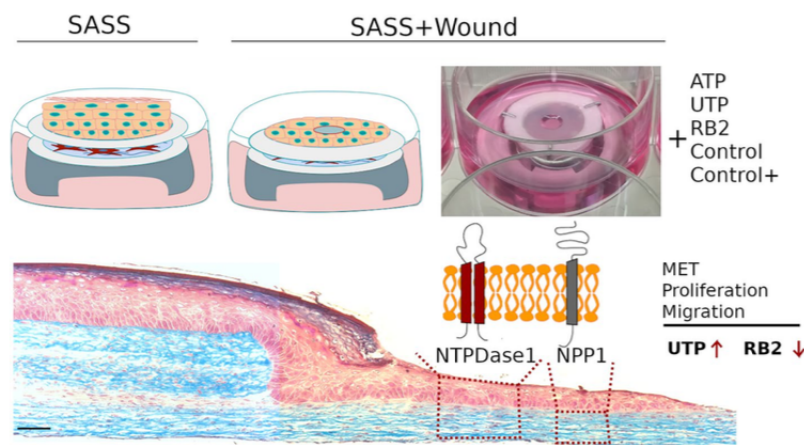
Liliana I. Sous Naasani^{1,4} · Jean Sévigny^{2,3} · Véronique J. Moulin^{3,4} · Márcia Rosângela Wink¹

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Abstract

The therapeutic potential of purinergic signaling has been explored for a wide variety of diseases, including those related to the skin. In this study, we used the self-assembled skin substitutes (SASS), a highly functional reconstructed human skin model, which shares many properties with normal human skin, to study the impact of purinergic receptors agonists, such as ATP, UTP and a P2Y receptor antagonist, Reactive Blue 2 during wound healing. After treating the wounded skins, we evaluated the wound area, reepithelialization, length of migrating tongues toward the wound, quality of the skins through the cytokeratin 10 and laminin-5 expression, epidermal and dermal cell proliferation. In addition, the expression of the main ectoenzymes capable of hydrolyzing nucleotides were investigated through the wounded SASS regions: unwounded region, wound margin, intermediate region and migrating epidermal tongue. After 3 days, under the UTP treatment, the wounded SASS showed an increase in the reepithelialization and in the proliferation of keratinocytes and fibroblasts, without altering the quality of the skin. We also identified the presence of the ectoenzymes NTPDase1 and NPP1 in the reconstructed human skin model, suggesting their involvement in wound healing. Considering the need for new therapies capable of promoting healing in complex wounds, although these results are still preliminary, they suggest the involvement of extracellular nucleotides in human skin healing and the importance to understand their role in this mechanism. New experiments it will be necessary to determine the mechanisms by which the purinergic signaling is involved in the skin wound healing.

Graphical Abstract



Véronique J. Moulin and Márcia Rosângela Wink have shared senior authorship

Extended author information available on the last page of the article

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Introduction

Wound healing is one of the most complex processes in the human body. It greatly depends on the extent and synchronization of a variety of cell types in the phases of hemostasis, inflammation, growth, re-epithelialization, and remodeling (Martin 1997; Singer and Clark 1999; Gurtner et al. 2008; Rodrigues et al. 2019).

The epidermis can self-regenerate due to the presence of stem cells (Blanpain and Fuchs 2006). In wounds, reepithelialization progresses by the growth and migration of keratinocytes from the surrounding wound margins toward the center, with the regeneration of an stratified and differentiated epidermis over the dermo-epidermal junction in reorganization (Stanley et al. 1981). In case of deep injuries and burns, the process of healing is not adequate, leading to a chronic wound as a consequence (Blanpain and Fuchs 2006; Vig et al. 2017). The development of chronic, non-healing wounds is a persistent medical problem that causes patient morbidity or even death and increases healthcare costs (Veith et al. 2019).

While several wound healing therapies are available, the effectiveness is only moderate. Therefore, there is a need for more efficient therapies to heal complex wounds. Reconstructed human skin models are upcoming alternatives to treat patients by promoting tissue regeneration (Proulx et al. 2011; Rodrigues et al. 2019). In addition, this 3D models can be used to understand the mechanisms of wound healing (Laplanche et al. 2001; Xie et al. 2010), skin aging (Diekmann et al. 2016; Weinmüller et al. 2020), psoriasis (Jean et al. 2011), hypertrophic scar (Moulin 2021), cutaneous squamous cell carcinoma (Commandeur et al. 2009) and melanoma (Hill et al. 2015). Therefore, skin models are promising tools to reach new advances that can be translated to benefit patients.

Several tissue engineered skin substitutes have been developed and optimized, based on culture conditions, cell sources, cell types and natural or synthetic dermal substrates, in order to better mimic the composition of the skin (Choudhury and Das 2021) or with the aim to improve the healing using mesenchymal stem cells (Naasani et al. 2018; Naasani et al. 2019). One example is the self-assembled skin substitutes (SASS) are a tissue-engineered skin composed by a natural collagen extracellular matrix, secreted by the own fibroblasts, without using any exogenous scaffold or biomaterial, underlying a fully differentiated epidermis (Beaudoin Cloutier et al. 2017). The self-assembly approach allows the reconstruction of

a highly functional bilayered skin substitute, which shares many properties with native human skin (Larouche et al. 2016). The capacity to preserve stem cells (Lavoie et al. 2013), make the SASS a suitable skin substitute for autologous graft in humans, without a host immune rejection when is produced with cells isolated from the patient, making it suitable for clinical use in burn treatment (Germain et al. 2018).

The purinergic signaling plays important and diverse roles in skin physiology and wound healing (Burnstock et al. 2012a). Extracellular nucleotides (ATP, ADP, AMP, UTP) are signaling molecules of the purinergic cascade and influence a variety of physiological processes, such as endocrine and exocrine secretions, immune responses, inflammation, proliferation, differentiation, migration, cell death and regeneration (Geoffrey Burnstock 2007a, b). Once released, nucleotides activate P2X ionotropic receptors (P2X₁₋₇) and P2Y metabotropic receptors (P2Y_{1,2,4,6,11-14}) responsive to tri/diphosphate nucleotides (Abbracchio et al. 2006; Burnstock 2007a, b). Whereas all P2X receptors are activated by Adenosine triphosphate (ATP), P2Y receptor subtypes differ from each other according to their nucleotide selectivity. These receptors are activated by several nucleotides including ATP, UTP, ADP and UDP (Huang et al. 2021).

Nucleotide-induced responses can be modulated by the ectonucleoside triphosphate diphosphohydrolase family (NTPDases 1–8); the nucleotide pyrophosphatase/phosphodiesterase family (NPP1-3) and ecto-5'-nucleotidase (CD73). (Zimmermann 2001; Robson et al. 2006). The NTPDases hydrolyze triphosphate (ATP and UTP) and diphosphate (ADP and UDP) nucleotides to their monophosphate derivatives (adenosine 5'-monophosphate (AMP) and uridine 5'-monophosphate (UMP) (Kukulski et al. 2005). AMP and UMP are then further hydrolyzed by CD73 to generate adenosine and uridine, respectively. The adenosine can activate the P1 type receptors (A₁, A_{2a}, A_{2b} and A₃). Therefore, these enzymes are powerful tools to control the effects mediated by extracellular purines and pyrimidines (Anderson and Parkinson 1997; Robson et al. 2006).

There is a growing interest in the therapeutic potential of purinergic compounds (Burnstock 2018), several studies evaluated the P1 and P2 purinergic receptors in various types of skin cells (White et al. 2009; Burnstock et al. 2012b). Additionally, it has become evident that P2 receptor agonists and antagonists may have therapeutic potential (Gendaszewska-Darmach and Kucharska 2011). However, the mechanisms involved in extracellular ATP signaling are poorly understood, particularly in the human dermis (Flores-Muñoz et al. 2021).

More information on these processes would be crucial for establishing future clinical applications using the nucleotides potential for tissue regeneration, without undesirable side effects. Therefore, in order to contribute with the

understanding of the role of purinergic signaling in wound healing, in this study we used the self-assembled skin substitutes (SASS) to investigate the effect of agonists ATP and UTP, and antagonist of P2Y purinergic receptors, Reactive Blue 2 (RB2) on a wound healing model. After the treatment of the wounded skins, we evaluate the wound area, reepithelialization, the length of migrating tongues toward the wound, the quality of the skins through the CK10 and laminin-5 expression, epidermal and dermal cell proliferation, and the NTPDase1 and NPP1 expression.

Materials and methods

Cell isolation and culture

Primary human Keratinocytes and fibroblast were isolated and cultured as described (Moulin 2021). The source of cells are small pieces of skin removed by surgery and following procedures approved by the institution's committee for the protection of human subjects (Moulin 2021).

Briefly, the epidermis and dermis were separated after a thermolysin (0.5 mg/mL, Sigma, St-Louis, MO, USA) incubation process. Then, keratinocytes were isolated using a 0.05% trypsin/ 0.01% EDTA solution. Fibroblasts were isolated from the dermis using collagenase H (Roche Diagnostics, Laval, Qc, Canada). Keratinocytes were grown on a feeder layer of irradiated human fibroblast, obtained as described previously (Moulin 2021) and then cultured in keratinocyte medium: Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 Medium (3:1) supplemented with 5% FetalClone II serum (HyClone, GEHealthcare Life Sciences, Mississauga, Ont, Canada), 10 ng/mL human epidermal growth factor, 5 µg/mL Insulin, 0.4 µg/mL Hydrocortisone, 0.212 µg/mL Isoproterenol, 100 IU/mL, penicillin G and 25 µg/mL Gentamicin. Before the keratinocytes reached confluence, they were detached and used until the third passage.

Dermal fibroblast were grown in DMEM supplemented with 10% Fetal calf serum (Wisent, Saint-Bruno, QC, Canada), 100 IU/mL Penicillin G, 25 µg/mL Gentamicin until they achieve the confluence.

The cells were counted with a cell counter (Beckman Coulter® Life Sciences). The cultures were maintained in a humidified incubator at 37 °C, 7% CO₂.

Self assembled skin substitute

The self assembled skin substitute (SASS) was produced as described previously (Germain et al. 2018). For the dermal sheet, 5×10^4 fibroblasts/well were seeded into 12 well plate containing a anchoring paper (Whatman® filter)

and cultured with fibroblast medium. When they reached confluence, cells were treated with 50 µg/ml ascorbic acid during one month in order to secrete an abundant extracellular matrix and to form the dermal sheet. Keratinocytes were then seeded on a first dermal sheet (DS1) at 8×10^5 cells/well with keratinocyte medium containing 50 µg/ml ascorbic acid and maintained for 4 days with daily media changes to form a keratinocyte sheet. After four days, the epidermal sheet was stacked with a second dermal sheet (DS2), and kept in place thanks to Ligacip®. Skin were then placed on the air–liquid interface and cultured in air–liquid medium (DMEM/Ham F12, supplemented with 5% FetalClone II serum, 5 µg/mL Insulin, 0.4 µg/mL Hydrocortisone, 0.212 µg/mL Isoproterenol, 100 IU/mL, penicillin G and 25 µg/mL Gentamicin). Medium was changed every 48 h for 14 days to achieve formation of a stratified epidermis.

Each skin substitute was produced with keratinocytes and fibroblast from the same patient and two different patients were used.

A normal human skin (NHS) specimen from the LOEX biological material bank (previously approved by the institutional ethics committee for utilization in research was used as control for histological analysis.

Wound healing model

The wound healing model was performed with some modifications of the model described by Laplante et al. (2001). The SASS was wounded with a 4 mm diameter biopsy punch, and stacked with a third dermal sheet (DS3) to allow the migration of the epidermal tongue. The wounded SASS was placed on the air–liquid interface and cultured with the different treatments that will be described below.

Treatments and wound area measure

To evaluate the effect of the nucleotides and the P2Y receptors on the wound healing, the wounded reconstructed human skin were cultured with DMEM/Ham F12, supplemented with 5% FetalClone II, 50 µg/ml ascorbic acid, penicillin G and 25 µg/mL Gentamicin (Control), which was used as a base medium for the different treatments: 100 µM Adenosine triphosphate (ATP); 100 µM Uridine triphosphate (UTP); 100 µM Reactive blue 2 (RB2). For the positive control (Control+), DMEM/Ham F12 media supplemented with 5% FetalClone II serum, and the additives: 5 µg/mL Insulin, 0.4 µg/mL Hydrocortisone, 0.212 µg/mL Isoproterenol, 50 µg/ml ascorbic acid, 100 IU/mL Penicillin G and 25 µg/mL Gentamicin was used.

Following 3 days of treatment, the wound was photographed with a phase contrast microscopy (Nikon Eclipse TS-100) and the wound area was calculated using ImageJ

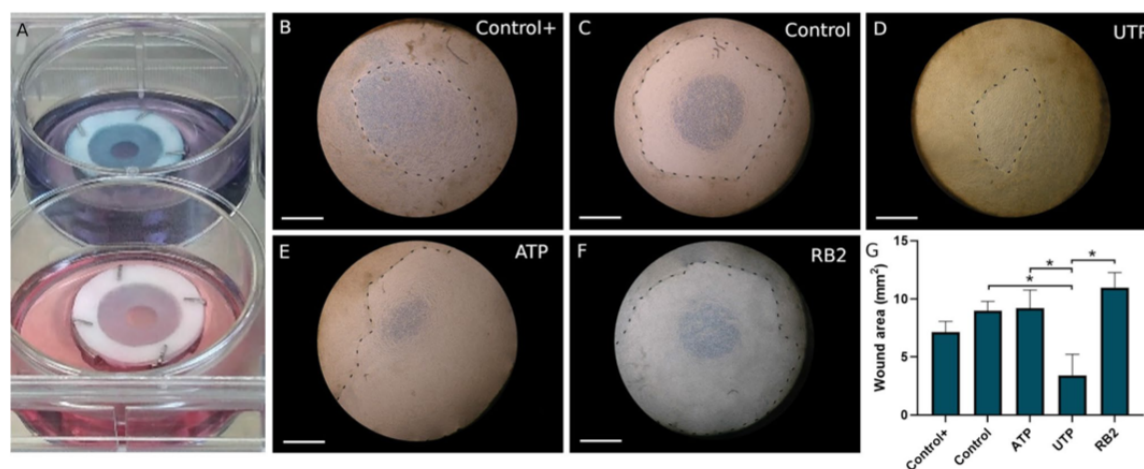


Fig. 1 Wound healing assay on the self-assembled skin substitute. **a** Macroscopic aspect of the wounded SASS on the air–liquid interface at day 0; note that RB2 is a blue dye (top) vs control (bottom). Wounds after 3 days according the different treatments, **b** control+ (see below), **c** control (see below), **d** 100 μ M UTP in control medium, **e** 100 μ M ATP in control medium, **f** 100 μ M RB2, the dotted black lines point the ring of reepithelialization from the border to the center of the wound. **g** The wound area (mm^2) following quantitative evalu-

ation. SASS was produced from cells obtained from two different patients, $N=2$, in triplicates. Data were analyzed statistically by One-way ANOVA followed by Tukey post-hoc test. Graph bars represent the mean with SD (* $p<0.05$). Control+: DMEM/Ham F12 + 5% FetalClone II serum, Insulin, Hydrocortisone, Isoproterenol, ascorbic acid; Control: DMEM/Ham F12 + 5% FetalClone II, ascorbic acid. Phase contrast micrograph. Scale bars: 500 μ m

software. Wounded SASS was then used for histological analysis. Two different patients were analyzed in triplicates.

Masson's Trichrome staining

The morphology of wounded SASS after 3 days of healing was evaluated using Masson's Trichrome staining performed on 5 μ m thick sections. Histological micrographs were performed with a Zeiss microscope equipped with Zeiss Axiocam HRm Rev 3 (Carl Zeiss Canada Ltd. Toronto, ON, Canada). The length of migrating tongues (part of the migrating epidermis into the wound) was measured using ImageJ software. Cells were derived from two different patients and analyzed in triplicates.

Immunofluorescence

Wounded tissues were embedded in Tissue-Tek® Optimal Cutting Temperature Compound (Sakura Finetek USA), frozen in liquid nitrogen and maintained at -80°C for cryostat sectioning. Indirect immunofluorescence assays were performed on 5- μ m-thick cryosections of skins following permeabilization with acetone (10 min at -20°C). The antibodies used were: mouse monoclonal anti-Laminin 5 ($\gamma 2$ chain) clone D4B5 (EMD Millipore; 1:400 dilution); mouse monoclonal anti-CK10 clone RKSE60 (Cedarlane, Burlington, ON, Canada; 1:100 dilution); rabbit polyclonal IgG anti-Ki67 Ab15580 (Abcam, 1:200 dilution);

Polyclonal guinea pig anti-human NTPDase1/CD39 (Name: hN1-1C; hN1-2C; hN1-3C) (Ectonucleotidases-ab, Canada; 1:300 dilution); Polyclonal guinea pig anti-human NPP1 (Name: hNPP1-1C; hNPP1-2C; hNPP1-3C) (Ectonucleotidases-ab, Canada; 1:300 dilution). Primary antibodies were replaced by PBS-BSA 1% or preimmune serum for NTPDase1 and NPP1 as negative controls. The tissue sections were incubated with the primary antibody for 1 h and 15 min at room temperature. After PBS washes the respective secondary antibody was added and incubated for 30 min in a humid chamber. The following secondary antibodies were used: Donkey anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A21206) (Thermo Fisher Scientific); Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 A11005 (ThermoFisher Scientific); Goat anti-Guinea Pig IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (ThermoFisher Scientific). The cell nuclei were counterstained with Hoechst reagent 33,258 (Sigma Chemicals). Fluorescent micrographs were observed and photographed under a Zeiss microscope equipped with Zeiss Axiocam ICc1 digital camera.

Ki67 positive cells were quantified using imageJ software and the percentage was calculated in relation to the cell nuclei in each field. The NTPDase1 and NPP1 relative fluorescence intensity was quantified as described by Jensen (2013). We adjusted the threshold in the positive control to select the fluorescence regions and the intensity

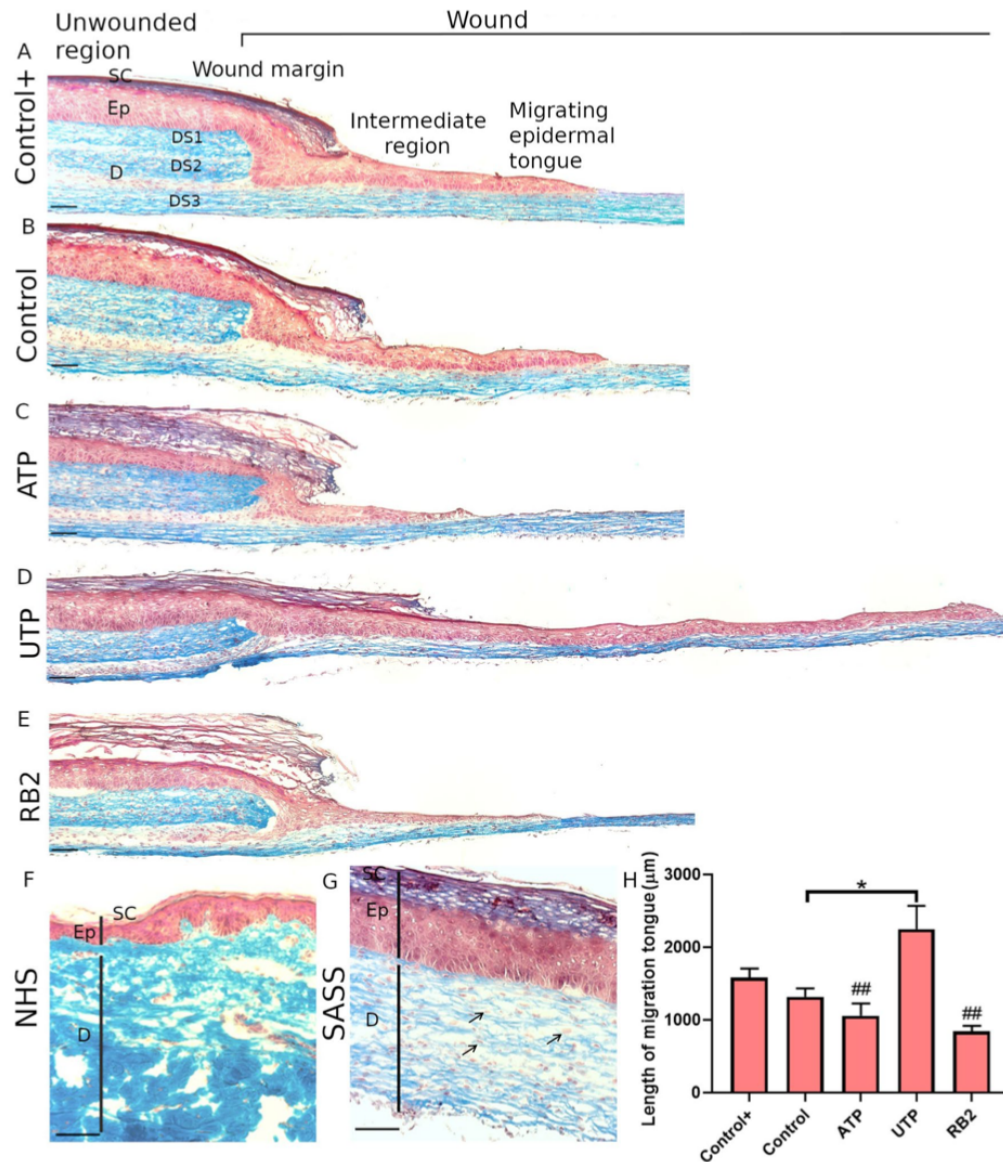


Fig. 2 Masson's trichrome staining in the wounded Self-Assembled Skin Substitute. Histological cross-section of the reconstructed human skin showing the unwounded region and the wound, composed by the wound margin, the intermediate region and the migrating epidermal tongue. It is possible to differentiate the epidermis layer (Ep) with the stratum corneum (SC) from the dermis (D) and the three dermal sheets (DS1,2,3), the third sheet belongs to the wound assay model, allowing the migration of the epidermal tongue; **a** Control+, **b** Control, **c** ATP, **d** UTP and **e** RB2. Similarity between

the normal human skin (NHS) (**f**) and the Self-Assembled Skin Substitute (SASS) (**g**) are shown, the fibroblasts embedded in the collagen fibers are indicated. **H**) The length of migrating tongues were measured. One-way ANOVA and Tukey post-hoc test (* $p < 0.05$: different from Control and Control+) (## $p < 0.01$: different from UTP) Graph bars represent the mean with SD. SASS was produced from cells obtained from two different patients, N=2, in triplicate. Scale bars 100 μm

measurements were performed within regions of interest (ROIs) to separately quantify intensity in the epidermis and in the dermis. The relative fluorescence intensity was

expressed as the integrated density (IntDen) by each total ROI area in μm^2 . Two different patients were analyzed in duplicates.

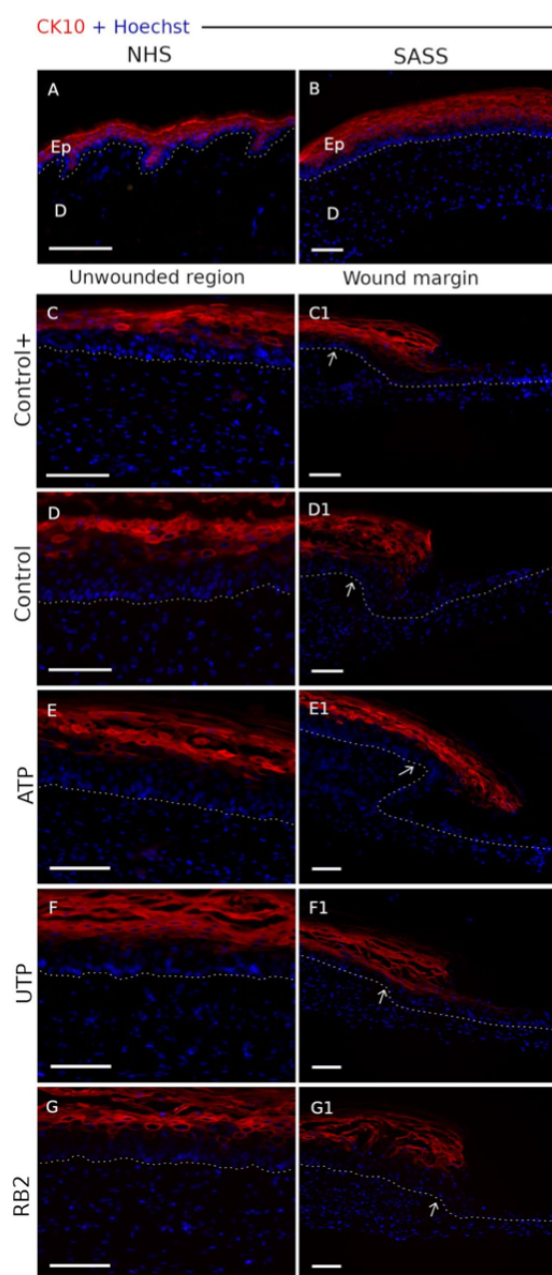


Fig. 3 Immunofluorescence labeling of Cytokeratin 10. Expression of Cytokeratin 10 (CK10) (red) in normal human skin (NHS) (a) and the self assembled skin substitute (SASS), the dotted white lines indicate the basement membrane. As expected, the basal layers of the epidermis were unlabeled. The CK10 labeling is shown in the unwounded region and the wound margin (indicated by white arrow) for the Control+ (c–c1), Control (d–d1), ATP (e–e1), UTP (f–f1) and RB2 (g–g1) treatments. The CK10 expression was unlabeled in the intermediate region and migrating epidermal tongue. Nuclei were stained with Hoechst dye (blue). Scale bars 100 μm

Scratch wound healing assay on normal human keratinocytes

The migration capabilities of normal human keratinocytes were assessed using a scratch wound healing assay. The keratinocytes were seeded into 12-well cell culture plates, containing a feeder layer of irradiated human fibroblast, at a concentration of 2×10^5 cells/well, and cultured in a keratinocyte medium to produce a nearly confluent cell layer. After 24 h, the cells were treated with 10 μg/ml Mitomycin C (m4287) for 3 h. A linear wound was generated in the layer using a sterile 200 μL plastic pipette tip to produce a scratch. Any cellular debris was removed with PBS before adding the following treatments: DMEM/Ham F12, supplemented with 5% FetalClone II, penicillin G and 25 μg/mL Gentamicin, as the control media used for the different treatments: 100 μM Adenosine triphosphate (ATP); 100 μM Uridine triphosphate (UTP); 100 μM Reactive blue 2 (RB2). Positive control (Control+) was DMEM/Ham F12 media supplemented with 5% FetalClone II serum, 10 ng/mL human epidermal growth factor, 5 μg/mL Insulin, 0.4 μg/mL Hydrocortisone, 0.212 μg/mL Isoproterenol, 100 IU/mL, Penicillin G and 25 μg/mL Gentamicin. The migration progress was monitored and photographed with a phase contrast microscopy (Nikon Eclipse TS-100) at 0 h, 24 h and 48 h. The area of the scratch in each image was estimated using imageJ software. Scratch area on 0th hour was considered 100%, for each respective image, and the reduction in area was calculated for each set in 24 and 48 h. Two different patients were analyzed in triplicates.

