Universidade Federal do Rio Grande do Sul
Programa de Pós-Graduação em Ciências Biológicas: Fisiologia

Tese de doutorado

DESENVOLVIMENTO DE UM SUBSTITUTO NANOESTRUTURADO A SER UTILIZADO EM ASSOCIAÇÃO COM CÉLULAS-TRONCO PARA A TERAPIA VASCULAR EM DOENÇA ARTERIAL PERIFÉRICA.

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Orientadora

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Tese realizada sob a orientação da Profª. Drª. Patricia Pranke, apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Fisiologia da Universidade Federal do Rio Grande do Sul em preenchimento parcial dos requisitos para a obtenção do título de Doutora em Fisiologia.

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Sumário

Apresentação

A presente tese foi desenvolvida no laboratório de Hematologia e Células-Tronco da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul e no laboratório de Células-Tronco do Instituto de Ciências Básicas da Saúde, da mesma universidade.

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Resumo

Atualmente, existe uma grande necessidade médica por enxertos vasculares de pequeno calibre (< 6 mm), que possam ser utilizados em cirurgias de reconstrução vascular. Nesse trabalho, dois tipos de biomateriais vasculares foram desenvolvidos pela técnica de *electrospinning*: biomateriais de policaprolactona (PCL) e biomateriais de poli(carbonato de trimetileno – *co* – ácido lático) (PTMCLLA). Os biomateriais de PCL foram funcionalizados com heparina e com VEGF (PCL/Hep/VEGF). Os biomateriais de PTMCLLA foram desenvolvidos a partir de três razões de carbonato de trimetileno/ ácido lático: 20/80, 30/70 e 40/60.

Os biomateriais de PCL apresentaram taxa de degradação lenta e alta elasticidade. A funcionalização dos biomateriais preveniu a coagulação do sangue e também favoreceu o crescimento de células-tronco mesenquimais (CTMs) e de células progenitoras endoteliais (CPEs) nessas estruturas. A análise de PCR demonstrou que o VEGF adsorvido aos biomateriais não foi suficiente para diferenciar as CTMs em células endoteliais. O cultivo das CPEs sobre os biomateriais aumentou a expressão de VE-caderina e a presença de VEGF nas estruturas manteve o nível de expressão de CD31 e CD34 nessas células. Após essas análises, os biomateriais de PCL/Hep/VEGF foram fabricados em formato tubular. As CPEs foram semeadas no lúmen do biomaterial, através de biorreatores de parede rotatória (BPR), e mantidas em cultivo, por biorreatores de perfusão (BP). O BPR favoreceu a distribuição homogênea das CPEs na parede luminal dos biomateriais enquanto que o BP estimulou seu crescimento e otimizou seu metabolismo energético.

Os biomateriais produzidos a partir dos copolímeros de PTMCLLA 30/70 e 40/60 exibiram uma alta flexibilidade. Porém, os biomateriais de PTMCLLA 40/60 tiveram um grande enrugamento. Os biomateriais de PTMCLLA 30/70 suportaram a adesão e o crescimento de CTMs, de CPEs e de células musculares lisas.

Os resultados obtidos no presente estudo demonstram que biomateriais de PCL/Hep/VEGF apresentam características físico-químicas compatíveis para o uso vascular. Ainda, previnem a formação de trombos em sua superfície e propiciam o desenvolvimento da camada endotelial em seu lúmen. Os biomateriais de PTMCLLA 30/70 exibem alta flexibilidade e suportam o desenvolvimento de células vasculares e de células-tronco mesenquimais. De acordo com esses resultados, é possível concluir que

biomateriais de PCL/Hep/VEGF e de PTMCLLA 30/70 são candidatos promissores para aplicação como enxertos vasculares.