Statistical analysis

Data were analyzed statistically by one-way ANOVA followed by Tukey post-hoc test for the wound healing assay and the length of migrating tongues. Two-way ANOVA followed by Tukey post-hoc test for the Ki67 expression, the scratch wound assay and relative fluorescence intensity was used. We also performed an unpaired t-test to compare the NHS versus the SASS in the Ki67 expression and relative fluorescence intensity. Statistically significant differences were accepted when $p < 0.05$.

Results

Wound healing assay on the self-assembled skin substitute

The Self-Assembled Skin Substitute was successfully produced from human keratinocytes seeded on dermal sheets. Fibroblasts secreted abundant extracellular matrix and formed macroscopically visible natural sheets, in which it

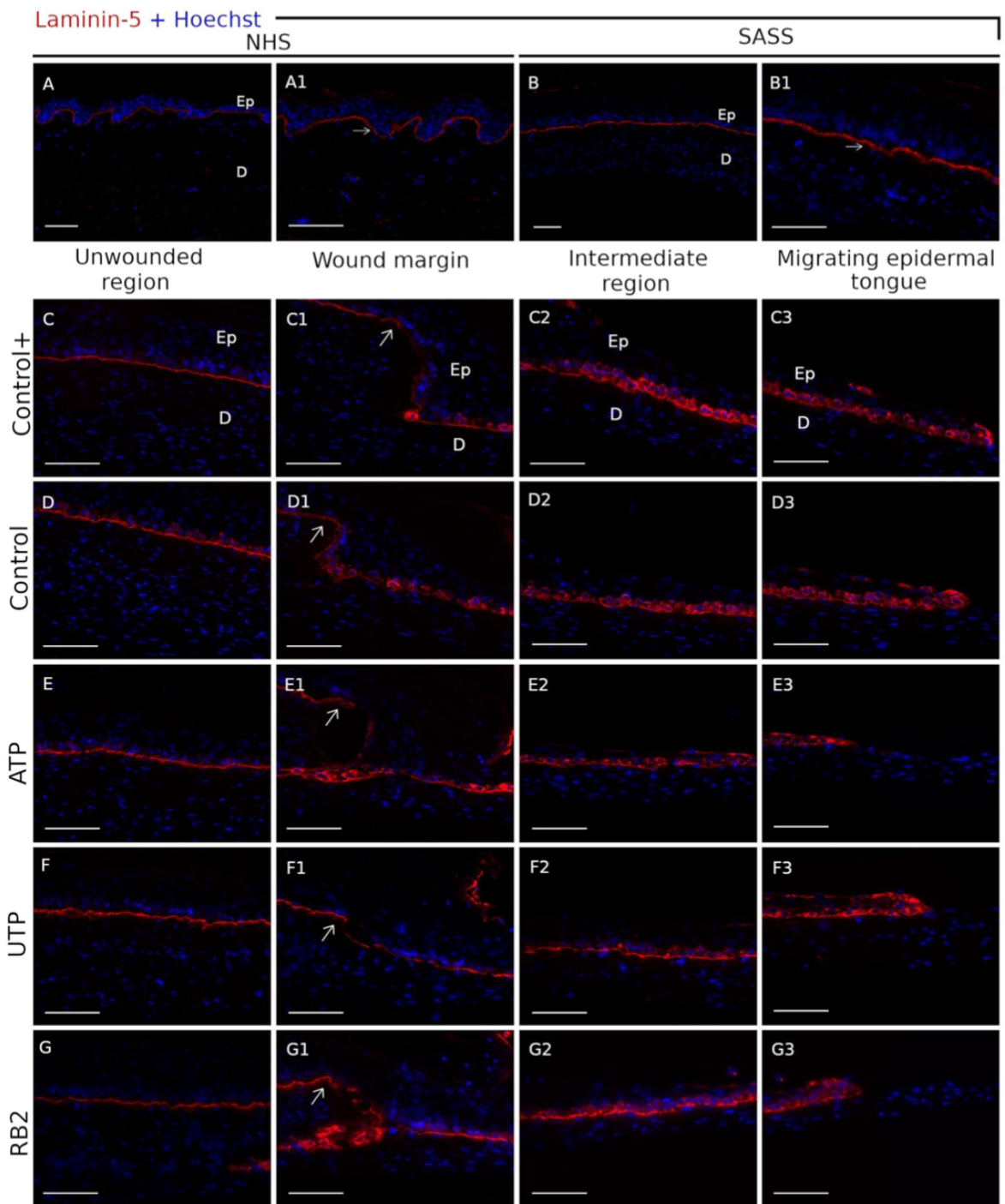


Fig. 4 Laminin-5 expression in the wounded SASS. Laminin 5 (red) expression in the basement membrane between epidermis and dermis in the normal human skin (a–a1) and the SASS (b–b1) is shown. The higher nuclei staining with Hoechst in epidermis allows to detect the difference between the epidermis (Ep) and dermis (D) layers. The laminin 5 expression in the basement membrane of the unwounded

region (c–g) and the wound margin (indicated by white arrows) (c1–g1) is exposed; the basal keratinocytes of the intermediate region (c2–g2) and migrating epidermal tongue (c3–g3) exhibit a cytoplasmic expression in all the treatments, Control+ (c–c3), Control (d–d3), ATP (e–e3), UTP (f–f3) and RB2 (g–g3). Nuclei were stained with Hoechst dye (blue). Scale bars 100 μm

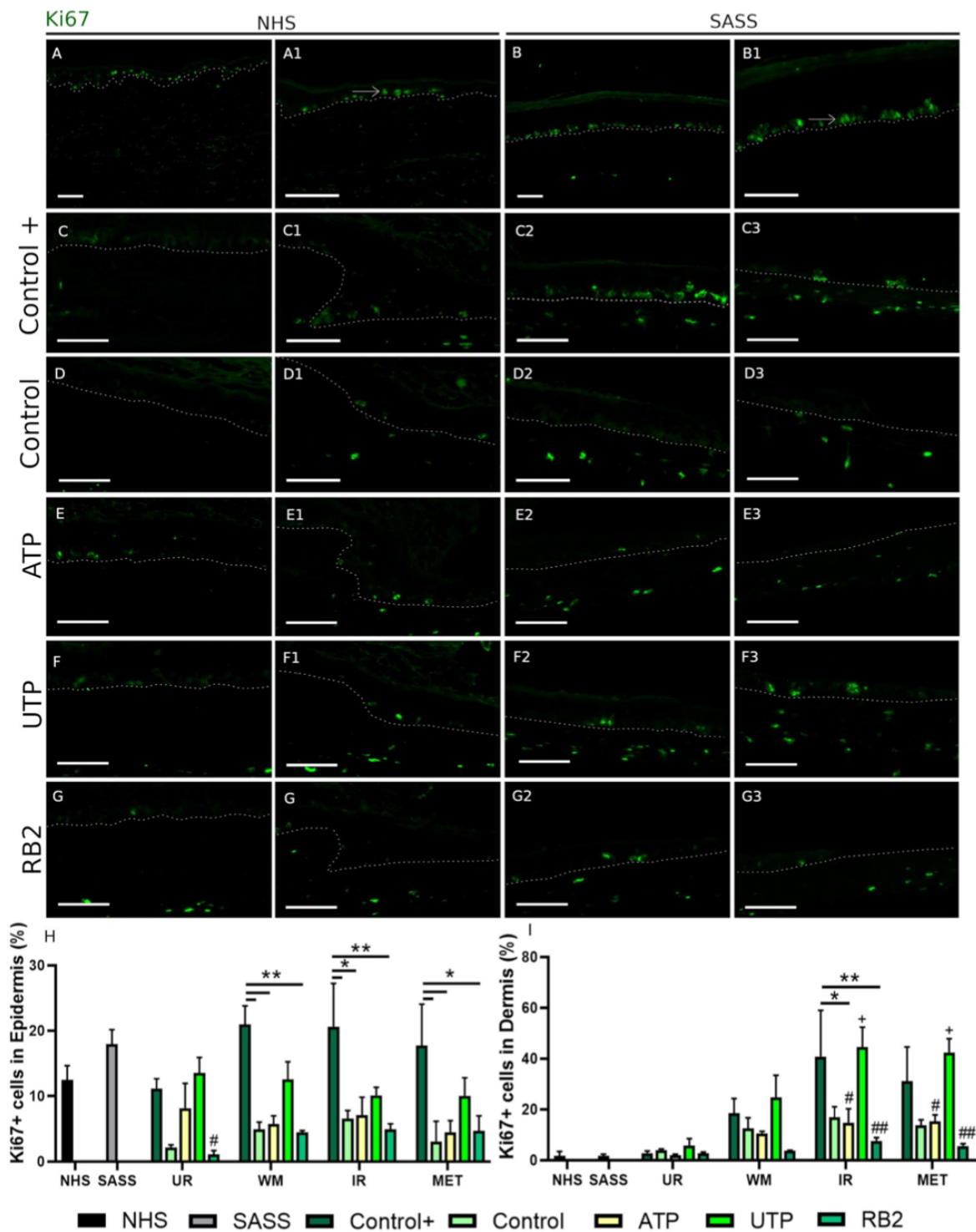


Fig. 5 Expression of the proliferative marker Ki67 in keratinocytes and fibroblasts of the wounded reconstructed human skin. The Ki67 positive cells (green) in NHS (a–a1) and SASS (b–b1) are indicated. The dotted white lines indicate the basement membrane of the epidermis. The Ki67 expression is shown in all the treatments; Control+ (c–c3), Control (d–d3), ATP (e–e3), UTP (f–f3) and RB2 (g–g3) and all the regions of the wounded SASS; unwounded region (UR), wound margin (WM), intermediate region (IR) and migrating epidermal tongue (MET). Quantification of the percentage of Ki67+ cells in the epidermis (H) and dermis (I) in each section for the different treatments shows the highest percent of Ki67 positive cells in Control+ and UTP treatments. Asterisk (*) indicates a significant difference versus Control+ group; Plus symbol (+) indicates significant difference versus control group; and hashtag (#) indicates a significant difference versus the UTP group. Two-way ANOVA followed by Tukey post-hoc test for the treatments and unpaired t-test to compare the NHS versus the SASS with no significant differences. (*, #, + $p < 0.05$), (**, ## $p < 0.01$). Graph bars represent the mean with SEM. SASS was produced from cells obtained from two different patients, N=2, in duplicates. Scale bars 100 μ m

was possible to seed the keratinocytes and stack to form the SASS, with uniform macroscopic aspect. A circular wound was produced on this reconstructed human skin. Following addition of a third dermal sheet, keratinocytes can migrate to allow the wound reepithelialization (Fig. 1a). From this model, it was possible to microscopically observe the wound closure through the ring of reepithelialization that progressed toward the wound center. The effect of the nucleotides and the P2Y receptors on the wound healing was analyzed using ATP, UTP, and RB2 treatments diluted in the medium, containing serum, but without additives (Insulin, Hydrocortisone and Isoproterenol), used as a control treatment. Therefore, we used the complete air–liquid medium as positive control. After 3 days in culture, the effect of diverse treatments on healing was observed by following the reepithelialization progress (Fig. 1b–f). The quantification was done by measuring the wound area, which was lower after the UTP treatment, when compared with the Control and RB2 treatments (Fig. 1g and supplementary Figure 2).

Histological cross-section of the wound healing model.

Histological analysis was performed to evaluate the integrity of the reconstructed human skin and the reepithelialization progress after treatment with agonists and antagonists of P2Y receptors. Following Masson's Trichrome staining it was possible to discriminate the epidermis from the dermis. An stratified epithelium until the stratum corneum was properly obtained. A dermis, composed of fibroblasts embedded into a rich extracellular matrix, colored in blue, displayed a microscopic structure similar to the normal human skin (Fig. 2a, f, g). The wounded reconstructed human skin was composed by the unwounded region and the wound. The

unwounded region contains a fully differentiated epidermis above the three dermal sheets that form the dermis, whereas, the wound is composed by the wound margin, the limit area that was injured, followed by the intermediate region and the migrating epidermal tongue, that represents the migrating keratinocytes, without stratum corneum, on the third dermal sheet. We observed differences in the length of migrating tongues according to treatments (Fig. 2a–e). The length of migrating tongues was measured and UTP treatment induced statistically significant increase in keratinocyte migration compared to Control, ATP and RB2 treatments. (Fig. 2h).

Immunofluorescence analysis

Expression of epidermal differentiation-specific protein markers, such as cytokeratin 10 (CK10), laminin-5, an important component of the basement membrane, and the proliferation marker, Ki67 were evaluated on the wounded reconstructed human skin. CK10 expression was restricted to the epidermal suprabasal layers on normal human skin, SASS and unwounded region of the wounded SASS (Fig. 3a–g). This expression was reduced in the wound margin in all the treatments (Fig. 3c1–g1). Laminin 5 formed a continuous line at the dermo–epidermal junction on normal human skin, SASS and in the unwounded region of the wounded SASS (Fig. 4a–g). From the wound margin, it was observed a keratinocyte cytoplasmic expression of laminin 5 in the basal layer, which was increased toward the migrating epidermal tongue (Fig. 4c1–g3). No change has been observed according to the treatments.

The proliferation marker Ki67 was expressed in basal cells of the epithelium as expected on normal human skin, SASS and in the unwounded region of the wounded SASS (Fig. 5a–g). Moreover, a larger number of ki67 positive fibroblasts were observed from the wound margin to the migrating epidermal tongue (Fig. 4c1–g3). Ki67 positive cells were quantified in each section of epidermis and dermis. In the epidermis, the use of serum and additives in the medium (positive control) induced the greatest number of Ki67+ positive cells in all sections of the wound. The use of serum alone (without additive) greatly reduced the number of positive cells in unwounded skin and in the wound compared to the positive control. Regarding the treatments, the addition of UTP in the control medium (without additive) promoted a strong proliferation in the unwounded skin and in the wound which was not observed when using RB2 or ATP. The number of Ki67-positive cells induced by the UTP treatment was not statistically different from the number of cells obtained with the use of additives in the medium.

In the dermis, the number of Ki67 positive cells increased in wounds, independently of the treatment. Again, addition of additives or UTP in the medium statistically increased the number of ki67 positive cells in the wound. In the wound

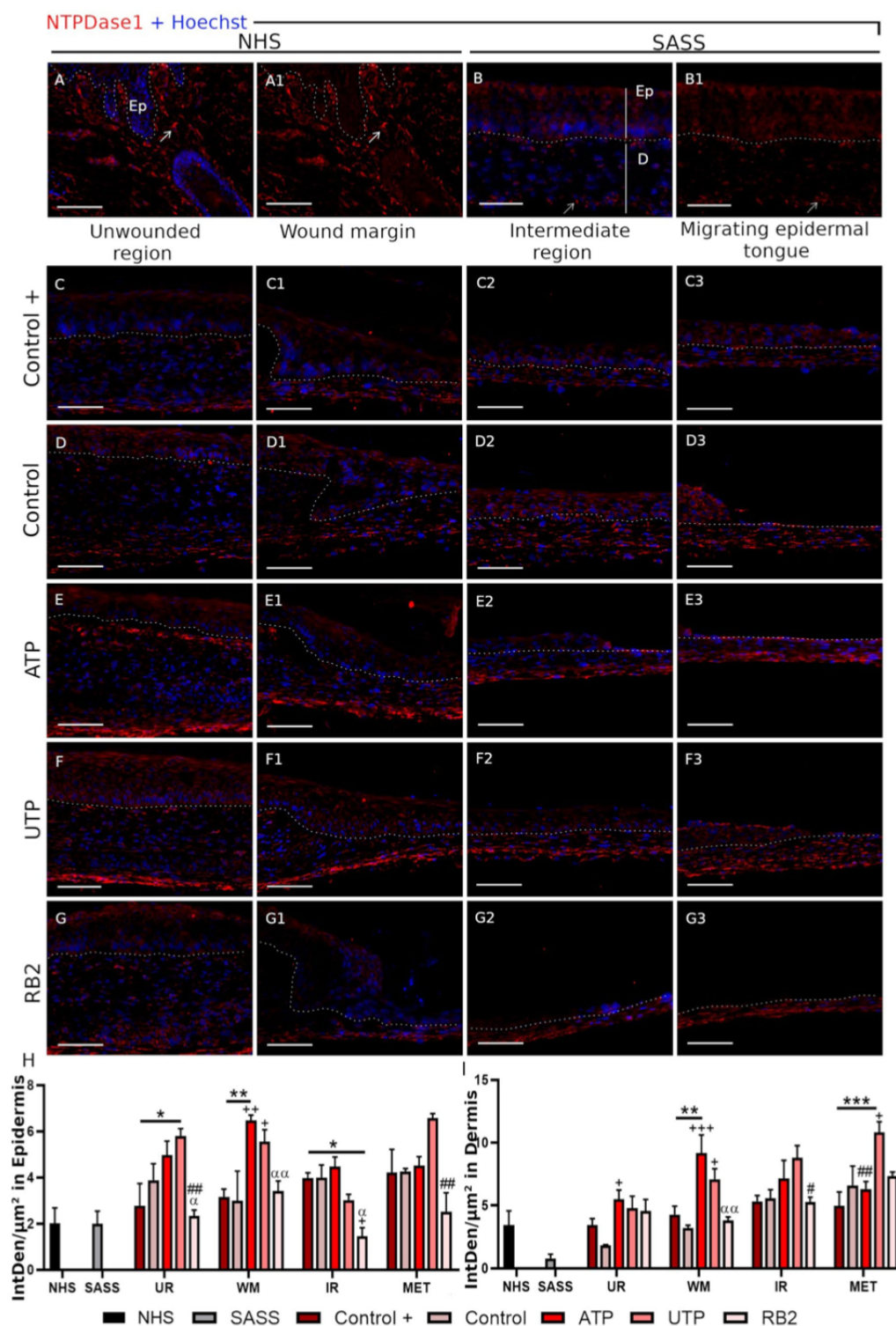


Fig. 6 NTPDase1 expression in normal and reconstructed human skin. Expression of the NTPDase1 in the normal human skin (a–a1) and in the Self-Assembled Skin Substitute, (b–b1) the positive expression in dermal cells are indicated (arrow). The dotted white lines indicate the basement membrane between the epidermis (Ep) and the dermis (d). The NTPDase1 expression was observed in keratinocytes of the SASS and wounded SASS and few positive NTPDase1 cells were observed in the NHS. NTPDase1 was also observed in all the regions of the wounded SASS: unwounded region (UR), wound margin (WM), intermediate region (IR) and migrating epidermal tongue (MET) and all the treatments: Control+ (c–c3), Control (d–d3), ATP (e–e3), UTP (f–f3) and RB2 (g–g3). Relative NTPDase1 fluorescence intensity (Integrated density/ μm^2) in Epidermis (h) and Dermis (i) The asterisk indicate the significant differences versus Control+ group, the plus symbol (+) indicate significant differences versus Control group, the hashtag (#) indicate significant differences versus the UTP group and the alpha (α) indicate significant differences versus the ATP group. Two-way ANOVA followed by Tukey post-hoc test for the treatments and unpaired t-test to compare the NHS versus the SAAS (*, +, $\alpha p < 0.05$), (**, ##, ++, $\alpha\alpha p < 0.01$), (***, $\alpha\alpha\alpha p < 0.001$). Graph bars represent the mean with SEM. SASS was produced from cells obtained from two different patients, N=2, in duplicates. Scale bars 100 μm

margin of SASS treated with UTP there was the highest percentage of Ki67 positive fibroblasts, which was different from RB2. In the intermediate region and the migrating epidermal tongue the result was similar, but in this case, the differences were also in comparison to the control, ATP and RB2 treatments. No significant differences between the normal human skin and the SASS, in both epidermis and dermis were detected.

NTPDase1 and NPP1 expression in normal and reconstructed human skin

The NTPDase1 and NPP1 expressions were evaluated in the normal human skin, the SASS and the wounded SASS. In normal human skin and SASS, the NTPDase1 was mostly observed in dermal cells and epidermal cells (Fig. 6a–b1) while NPP1 was only expressed in the dermal part of the skin (Fig. 7a–b1). For both stainings, there was no immunolabeling when the respective pre-immune sera were used (Supplementary Figure 1A–B1 and C–D1).

During wound healing, NTPDase1 was observed in all regions of the wound, independently of the treatments in epidermis and dermis. By quantifying the fluorescence intensity, we identified a higher expression of NTPDase1 in dermis and epidermis following the ATP and UTP treatments, in comparison with both control and positive control. By contrast, NPP1 was not detected in epidermis during healing, but NPP1 was increased in the intermediate region and migrating epidermal tongue, after UTP treatment (Fig. 7h). Interestingly, we detected a higher NPP1 intensity in the superficial dermis, near to the basement membrane, in comparison with the inner dermis, in the wound margin and intermediate region (Fig. 7c–g3, i).

Scratch wound assay in primary human keratinocytes

The migration capabilities of primary human keratinocytes in contact with Control+, Control, ATP, UTP and RB2 were analyzed following scratch formation on confluent keratinocytes (Fig. 8a–e). Mitomycin C was added before the treatments to inhibit cell proliferation. After 24 h, among all the treatments, the UTP group showed best results (Fig. 8a1–e1, f). Complete closure of the scratch area was observed in Control+ and UTP groups in 48 h (Fig. 8a2–e2, f) whereas the other treatments did not stimulate migration.

Discussion

Considering the need for more therapies to heal complex wounds and the role of the purinergic signaling in the skin, more information on these processes are necessary to establish future clinical applications, using the nucleotides potential for tissue regeneration. Therefore, in this study, we used the self-assembled skin substitutes (SASS), to study the effect of ATP, UTP and a P2Y receptor antagonist, Reactive Blue 2 (RB2), on a wound healing model.

Following the isolation of human skin fibroblasts, keratinocytes and their expansion in vitro, the self assembly approach allows the production of skin substitutes. They are very similar to the normal human skin, composed of a dermis, a functional basement membrane and an epidermis, without the use of any synthetic material (Beaudoin Cloutier et al. 2017). This wound healing model offers an accurate tool to compare, under standardized conditions, the effect of various factors on the rate of reepithelialization, by macroscopically following the wound closure (Laplanche et al. 2001). Three days post-wounding, UTP treatment significantly accelerated wound closure, when compared to controls, whereas ATP and RB2 did not modify it.

The histological analysis of SASS and the unwounded region showed a well-structured epidermis from the stratum basale to the stratum corneum, produced by keratinocytes. Below there was an organized dermis, entirely produced from fibroblasts that increased the release of growth factors and extracellular matrix, by adding the ascorbic acid. The wound margin represented the limit of that differentiated epidermis above the dermis, since the stratum corneum was not produced in 3 days after wound in the neodermis. Therefore, the cytokeratin 10 expression was restricted to the epidermal suprabasal layers on SASS, unwounded region and wound margin, similar to the human skin. The unlabeled keratinocytes had an undifferentiated morphology and were located in the deep layers (Laplanche et al. 2001). Accordingly, the migrating keratinocytes on the third dermal sheet,

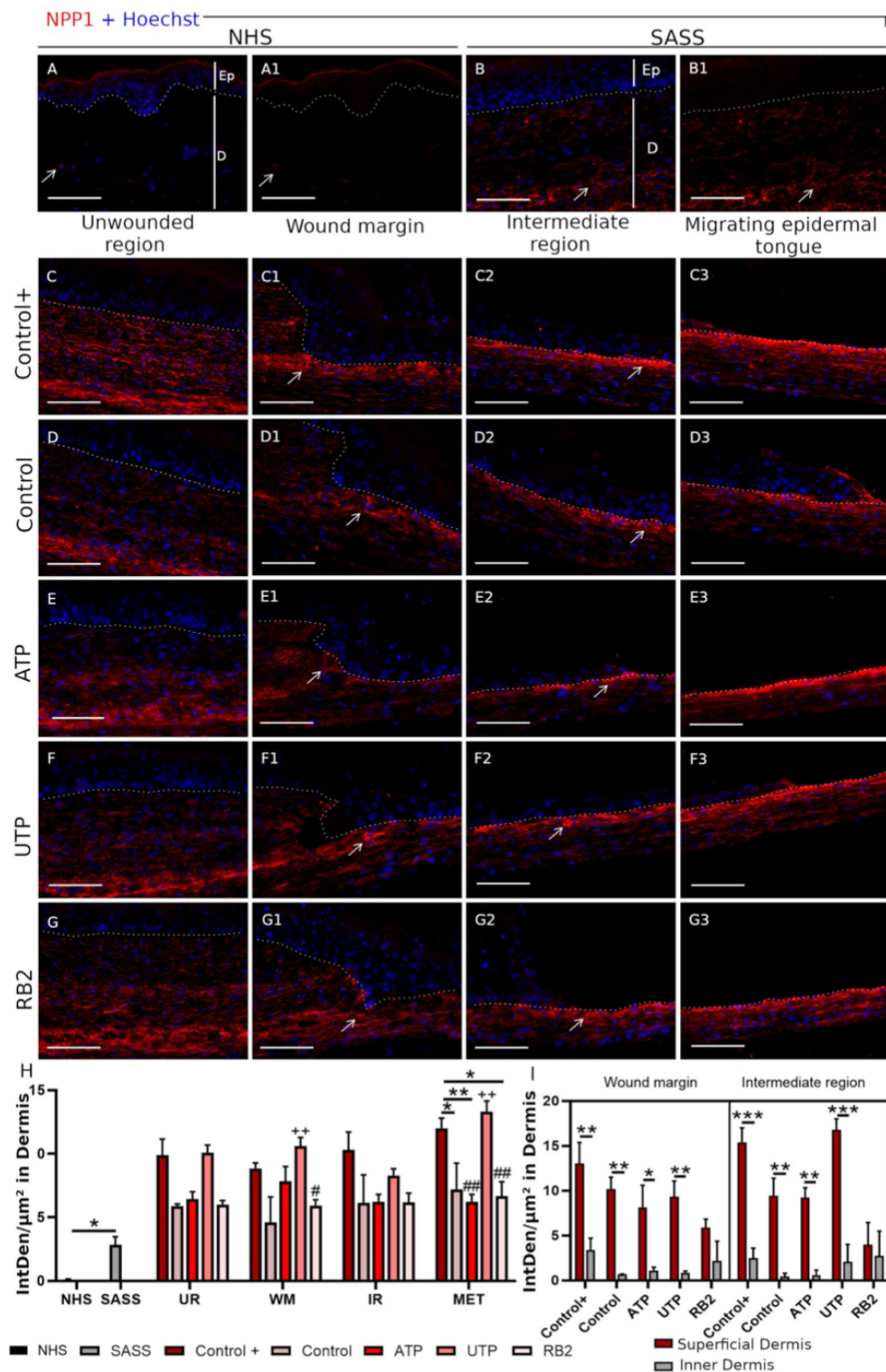


Fig. 7 NPP1 expression in normal and reconstructed human skin. The positive expression in fibroblasts in NHS (a–a1) and SASS (b–b1) is indicated (arrow). The dotted white lines indicate the basement membrane between the epidermis (Ep) and the dermis (D). NPP1 labeling was observed in fibroblasts from the wounded SASS in the Control+ (c–c3), Control (d–d3) and following ATP (e–e3), UTP (f–f3) and RB2 (g–g3) treatment and in all the regions of the wounded SASS: unwounded region (UR), wound margin (WM), intermediate region (IR) and migrating epidermal tongue (MET); we observed a higher intensity near the basement membrane in the wound margin and intermediate region, indicated by the white arrow. Relative NTP-Dase1 fluorescence intensity (Integrated density/ μm^2) in dermis (h) and superficial and inner part of the dermis (i). Significant differences versus the Control+ (*); Control (+); UTP treatment (#) and superficial versus inner dermis (*). Two-way ANOVA followed by Tukey post-hoc test for the treatments and unpaired t-test to compare the NHS versus the SASS (*, # $p < 0.05$), (**, ## $p < 0.01$), (***, ### $p < 0.001$). Graph bars represent the mean with SEM. SASS was produced from cells obtained from two different patients, N=2, in duplicates. Scale bars 100 μm

in the intermediate region and migrating epidermal tongue, were unlabeled for cytokeratin 10.