Palavras-chaves: engenharia de tecidos vasculares, células progenitoras endoteliais, biomateriais vasculares, poli(carbonato de trimetileno – *co* – ácido lático), poli(caprolactona)

Abstract

Currently, there is a great medical need for small caliber vascular grafts (<6 mm), which can be used in vascular replacement surgeries. In this work, two types of vascular biomaterials were developed by the electrospinning technique: biomaterials of polycaprolactone (PCL) and biomaterials of poly(trimethylene carbonate-*co*-L-lactide) (PTMCLLA). PCL biomaterials were functionalized with heparin and VEGF (PCL / Hep/VEGF). The PTMCLLA biomaterials were developed from three ratios of trimethylene carbonate/lactide: 20/80, 30/70 and 40/60. The PCL biomaterials presented a slow degradation rate and high elasticity. The functionalization of the biomaterials prevented the blood from clotting and also favored the growth of mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) in these structures. PCR analysis demonstrated that VEGF adsorbed by the biomaterials was not sufficient to differentiate the MSCs into endothelial cells. The cultivation of CPEs on the biomaterials increased their expression of VE-cadherin and the presence of VEGF in the structures maintained the cell expression of CD34 and CD31. After these analyzes, the PCL/Hep/VEGF biomaterials were produced in a tubular geometrical form. The CPEs were seeded into their lumen by rotating bioreactors (RB) and maintained in culture by perfusion bioreactors (PB). The RB favored the homogeneous distribution of the CPEs in the luminal wall of the biomaterials while the BP stimulated their growth and optimized their energetic metabolism.

The biomaterials produced from the PTMCLLA 30/70 and 40/60 copolymers exhibited high flexibility. However, the PTMCLLA 40/60 biomaterials exhibited substantial wrinkling. The PTMCLLA 30/70 biomaterials supported the adhesion and growth of MSCs, CPEs and smooth muscle cells.

This study has demonstrated that PCL/Hep/VEGF biomaterials have physicochemical characteristics compatible with vascular use. Furthermore, they prevent thrombus formation on their surfaces and promote the development of the endothelial layer in their lumen. Biomaterials of PTMCLLA 30/70 exhibit high flexibility and support the development of vascular and mesenchymal stem cells. According to these results, it can be concluded that PCL/Hep/VEGF and PTMCLLA 30/70 biomaterials are promising candidates for use as vascular grafts.

Key-words: vascular tissue engineering, vascular biomaterials, endothelial progenitor cells, poly(trimethylene carbonate-*co*-L-lactide), poly(caprolactone)

Lista de abreviaturas e siglas

Ac-LDL	Lipoproteína de baixa densidade acetilada
ANOVA	Análise de variância
APTT	do inglês *Activated partial thromboplastin time*
ATP	Adenosina trifosfato
CACs	do inglês C*irculating angiogenic cells*
CCK-8	do inglês *Cell Counting Kit-8*
CPEs	Células progenitoras endoteliais
CT	Células-tronco
CTEs	Células-tronco embrionárias
CTMs	Células-tronco mesenquimais
DAPI	do inglês *4,6-diamidino-2-fenylindole dihydrochloride*
DAPs	Doenças arteriais periféricas
DCM	Diclorometano
DMA	do inglês *Dynamic mechanical analysis*
DMEM	Meio de Eagle modificado por Dulbecco
early EPCs	do inglês *Early endothelial progenitor cells*
ECM	do inglês *Extracelular matrix*
EDC	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide
EGM	do inglês *Endothelial cell growth medium*
EPCs	do inglês *Endothelial progenitor cells*
e-PTFE	Politetrafluoretileno expandido
FBS	do inglês *Fetal bovine serum*
FDA	do inglês *Food and Drug Administration*
FGF-2	do inglês *Fibroblast growth factor 2*
FITC	do inglês *Fluorescein Isothiocyanate*
fvW	Fator de von Willebrand
G-CSF	do inglês *Granulocyte-colony stimulating factor*
GPC	do inglês *Gel Permeation Chromatograph*
HCl	Ácido clorídrico
Hep	Heparina
Hepes	n-2 hidroxietil piperazine- n'- 2 ácido sulfônico etano