The basement membrane is essential for cohesion at the dermoepidermal junction and an important factor in successful healing. Also, the basement membrane participates in the regulation and anchoring of the stem cells (Lavoie et al. 2013). In SASS and unwounded region, the basement membrane, observed by the laminin 5 expression, formed a continuous line at the dermo-epidermal junction; similarly to the normal human skin, as expected. From the wound margin, it was observed a keratinocyte cytoplasmic expression of laminin 5 in the basal layer. It was increased toward the migrating epidermal tongue, since the deposition of laminin 5 over exposing dermal collagen in epidermal wounds is an early event in repair of the basement membrane (Nguyen et al. 2000).

Reepithelialization is a critical step in wound healing. Is the resurfacing of a wound with new epithelium and consists of both migration and proliferation of keratinocytes at the wound edges (Santoro and Gaudino 2005). The length of the migrating epidermal tongues, analyzed histologically in this study, showed an increase of the reepithelialization with UTP treatment. Therefore, a regenerative effect on wound healing after 3 days of treatment, meanwhile, a committed reepithelialization with RB2 treatment was observed. We confirmed these results observed in a 3D skin model, analyzing a scratch wound assay with primary human keratinocytes. As expected, UTP treatment showed the best results at 24 h, and similar results with the positive control in 48 h were observed. In agreement, similar results were obtained in corneal wound healing by Pintor et al. (2004), where the UTP treatment accelerated wound healing in the rabbit cornea, whereas purinoceptor antagonists, such as suramin and RB2, blocked the effect of UTP (Pintor et al. 2004). Jokela et al. (2017) explored the relationship between the UTP and

hyaluronan synthase (HAS) metabolism in human HaCaT keratinocytes, in a tentative to explain the the rapid activation of hyaluronan metabolism, in response to tissue trauma, or ultraviolet radiation. They demonstrated, for the first time, that UTP strongly regulates HAS2 expression, leading to hyaluronan accumulation, providing an explanation for the rapid increase in hyaluronan synthesis detected after injury (Jokela et al. 2017).

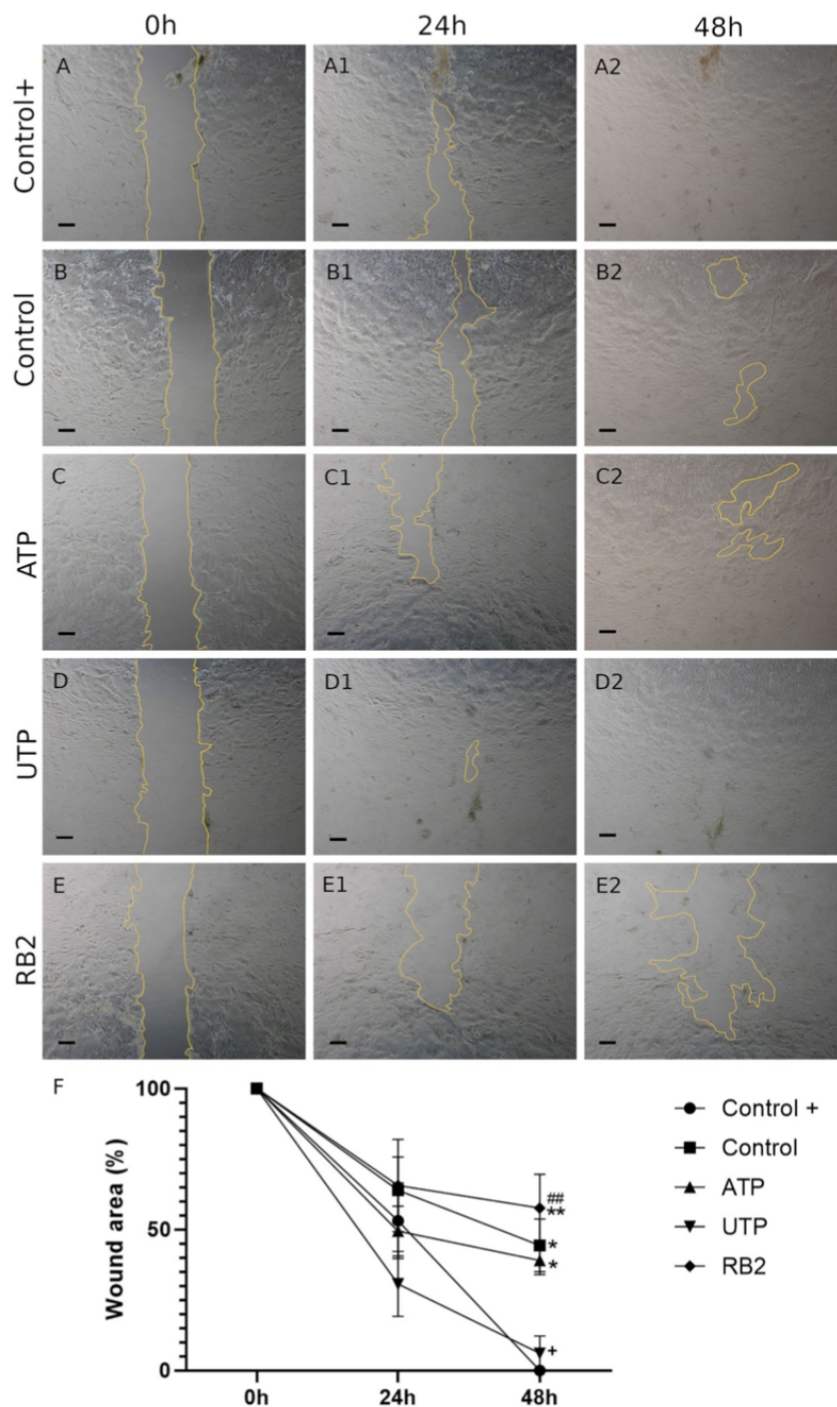
Even though, no significative differences were observed in the wound closure area analyzed between the positive control (with additives) and the control, the presence of the positive control was important, as there was an increase in the proliferation of keratinocytes and fibroblasts in this group, as determined by the presence of ki67. Although the treatments with nucleotides were done in medium without the additives, the presence of UTP was capable to induce the increase in proliferation in both keratinocytes and fibroblasts.

In the wound microenvironment, keratinocytes will be exposed to ADP, ATP and UTP released from dead or damaged cells as well as from platelets and inflammatory cells. Our results showed that when SASS or keratinocytes were treated with RB2, there was a decrease in the proliferation and migration suggesting that the endogenous nucleotides have an effect on skin wound healing through the P2Y receptors. There are few studies in the literature showing that keratinocytes express specific receptors for these nucleotides. However, the extent to which the nucleotides ATP and UTP are differentially regulated and act as independent messengers through purinergic receptors in cells from healthy and damaged skins is still unknown (Boarder and Hourani 1998; Anderson and Parkinson 1997; Lazarowski et al. 2000; Dixon et al. 1999).

The expression of P2Y2 receptor was reported in basal and parabasal primary human keratinocytes by Greig et al. (2003). They suggested that P2 purinergic receptor agonists altered keratinocyte cell number. In addition, UTP and low concentrations of ATP caused an increase in cell number, probably due a direct proliferative effect on basal cells, via P2Y2 receptors (Greig et al. 2003). Likewise, Boucher et al. (2010) reported the involvement of the P2Y2 receptors in the epithelial injury response and cell migration of corneal epithelial cells (Boucher et al. 2010). Jin et al. (2014) using human fibroblast and a mice model showed that P2Y2 receptors, activated by ATP and UTP, have an important role in the skin wound healing process, in vitro and in vivo, by increasing cell proliferation, cell migration and the expression of extracellular matrix proteins. Thus, suggesting this receptor as a potential therapeutic target in chronic wound diseases (Jin et al. 2014).

P2Y2 is potentially activated by both ATP and UTP, whereas human P2Y4 is activated by UTP. However, there are differences among species as the P2Y4 ortholog can also be activated by ATP (Brunschweiler and Muller 2006).

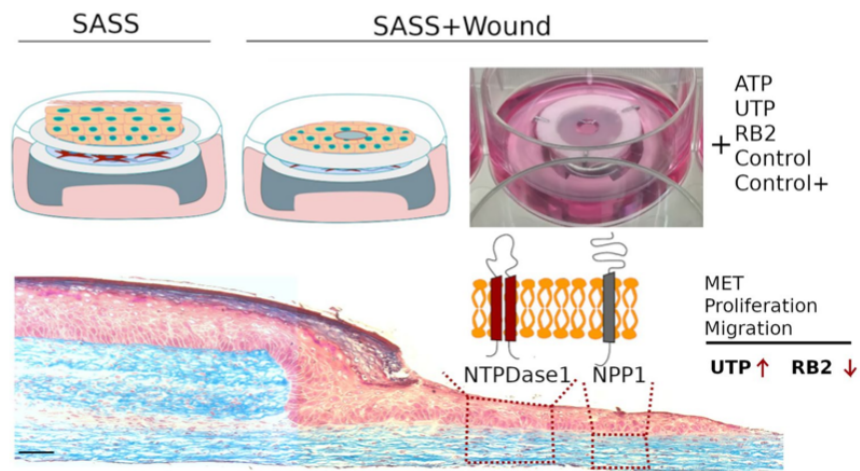
Fig. 8 Scratch wound assay in primary human keratinocytes. Migration of primary human keratinocytes at 0 h, 24 h and 48 h in presence of the treatments Control+ (a–a2), Control (b–b2), ATP (c–c2), UTP (d–d2) and RB2 (e–e2). Wound area percentage at each time point compared with each original wound area at time 0 h are shown. Two-way ANOVA followed by Tukey post-hoc test for the treatments. Significant differences versus the Control+ (*); Control (+); and UTP treatment (#); (*, + < 0.05), (**, ## $p < 0.01$). All values represent mean $\pm$ SEM. Primary human keratinocytes were obtained from two different patients, N=2, in triplicates. Scale bar 250 μ m



Human P2Y6 receptors are preferentially activated by UDP (von Kügelgen and Wetter 2000), but in mice also by UDP (Kauffenstein et al. 2010). The expression and functionality

of P2Y receptors involved in the regulation of proliferation of human keratinocytes and the HaCaT cell line was investigated. In both cell types there was the expression of

Fig. 9 Schematic representation of the UTP role in a reconstructed human skin model. UTP treatment, the wounded SASS showed an increase in the reepithelialization, migration and in proliferation of keratinocytes and fibroblasts, different from RB2, when a committed reepithelialization was observed. We also identified the presence of the ectoenzymes NTPDase1, in keratinocytes and fibroblasts and NPP1 in fibroblasts along the wound, suggesting their involvement in wound healing



P2Y1, P2Y2, P2Y4, and P2Y6 (Burrell et al. 2003). However, in primary human astrocytes when cells were cultured in the presence of calcium (200 mM and 1 mM) to mimic the differentiation, there was P2Y2 and P2Y4 downregulation. Although P2Y1, P2Y2, P2Y4 and P2Y6 receptor mRNA was expressed, only the P2Y2 receptor subtype was activated by ATP and UTP leading to intracellular calcium release and cell proliferation (Burrell et al. 2003). In addition, in situ hybridization studies showed that P2Y2 receptors were localized in the proliferative basal layer of the epidermis (Dixon et al. 1999). The actions of UTP observed in our study, improving the wound healing can be mediated by P2Y4 receptors as the ATP did not have any effects. However the participation of other P2Y receptors contributing to UTP proliferation can not be ruled out.

Burrell et al. (2003) showed that there was no response when treating human primary keratinocytes with 2MeSADP (P2Y1 agonist), ADP or UDP. Thus, suggesting that the P2Y1 and P2Y6 receptors, respectively, were not functional in this cell type (Burrell et al. 2003). The purinergic ectoenzyme NTPDase1 hydrolyzes nucleoside diphosphate and triphosphate equally. Thus, it is possible that UTP added to treat SASS can be hydrolyzed to UDP, which could in turn stimulate the P2Y6 receptor, promoting at least part of the observed results, considering that in the reconstructed skin model analyzed was composed also by dermal fibroblasts that express high levels of P2Y6 (Gendaszewska-Darmach and Kucharska 2011).

NTPDase1/CD39 can hydrolyze ATP and ADP to AMP, and UTP to UDP and UMP. This can reflect the higher intensity of this enzyme as observed after the ATP and UTP treatments in the 3D skin model. In agreement, the ATP exposure was shown to increase ectonucleotidase activity in a lineage of human epithelioid carcinoma cells (Wiendl et al. 1998). We identified the NTPDase1 expression in

fibroblasts and keratinocytes of normal human skin and the reconstructed human skin. This enzyme is expressed on different immune cell types (Giuliani et al. 2020). Fausther et al. (2010) identified the NTPDase1 expression throughout the respiratory system, including superficial epithelia and fibroblasts (Fausther et al. 2010). However, Ho et al. (2013) observed low NTPDase1 levels in HACAT cells (Ho et al. 2013). Otherwise, NTPDase1/CD39 was identified to mark normal murine fibroblasts from healthy tissues (Agorku et al. 2019). Moreover, fibroblasts may express a number of mesenchymal markers (LeBleu and Neilson 2020). We previously reported the ENTPDase1 and ENPP1 gene expression in mesenchymal stem cells isolated from skin and cornea. Dermal mesenchymal stem cells expressed higher levels of ENTPDase1, leading to adenosine release that could be associated with the immunomodulatory properties of these cells (Naasani et al. 2017).

Recently, Peng et al. (2021) investigated the correlation between the NTPDase1 expression level and the degree of skin graft injuries in a mice model. They found a negative correlation showing that the higher the expression of the NTPDase1, the lower the concentration of extracellular ADP, proliferation and activation of macrophages and B cells activity. Thus, the activity of NTPDase1 was able to decrease the degree of skin graft injuries, providing a foundation for new studies investigating the use of NTPDase1 to alleviate organ damage, during the acute Antibody-mediated rejection (Peng et al. 2021). Therefore, NTPDase1 expression could be associated with immunogenic events in tissue regeneration. Accordingly, Mizumoto et al. (2002) reported the modulatory roles in inflammation and immune responsiveness of NTPDase1 on mice skin, as responsible for the Langerhans cells ecto-NTPDase activity and the augmented inflammatory responses in skin CD39^{-/-} mice (Mizumoto et al. 2002).

Extracellular ATP can be directly hydrolysed to AMP by NPPs, as reviewed in Giuliani et al. (2020). The expression and role of NPP1 is still unknown in tissue regeneration. No labeled keratinocytes in normal human skin and in the reconstructed human skin were identified. This observation is in accordance with the work of Chourabi et al. (2018), which detected *ENPP1* transcript in primary human fibroblasts and melanocytes but not in keratinocytes (Chourabi et al. 2018). A pathogenic role of this enzyme was reported observing its overexpression in cultured skin fibroblasts from insulin-resistant humans (Goldfine et al. 1999). Moreover, NPP1 expression has been reported to be increased in neural brain tumors (Lee 2015). Thus, the NPP1 overexpression could be associated with pathological events or at least, when cells are not in homeostasis state, as observed during wound healing.

In conclusion, as a novelty, we have studied the role of two important nucleotides of the purinergic signaling, ATP and UTP, in a reconstructed human skin model. UTP treatment showed a therapeutic effect on wound healing, improving the migration and proliferation of human keratinocytes, an effect that could be blocked by the antagonist of P2Y purinergic receptors, Reactive Blue 2 (RB2). In addition, the expression of the enzymes NTPDase1 and NPP1 was observed for the first time in a reconstructed human skin model, showing their modulation in the wounded SASS regions (Fig. 9). Nevertheless, new experiments will still be necessary to understand how the purinergic signaling is involved in the skin physiology and wound healing process.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare.

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Authors and Affiliations

Liliana I. Sous Naasani^{1,4} · Jean Sévigny^{2,3} · Véronique J. Moulin^{3,4} · Márcia Rosângela Wink¹ 

✉ Márcia Rosângela Wink
mwink@ufcspa.edu.br; marciawink@yahoo.com.br

¹ Laboratório de Biologia Celular, Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rua Sarmento Leite 245, Porto Alegre, RS CEP 90050-170, Brazil

² Département de Microbiologie-Infectiologie Et d'Immunologie, Faculté de Médecine, Université Laval, Québec, QC G1V 0A6, Canada

³ Centre de recherche du CHU de Québec-Université Laval, Québec City, QC G1V 4G2, Canada

⁴ Département de Chirurgie, Faculté de Médecine, Université Laval, Québec, QC G1V 0A6, Canada

9 CAPÍTULO V - Artigo 5

CD73 is overexpressed during the wound healing process in bilayered self assembled skin substitute (SASSs) model

Manuscrito em preparação

Após o nosso grupo de pesquisa ter demonstrado que as MSCs liberam altas concentrações de adenosina, produto da alta atividade hidrolítica do AMP em ADO pela CD73, o papel desta enzima no processo de regeneração tecidual tem ganhado o nosso interesse. A CD73 é expressa na superfície celular de uma variedade de tecidos e a alta expressão desta proteína tem sido implicada com uma variedade de tumores, no entanto o papel da CD73 na regeneração tecidual ainda é desconhecido. Os substitutos de pele são uma ferramenta valiosa para estudar a biologia da pele, incluindo o efeito de compostos na cicatrização de feridas. Neste capítulo será analisada a expressão da CD73 durante a cicatrização de feridas no modelo de substitutos de pele auto-montados (SASS) desenvolvido pelo grupo de pesquisa do LOEX da Universidade Laval na cidade de Quebec, Canadá. A expressão da CD73 também será analisada no modelo de cicatrização de feridas, após estimulação com AMP e ADO e a inibição farmacológica com o antagonista da CD73, Adenosina 5'- (α , β -metileno) difosfato (APCP). Uma compreensão profunda do papel da enzima CD73 e da ADO na regeneração tecidual poderá levar a novas formas de tratamento que permitam modular a via da sinalização purinérgica, seja através da utilização de nucleotídeos ou agonistas/antagonistas de receptores e enzimas, e desta forma reduzir processos de inflamação crônica e melhorar a cicatrização, processos que são decisivos na recuperação de grandes lesões cutâneas.

CD73 overexpression during the wound healing process in skin

Liliana Ivet Sous Naasan^{1,4}, Jean Sévigny^{2,3}, Véronique J. Moulin^{3,4*}, Márcia Rosângela Wink^{1*}

***These authors shared senior authorship**

1 Laboratório de Biologia Celular, Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rua Sarmento Leite 245, 90050-170, Porto Alegre, RS, Brazil

2 Département de Microbiologie-Infectiologie et d'Immunologie, Faculté de Médecine, Université Laval, Québec, QC G1V 0A6, Canada

3 Centre de recherche du CHU de Québec-Université Laval, Québec City, QC G1V 4G2, Canada

4 Département de chirurgie, Faculté de Médecine, Université Laval, Québec, QC G1V 0A6, Canada

Corresponding author: Wink, MR

E-mails: mwink@ufcspa.edu.br; marciawink@yahoo.com.br

Universidade Federal de Ciências da Saúde de Porto Alegre – UFCSPA

245, Sarmento Leite. Porto Alegre, RS, Brasil, CEP 90050-170

Telephone: +55 (51) 3303.8762; fax: +55 (51) 3303.8810

Abstract

The key role of the CD73 during tumor progression has been studied, but their involvement during the wound healing is poorly understood. The tissue engineered skin substitutes represent a valuable tool for experimental research to study skin biology. The Self-assembled skin substitutes (SASS) are a highly functional bilayered skin substitute which has been demonstrated as a good model to study the healing process. In this study we analyzed CD73 expression during healing, following CD73 stimulation with AMP and adenosine (ADO) and pharmacological inhibition with Adenosine 5'-(α,β -methylene)diphosphate (APCP). ADO significantly stimulated reepithelialization whereas no alterations in the structural skin proteins, cytokeratin 10 and laminin-5 expression, were identified after treatments. The cell proliferation was also analyzed according to the treatments and the regions of the wounded SASS: unwounded region, wound margin, intermediate region and migrating epidermal tongue. There was an increase in the cell number following AMP and ADO treatments mainly in the dermis in the intermediate region and in migrating epidermal tongue regions. In addition, CD73 expression was increased, mainly in basal keratinocytes, during wound healing. This expression was also observed in injured keratinocytes cultured in monolayer. The signaling pathways involving CD73 in wound healing may provide new insights into therapeutic strategies.

Introduction

Skin repair greatly depends on the extent and mechanism of injury. It involves a variety of cell types with different functions in the phases of hemostasis, acute inflammation, proliferation and maturation, making the skin wound healing one of the most complex processes in the human body that still remains unclear (Veith et al. 2019; Das and Baker 2016; Rodrigues et al. 2019).

There are considerations about how surrounding factors can affect cellular function in wound healing. A thorough discovery of cellular diversity and function, as well as, the identification of cell surface markers to isolate the most effective cells will aid our understanding of skin biology and to generate effective therapies for skin injuries (Rodrigues et al. 2019).

After a cell damage, nucleotides and nucleosides can be released from the intracellular environment to be degraded by ectoenzymes and/or act on different purinergic receptors (ionotropic P2X or metabotropic P2Y receptors for ATP/ADP and P1 selective for adenosine) (Abbracchio et al. 2009; Burnstock 2007). Extracellular ATP has a short half-life before being converted to adenosine by hydrolysis or by dephosphorylation of ATP to AMP (Regateiro, Cobbold, and Waldmann 2013). Tri/diphosphate nucleotides are degraded by ecto-NTPDases (NTPDases1-8) and ecto-5'nucleotidase (CD73) hydrolyzes AMP to adenosine (ADO), being the major contributor to the pathway that completely hydrolyses extracellular ATP to adenosine (Zimmermann 2001; Robson, Sévigny, and Zimmermann 2006; Bavaresco et al. 2008). This physiological release and degradation must be regulated to avoid a prolonged immune response and consequent increase in inflammatory mediators that regulate tissue damage, induce excessive recruitment of immune cells and establish chronic inflammatory conditions accompanied by fibrosis (Ferrari et al. 2016).

The CD73 is linked to the plasma membrane with its catalytic site exposed to the extracellular space and is expressed on the cell surface of a variety of tissues (Bavaresco et al. 2008; Resta, Yamashita, and Thompson 1998; Colgan et al. 2006). The CD73 expression was observed in human intestinal epithelial cells (Strohmeier et al. 1997), endothelial cells (Airas et al. 1997), dermal mesenchymal stromal cells (Naasani et al. 2017), and by adaptive immune cells (Bono et al. 2015; Thomson et al. 1990). In hypoxic conditions and inflammatory mediators, the CD73 expression and function is increased (Antonioli et al. 2016). Upregulation of this protein has been implicated in a variety of tumors, such as colon, lung, pancreatic, and ovarian carcinomas (Jin et al. 2010). Indeed, elevations of CD73 in human carcinomas correlate with increasing tumor invasion, propensity for metastasis, vascularity, and shorter survival (Spychala 2000; Zhang 2010; Wladis, Weller, and Adam 2020). However, the role of CD73 in wound healing is poorly understood.

Reconstructed human skin models are upcoming alternatives to traditional wound healing strategies and tissue regeneration. Several tissue engineered skin substitutes have been developed and optimized, based on culture conditions, cell sources, cell types and natural or synthetic dermal substrates, in order to better mimic the composition of the skin (Choudhury and Das 2021). They are a valuable tool for experimental research to study skin biology (Mieremet et al. 2019). The Self-assembled skin substitutes (SASS) are a highly functional bilayered skin substitute which shares many properties with native human skin (Beaudoin Cloutier et al. 2017), and can be useful to study the skin wound healing (Laplanche et al. 2001).

The signaling pathways involving adenosine and CD73 in wound healing need to be elucidated for the development of targeted therapies. In this study we analyzed the CD73 expression on an *in vitro* wound healing model, and the impact of its stimulation with AMP and ADO and its pharmacological inhibition with Adenosine 5'-(α,β -methylene)diphosphate (APCP) on healing.

Material and Methods

SASS production

Small pieces of skin removed by surgery and following procedures approved by the institution's committee for the protection of human subjects was the source of cells. Primary human keratinocytes and fibroblast were isolated and cultured as described (Moulin 2021). The Self Assembled Skin Substitute (SASS) was produced as described previously (Germain et al. 2018). For the dermal sheet, 5×10^4 fibroblasts/well were seeded into 12 well plate containing a anchoring paper (Whatman® filter) and cultured with fibroblast medium: 10% Fetal calf serum (Wisent, Saint-Bruno, QC, Canada), 100 IU/mL Penicillin G, 25 µg/mL Gentamicin.

When fibroblasts reached confluence, they were treated with 50 µg/ml ascorbic acid during one month in order to secrete an abundant extracellular matrix and to form the dermal sheet. Keratinocytes were then seeded on a first dermal sheet (DS1) at 8×10^5 cells/well with keratinocyte medium: Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 Medium (3:1) supplemented with 5% FetalClone II serum (HyClone, GEHealthcare Life Sciences, Mississauga, Ont, Canada), 10 ng/mL human epidermal growth factor, 5 µg/mL Insulin, 0.4 µg/mL Hydrocortisone, 0.212 µg/mL Isoproterenol, 100 IU/mL, penicillin G and 25 µg/mL Gentamicin, and maintained for 4 days with daily media changes to form a keratinocyte sheet. After four days, the epidermal sheet was stacked with a second dermal sheet (DS2), and kept in place thanks to Ligaclip®. Skin were then placed on the air-liquid interface and cultured in air-liquid medium (DMEM/Ham F12, supplemented with 5% FetalClone II serum, 5 µg/mL Insulin, 0.4 µg/mL Hydrocortisone, 0.212 µg/mL Isoproterenol, 100 IU/mL, penicillin G and 25 µg/mL Gentamicin). Medium was changed every 48 hours for 14 days to achieve formation of a stratified epidermis. The cultures were maintained in a humidified incubator at 37 °C, 7% CO₂.

Wounded SASS and wound area measure

To reproduce a wound healing model on the SASS, the model described by Laplante and colleagues (2001) (Laplante et al. 2001) was performed. The SASS was wounded with a 4mm diameter biopsy punch, and stacked with a third dermal sheet (DS3) to allow the migration of the epidermal tongue. The wounded SASS was placed on the air-liquid interface and cultured with the different treatments described on the next section.

To measure the wounded area, after 3 days the wound was photographed with a phase contrast microscopy (Nikon Eclipse TS-100) and the wound area was calculated using ImageJ software.

AMP, ADP and APCP treatments

DMEM/Ham F12 media supplemented with 5% FetalClone II serum, and the additives: 5 µg/mL Insulin, 0.4 µg/mL Hydrocortisone, 0.212 µg/mL Isoproterenol, 50µg/ml ascorbic acid, 100 IU/mL Penicillin G and 25 µg/mL Gentamicin was used as positive control (Control+). DMEM/Ham F12, supplemented with 5% FetalClone II, 50µg/ml ascorbic acid, penicillin G and 25 µg/mL Gentamicin was used as Control and as medium to dilute tested molecules: 100 µM Adenosine monophosphate (AMP), 100 µM Adenosine (ADO) and 100 µM Adenosine 5'-(α,β -methylene)diphosphate (APCP). Tissues were fixed after the third day.

Masson's Trichrome staining

The tissues were embedded in Tissue-Tek® Optimal Cutting Temperature Compound (Sakura Finetek USA), frozen in liquid nitrogen and maintained at -80°C for cryostat sectioning. Masson's Trichrome staining was performed on 5µm thick sections. Histological micrographs were performed with a Zeiss microscope equipped with Zeiss Axiocam HRm Rev 3 (Carl Zeiss Canada Ltd. Toronto, ON, Canada). The length of migrating tongues (part of the migrating epidermis into the wound) was measured using ImageJ software.

A normal human skin (NHS) specimen from the LOEX biological material bank (previously approved by the institutional ethics committee for utilization in research) was used as control for histological analysis.

Immunofluorescence assays

Indirect immunofluorescence assays were performed on 5-µm-thick cryosections of skins following permeabilization with acetone (10 min at -20 °C). Primary antibodies were replaced by PBS-BSA 1% or preimmune serum for CD73 as negative controls. The tissue sections were incubated with the primary antibody for 1 hour and 15 min at room temperature. After PBS washes the respective secondary antibody was added and incubated for 30 min in a humid chamber.

The primary antibodies used were: mouse monoclonal anti-Laminin 5 (γ 2 chain) clone D4B5 (EMD Millipore; 1:400 dilution); mouse monoclonal anti-CK10 clone RKSE60 (Cedarlane, Burlington, ON, Canada; 1:100 dilution); rabbit polyclonal IgG anti-Ki67 Ab15580 (Abcam, 1:200 dilution); anti-vimentin ab8978 (Abcam, 1:300 dilution); Polyclonal rabbit anti-human ecto-5'-Nucleotidase/CD73 antibodies Name: h5'NT-1L(I4,I5); h5'NT-2L(I4,I5); h5'NT-3L(I4,I5) (Ectonucleotidases-ab, Canada; 1:300 dilution). The secondary antibodies used were: Donkey anti-Rabbit IgG (H+L) Highly CrossAdsorbed Secondary Antibody, Alexa Fluor 488 (A21206) (Thermo Fisher Scientific); Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 A11005 (ThermoFisher Scientific). The cell nuclei were counterstained with Hoechst reagent 33258 (Sigma Chemicals). Fluorescent micrographs were observed and photographed under a Zeiss microscope equipped with Zeiss Axiocam ICc1 digital camera.

Fluorescence intensity and proliferation measures

The relative CD73 fluorescence intensity was quantified as described (Jensen 2013) using ImageJ software. The threshold was adjusted in the positive control in order to select the fluorescence regions, then, the intensity measurements were performed within regions of interest (ROIs) to separately quantify intensity in the epidermis and in the dermis. Differences in the CD73 fluorescence intensity between intensity NHS, SASS and wounded SASS (using the positive control) were analyzed in epidermis and dermis and differences in the CD73 fluorescence intensity between the treatments were analyzed for each region of the wounded SASS. The relative fluorescence intensity was expressed as the integrated density (IntDen) by each total ROI area in μm^2 . The proliferative cells (KI67+ cells) were quantified using imageJ software, by quantifying the percentage of positive cells in relation to the cell nuclei in each field.

CD73 expression in primary human keratinocytes in monolayer

The CD73 expression was analyzed in keratinocytes cultured in monolayer. The keratinocytes were seeded into 6-well cell culture plates, containing a feeder layer of irradiated human fibroblast, at a concentration of 5×10^4 cells/well to obtain a confluent monolayer to produce a scratch wound. The cultures were treated with and without $10\mu\text{g/ml}$ Mitomycin C (m4287) for 3 hours before generating a linear wound using a sterile 200 μL plastic pipet tip. Cells were then culture in a keratinocyte medium: Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 Medium (3:1) supplemented with 5% FetalClone II serum (HyClone, GEHealthcare Life Sciences, Mississauga, Ont, Canada), 10 ng/mL human epidermal growth factor, 5 $\mu\text{g/mL}$ Insulin, 0.4 $\mu\text{g/mL}$ Hydrocortisone, 0.212 $\mu\text{g/mL}$ Isoproterenol, 100 IU/mL, penicillin G and 25 $\mu\text{g/mL}$ Gentamicin. An indirect immunofluorescence was performed on the slides with Polyclonal rabbit anti-human ecto-5'-Nucleotidase/CD73, using the same conditions described above.

Statistical analysis

The wound healing assay, the length of migrating tongues, Ki67 expression and fluorescence intensity between the regions were analyzed statistically by one-way ANOVA followed by Tukey post-hoc test. Unpaired t-test was used to compare the NHS versus the SASS. The relative fluorescence intensity between treatments were analyzed statistically by two-way ANOVA followed by Tukey post-hoc test. Statistically significant differences were accepted when $p < 0.05$.

Results

Wound healing assay on SASS

The wound healing model was made from an *in vitro* produced skin called Self Assembled Skin Substitute constituted of a dermis and a differentiated epidermis (Figure 1 A-D). The wound closure was observed through the ring of reepithelialization that progressed toward the wound center, after the third day in culture with the treatments (Figure 1 E-I). The wound area was measured and whereas control+, control, AMP and APCP treatments showed similar wound closure, the treatment with ADO significantly stimulated the reepithelialization rate in comparison with the control and the APCP treatment (Figure 1J).

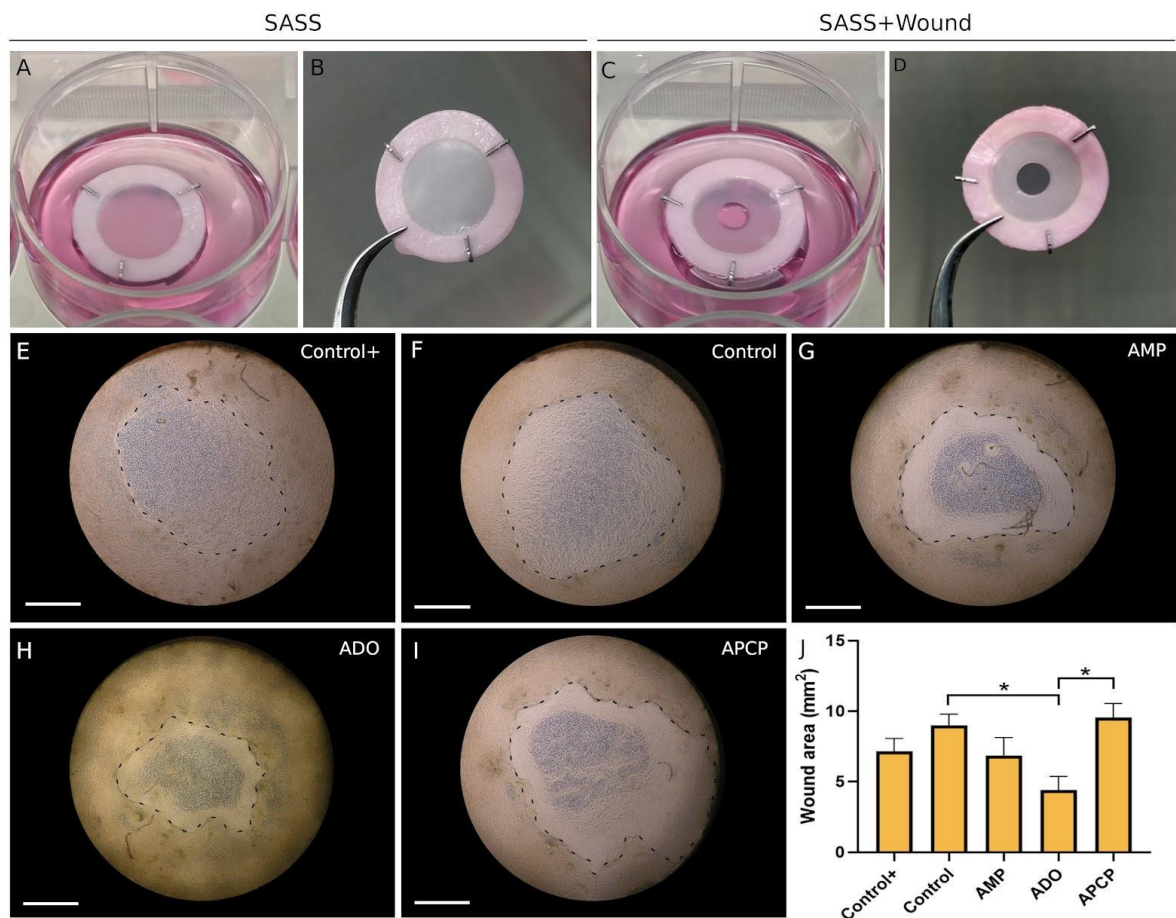


Figure 1. Self Assembled Skin Substitute and wound healing assay. A-B) The SASS cultured at the air-liquid interface. C-D) The wounded SASS. E) Micrography of the wounded skin after 3 days with the treatments E) Control+, F) Control, G) AMP, H) ADO, I) APCP. Dotted black lines indicate the migrating epidermal tongue. J) Quantification of the surface area of the wound(mm²) after 3 day-treatments. Data were analyzed statistically by one-way ANOVA followed by Tukey post-hoc test. Graph bars represent the mean with Standard error of mean (SEM), N=2, (**p* < 0.05). Phase contrast micrograph. Scale bars: 500 μ m.

Histological analysis of Self-Assembled Skin Substitute

To analyze the histology of the SASS and the reepithelialization progress after the treatments, Masson's Trichrome staining was performed. In these slides, it was possible to identify the epidermis and the dermis on NHS, SASS and wounded SASS. As expected, a stratified epithelium, with the stratum corneum, and a dermis composed of extracellular matrix formed by fibroblast were observed in SASS, similarly to normal human skin (Figure 1 A-B1). The wounded SASS was divided into four regions: The Unwounded region (UR), formed by the complete reconstructed skin plus the third dermal sheet added after wound formation; the wound margin (WM) performed on day 0; the intermediate region (IR) of the migrating epidermal tongue and the tip of the migrating epidermal tongue (MET). It is possible to observe that the stratum corneum was visible only at the level of the epithelium of the skin and up to the edge of the wound (Figure 2 C). The length of migrating epidermal tongues were measured for all the treatments, and only showed a significant increase when ADO was added compared to the control (Figure 2 C-H).

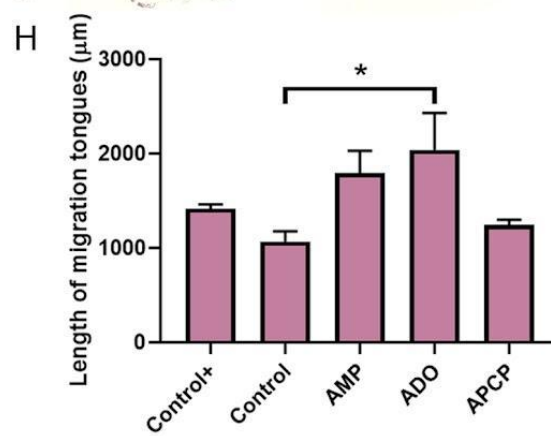
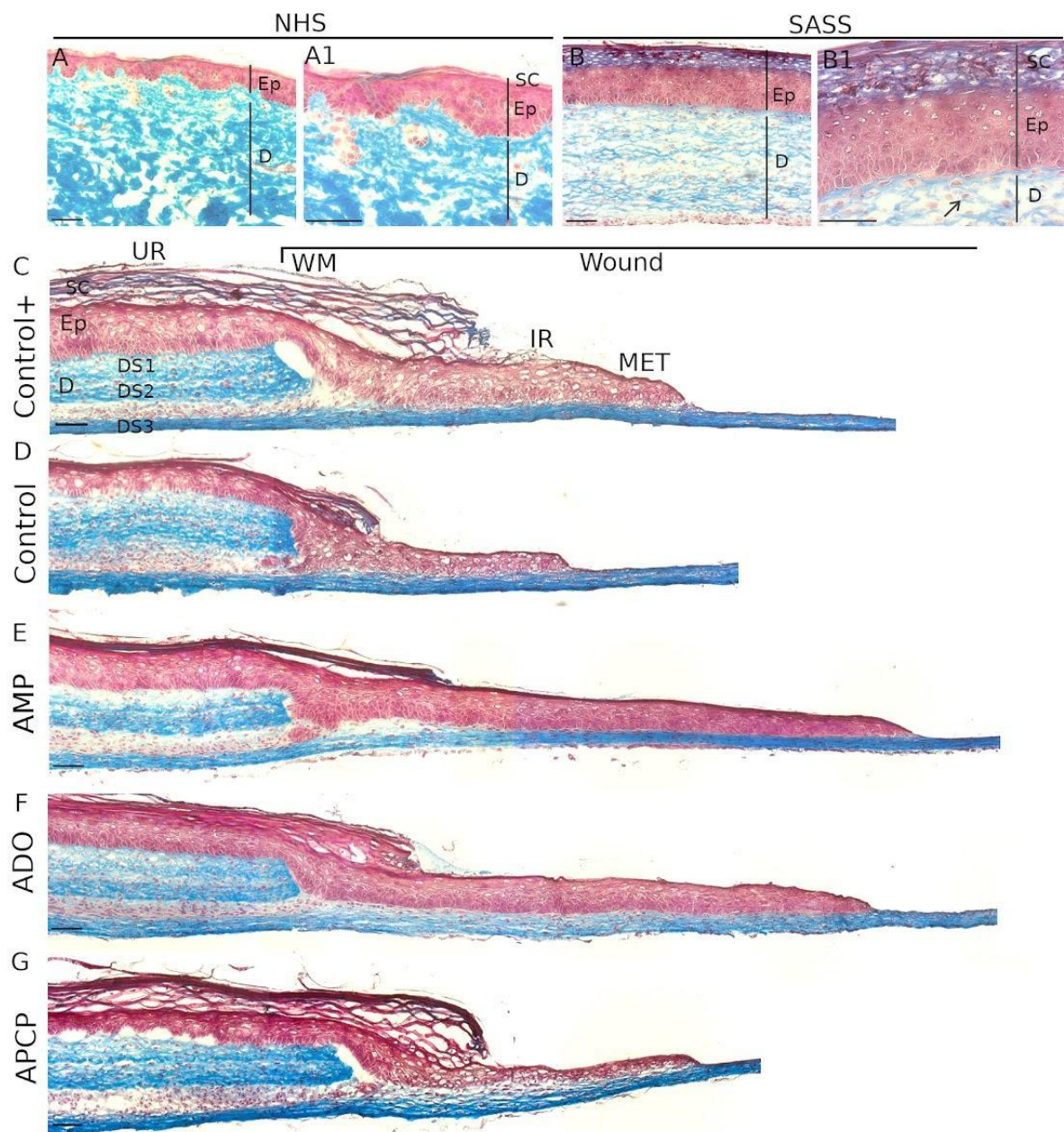


Figure 2. Masson's trichrome staining of wounded SASS. Cross-section of the normal human skin (NHS) (A-A1) and the Self Assembled Skin Substitute (SASS) (B-B1). The epidermis (Ep) with the stratum corneum (SC) and the dermis (D) are shown, fibroblasts embedded into matrix are indicated (arrow). Histological sections of wounded SASS according to treatment C) Control+, D) Control, E) AMP, F) ADO and G) APCP. Different regions in wounded SASS were named (C): the unwounded region (UR), followed by the wound that is composed by the wound margin (WM), the intermediate region (IR) and the migrating epidermal tongue (MET). The first and second dermal sheets in the dermis (DS1,2) represent the dermis of the SASS, the third dermal sheet (DS3) belongs to the wound assay model, allowed the migration of the epidermal tongue. The length of the migrating epidermal tongue between the WM and the tip of MET was measured (H) and one-way ANOVA and Tukey post-hoc test were done $*p < 0.05$. $N=2$. Graph bars represent the mean with SEM. Scale bars 100 μm .

Cell proliferation in wounded Self-Assembled Skin Substitute

The proliferative cells through the regions of the wounded SASS were identified following Ki67 labeling in epidermis and dermis. In normal human skin and SASS, the proliferative cells were observed mainly in the basal layer of the epidermis. The percentage of positive cells labeling out of the total number of nuclei evaluated in normal human skin and in the SASS, in both epidermis and dermis was not statistically different. (Figure 3 A-B1, H). In all regions of the epidermis of wounded SASS, the use of supplemented medium (control+) increased the number of Ki67 positive cells compared to medium without additive (Control). Furthermore, in both cases, Ki67 staining of epidermal cells increased in the wound regions compared to unjuried skin. By contrast to the epidermis, very few Ki67 positive cells were observed in the unwounded part of the dermis. The number of positive cells strongly increased in all the wound parts with, again, a higher number of positive cells when control+ medium was used.

Following treatments with AMP or ADO, the number of Ki67 positive cells in epidermis increased in the wound margin region. In the dermal part, AMP stimulated growth level in the center of the wound (intermediate and MET regions). ADO also increased the number of Ki67 positive cells in the MET region of the dermis. The addition of the antagonist of CD73, APCP, never modified the number of growing cells in both parts of the skin. (Figure 3 C-H).

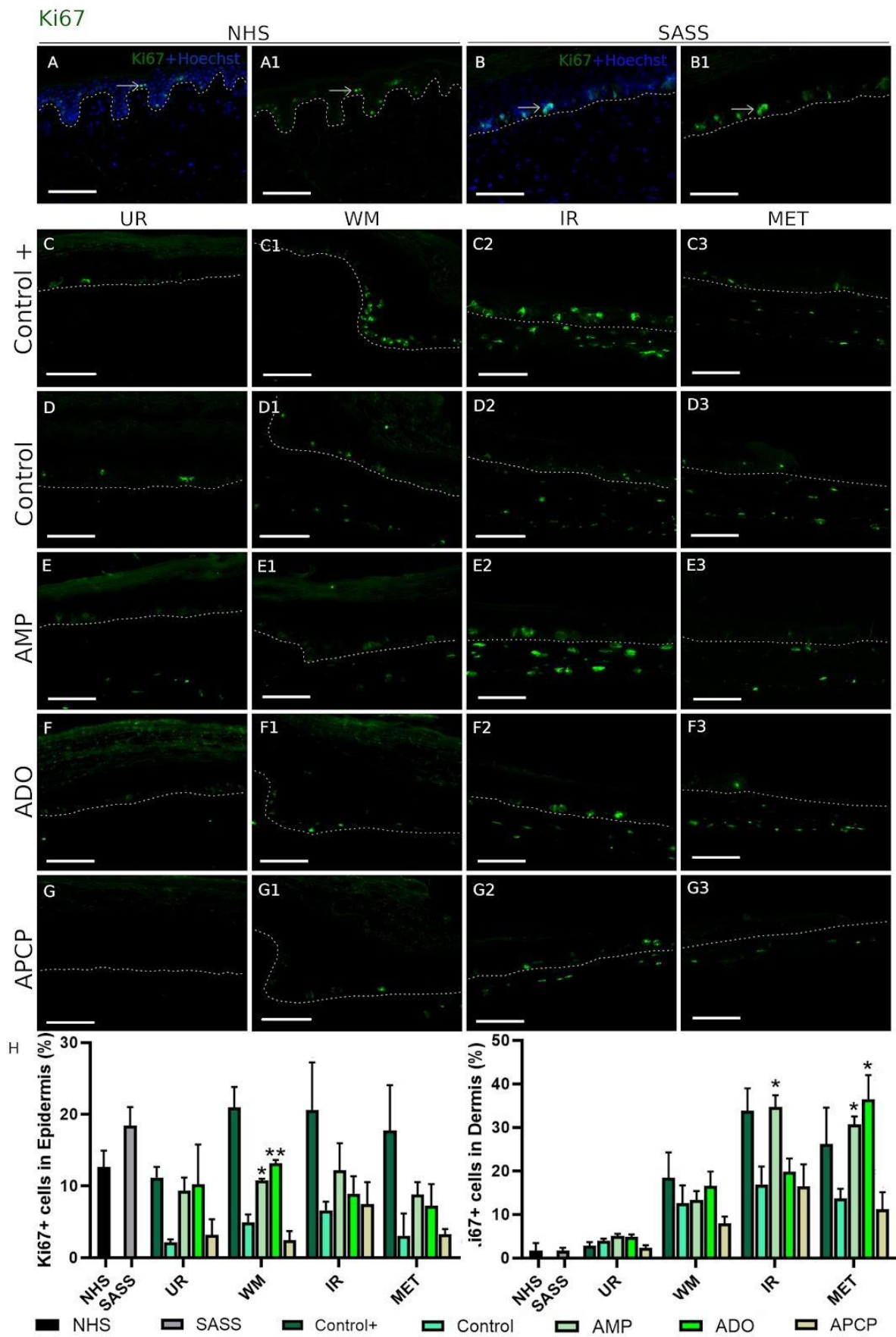


Figure 3. Ki67 staining in the epidermis and dermis of wounded SASS. Merged immunofluorescent staining for Ki67 (green) and nuclei (blue) in NHS (A-A1) and SASS (B-B1), white arrows indicate Ki67+ cells (A1-B1) respectively. The dotted white lines indicate the basement membrane of the epidermis. Immunostaining sections of wounded SASS according to treatment Control+ (C-C3), Control (D-D3), AMP (E-E3), ADO (F-F3) and APCP (G-G3). Ki67 positive cells were observed in the unwounded region (UR), wound margin (WM), intermediate region (IR) and in migrating epidermal tongue (MET). (H) The Ki67 positive cells were quantified in relation to the number of nuclei in the epidermis and the dermis. Asterisk indicates significant differences versus Control group. One-way ANOVA followed by Tukey post-hoc test for the treatments in each region and unpaired t-test to compare the NHS versus the SASS with no significant differences. (* $p < 0.05$), (** $p < 0.01$). Graph bars represent the mean with SEM. N=2. Nuclei were stained with Hoechst dye (blue). Scale bars 100 μm .

Expression of Cytokeratin 10 and Laminin-5 in wounded Self Assembled Skin Substitute

To identify any changes in the structure of the Self Assembled Skin Substitute following treatments, we analyzed the expression of laminin-5 as a marker of the basement membrane structure and cytokeratin 10 (CK10) as a marker of the epidermal differentiation. CK10 was expressed by epidermal suprabasal layers in SASS similarly to NHS (Figure 4 A-B). From the wound margin, the CK10 staining was reduced, being absent in the intermediate region and in migrating epidermal tongue (Figure 4 C-G1). The laminin-5 expression was observed as a continuous line between the epidermis and the dermis, on normal human skin, SASS, in the unwounded region and in the wound margin (Figure 5). Approaching the tip of the migrating tongue, the labeling was mainly found in the cytoplasm of basal keratinocytes (Figure 5 C1-G3). The expression of laminin-5 and cytokeratin 10 was similar for all treatments.