HGF	do inglês *Hepatocyte growth factor*
ICMI	Isquemia crítica dos membros inferiores
IGF-1	do inglês *Insulin-like growth factor*
IL-1	Interleucina 1
IL-6	Interleucina 6
iNOS	do inglês *Inducible nitric oxide synthase*
iPSCs	do inglês *Induced pluripotent stem cells*
KDR	do inglês *Kinase insert domain receptor*
Klf4	do inglês *Kruppel-like factor 4*
LDH	do inglês *Lactate dehidrogenase*
LE	do inglês *Loading efficiency*
MEC	Matriz extracelular natural
MSCs	do inglês *Mesenchymal stem cells*
NaCl	Cloreto de sódio
NaOH	Hidróxido de sódio
NHS	N-hydroxysuccinimide
NO	do inglês *Nitric oxid*
Oct-3/4	do inglês *Octamer-binding transcription factor 3/4*
OECs	do inglês *Late outgrowth endothelial cells*
PAD	do inglês *Peripheral arterial disease*
PBS	do inglês *Phosphate buffer solution*
PCL	Poli(caprolactona)
PCR	do inglês *Polymerase Chain Reaction*
PDGF	do inglês *Platelet-derived growth factor*
PIGF	do inglês *Placental growth factor*
PLLA	poli(L-ácido lático)
PTMC	do inglês *Poly(trimethylene carbonate)*
PTMCLLA	do inglês *Poly(trimethylene carbonate – co –L- lactide)*
rpm	Rotações por minuto
SDF-1	do inglês *Stromal cell-derived gactor 1*
SEM	do inglês *Scanning electron microscopy*
SOX2	do inglês *(Sex determining region Y)-box 2*
TGA	do inglês *Thermogravimetric analysis*
UCB	do inglês *Umbilical cord blood*

UEA	do inglês *Ulex europaeaus agglutinin I*
UV	Ultravioleta
VEGF	do inglês *Vascular endothelial growth factor*
VEGFR	do inglês *Vascular endothelial growth factor receptor*
Wnt	do inglês *Wingless-related integration site*
7AAD	7-Amino Actinomycin D

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1. Introdução

As doenças arteriais periféricas são caracterizadas pela obstrução de artérias periféricas e redução do aporte sanguíneo aos tecidos. Em casos graves, procedimentos cirúrgicos são realizados com o objetivo de restaurar o fluxo sanguíneo (1). Nesses procedimentos, homoenxertos ou implantes sintéticos são utilizados como substitutos do vaso lesado. Contudo, quando vasos de pequeno diâmetro (diâmetro interno < 6 mm) são acometidos, a taxa de falha desses substitutos é bastante alta, principalmente para os enxertos sintéticos (2). Dessa forma, novos enxertos vasculares devem ser desenvolvidos para o tratamento desse quadro.

A engenharia de tecidos é uma ciência que visa à reconstrução de tecidos lesados através da associação de biomateriais, células e moléculas bioativas. Os biomateriais atuam como suportes para o crescimento celular, sendo chamados de matrizes ou *scaffolds.* Essas matrizes fornecem a estrutura para que as células possam se desenvolver, secretar matriz extracelular e organizar o tecido a ser regenerado. Enquanto isso, as moléculas bioativas auxiliam as células a desempenharem suas funções (3). Devido a essas características, a engenharia de tecidos tem atraído grande atenção como uma ferramenta para o desenvolvimento de substitutos vasculares.

O endotélio vascular, camada mais interna dos vasos sanguíneos, desempenha diferentes funções relacionadas à homeostasia vascular (4). Dessa forma, o desenvolvimento da camada endotelial no interior do biomaterial vascular é crucial para o bom desempenho do mesmo. As células-tronco mesenquimais e as células progenitoras endoteliais têm sido bastante investigadas como fontes celulares para a formação do endotélio na parede luminal de substitutos vasculares. As células-tronco mesenquimais são células-tronco adultas que apresentam facilidade de obtenção e cultivo. Essas células apresentam grande plasticidade e, se adequadamente estimuladas, podem se diferenciar em células endoteliais (5). Enquanto isso, as células progenitoras endoteliais são células que apresentam uma alta taxa de proliferação e um grande potencial de vasculogênese. Elas contribuem direta e indiretamente para a formação e reparo do endotélio vascular (6).