CK10 + Hoechst

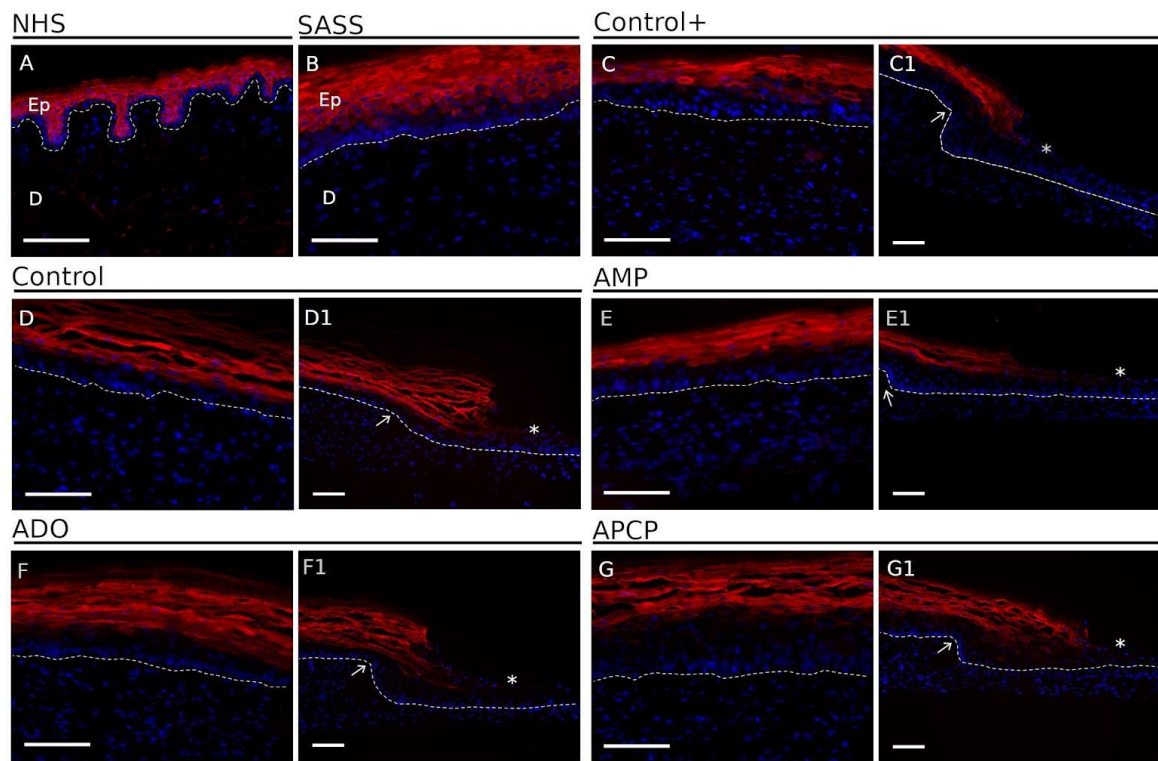


Figure 4. Cytokeratin 10 expression in wounded SASS. Cytokeratin 10 (CK10) expression (red) in normal human skin (NHS) A) and SASS B); dotted white lines indicate the basement membrane, under the basal layer of epidermis, which was unlabeled. The CK10 expression is shown in the unwounded region and at the wound margin for all the treatments; Control+ (C-C1), Control (D-D1), AMP (E-E1), ADO (F-F1) and APCP (G-G1). The white asterisk indicates positive keratinocytes away from the wound margin. Nuclei were stained with Hoechst dye (blue). White arrow indicates the wound margin. Scale bars 100 μ m.

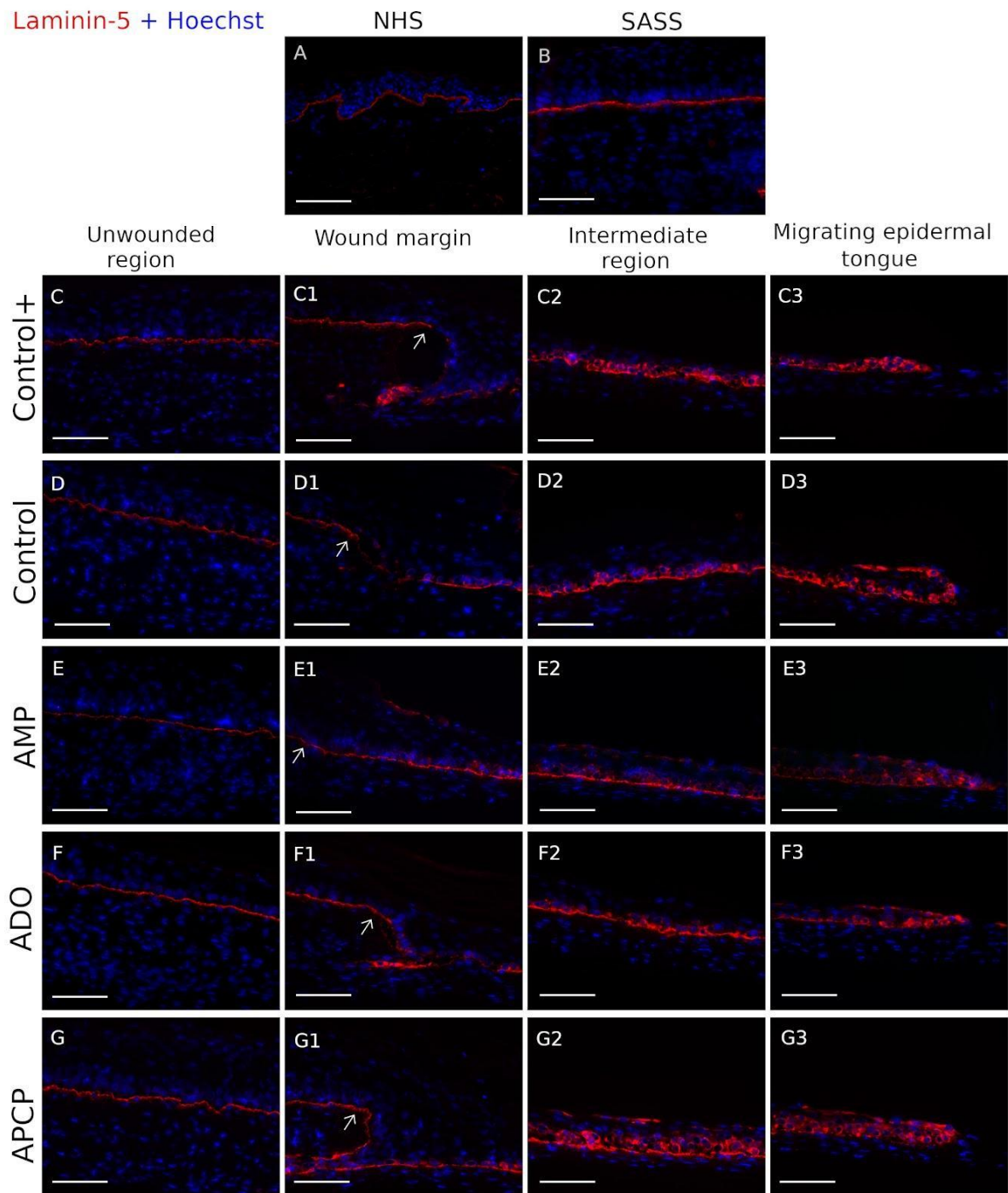


Figure 5. Laminin-5 expression in the wounded SASS. Immunofluorescence staining of the laminin 5 (red) expression in the basement membrane between epidermis (Ep) and dermis (D) in the normal human skin A) and the SASS B). The laminin 5 is present in the basement membrane of the unwounded region (C-G), the wound margin (C1-G1), the intermediate region (C2-G2) and the migrating epidermal tongue (C3-G3) in all the treatments. Cell nuclei (blue) stained by Hoechst dye. White arrow indicates the wound margin. Merged images, Scale bars 100 μ m.

CD73 expression in wounded Self Assembled Skin Substitute

To identify the epidermis and dermis sections in the wounded SASS we analyzed the vimentin expression, which was expressed by fibroblasts in the wounded SASS, similarly to human skin (Figure 6 A-C). In both human and SASS skins, we observed a strong CD73 labeling of cells in the dermis (Figure 6 D-E1). Interestingly, in the wounded SASS, the CD73 labeling was observed in fibroblast but also in basal keratinocytes, a staining that increased from the wound margin to the migrating epidermal tongue. The CD73 expression was also observed in more differentiated keratinocytes but it was with a lower intensity compared with the basal cell one in the wounded SASS (Figure 6 F-F4a). All these results were compared with the preimmune serum staining that was negative (Supplementary Figure 1).

By quantifying the fluorescence intensity, we observe that the CD73 expression in the epidermis and dermis was higher in wounded SASS (Figure 8 A-B).

Following treatments, CD73 staining also showed a similar pattern with an increase of expression from the margin to the center of the wound in basal keratinocytes for the different treatments (Figure 7). However, in the epidermis, the addition of AMP significantly increased CD73 expression in migrating epithelial tongue compared to other treatments (Figure 8 C-D). Concerning the dermal part, CD73 expression in fibroblasts was slightly increased in wound but only significantly increased following treatment with AMP in wound margin (Figure 7 A-E4 and Figure 8 C-D).

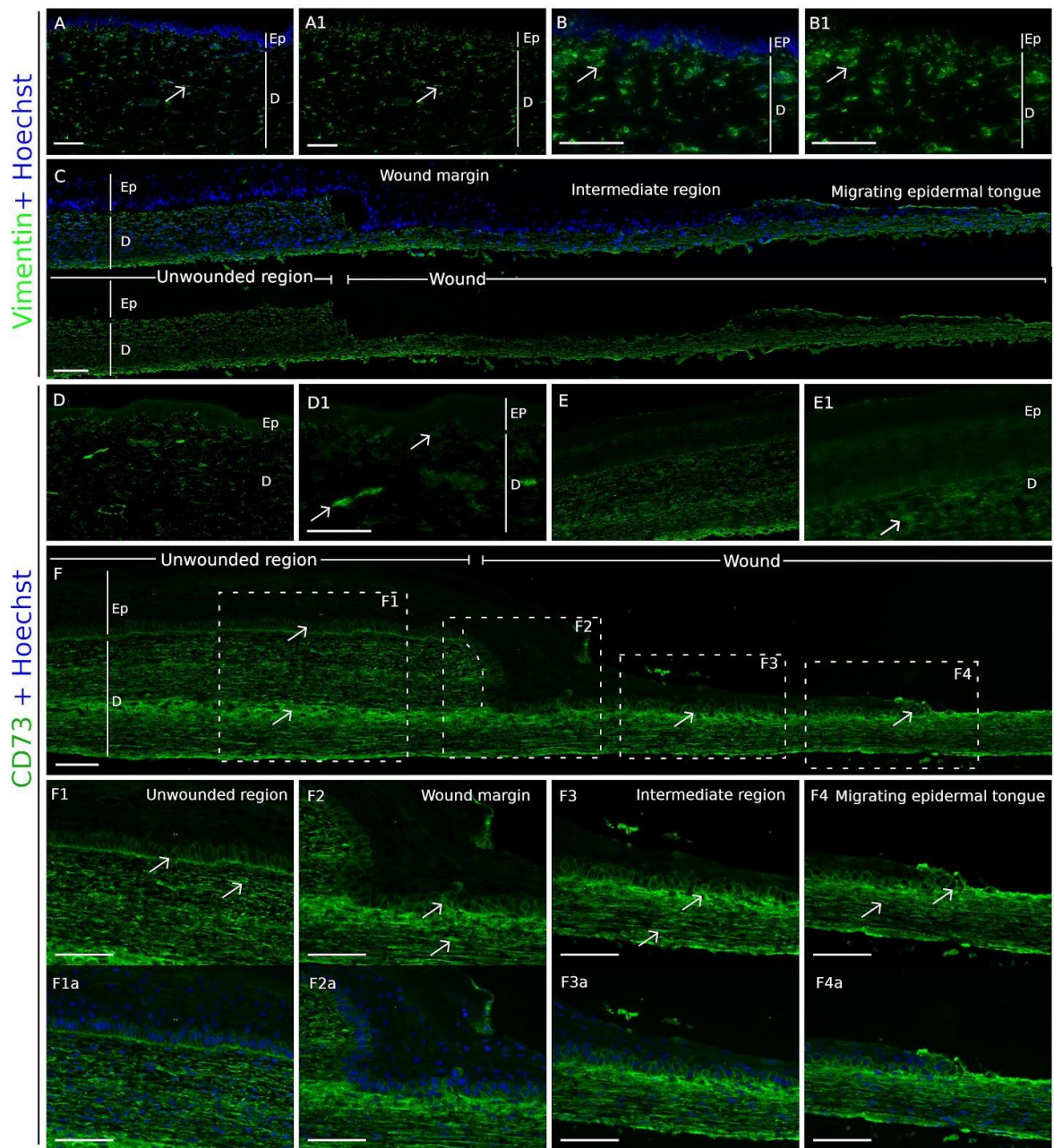


Figure 6. Vimentin and CD73 expression in wounded SASS. Vimentin positive cells in normal human skin (NHS) (A-A1), in SASS (B-B1) and in wounded SASS (C) are indicated with arrows). CD73 expression in dermal and epidermal cells in NHS (D-D1) and SASS (E-E1). In the wounded SASS F), the CD73 labeling was observed in fibroblasts. In epidermis, the CD73 labeling was more intense in basal keratinocytes, compared to normal skin epidermis and SASS. The regions of the wounded SASS are indicated, showing with more details the expression in fibroblast and basal keratinocytes. Scale bars 100 μ m.

CD73+Hoechst

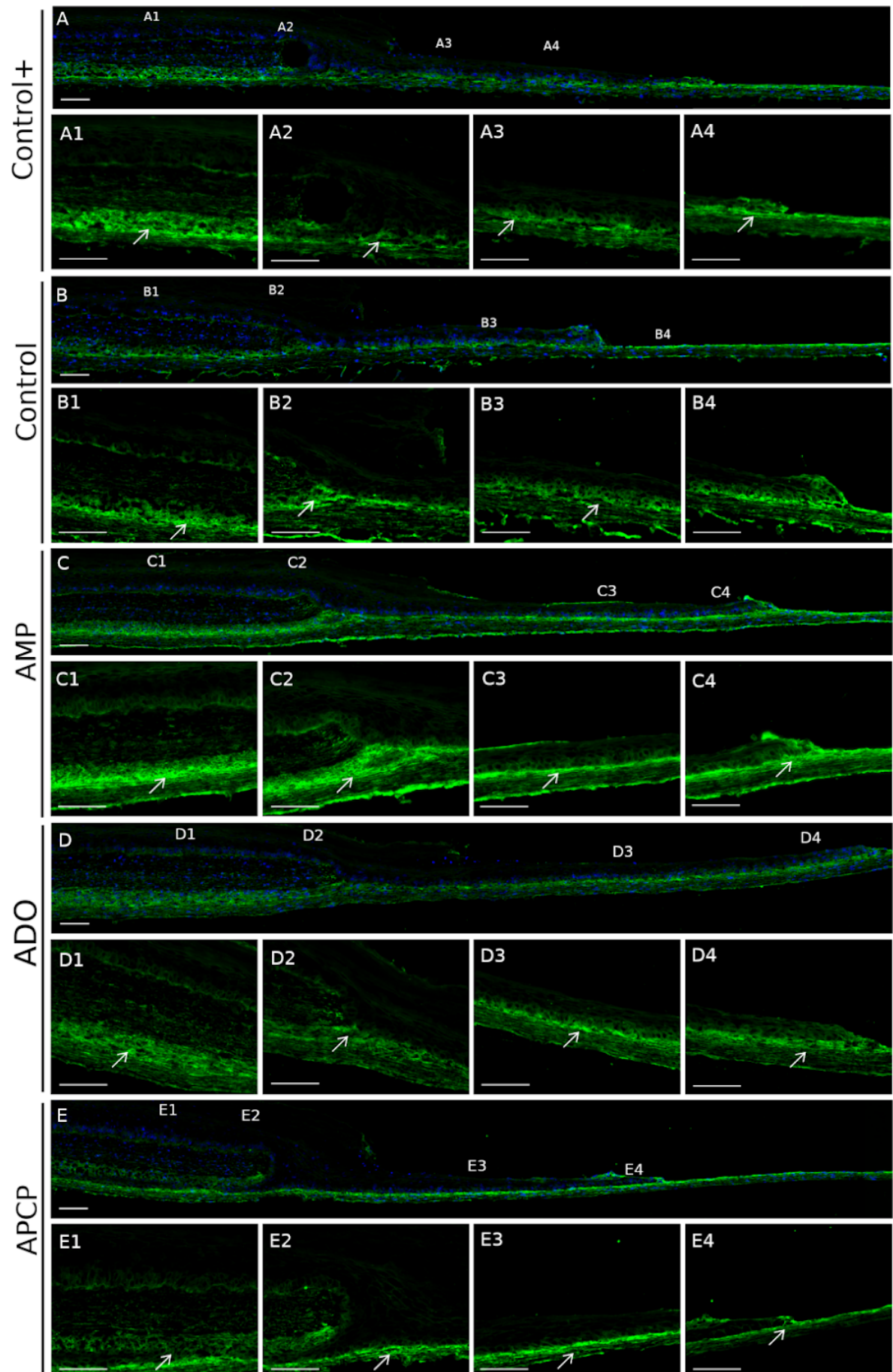


Figure 7. CD73 expression in wounded SASS after treatments. Expression of CD73 in the skin substitute after the treatments Control+ (A-A4), Control (B-B4), AMP (C-C4), ADO (D-D4) and APCP (E-E4). The CD73 labeling is indicated in each region, unwounded region (A1-E1), wound margin (A2-E2), intermediate region (A3-E3) and migrating epidermal tongue (A4-E4). Scale bars 100 μm .

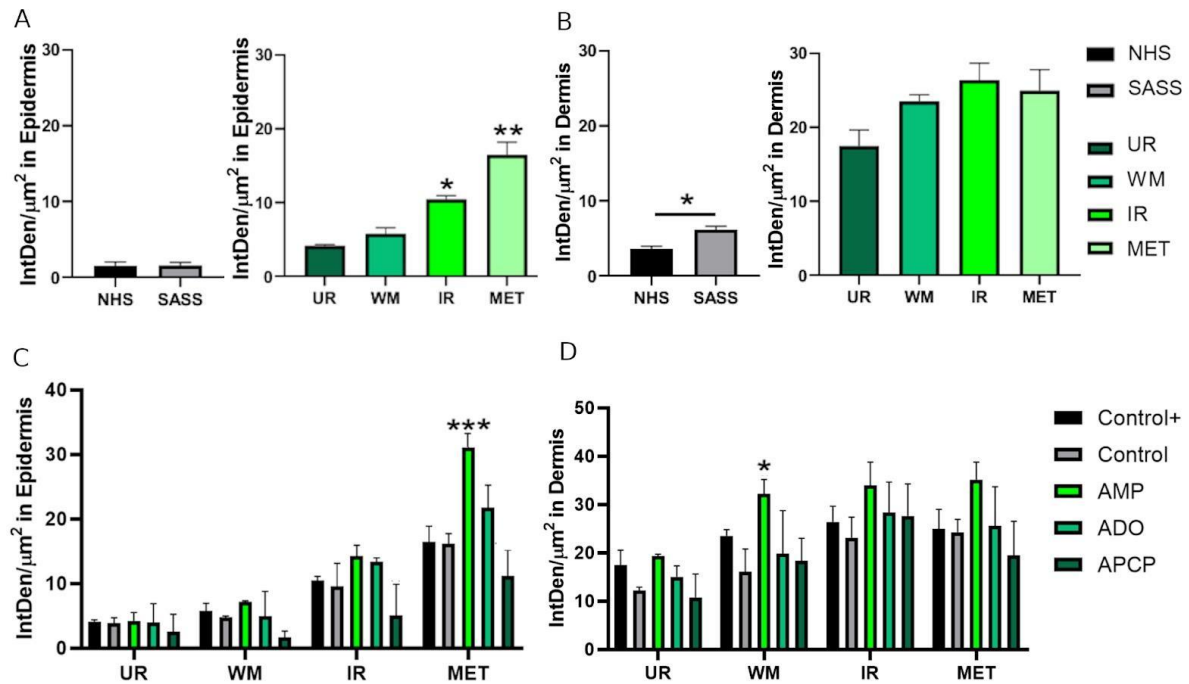


Figure 8. Relative CD73 fluorescence intensity in wounded SASS. Fluorescence intensity (Integrated density/ μm^2) in epidermis A), and dermis B) on NHS, SASS and all the regions. Integrated density/ μm^2 in epidermis C) and dermis D) on all the regions after treatments. (A-B) Unpaired t-test to compare the NHS versus the SASS; one-way ANOVA followed by Tukey post-hoc test, significant differences versus unwounded region (UR) (*). (C-D) Two-way ANOVA followed by Tukey post-hoc test, significant differences versus Control (*). Graph bars represent the mean with SEM, N=2 (* <0.05); (** $p<0.01$), (***) $p<0.001$).

CD73 expression in primary human keratinocytes in monolayer

The CD73 was observed in keratinocytes cultured in monolayer and then scratched to mimic a wound. The keratinocytes showed a strongest CD73 expression in the scratch region compared to the unwounded region that showed a very weak labeling (Figure 9). No differences were observed with and without the mitomycin C treatment (data not shown). The labeling was compared with the unlabeled preimmune serum staining (Supplementary Figure 1 D-D1).

CD73 + Hoechst

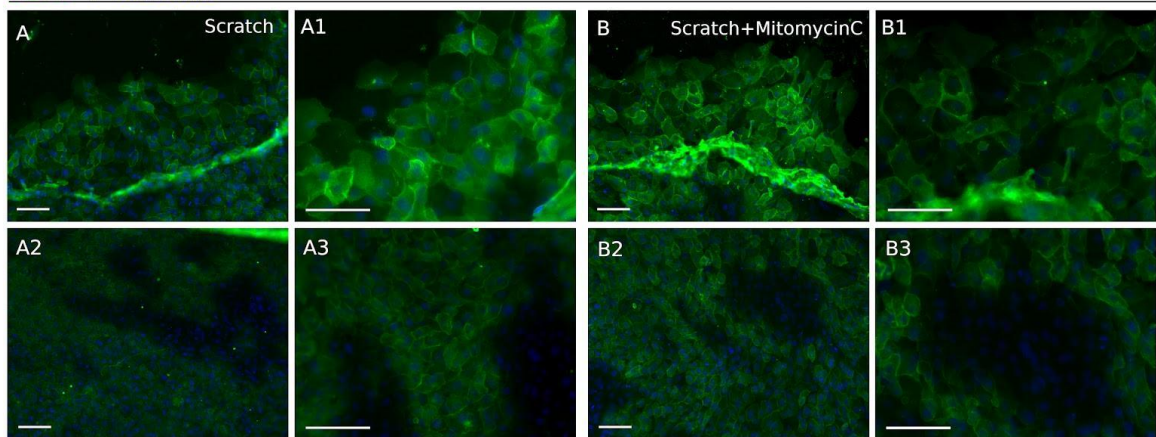
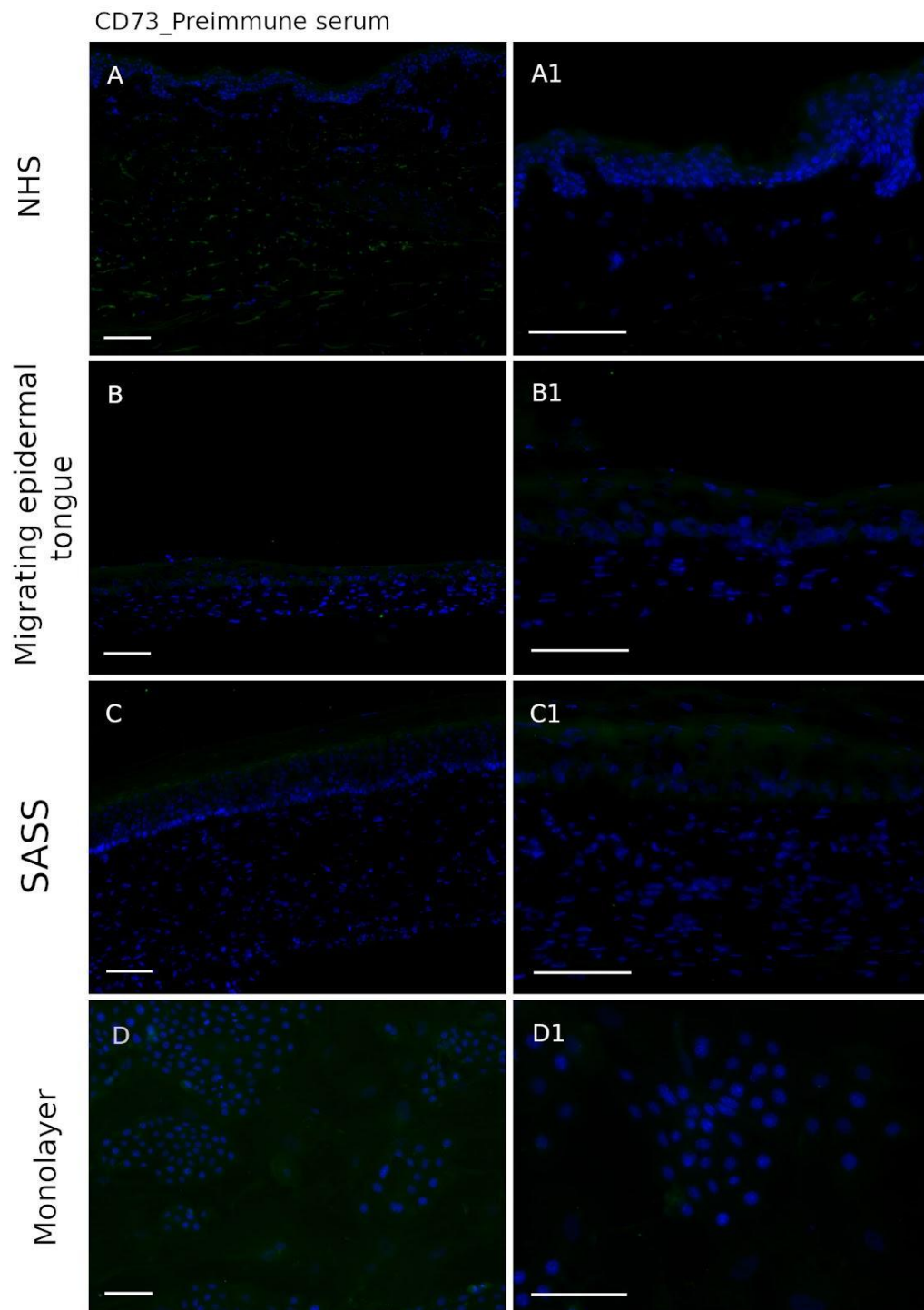


Figure 9. CD73 expression in primary human keratinocytes in monolayer. CD73 expression in primary human keratinocytes in monolayer after scratch, without mitomycinC (A-A3) on the scratch region (A-A1) and near the scratch region (A2-A3); and with the mitomycinC treatment (B-B3), on the scratch region (B-B1) and near the scratch region (B2-B3). Cell nuclei (blue) stained by Hoechst dye. Scale bars 100 μ m.



Supplementary Figure 1. Unlabeled labeling of CD73 using preimmune serum instead of antibody in normal human skin (A-A1), wounded SASS on migrating epidermal tongue (B-B1), SASS (C-C1) and keratinocytes in monolayer (D-D1). Scale bars 100 μ m.

Discussion

The wound healing model produced from the Self-Assembled Skin Substitute was an useful tool to analyze the CD73 involvement during the wound healing process, as well as, the effect of ADO and AMP on the reepithelialization process. Likewise, the analysis of proliferation during the wound healing showed the involvement of AMP and ADO in both parts of the skin, but not in the same place: on the wound margins for epidermis and on the center of the wound for the dermis. The expression of structural components of the skin, such as cytokeratin 10 and laminin-5, was observed in the different regions of the wounded SAAS, without detecting alterations with the different treatments.

The role of ADO in wound healing has already been studied. ADO has been used as a topical application to reduce wrinkles (Legendre et al. 2007), perhaps by promoting collagen production by dermal fibroblasts via the adenosine A2A receptor (Chan et al. 2006). The nucleoside promotes wound neovascularization by stimulating the A2A receptor (Montesinos et al. 2004). However, an excessive ADO generation by CD39 and CD73 promotes dermal fibrosis (Lu and Insel 2014; Fernández et al. 2013). In accordance, studies involving tumor biology have demonstrated a positive association of CD73/ADO expression with extracellular matrix organization and TGF- β expression (Turcotte et al. 2017). The role of TGF- β is already demonstrated by its capacity to stimulate the proliferation and migration of fibroblasts, increased collagen production and by the profibrogenic activity, which are the basis for the development of tissue fibrosis (Ferrari et al. 2016).

Shaked et al. demonstrated in burn patients that there is an accumulation of ADO in the fluid of burn blisters, and that this could exert effects depending on its concentration, which may have beneficial anti-inflammatory effects but also lead to systemic dysfunction of the immune system and hair loss syndrome (Shaked et al. 2007). According to Andrés and colleagues (2017), ADO plays an important role in the regulation of epidermal inflammation and the function of keratinocytes and, therefore, can constitute an interesting therapeutic strategy for hyperproliferative inflammatory skin diseases such as psoriasis (Andrés et al. 2017).

Our group previously showed that mesenchymal stromal cells (MSCs) isolated from human dermis and cultured in monolayer on plastic release high concentrations of ADO (Naasani et al. 2017). This increase may be related to the high expression of CD73, considered one of the MSCs markers, resulting in a high hydrolytic activity of AMP in ADO (Roszek and Wujak 2018). The accumulation of ADO can help control the recruitment of immune cells during the healing process. This is extremely relevant, since these cells can direct different types of tissue regeneration depending on their polarization, and may have anti-fibrotic or inflammatory effects, which would lead to a process of regeneration or fibrosis formation, respectively (Larouche et al. 2018).

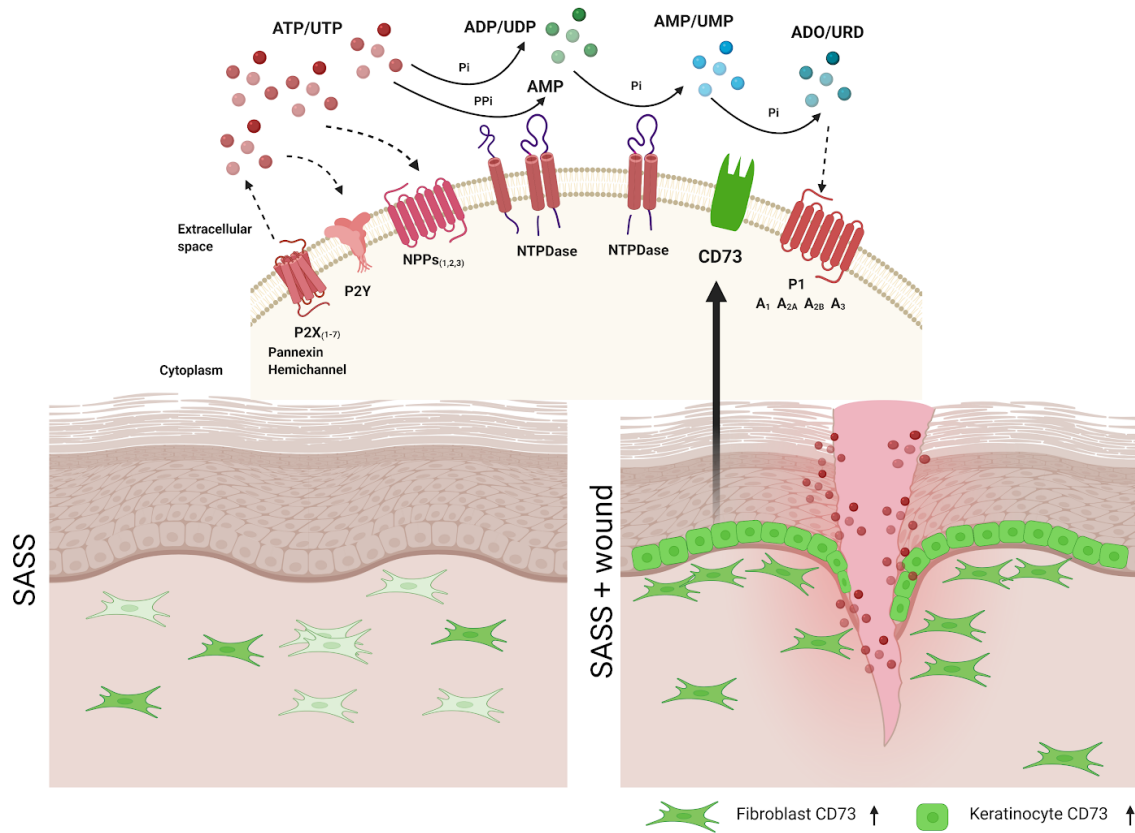
In order to analyze the CD73 involvement during the wound healing process, we identified the dermis from the epidermis by using vimentin labeling, expressed by fibroblast in the wound. While CD73 was very weakly expressed in unwounded skin, its expression strongly increased during the wound healing process, mainly in basal keratinocytes and, in dermis, below the basement membrane region of the neoepidermis. Same observation was made with monolayer-cultured keratinocytes that were injured, which suggests the involvement of CD73 during human skin repair. The CD73 overexpression during wound healing was further

increased following AMP treatment. This result was expected, since the AMP is hydrolyzed to ADO by CD73. Once inside the cell, ADO is converted to inosine through adenosine deaminase or in AMP by adenosine kinase (Galgaro et al. 2021).

CD73 associated with ADO metabolism promotes tumor progression by suppressing the antitumor immune response and promoting angiogenesis (Reviewed in (Chen et al. 2019)). According to Azambuja and colleagues (2020), the CD73 knockdown can regulate the glioblastoma immune microenvironment and delay tumor growth by inducing apoptosis (Azambuja et al. 2020). A distinct perspective on the role of CD73-generated adenosine in tumor development was observed in endometrial carcinomas. Bowser and colleagues (2016) reported that the loss of CD73 in endometrial carcinoma is crucial for tumor progression, since it cancels the physiological effect of extracellular adenosine to protect epithelial integrity. Adenosine generated by CD73 appears to be necessary to sustain a physiological balance that maintains epithelial integrity in endometrial carcinomas with stable cell-cell adhesions mediated by A1R (Bowser et al. 2016). As epithelial barrier function is essential for the inhibition of endometrial carcinomas with fewer intervening stromal or inflammatory cells in interconnected malignant glands (Silverberg 2000), Wang and colleagues (2021) provided a novel mechanism for adenosine-mediated barrier protection, showing that enhancing extracellular adenosine levels restores barrier function in acute lung injury, through expression of focal adhesion proteins (Wang et al. 2021). Bowser and Broaddus (2016) in their review discussed the role of CD73 in protecting epithelial integrity. Furthermore, they argue that CD73 is not uniformly expressed by all epithelial cells in all tissues and epithelial cell compartments, such as pancreas, breast and gastrointestinal tract (Bowser and Broaddus 2016). Considering that, the generation of extracellular adenosine by CD73 is crucial for the maintenance of the epithelial barrier, similarly to endothelial cells, adenosine generated by CD73 decreases permeability in intestinal epithelial cells and regulates cell adhesion molecules (Bowser and Broaddus 2016).

Interestingly, CD73, as an adhesion molecule, can bind various components of the extracellular matrix, including laminin (Olmo et al. 1992; Stochaj et al. 1989; Minor et al. 2019). As we have observed in our wound healing model, there is an increase in laminin-5 expression during the wounded regions. Laminin 5 induces the adhesion, spreading, and migration of human keratinocytes and during skin wound healing, its deposition in migratory keratinocytes occurs early, followed by hemidesmosomes and then basement membrane formation (Amano et al. 2004). According to Minor and colleagues (2019), the importance of CD73 is especially evident in the context of acute and chronic types of injury, where its activity is vital to maintain tissue integrity and improve recovery (Minor et al. 2019). Therefore, the CD73 increase during wound healing could be related to the protection of the epithelial barrier.

In conclusion, in this study we found that the CD73 expression is modulated during the wound healing, after stimulation with AMP and ADO in the Self Assembled Skin Substitute (SASS) model. The increase in CD73 expression in the wounded SASS regions suggests its protective role during the wound healing by controlling the adenosine levels, which should lead to a process of regeneration, without fibrosis formation.



Graphical abstract.
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10 DISCUSSÃO

O potencial terapêutico associado às células estromais mesenquimais está aumentando constantemente. Devido ao potencial de diferenciação e capacidade imunomoduladora e de quimioatração, as MSCs têm sido consideradas como uma fonte potencial de células para seu uso em bioengenharia de tecidos e terapias celulares (Paim and Wink 2022). Atualmente, o secretoma das MSCs também tem sido considerado para o tratamento da COVID (Schultz, Bertoni, and Wink 2021). No entanto, mais estudos e ensaios clínicos serão necessários para demonstrar a segurança e eficácia destas células, até que possam ser utilizadas em pacientes.

Uma das principais vantagens das MSCs é que atualmente podem ser facilmente isoladas a partir de materiais biológicos, que geralmente são descartados, como pele, após uma cirurgia de abdominoplastia, tecido adiposo, após uma cirurgia de lipoaspiração e limbo esclero-corneal após um transplante de córnea, a partir de um doador cadáver. Segundo a Sociedade Internacional de Cirurgia Plástica Estética, o Brasil superou os Estados Unidos em relação aos procedimentos cirúrgicos estéticos como abdominoplastias e lipoaspirações (Gomes et al. 2021), aumentando assim, a disponibilidade de tecidos nos principais centros hospitalares. Neste trabalho efetivamente isolamos populações de células estromais mesenquimais a partir de lipoaspirado humano (hAD-MSCs), tecido esclero-corneal humano (L-MSCs) e tecido adiposo murino (mAD-MSCs), as quais apresentaram morfologia fusiforme, adesão ao plástico, potencial de auto-renovação e diferenciação de linhagem, característico de células tronco mesenquimais.

Uma das abordagens para que as células estromais mesenquimais possam ser transplantadas é através da utilização de um *scaffold*. A produção de *scaffolds* é um dos principais desafios na área de bioengenharia de tecidos. Dependendo da abordagem, esses *scaffolds* podem carregar células diferenciadas no tecido alvo ou permitir que as MSCs mantenham seu potencial indiferenciado e expressão de marcadores. A última opção seria a mais adequada para a regeneração de tecidos como pele ou córnea, onde possam migrar para a área lesionada e auxiliar no reparo.

Não é um acaso que a membrana amniótica humana tenha sido considerada como um *scaffold* versátil para engenharia de tecidos. A membrana amniótica é uma fonte natural de colágeno, uma das principais proteínas estruturais do corpo, que por conter a sequência RGD favorece a adesão celular. Após uma cirurgia de cesárea, a placenta, que geralmente é descartada,

também é consideradas uma fonte tecidual para engenharia de tecidos, por ser rica em células estromais mesenquimais do cordão umbilical, membranas placentárias como o córion e a membrana amniótica e demais tecidos placentários.

Neste trabalho demonstramos que foi possível separar manualmente a hAM da placenta e outras membranas fetais e de preservá-la em glicerol a 4° C, o que facilita o processo de conservação deste tecido. Após avaliar o impacto do processo de descelularização da hAM, confirmamos que não foram afetadas as principais proteínas de matriz, o que é fundamental para a estruturação de um *scaffold*. Com a análise termogravimétrica foi possível identificar que a membrana amniótica é estável a altas temperaturas, importante característica para o tratamento de queimaduras.

Após o cultivo das AD-MSCs na DhAM, foi possível observar que não foi alterada a viabilidade e senescência e que as células não perderam seu potencial de células tronco, tanto do potencial de diferenciação tri-linhagem como da manutenção dos marcadores de células mesenquimais.

A membrana amniótica humana também tem sido estudada como forte alternativa para o tratamento de lesões na córnea. No segundo capítulo foi feita a comparação do potencial da membrana amniótica humana descelularizada (DhAM) e da submucosa do intestino delgado porcino (SIS) como *scaffolds* para o cultivo de células mesenquimais limvais. Ambos *scaffolds* mantiveram a viabilidade, morfologia, densidade e expressão de marcadores das células mesenquimais limvais, no entanto, devido a sua transparência e a preservação dos marcadores de caracterização de mesenquimais, a hAM representa uma grande vantagem para sua utilização como suporte de células mesenquimais, criando uma espécie de curativo temporário que permita a liberação de moléculas bioativas, como citocinas e fatores de crescimento e, em seguida, promova a regeneração dos tecidos, tanto em medicina humana e veterinária.

Com a finalidade de utilizar o constructo de membrana amniótica com MSCs como um curativo para o tratamento de queimaduras, foi padronizado o modelo murino de lesão cutânea. Os resultados obtidos demonstraram que a utilização da membrana amniótica descelularizada para o tratamento de queimaduras pode ser igualmente eficaz como o tratamento comercial e ainda melhor quando combinada com células estromais mesenquimais, em comparação com a utilização de gaze.

Na análise macroscópica das lesões observamos uma menor quantidade de tecido inviável, e aparentemente uma maior densidade capilar e melhor aspecto visual da cicatriz no grupo DhAM+AD-MSCs. A análise histológica mostrou que a DhAM foi segura e eficaz, pois não causou alteração nos órgãos. Também, a DhAM foi capaz de cicatrizar as feridas melhor que o controle positivo (curativo comercial), e a presença de AD-MSCs melhorou a cicatrização, em comparação com outros grupos. A liberação do uso da membrana amniótica no Brasil, teve um grande avanço com o parecer positivo do CFM quanto ao uso deste rico tecido no tratamento de queimaduras e em outras tantas áreas de aplicação no país, no entanto ainda é necessário aguardar a regulamentação da utilização deste tecido junto aos órgãos de saúde e vigilância do Brasil. Este projeto teve financiamento do PPSUS, portanto, os resultados da eficácia e segurança deste *bioscaffold*, de baixo custo e elevada disponibilidade, podem representar um dos caminhos para a futura aplicação clínica como terapia para o tratamento de queimaduras pelo SUS.

O aumento de células CD73 positivas nas primeiras duas semanas da lesão no modelo de queimadura, sugere o envolvimento desta enzima no processo de cicatrização de feridas. As AD-MSCs poderiam promover a regeneração modulando o sistema imunológico, visto que a enzima CD73 produz adenosina que é um imunossupressor, o que foi reforçado pela menor expressão do marcador CD11b no grupo de tratamento com AD-MSC.

O envolvimento da CD73 na regeneração de tecidos também foi observado no modelo de cicatrização de feridas realizado no substituto de pele auto-montados (SASS). Curiosamente, foi identificado um aumento na expressão da CD73 no modelo de SASS com injúria, nos queratinócitos da membrana basal e nos fibroblastos perto da membrana basal, em comparação com a pele humana e o SASS. Esse aumento na expressão da CD73 também foi observado em queratinócitos em monocamada. Como esperado, o aumento na expressão da CD73 também foi observado após o tratamento com AMP. Nos estudos relacionados com câncer, o papel da CD73 tem sido associado na proteção da integridade da barreira epitelial (Bowser and Broaddus 2016). Esse fenômeno poderia estar ocorrendo durante a cicatrização de feridas, sendo que a CD73 pode ligar vários componentes da matriz extracelular, incluindo laminina, cuja expressão também foi observada nos queratinócitos da membrana basal.

O potencial terapêutico da sinalização purinérgica tem sido explorado numa ampla variedade de doenças, incluindo as da pele, mas o desenvolvimento de compostos purinérgicos ainda está na sua infância. No entanto, como acreditava o Dr. Burnstock, "podemos olhar para o

futuro dos compostos purinérgicos como agentes terapêuticos com otimismo" (Geoffrey Burnstock and Knight 2018).

O SASS é uma excelente ferramenta para o estudo de sistemas biológicos, que tornou possível analisar o papel agonistas e antagonistas de sinalização purinérgicos, como ATP, UTP e um antagonista do receptor P2Y, Reactive Blue 2 (RB2). No SASS, o tratamento com UTP mostrou um efeito terapêutico na cicatrização de feridas. O potencial terapêutico do UTP tem sido demonstrado em sistemas como córnea de coelho, queratinócitos humanos HaCaT e fibroblastos (Pintor et al. 2004; Jokela et al. 2017), no entanto, esse potencial nunca foi estudado em um modelo de pele humana.

A expressão das enzimas NTPDase1 e NPP1 foi observada pela primeira vez em um modelo de pele humana reconstruída, mostrando sua modulação nas regiões SASS com injúria. O nosso grupo de pesquisa relatou a expressão dos genes ENTPDase1 e ENPP1 em células estromais mesenquimais isoladas da pele e da córnea. As células estromais mesenquimais dérmicas expressaram níveis mais elevados de ENTPDase1, levando à liberação de adenosina que pode estar associada às propriedades imunomoduladoras dessas células (Naasani et al. 2017).

Portanto, esperamos que estes estudos possam contribuir com a área de biomateriais e regeneração, apresentando uma estratégia eficiente para o tratamento de queimaduras que também seja economicamente viável, pois pode ser engenheirada com tecidos humanos que normalmente são descartados nos hospitais. Também, que o envolvimento de nucleotídeos extracelulares e principais ectoenzimas, na cicatrização da pele humana, possa contribuir nas futuras aplicações clínicas utilizando o potencial de nucleotídeos para o desenvolvimento de novas abordagens para o tratamento farmacológico de feridas complexas.

11 CONCLUSÕES

1. Populações de células estromais mesenquimais a partir de lipoaspirado humano (hAD-MSCs), tecido esclero-corneal humano (L-MSCs) e tecido adiposo murino (mAD-MSCs), as quais apresentaram morfologia fusiforme, adesão ao plástico, potencial de auto-renovação e diferenciação de linhagem, foram efetivamente isoladas.
2. Foi efetivamente padronizada a descelularização da membrana amniótica humana, garantido a preservação das principais proteínas de matriz extracelular como colágeno e fibronectina.
3. A membrana amniótica humana foi caracterizada física e histologicamente na presença e ausência de células estromais mesenquimais.
4. O cultivo das células estromais mesenquimais na membrana amniótica não alterou a viabilidade e senescência, manutenção dos marcadores de células mesenquimais, potencial de células tronco e diferenciação tri-linhagem.
5. A membrana amniótica humana foi um *scaffold* mais adequado para as células estromais mesenquimais do limbo esclerocorneal humano, do que a submucosa de intestino delgado de suínos (SIS), disponível comercialmente.
6. O modelo murino de lesão cutânea por queimaduras de segundo grau profundo foi padronizado, podendo-se identificar que a queimadura atingiu os folículos pilosos e glândulas sebáceas.
7. Foi avaliado o efeito da membrana amniótica descelularizada na presença e ausência de células estromais mesenquimais derivadas de tecido adiposo de ratos na regeneração tecidual no modelo pré-clínico de queimadura de segundo grau profundo, através de análises histológicas e de imunofluorescência.
8. As análises macroscópicas e histológicas demonstraram a eficácia e segurança da membrana amniótica associada com células estromais mesenquimais derivadas de tecido adiposo de ratos no modelo pré-clínico de queimadura de segundo grau profundo.
9. O envolvimento da CD73 na regeneração da pele foi observado *in vitro*, através do substituto cutâneo SASS e *in vivo*, no modelo pré-clínico de queimadura.
10. O papel dos componentes da sinalização purinérgica na regeneração tecidual foi analisado através do substituto cutâneo substituto cutâneo SASS, demonstrando o

envolvimento das enzimas CD73, NTPDase1 e NPP1 e do nucleotídeo UTP durante a cicatrização de feridas.

12 PERSPECTIVAS

1. Avaliar a expressão das enzimas NTPDase1 e NPP1 nas peles dos ratos do modelo pré-clínico de queimadura de segundo grau profundo.
2. Avaliar o perfil de células imunes nas peles dos ratos do modelo pré-clínico de queimadura de segundo grau profundo.
3. Estudar a expressão das enzimas CD73, NTPDase1 e NPP1 em peles com cicatrizes hipertróficas.

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ANEXO A: CEP

Parecer de aprovação do Comitê de Ética e Pesquisa com Seres Humanos (CEP)

IRMANDADE DA SANTA CASA
DE MISERICORDIA DE PORTO
ALEGRE - ISCMPA



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Isolamento de células a partir de material de descarte humano para estudos em medicina regenerativa

Pesquisador: Marcia Rosangela Wink

Área Temática:

Versão: 2

CAAE: 98290318.5.0000.5335

Instituição Proponente: ISCMPA

Patrocinador Principal: Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul

DADOS DO PARECER

Número do Parecer: 3.029.141

Apresentação do Projeto:

Projeto de pesquisa a ser realizado em parceria com o Laboratório de Biologia Celular da Universidade Federal de Ciências da Saúde de Porto Alegre e as Unidades de Obstetrícia, Oftalmologia e Cirurgia Plástica da Santa Casa de Porto Alegre, utilizando material de descarte hospitalar como lipoaspirado, pele, limbo esclero-corneal, membrana amniótica e cordão umbilical, serão extraídos diferentes tipos celulares humanos (queratinócitos, melanócitos, células estromais mesenquimais e células endoteliais) para o desenvolvimento de novas terapias celulares para medicina regenerativa, como a aplicação de células estromais mesenquimais em sítios lesionados e também para a verificação da biocompatibilidade de materiais propostos para uso clínico, tais como filmes de quitosana e colágeno, stents cardíacos de ferro e geopolímeros.

Objetivo da Pesquisa:

Já referido em parecer anteriormente emitido.

Avaliação dos Riscos e Benefícios:

Já referido em parecer anteriormente emitido.

Comentários e Considerações sobre a Pesquisa:

Já referido em parecer anteriormente emitido.

Endereço: R. Profª Annes Dias, 295 Hosp. Dom Vicente Scherer

Bairro: 6º andar - Centro

CEP: 90.020-090

UF: RS

Município: PORTO ALEGRE

Telefone: (51)3214-8571

Fax: (51)3214-8571

E-mail: cep@santacasa.tche.br

IRMANDADE DA SANTA CASA
DE MISERICORDIA DE PORTO
ALEGRE - ISCMPA



Continuação do Parecer: 3.029.141

Considerações sobre os Termos de apresentação obrigatória:

Já referido em parecer anteriormente emitido.

Recomendações:

Inserido nº do CNPJ na Folha de Rosto para Pesquisa envolvendo seres humanos.

Conclusões ou Pendências e Lista de Inadequações:

As pendências solicitadas foram atendidas conforme carta e documentos anexados, a pesquisa está de acordo com a resolução vigente.

Considerações Finais a critério do CEP:

Após reavaliação do protocolo acima descrito, o presente comitê não encontrou óbices quanto ao desenvolvimento do estudo em nossa Instituição e poderá ser iniciado a partir da data deste parecer.

Obs.: 1 - O pesquisador responsável deve encaminhar à este CEP, Relatórios de Andamento dos Projetos desenvolvidos na ISCMPA. Relatórios Parciais (pesquisas com duração superior à 6 meses), Relatórios Finais (ao término da pesquisa) e os Resultados Obtidos (cópia da publicação).

2 – Para o início do projeto de pesquisa, o investigador deverá apresentar a chefia do serviço (onde será realizada a pesquisa), o Parecer Consubstanciado de aprovação do protocolo pelo Comitê de Ética.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1200535.pdf	12/11/2018 16:54:02		Aceito
Recurso Anexado pelo Pesquisador	CARTA_RESPOSTA_AO_CEP.pdf	12/11/2018 16:53:24	Samlai Vedovatto	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO_MATERIAIS_DE_DESCARTE.pdf	12/11/2018 16:50:59	Samlai Vedovatto	Aceito
Folha de Rosto	FOLHA_DE_ROSTO.pdf	12/11/2018 16:49:40	Samlai Vedovatto	Aceito
TCLE / Termos de Assentimento / Justificativa de	TCLEs_materiais_descarte_e_justificativas.pdf	12/09/2018 17:57:45	THAIS CASAGRANDE PAIM	Aceito

Endereço: R. Profª Annes Dias, 295 Hosp. Dom Vicente Scherer

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UF: RS

Município: PORTO ALEGRE

Telefone: (51)3214-8571

Fax: (51)3214-8571

E-mail: cep@santacasa.tche.br

**IRMANDADE DA SANTA CASA
DE MISERICORDIA DE PORTO
ALEGRE - ISCMPA**



Continuação do Parecer: 3.029.141

Ausência	TCLEs_materiais_descarte_e_justificativas.pdf	12/09/2018 17:57:45	THAIS CASAGRANDE	Aceito
Outros	Formulario_Cadastro_Projetos_Unidade_Pesquisa.pdf	16/08/2018 16:02:38	Samlai Vedovatto	Aceito
Declaração de Instituição e Infraestrutura	Termo_responsavel_setor_onde_realiza_da_pesquisa.pdf	16/08/2018 16:00:56	Samlai Vedovatto	Aceito
Declaração de Pesquisadores	Termo_entrega_relatorios.pdf	16/08/2018 15:59:54	Samlai Vedovatto	Aceito
Outros	Declaracao_de_autorizacao_da_chefia_responsavel.pdf	16/08/2018 15:56:09	Samlai Vedovatto	Aceito
Outros	Formulario_Inscricao_Projetos_pesquisa.pdf	16/08/2018 15:52:48	Samlai Vedovatto	Aceito
Declaração de Pesquisadores	Declaracao_utilizacao_dados_prontuario.pdf	16/08/2018 15:51:29	Samlai Vedovatto	Aceito
Declaração de Instituição e Infraestrutura	Declaracao_isencao_onus_instituicao.pdf	16/08/2018 15:50:45	Samlai Vedovatto	Aceito
Declaração de Pesquisadores	Declaracao_confidencialidade_sujeito_estudo.pdf	16/08/2018 15:49:41	Samlai Vedovatto	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Declaracao_utilizacao_dados_material_biologico.pdf	16/08/2018 15:48:11	Samlai Vedovatto	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

PORTO ALEGRE, 21 de Novembro de 2018

Assinado por:
ELIZETE KEITEL
(Coordenador(a))

Endereço: R. Profª Annes Dias, 295 Hosp. Dom Vicente Scherer

Bairro: 6º andar - Centro

CEP: 90.020-090

UF: RS

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Telefone: (51)3214-8571

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E-mail: cep@santacasa.tche.br

ANEXO B: CEUA

Parecer de aprovação da Comissão de Ética no Uso de Animais (CEUA)



CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 206/17 Parecer 539/17

2) DATA DO PARECER: 13/12/17

3) TÍTULO DO PROJETO:

Desenvolvimento de modelo pré-clínico para avaliação da membrana amniótica humana para tratamento de queimaduras de diferentes graus.

4) PESQUISADOR RESPONSÁVEL:

Márcia Wink

5) RESUMO DO PROJETO:

O tratamento padrão para em casos de queimadura de pele de grandes áreas é de enxerto de pele autóloga. Propõem-se a utilização de membrana amniótica de doadoras de placenta como curativo para as queimaduras de pele. Esse tratamento será comparado com a utilização de células tronco mesenquimais obtidas de cultura de células a partir de tecido adiposo retirado de cirurgia de lipossucção. No estudo será feita uma comparação entre o curativo comercial, a utilização de membrana amniótica sozinha, membrana amniótica + células tronco mesenquimais e membrana amniótica aderida com fibroblastos de ratos.

6) OBJETIVOS DO PROJETO:

Caracterizar a membrana amniótica humana como curativo biológico regenerador de lesões cutâneas superficiais e profundas, na ausência ou presença de células tronco mesenquimais.

- 1- Padronizar protocolos de decelularização, criopreservação e preservação em glicerol da MA.
- 2- Padronizar protocolos para isolamento, cultivo e caracterização de MSCs derivadas de tecido adiposo human. (MSCs).
- 3- Caracterizar a MA decelularizada como scaffold para MSCs avaliando-se adesão, viabilidade, citotoxicidade, migração e morfologia celular.
- 4- Avaliar o efeito da MA na presença ou ausência de MSCs na regeneração tecidual em modelo murinho de lesão cutânea, por queimadura de diferentes graus, através de análises histológicas e de marcadores de inflamação.



UFCSPA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE
Rua Sarmento Leite, 245 - Fones: 0 xx 51 3303 9000 - Fax: 0 xx 51 3303.8810
CEP 90050-170 - Porto Alegre - RS - www.ufcspa.edu.br

7) FINALIDADE DO PROJETO: ☐ Ensino ☒ Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título ☒ Adequado ☐ Comentários

Introdução ☒ Adequada ☐ Comentários

Objetivos ☒ Adequados ☐ Comentários

Relevância e Justificativa ☒ Adequados ☐ Comentários

Materiais e Métodos ☒ Adequados ☐ Comentários

Cronograma para execução da pesquisa ☒ Adequado ☐ Comentários

Orçamento e fonte financiadora ☒ Adequados ☐ Comentários

Referências Bibliográficas ☒ Adequadas ☐ Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:

☒ Sim ☐ Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: B | C ☐ D ☐ E ☐

Justifique:

Grau de invasividade = 3

Espécie: Ratos Wistar

Número Amostral: 190



UFCSPA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE
Rua Sarmento Leite, 245 - Fones: 0 xx 51 3303 9000 - Fax: 0 xx 51 3303.8810
CEP 90050-170 - Porto Alegre - RS - www.ufcspa.edu.br

Redução Amostral:

☐ Sim

☒ Não

Justifique:

Substituição de Metodologia:

☐ Sim

☒ Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia:

☐ Sim

☒ Não

Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais:

☒ Adequada

☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais:

☒ Adequada

☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável):

☒ Adequada

☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Anestesia dos animais (se aplicável):

☒ Adequada

☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Eutanásia dos animais (se aplicável):

☒ Adequada

☐ Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Laboratório):

Rua Sarmento Leite, 245 • Porto Alegre, RS • CEP 90050-170 • Fone: 0 xx 51 3303 9000 • Fax: 0 xx 51 3303.8810 • www.ufcspa.edu.br



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Outra instituição. Qual?

UFCSPA – Laboratório de Biologia Celular

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
------	---------	------	------------

12) RECOMENDAÇÃO:

- ☒ Aprovado
☐ Com Pendência
☐ Não aprovado

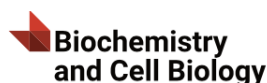
Data de início 01/09/2017 Data de Término 31/12/2021

Comentários gerais sobre o projeto:

- | |
|---|
| 1- As questões foram adequadamente respondidas.
2- A CEUA considerou o animais em 190. |
|---|

APÊNDICE A: PRODUÇÃO CIENTÍFICA - AUTORIA

Produção científica realizada durante o doutorado, além da produção da tese - com autoria:



Note

Epidermal melanocytes metabolize extracellular nucleotides by purinergic enzymes

Liliana Ivete Sous Naasani ^a, Jéssica Gonçalves Azevedo ^a, Jean Sévigny ^{b,c}, Tiago Franco de Oliveira ^d, Silvyia Stuchi Maria-Engler ^e, and Márcia Rosângela Wink ^a

^aLaboratório de Biologia Celular, Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), 90050-170 Porto Alegre, RS, Brasil; ^bDépartement de Microbiologie-Infectiologie et d'Immunologie, Faculté de Médecine, Université Laval, Québec city, QC G1V 0A6, Canada; ^cCentre de recherche du CHU de Québec, Université Laval, Québec city, QC G1V 4G2, Canada; ^dPrograma de Pós Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), 90050-170 Porto Alegre, RS, Brasil; ^eSkin Biology and Melanoma Lab, Department of Clinical Chemistry & Toxicology, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo (USP), São Paulo, Brasil

Corresponding author: Márcia Rosângela Wink (emails: marciawink@yahoo.com.br; mwink@ufcspa.edu.br)

Abstract

The human epidermal melanocyte (hEM) are melanin-producing cells that provide skin pigmentation and protection against ultraviolet radiation. Although purinergic signaling is involved in skin biology and pathology, the presence of NTPDase members, as well as the rate of nucleotides degradation by melanocytes were not described yet. Therefore, in this study, we analyzed the expression of ectonucleotidases in hEM derived from discarded foreskin of male patients. The expression of purinergic enzymes was confirmed by mRNA and flow cytometry. Among the ectonucleotidases, ectonucleoside triphosphate diphosphohydrolase1 (NTPDase1) and ecto-5'-nucleotidase were the ectoenzymes with higher expressions. The hydrolysis rate for ATP, ADP, and AMP was low in comparison to other primary cells already investigated. The amount of ATP in the culture medium was increased after a scratch wound and decreased to basal levels in 48 h, while the NTPDase1 and P2X7 expressions increased. Therefore, it is possible to suggest that after cell injury, the ATP released by hEM into the extracellular space will be hydrolyzed by ectonucleotidases as the NTPDase1 that will control the levels of nucleotides in the skin micro-environment.

Key words: primary human epidermal melanocytes, NTPDases, CD73, NPPs, extracellular nucleotides

Introduction

Melanocytes are melanin-producing neural crest-derived cells, present in the basal layer of human epidermis, in hair, eye, inner ear, brain, lungs, heart, and adipose tissue (Quevedo and Fleischmann 1980; Otreba et al. 2015).

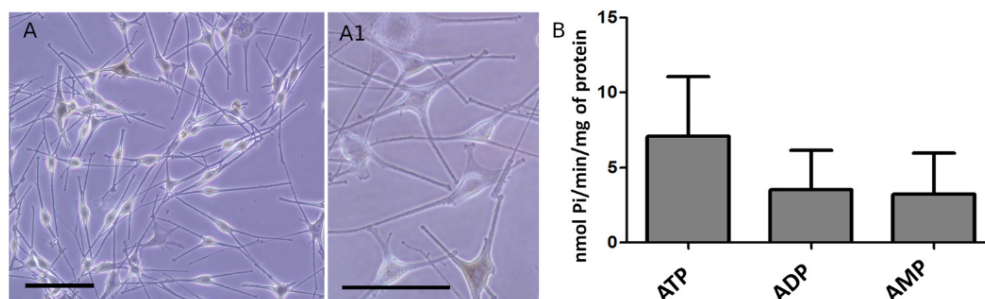
The maintenance of the melanocyte is dependent on a population of melanocyte stem cells, which after migration into epidermis, give rise to differentiated pigment-producing melanocytes (Lee and Fisher 2014). Besides, specific melanocyte traits provide unique vulnerabilities for these cells, leading to pathologies involving human melanocytic tissues, such as pigmentation defects, melanocyte-specific immune disorders, and melanomas (Tolleson 2005). For this reason, the elucidation of molecular and physiological mechanisms that occur in melanocytes is important for the development of new therapeutic strategies for melanocyte malignancies.

Purinergic signaling is involved in skin pathology, including inflammation, wound healing, and skin cancer (Burnstock et al. 2012). There is a growing interest in the therapeutic potential of purinergic compounds (Burnstock and Di Virgilio 2013), leading to several studies that eval-

uated the P1 and P2 purinergic receptors in various types of skin cells (Burnstock et al. 2012). However, there are relatively little data in the literature regarding the ATP metabolism on melanocytes surface, and no information about the ectonucleotidases profile expression (Holzer and Granstein 2004; Burnstock et al. 2012). The main families of enzymes that hydrolyze the nucleotides are the ectonucleoside triphosphate diphosphohydrolase (NTPDase), the nucleotide pyrophosphatase/phosphodiesterase (NPP), and the ecto-5'-nucleotidase (CD73) that generates adenosine from AMP (Galgaro et al. 2021). Together, these ectonucleotidases control the extracellular nucleotides and nucleosides levels, hence regulating the signaling events induced through the purinergic receptors (Zimmermann 2001; Robson et al. 2006; Knowles 2011).

Therefore, considering the limited information describing the purinergic signaling players in skin, in this study, we evaluate a candidate reference gene for epidermal melanocytes derived from discarded human foreskin, and further analyzed the level of mRNA expression of ectonucleotidases and their protein expressions and enzymatic functionalities for ATP, ADP, and AMP degradation on the cell surface.

Fig. 1. Ectonucleotidase activities and expressions in human epidermal melanocytes culture. (A) Subconfluent primary culture of hEM. (A1) Higher magnification of images shows the bipolar spindle-shaped morphology of the melanocytes. (B) hEM hydrolyzed ATP, ADP, and AMP with no statistically significant difference among them. The results are expressed as specific activity (nmol Pi/min/mg of protein). Bars represent mean $\pm$ SD ($n = 3$). Phase contrast micrograph. Scale bars: 100 μ m.



Materials and methods

Melanocyte cultures

Normal human skin cells were obtained from donated foreskin samples from male patients from the University of São Paulo Hospital. Epidermal melanocytes were isolated by Dr. Silvy Maria Engler group of Departamento de Análises Clínicas—Faculdade de Ciências Farmacêuticas, Universidade de São Paulo-USP, and cultivated as described previously (Brohem et al. 2012; Pennacchi et al. 2015). Ultrapure primary melanocyte cultures are considered phototype 4 according to the Fitzpatrick scale. We have worked routinely with these melanocytes as described in Brathwaite et al. (2022).

To this end, the project has undergone review and approval by the Ethics Committee of USP (CEP/FCF/169/2009) and UFCSPA (CEP n° 1056/10). The melanocytes were maintained in 254CF media (SKU #M-500-254CF, Cascade Biologics) supplemented with human melanocyte growth supplement (HMGS—SKU # S-002-5, Cascade Biologics, Portland, OR, USA). Medium was changed every day. Cultures were maintained in 5% CO₂/95% air at 37 °C and allowed to grow to confluence (80%). Photomicrographs were performed using a BX-50 Olympus microscope with optical lens (10X/0.30 Ph1-UplanFI), coupled to a camera Motican 2500 (Olympus, Hamburg, Germany).

ATP, ADP, and AMP hydrolysis assay

The hydrolysis assay was evaluated from primary cultures derived from three different foreskin donors, and the experiments were performed in triplicates. To determine the nucleotide hydrolysis, the enzymatic assay on the cell surface was performed as already described (Wink et al. 2003b). The nucleotide concentrations were chosen from substrate curves to assure the enzyme saturation and the linearity of the reaction (1 mmol/L ATP, ADP, and AMP) for 30 min.

RNA extraction and cDNA synthesis

Total RNA was extracted using the RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The reverse transcription reactions were performed using the high-capacity cDNA reverse transcription kit (ThermoFisher Scientific).

Validation of housekeeping gene expression in human epidermal melanocyte

For real-time (RT)-qPCR, we used three reference genes: beta-2-microglobulin (β 2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and TATA box binding protein (TBP). Primers were designed using the primer-BLAST program (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and were analyzed using Integrated DNA Technologies Oligo Analyzer 3.1 software (<http://www.idtdna.com/calc/analyser/>) (Supplementary Tables I, II, and III).

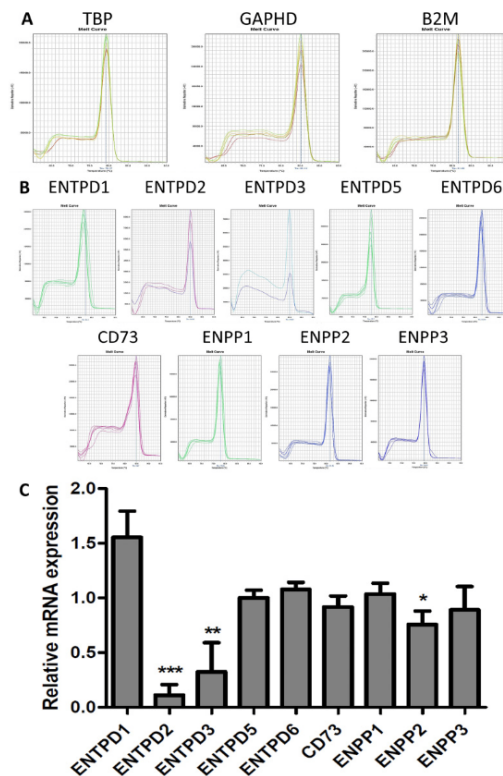
Ectonucleotidases gene expressions in human epidermal melanocyte

The expressions of the genes *ENTPD1*, *ENTPD2*, *ENTPD3*, *ENTPD5*, *ENTPD6*, *ENTPD8*, *ENPP1*, *ENPP2*, *ENPP3*, *Ecto-5'NT/CD73*, and alkaline phosphatase (*ALP*) were evaluated from primary cultures derived from three different foreskin donors (The method description is available in the legend of Supplementary Table I).

Flow cytometry analysis

The surface markers of all potential ectonucleotidases were analyzed by flow cytometry using the following antibodies: NTPDase1 (hN1-1cl5), NTPDase2 (hN2-H9s), NTPDase3 (hN3-H10s), NTPDase8 (hN8-D7s), ENPP1 (hNPP1-2cl6), ENPP3 (hNPP3-4cl5), and CD73 (h5'NT-2LI5). These antibodies were obtained from <http://ectonucleotidases-ab.com>. Cells were dissociated with trypsin, centrifuged, and then 2×10^5 cells were incubated for 1 h with the indicated primary antibody (1:100) followed by 30 min incubation with the correspond-

Fig. 2. Quantification of ENTPD and NPP mRNA expressed in hEM. (A) Melt curve of reference genes, collected using the StepOnePlus™ (Applied Biosystems). (B) Melting curves of ectonucleotidase genes. (C) The hEM expressed the genes *ENTPD1*, *ENTPD2*, *ENTPD3*, *ENTPD5*, *ENTPD6*, *CD73*, *ENPP1*, *ENPP2*, and *ENPP3*. The expression of *ENTPD8* and *ALPL* was not observed. Bars represent mean $\pm$ SEM ($n = 3$). Data were analyzed statistically by one-way ANOVA followed by Tukey's post-hoc test. *Different from *ENTPD1* hydrolysis.



ing secondary antibody (1:1000) conjugated to Alexa Fluor 488 green-linked secondary goat anti-rabbit to CD73, anti-mouse to NTPDase 2, 3, and 8, and anti-guinea pig to NTPDase1, ENPP1, and ENPP3. As positive control, cell line SIHA was used for NTPDase2 and CD73. As negative control, cells were incubated with preimmune antibodies, ENPP1(hNPP1-2cPi) and ENPP3 (hNPP3-4cPi), to discount the value of non-specific reaction (Supplementary Fig. 1). For analysis, at least 10 000 events were used, and they were performed using a FACSCalibur flow cytometer equipped with a 488-nm argon laser, using the BD CellQuest software (Becton-Dickinson, San Diego, USA).

ATP determination by liquid chromatography-tandem mass spectrometry

The extracellular ATP was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in a normal human epidermal melanocyte (hEM) primary culture and after a scratch wound. The scratch wound was performed and the media was collected in 0, 24, and 48 h. An aliquot of 50 μ L of the sample was centrifuged for 10 min at 9000g, the supernatant was collected and a 5 μ L aliquot was directly injected into the LC-MS/MS system. A Nexera-i LC-2040C Plus system coupled to a LCMS-8045 triple quadrupole mass spectrometer (Shimadzu, Japan) was used for the analysis. Separation was performed on an Acquity UPLC[®] BEH C18 (1.7 μ m, 2.1 mm $\times$ 50 mm) column (Waters, USA) using 0.1% tributylamine and 0.03% acetic acid in water (solution A) and acetonitrile (solution B) at a flow rate of 300 μ L/min and a temperature of 60 $^{\circ}$ C as follows: 0–1.5 min, 15%–90% of B; 1.5–3.0 min, 90% of B; 3.0–3.2 min, 90%–15% of B; and 3.2–8 min, 15% of B. The analyses were conducted with electrospray ionization in the negative mode (ESI[−], [M-H][−]), employing the following optimized parameters: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; interface temperature, 300 $^{\circ}$ C; desolvation temperature, 525 $^{\circ}$ C; DI temperature, 250 $^{\circ}$ C; heat block temperature, 400 $^{\circ}$ C; and drying gas flow, 10 L/min. Multiple reaction monitoring-extracted ion chromatograms were generated using the following transitions for ATP detection: m/z 506.0 $\rightarrow$ 158.9, quantifier transition; m/z 506.0 $\rightarrow$ 407.9, qualifier transition 1; and m/z 506.0 $\rightarrow$ 78.9, qualifier transitions 2. Data treatment analysis was performed using the LabSolutions software (Shimadzu, Japan).

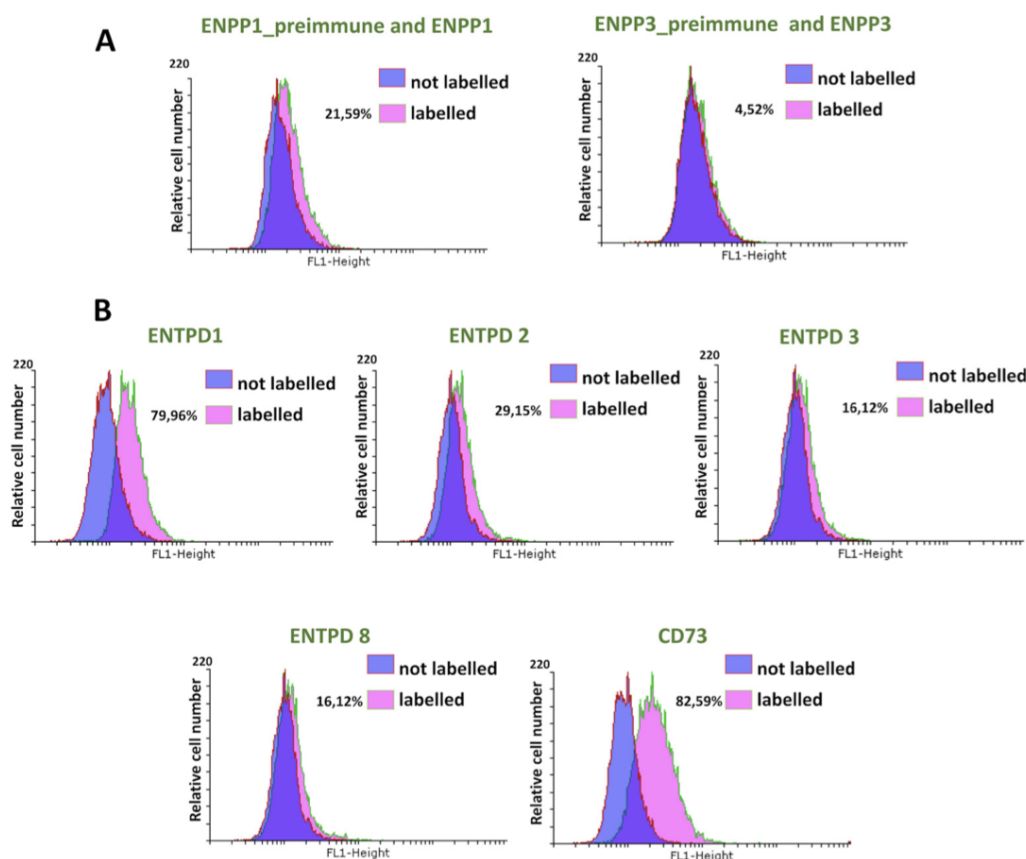
Immunofluorescence assay

Indirect immunofluorescence assays were performed in hEMs after a scratch wound. The NTPDase1 and P2X₇ receptor expressions were analyzed in a normal hEM culture and after 0, 24, and 48 h of a scratch wound. Following permeabilization with acetone (10 min at -20° C), the cultures were incubated with the primary antibody for 1 h and 15 min at room temperature. After PBS washes, the respective secondary antibody was added and incubated for 30 min in a humid chamber. The primary antibodies used were rabbit anti-NTPDase1 (Proteintech, 192 291) and rabbit anti-P2X₇ receptor (Alomone, APR-004). The secondary antibody used was goat anti-rabbit IgG (H + L) Alexa Fluor 488 (molecular probes). The cell nuclei were counterstained with DAPI (molecular probes). Images were obtained with a fluorescence microscope (Olympus IX51/Olympus U-RFLT) coupled with 460–490 and 372 nm band-pass excitation filters, respectively.

Statistical analysis

The mean C_q values, standard deviation (SD), coefficient of variation (CV), and maximum fold change (MFC, the ratio of the maximum and minimum values observed within the dataset) were calculated for RT-qPCR analysis. Data were analyzed statistically by one-way ANOVA followed by Tukey's post-hoc test using the statistical program GraphPad Prism 5.

Fig. 3. Ectonucleotidases expression on epidermal melanocytes cell surface. (A) Representative flow cytometry histograms of the ENPP1 and ENPP3 with preimmune antibodies, after discounting the unspecific positivity. (B) ENTPD1, ENTPD2, ENTPD3, ENTPD8, and CD73 on melanocytes; the blue color shows not labeled cells incubated only with secondary antibodies and purple shows labeled cells with primary and secondary antibodies.



Differences between mean values were considered significant when $p < 0.05$.

Results and discussion

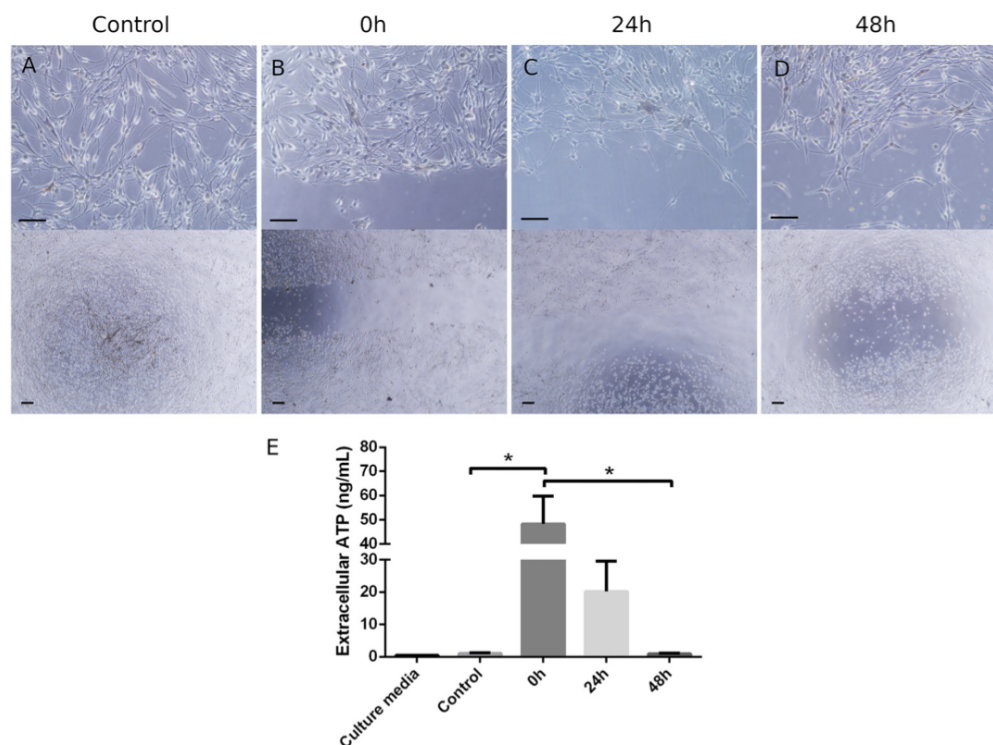
The culture of primary cells derived from foreskin samples showed a bipolar spindle-shaped morphology characteristic of normal epidermal melanocytes (Figs. 1A and 1A1). We evaluate the nucleotide hydrolysis on the melanocyte surface. The enzymatic assay showed that melanocytes hydrolyzed the extracellular nucleotides ATP, ADP, and AMP at low rates (7.65 ± 4.22 ; 5.17 ± 2.35 ; and 4.80 ± 2.65 nmol Pi/min/mg, respectively), without statistically significant differences among them (Fig. 1B).

We observed that the rate of ATP hydrolysis was very low on hEM, when compared with other primary cell types already

investigated, such as astrocytes (Wink et al. 2003a, 2006), neurons (Boeck et al. 2002), cardiomyocytes (Barreto-Chaves et al. 2006), endothelial cells (Koziak et al. 1999), vascular smooth muscle cells (Bertoni et al. 2020a), macrophages (Zanin et al. 2012), and salivary gland cells (Henz et al. 2006). In accordance with Dzhandzhugazyan et al. (1998), found an ATP hydrolysis rate of around 2 nmol of Pi/min per 10^6 cells on primary melanocytes from Clonetics (USA) (Dzhandzhugazyan et al. 1998).

The rate of ATP and ADP degradation on melanocyte cells was similar to that measured on human dermal and limbal mesenchymal stem cells (MSCs) (0.7 and 0.8 nmol Pi/min/mg of protein, respectively). However, while dermal and limbal MSCs hydrolyze AMP (20 and 21 nmol Pi/min/mg of protein, respectively) with a rate around 30 times higher than ATP (Naasani et al. 2017), hEM presented a low AMPase activity

Fig. 4. Extracellular ATP determination by LC-MS/MS on melanocytes after a scratch wound. (A) The extracellular ATP was measured on a normal melanocyte culture (control) and (B) after 0 h, (C) 24 h, and (D) 48 h of a scratch wound. Graph bars show the mean of extracellular ATP (ng/mL) on a normal culture and after a scratch wound. Scale bars: higher magnification 100 μ m; 200 μ m.



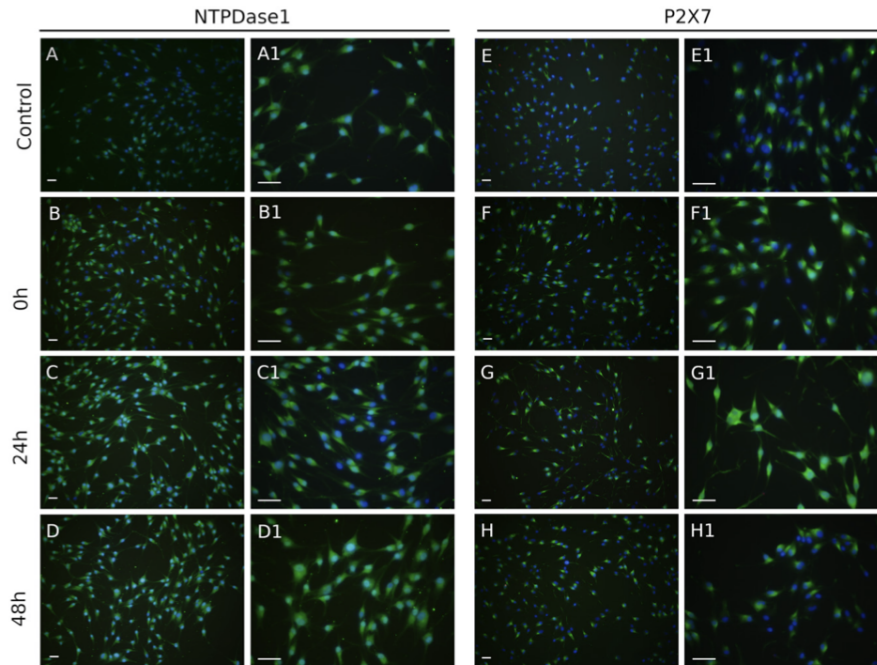
at the same levels as for ATPase and ADPase activities. Thus, at physiological conditions, melanocytes hydrolyze ATP, ADP, and AMP at low rates.

Considering that NTPDase genes were expressed in low levels on melanocytes, the quantification of these genes with RT-qPCR has to be performed with caution. Regarding this methodological approach, normalization is one of the pivotal steps in the data analysis, since it can control crucial variability on experimental conditions such as cDNA and amplicon synthesis efficiencies and errors of pipetting (Iser et al. 2015; Naasani et al. 2017; Bertoni et al. 2020b). Therefore, considering the absence of a valid reference gene in the literature for the RT-qPCR analysis on human melanocyte primary culture and the necessity of its proper validation, we evaluated three gene candidates in hEM. The *TBP* and *GAPDH* showed the efficiency and slope within the parameters of a good reaction (efficiency: 90%–110%; slope: 3.10–3.58) and the R^2 was greater than 0.99 for *TBP* and *B2M*. The *TBP* exhibited the lowest SD, CV, and MFC among the three and therefore, it was chosen as a reference gene for normalization of ectonucleotidases expression (Supplementary Table I).

ENTPD1 was the ectonucleotidase with the highest expression in hEM, followed by a similar level of mRNA expression of *ENTPD5*, *ENTPD6*, *CD73*, *ENPP1*, and *ENPP3*. The ectonucleotidases *ENTPD2*, *ENTPD3*, and *ENPP2* presented lower mRNA expression levels when compared with *ENTPD1* (Fig. 2). To confirm the mRNA expression result, the expression of NTPDases, NPPs, and CD73 was analyzed on the melanocyte surface by flow cytometry (Fig. 3). The result showed that the melanocyte cells expressed low levels of *ENPP1* and *ENTPD2*; they did not express *ENPP3*, *ENTPD3*, and *ENTPD8*. In accordance with mRNA results, hEM expressed higher levels of *ENTPD1*(CD39) and *CD73* proteins.

The ATP hydrolysis rate on the melanocyte surface was very low, when compared with other primary culture cells. One of the possibilities is that the enzyme has its expression increased when there is an injury that could induce the extracellular release of ATP. Thus, to investigate this possibility, we did a scratch wound in the melanocyte monolayer and analyzed the release of ATP concentration in the medium in 0, 24, and 48 h, in comparison with control cells in the culture medium (Figs. 4A–4C). The data suggest that after the injury,

Fig. 5. NTPDase1 and P2X₇R expressions in human epidermal melanocytes. NTPDase1 expression on normal human epidermal melanocytes (A, A1); after 0 h (B, B1), 24 h (C, C1), and 48 h (D, D1) of a scratch wound. P2X₇R expression on normal human epidermal melanocytes (E, E1); after 0 h (F, F1), 24 h (G, G1), and 48 h (H, H1) of a scratch wound. Scale bars (A–H): 200 μ m; (A1–H1): 100 μ m.



the ATP is released and it is gradually hydrolyzed (Fig. 4E), probably by NTPDase1, being the NTPDase with the highest expression on hEM (Figs. 2 and 3) and it was modulated after the scratch wound (Fig. 5). Therefore, it is possible that the NTPDase1 would be important to decrease the levels of extracellular ATP to the basal level after injuries. However, it is not possible to rule out that other ectoenzymes also contribute to ATP hydrolysis on hEM surface.

The NTPDase1 is expressed by Langerhans cells and could be responsible for hydrolyzing the extracellular nucleotides as a protective role in this pathway (Mizumoto et al. 2002). In addition, keratinocytes also express ectoenzymes that can hydrolyze the extracellular ATP. Ho et al. (2013) showed that the human keratinocyte cell line can release ATP and also convert it to ADP by the enzymatic activity of the NTPDase2. The hEM expressed higher levels of ENTPD1 in comparison with other NTPDases, which is capable of hydrolyzing ATP and ADP to AMP. Our data are in accordance with the study of Dzhandzhugazyan et al. (1998) showing that melanocytes express the NTPDase1 at mRNA, as well as at protein level (Dzhandzhugazyan et al. 1998).

Also, they express CD73 that converts the AMP to adenosine. Kim et al. (2014) suggested that adenosine may have an inhibitory effect on pigmentation by negatively regulating tyrosinase enzyme activity. By using B16 murine melanoma

cells and the zebrafish model, they showed in vitro and in vivo that the pigmentation decreased, when cells were exposed to a high concentration of adenosine (Kim et al. 2014).

Besides the NTPDases, we also observed the expression of the ENPP1, one of the members of the ENPP family on hEM. The ENPP1 transcript was detected in primary fibroblasts and melanocytes, but not in keratinocytes by Chourabi et al. (2018). These researchers reported that ENPP1 mutation causes recessive cole disease, a genodermatosis of pigmentation, following a strict dominant mode of inheritance by altering melanogenesis. In this study with eight patients affected by an overlapping genodermatosis, the mutation in ENPP1 was identified in all patients, suggesting that it was likely to originate from defective melanocytes (Chourabi et al. 2018).

We analyzed the expression of P2X₇ on the melanocyte monolayer after the scratch in the wound in 0, 24, and 48 h (Fig. 5). It is possible that when melanocytes are exposed to injuries that lead to ATP release, the P2X₇ receptors have their expressions modulated (Fig. 5E–5H1). The importance of purinergic signaling in the physiology of skin is reinforced by a study from Lee et al. (2019). Although melanocytes expressing P2X₂, P2X₄, P2X₇, P2Y₁, and P2Y₁₁ mRNA, the authors reported an important role of extracellular ATP through P2X₇ receptors in melanogenesis (Lee et al. 2019). They showed that

extracellular ATP released from keratinocytes, in response to UVB radiation, promotes melanin production in primary hEM. The blockage of the P2X₇R inhibits the melanin synthesis, expression of melanocyte inducing transcription factor (MITF), tyrosinase, and Ca²⁺ influx induced by extracellular ATP. In addition, the knockout of P2X₇R in MNT1 cells (a human malignant melanoma cell line), using the CRISPR/Cas9 system, avoided the increase of MITF expression when cells were co-cultured with UVB-irradiated keratinocytes. In addition, the same group demonstrated that AZD-9056, a P2X₇R antagonist, used at 10 nmol/L was able to revert the effect of ATP in upregulating the expression of melanogenesis-related molecules, such as MITF, tyrosinase, and gp100, as well as the melanin contents in melanocytes (Kim et al. 2020). Thus, the authors suggest that AZD-9056 can be therapeutically considered in dermatology as an anti-melanogenic compound to whiten the skin in disorders such as melasma and those related to age.

P2X₇R has also been suggested as a target to treat other skin disorders, such as vitiligo (Ahn et al. 2020) and the irritant contact dermatitis (Silva et al. 2013; Zanin et al. 2015) through the reduction of inflammasome activation. The mechanism by which high concentration of extracellular ATP released from primary human epidermal keratinocytes, submitted to oxidative stress, affects primary hEM survival in vitiligo skin as shown recently by Ahn et al. (2020). A high concentration of extracellular ATP induced cell death by apoptosis in melanocytes. The vitiligo skins showed higher levels of ATP and an upregulation of active caspase-1. Also, authors observed the production of active forms of IL-1b and IL18, via P2X₇R in keratinocytes and melanocytes after the treatment with ATP. Thus, P2X₇R inhibition was able to suppress ATP-dependent inflammasome activation and melanocyte apoptosis, leading the antagonists as potential therapeutic candidates for vitiligo treatment (Ahn et al. 2020).

In conclusion, in this study, we analyzed the expression of the NTPDases and NPP members by hEMs as well as their capacity to metabolize the extracellular nucleotides on cell surface. The results showed that ATP, ADP, and AMP are hydrolyzed at low levels by melanocytes. Considering that the extracellular ATP can be released after cell injury promoting signaling through P2X receptors, it is possible that the capacity to metabolize nucleotides can be modulated by increasing the expression of ectonucleotidases, as a way to decrease the high levels of ATP, thus avoiding skin disorders and cell death by apoptosis.

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Data availability

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Author information

Author ORCIDs

Liliana Ivet Sous Naasani <https://orcid.org/0000-0001-9645-2412>

Tiago Franco de Oliveira <https://orcid.org/0000-0003-2594-8415>

Márcia Rosângela Wink <https://orcid.org/0000-0001-5987-3509>

Author notes

Liliana Ivet Sous Naasani and Jéssica Gonçalves Azevedo contributed equally to this work.

Author contributions

Conceptualization: LISN, MRW

Data curation: LISN, JGA, TFDO

Formal analysis: LISN, JGA, TFDO

Funding acquisition: JS, MRW

Investigation: LISN, JGA, JS, SSM

Methodology: LISN, JGA, JS, SSM

Project administration: MRW

Resources: JS, SSM, MRW

Software: JGA

Supervision: MRW

Validation: SSM

Writing – original draft: LISN, JS, MRW

Writing – review and editing: LISN, MRW

Competing interests

The authors have no conflict of interest to declare.

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Supplementary material

Supplementary data are available with the article at <https://doi.org/10.1139/bcb-2022-0058>.

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APÊNDICE B: PRODUÇÃO CIENTÍFICA - COAUTORIA

Produção científica realizada durante o doutorado - colaboração:

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Bioglass 45S5: Structural characterization of short range order and analysis of biocompatibility with adipose-derived mesenchymal stromal cells *in vitro* and *in vivo*



Cristiano Rodrigues^a, Liliana Ivet Sous Naasani^a, Carla Zanatelli^a, Thaís Casagrande Paim^a,
Jéssica Gonçalves Azevedo^a, João Cardoso de Lima^b, Marilda da Cruz Fernandes^c,
Sílvio Buchner^{d,1}, Márcia Rosângela Wink^{a,*,1}

^a Laboratório de Biologia Celular, Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, 90050-170 Porto Alegre, RS, Brazil

^b Programa de Pós-Graduação em Ciência e Engenharia de Materiais, Departamento de Engenharia Mecânica, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, SC, Brazil

^c Laboratório de Pesquisa em Patologia, Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, 90050-170 Porto Alegre, RS, Brazil

^d Instituto de Física, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, RS, Brazil

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Short range order
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Bioactivity
Mesenchymal stromal cell
F-actin

ABSTRACT

Bioactive glasses have potential applications in the field of regenerative medicine due to their bioactivity that permits interaction with both hard and soft tissues. In the same way, mesenchymal stromal cells (MSCs) have been experimentally tested as part of engineered constructs considering their self-renewal and multipotent capacities. However, to design an association, it is crucial to investigate the physical properties of bioglass 45S5, as well as its biocompatibility. Therefore, we investigated the structural short range order of the stoichiometric 45S5, by obtaining its total structure factors $S(K)$ and total pair distribution function $G(r)$. The *in vitro* compatibility of human MSCs with 45S5 was verified by viability, morphometry and osteoinduction assays, F-actin staining and scanning electron (SEM) analysis. The compatibility outcome was verified through a subcutaneous implantation in a murine model by grafting the 45S5 as a scaffold for allogeneic MSCs. The cell-substrate modulation includes the maintenance of the MSC viability and osteoinduction potential after being exposed to the 45S5 extract. A low spreading during cell adhesion was detected. Both normal actin cytoskeleton organization and nuclei irregularities were observed, besides an increase of hydroxyapatite (HA) depositions around cells. Cells showed satisfactory compatibility patterns when growing over 45S5 for 7, 30 and 90 days. The implant did not show any apparent toxicity for organs, or strong immunogenic reactions, and it was accompanied by a dense capsule formation around the graft. Our results indicate that MSCs can grow in the long term on the 45S5 while maintaining their characteristics. This fact, together with a non-toxicity to animals means that the 45S5 can be implemented in pre-clinical trials aiming MSC's transplantation leading to further bone and tissue repair.

1. Introduction

Biomaterials for bone tissue engineering should not only be biocompatible, biodegradable, bioactive and mechanically competent, but also, they should modulate over target cells and their microenvironment promoting cell adhesion, viability, proliferation, differentiation and mineralization [1–6]. Other desirable characteristic is anti-

bacterial function to avoid implant-related infections [7].

MSC are strong candidates in this field due to its ability to differentiate into specialized cells, naturally contributing to all processes of healing [8]. They can also restore the bone tissue by participating in the formation of bone callus in cases of fractures [9]. MSC subpopulations can be isolated and expanded in culture from various sites of adult tissues, including the bone marrow, skin, sclerocorneal limbus,

* Corresponding author at: Universidade Federal de Ciências da Saúde de Porto Alegre – UFCSPA, 245, Sarmento Leite, Porto Alegre, RS CEP 90050-170, Brazil. E-mail address: mwink@ufcspa.edu.br (M.R. Wink).

¹ These authors shared senior authorship.

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journal homepage: www.elsevier.com/locate/msecEvaluation of *in vitro* and *in vivo* biocompatibility of iron produced by powder metallurgy

Thaís Casagrande Paim^{a,1}, Diego Pacheco Wermuth^{b,1}, Isadora Bertaco^a, Carla Zanatelli^a,
Liliana Ivet Sousa Naasani^a, Mônica Slaviero^c, David Driemeier^c, Lirio Schaeffer^{b,2},
Márcia Rosângela Wink^{a,*,2}

^a Laboratório de Biologia Celular, Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rua Sarmento Leite 245, 90050-170 Porto Alegre, RS, Brazil

^b Laboratório de Transformação Mecânica, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, RS, Brazil

^c Setor de Patologia Veterinária, Faculdade de Veterinária (FAVET), Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, 91540-000 Porto Alegre, RS, Brazil

A B S T R A C T

Biodegradable metallic materials (BMMs) are expected to corrode gradually *in vivo* after providing the structural support to the tissue during its regeneration and healing processes. These characteristics make them promising candidates for use in stents. These endoprostheses are produced from metal alloys by casting and thermomechanical treatment. Since porous alloys and metals have less corrosion resistance than dense ones, the use of powder metallurgy becomes an option to produce them. Among the metals, iron has been proposed as a material in the manufacturing of stents because of its mechanical properties. However, even then it is unclear what toxicity threshold is safe to the body. Thus, the objective of this research was to verify the biocompatibility of sintered 99.95% and 99.5% pure iron by powder metallurgy *in vitro* with Adipose-derived mesenchymal stromal cells (ADSCs) and *in vivo* with a Wistar rat model. Herein, characterizations of iron powder samples produced by the powder metallurgy and the process parameters as compression pressure, atmosphere, sintering time and temperature were determined to evaluate the potential of production of biodegradable implants. The samples obtained from pure iron were submitted to tests of green and sintered density, porosity, microhardness, hardness and metallography. The biocompatibility study was performed by indirect and direct cell culture with iron. The effects of corrosion products of iron on morphology, viability, and proliferation of ADSCs were evaluated *in vitro*. Hemolysis assay was performed to verify the hemocompatibility of the samples. *In vivo* biocompatibility was evaluated after pure iron discs were implanted subcutaneously into the dorsal area of Wistar rats that were followed up to 6 months. The results presented in this paper validated the potential to produce biodegradable medical implants by powder metallurgy. Both iron samples were hemocompatible and biocompatible *in vitro* and *in vivo*, although the 99.95% iron had better performance *in vitro* than 99.5%.

1. Introduction

Biodegradable metallic materials (BMMs) are expected to corrode gradually *in vivo* after providing the structural support to the tissue during its regeneration and healing processes. The major components of BMMs should be essential metallic elements that can be metabolized by the organism with appropriate degradation rates to avoid overload. Magnesium-based BMMs and Iron-Based BMMs are the most commonly proposed metals to be used in cardiovascular or orthopedic implants [1,2].

In cardiovascular applications, BMMs are promising candidates for use in stents, whose main function is to keep the lumen open after the angioplasty procedure [3,4]. The ideal biodegradable cardiovascular stent must be biocompatible, prompt to disappear without developing an inflammatory response and it should have mechanical properties providing the required support to the vessel during the necessary period. The degradation must

begin at a very slow rate to keep the mechanical integrity of the stent until the arterial vessel remodeling, which is expected to conclude in a period of 6–12 months [5–7].

Most of the cardiovascular stents including BMMs are produced from metallic ingots fabricated using both casting and thermomechanical treatment. Recently, the use of new processes in the fabrication of biodegradable metallic stents, such as powder metallurgy, has been investigated. Because porous alloys and metals normally have less corrosion resistance than fully dense alloys, the use of powder metallurgy became a promising option in the conception of biodegradable stents [3,8].

The conventional process of powder metallurgy consists of metal forming metallic powders, alloy powders and non-metallic materials or ceramic powders in reliable parts, within a tool of the desired shape by applying pressure. The parts made by powder metallurgy have a certain porosity, which can be controlled by factors such as compaction pressure, sintering temperature and time, size and shape of the powder particles [4].

* Corresponding author at: Universidade Federal de Ciências da Saúde de Porto Alegre – UFCSPA 245, Sarmento Leite, Porto Alegre, RS CEP 90050-170, Brazil.

E-mail address: mwink@ufcspa.edu.br (M.R. Wink).

¹ These authors contributed equally to this work.

² These authors share senior authorship.

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The adenosinergic pathway in mesenchymal stem cell fate and functions

Bruna C. Galgaro¹ | Liziane R. Beckenkamp¹ | Martha van den M. Nunnenkamp¹ | Vitória G. Korb¹ | Liliana I. S. Naasani¹ | Katarzyna Roszek² | Márcia R. Wink¹

¹Laboratório de Biologia Celular, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Rio Grande do Sul, Brazil

²Department of Biochemistry, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, Toruń, Poland

Correspondence

Márcia R. Wink, Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rua Sarmento Leite, 245, Sala 304, Centro, Porto Alegre, RS 90050-170, Brazil.
Email: mwink@ufcspa.edu.br and marciawink@yahoo.com.br

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Abstract

Mesenchymal stem cells (MSCs) play an important role in tissue homeostasis and damage repair through their ability to differentiate into cells of different tissues, trophic support, and immunomodulation. These properties made them attractive for clinical applications in regenerative medicine, immune disorders, and cell transplantation. However, despite multiple preclinical and clinical studies demonstrating beneficial effects of MSCs, their native identity and mechanisms of action remain inconclusive. Since its discovery, the CD73/ecto-5'-nucleotidase is known as a classic marker for MSCs, but its role goes far beyond a phenotypic characterization antigen. CD73 contributes to adenosine production, therefore, is an essential component of purinergic signaling, a pathway composed of different nucleotides and nucleosides, which concentrations are finely regulated by the ectoenzymes and

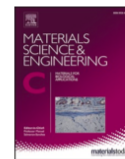
Abbreviations: A1R, adenosine 1 receptor; A2AR, adenosine 2A receptor; A2BR, adenosine 2B receptor; A3R, adenosine 3 receptor; ADA, adenosine deaminase; ADK, adenosine kinase; Ado, adenosine; ADP, adenosine 5'-diphosphate; AKT, protein kinase B (PKB); ALP, alkaline phosphatase; AMP, adenosine 5'-monophosphate; APCP, adenosine 5'-(α , β -methylene)-diphosphate; Arg-1, arginase1; ATP, adenosine triphosphate; AT-MSC, adipose tissue-derived MSC; BM-MSC, bone marrow-derived MSC; C/EBP α and C/EBP β , CCAAT/enhancer-binding protein α and β ; cAMP, cyclic AMP; CFU-F, colony-forming unit-fibroblast; CNT, concentrative nucleoside transporter; CREB, cAMP-response element binding protein; DC, dendritic cell; DP-MSC, dental pulp-derived MSC; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; ENT, equilibrative nucleoside transporter; ERK, extracellular signal-regulated kinase; GAG, glycosaminoglycan; GMSC, gingiva-derived MSC; GVHD, graft-versus-host disease; IFN- γ , interferon-gamma; JNK, c-Jun N-terminal kinase; Klf4, Krüppel-like factor 4; MHC, major histocompatibility complex; MMP, matrix metalloproteinases; MSC, mesenchymal stem cell; MSCA-1, mesenchymal stem cell antigen 1; M-CSF, macrophage colony-stimulating factor; NAD⁺, nicotinamide adenine dinucleotide; NF- κ B, nuclear factor- κ B; NK, natural killer cell; Nlrp3, NLR family pyrin domain containing 3; NO, nitric oxide; NPP1, ecto-phosphodiesterase/phosphodiesterase family member 1; PDE, phosphodiesterase; PGE2, prostaglandin E2; PI3K, phosphoinositide 3-kinase; PKA, cAMP-dependent protein kinase; PPAR γ , peroxisome proliferator-activated receptor- γ ; RANKL, receptor activator for nuclear factor κ B ligand; ROS, reactive oxygen species; TGF- β , transforming growth factor beta; TNAP, tissue-nonspecific alkaline phosphatase; TNF- α , tumor necrosis factor- α ; Treg, T regulatory lymphocyte; UC-MSC, umbilical cord-derived MSC; VEGF, vascular endothelial growth factor.

Bruna C. Galgaro and Liziane R. Beckenkamp contributed equally to this study.



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Mechanical properties, *in vitro* and *in vivo* biocompatibility analysis of pure iron porous implant produced by metal injection molding: A new eco-friendly feedstock from natural rubber (*Hevea brasiliensis*)

Diego Pacheco Wermuth^{a,1}, Thaís Casagrande Paim^{b,1}, Isadora Bertaco^b, Carla Zanatelli^b, Liliana Ivet Sous Naasani^b, Mônica Slaviero^c, David Driemeier^c, André Carvalho Tavares^a, Vinicius Martins^f, Camila Ferreira Escobar^e, Luis Alberto Loureiro dos Santos^d, Lirio Schaeffer^{a,2}, Márcia Rosângela Wink^{b,*,2}

^a Laboratório de Transformação Mecânica, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, RS, Brazil

^b Laboratório de Biologia Celular, Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rua Sarmento Leite 245, 90050-170 Porto Alegre, RS, Brazil

^c Setor de Patologia Veterinária, Faculdade de Veterinária (FAVET), Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, 91540-000 Porto Alegre, RS, Brazil

^d Laboratório de Biomateriais & Cerâmicas Avançadas, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, RS, Brazil

^e Centro de Ciência e Tecnologia em Energia e Sustentabilidade, Universidade Federal do Recôncavo da Bahia, Av. Centenário 697, 44.085-132 Feira de Santana, BA, Brazil

^f Laboratório de Metalurgia do Pó, Instituto Federal Sul-rio-grandense Campus Sapucaia do Sul, Av. Copacabana 100, 93216-120 Sapucaia do Sul, RS, Brazil

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Adipose-derived mesenchymal stromal cells (ADSCs)
In vivo model of subcutaneous implant

ABSTRACT

Metal injection molding (MIM) has become an important manufacturing technology for biodegradable medical devices. As a biodegradable metal, pure iron is a promising biomaterial due to its mechanical properties and biocompatibility. In light of this, we performed the first study that manufactured and evaluated the *in vitro* and *in vivo* biocompatibility of samples of iron porous implants produced by MIM with a new eco-friendly feedstock from natural rubber (*Hevea brasiliensis*), a promisor binder that provides elastic property in the green parts. The iron samples were submitted to tests to determine density, microhardness, hardness, yield strength, and stretching. The biocompatibility of the samples was studied *in vitro* with adipose-derived mesenchymal stromal cells (ADSCs) and erythrocytes, and *in vivo* on a preclinical model with Wistar rats, testing the iron samples after subcutaneous implant. Results showed that the manufactured samples have adequate physical, and mechanical characteristics to biomedical devices and they are cytocompatible with ADSCs, hemocompatible and biocompatible with Wistar rats. Therefore, pure iron produced by MIM can be considered a promising material for biomedical applications.

1. Introduction

The study of new degradable biomaterials has been one of the most attractive topics in the materials research field [1]. These materials are expected to safely and gradually degrade in the body after their function is served [2]. Metals comprise an important class of degradable biomaterials, because of their suitable mechanical properties in structural

components. Iron (Fe), Magnesium (Mg), Zinc (Zn), Molybdenum (Mo), and Tungsten (W) have been studied to be used as temporary orthopedic, vascular implants, and electronic systems [3].

Previous studies have shown that pure iron can be an appropriate material for biomedical applications [4–6]. Iron has great radial strength due to its high elastic modulus [7] and it is biocompatible. Studies have shown that pure iron stents produced by laser cutting did

* Corresponding author at: Universidade Federal de Ciências da Saúde de Porto Alegre–UFCSPA, 245, Sarmento Leite, Porto Alegre, RS CEP 90050-170, Brazil. E-mail address: mwink@ufcspa.edu.br (M.R. Wink).

¹ These authors contributed equally to this work

² These authors shared senior authorship

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