Diferentes técnicas têm sido empregadas para a produção dos biomateriais. O método de *electrospinning* caracteriza-se pela produção de biomateriais poliméricos formados por fibras que mimetizam fisicamente a matriz extracelular natural, proporcionando um microambiente favorável ao desenvolvimento celular (7). Os

polímeros utilizados nessa técnica devem ser escolhidos de acordo com as características que os biomateriais devem apresentar. Para a construção de biomateriais vasculares, devem ser utilizados polímeros que confiram resistência mecânica e também elasticidade aos mesmos. A poli(caprolactona) (PCL) é um polímero sintético, biodegradável e biocompatível, já aprovado pelo órgão americano que regulamenta o uso de alimentos e medicamentos, *Food and Drug Administration* (FDA), para uso em humanos. A PCL apresenta características físico-químicas como elasticidade, resistência mecânica e uma taxa de degradação relativamente lenta, compatíveis para aplicação na engenharia de tecidos vasculares (8). Contudo, assim como outros polímeros sintéticos, a PCL apresenta uma baixa bioatividade. Para minimizar essa limitação, moléculas bioativas podem ser ligadas aos biomateriais produzidos com esse polímero (9).

A heparina é um anticoagulante natural que pode ser ligada a biomateriais sintéticos para conferir propriedade antitrombogênica aos mesmos (10). Enquanto isso, o fator de crescimento vascular e endotelial (VEGF, do inglês *vascular endothelial growth factor*) é um peptídeo com funções angiogênicas e vasculogênicas. O VEGF ocasiona a quimiotaxia de células endoteliais e aumenta a sua proliferação (11). Assim, a funcionalização de biomateriais com heparina e com o VEGF propicia a regeneração vascular e o sucesso da aplicação dos mesmos. A heparina evita a formação de trombos e a obstrução do lúmen do biomaterial e o VEGF favorece a sua endotelização.

Devido à grande necessidade clínica por enxertos vasculares de pequeno diâmetro, novos materiais devem ser desenvolvidos. O poli(carbonato de trimetileno) é um polímero biodegradável do tipo elastômero, isto é, apresenta um elevado grau de elasticidade. Em função disso, os copolímeros de carbonato de trimetileno (TMC) têm sido bastante estudados para produção de materiais que sofrem deformações cíclicas constantes (12). O poli(carbonato de trimetileno-*co*-L-ácido lático) (PTMCLLA) é um copolímero formado por unidades de TMC e de ácido lático, que conferem, respectivamente, grande elasticidade e resistência aos materiais (13). Devido a essas características, o PTMCLLA é um polímero interessante para a produção de enxertos vasculares.

O uso de biorreatores para cultivo *in vitro* de células em biomateriais vasculares melhor simula o microambiente natural encontrado nos vasos sanguíneos (14). Os biorreatores possibilitam a passagem de meio de cultivo pelo lúmen desses biomateriais, expondo as células às forças mecânicas normalmente encontradas nas paredes dos vasos

(15). Assim, o uso desses dispositivos contribui para a maturação e para a funcionalidade dos substitutos vasculares desenvolvidos pela engenharia de tecidos.

Nesse contexto, o presente trabalho científico visa ao desenvolvimento de biomateriais endotelizados, a partir dos polímeros PCL ou PTMCLLA, que possam ser aplicados como substitutos vasculares em quadros graves de doença arterial periférica, utilizando os princípios da engenharia de tecidos.

2. Revisão bibliográfica

2.1. Doenças arteriais periféricas

As doenças arteriais periféricas (DAPs) são manifestações clínicas secundárias à diminuição do aporte sanguíneo aos tecidos devido à obstrução da artéria aorta e/ou de seus ramos. As artérias mais comumente afetadas são aquelas localizadas nos membros inferiores, contudo, os vasos renais e de outros órgãos também podem ser acometidos (1). A principal causa da DAP é a lesão por arteriosclerose, a qual ocasiona a obstrução do fluxo sanguíneo no leito vascular e, consequentemente, privação de oxigênio e nutrientes nos tecidos irrigados (16). Esse quadro origina o aparecimento de sintomas, como dores e lesões nos membros afetados (1).

As DAPs apresentam diferentes graus clínicos de gravidade, que podem variar entre zero e seis, de acordo com escala de *Rutherford*. Conforme a intensidade da obstrução do vaso arterial, as DAPs podem ser assintomáticas (categoria zero) ou até mesmo causar lesões tróficas nos membros acometidos (categoria seis) (17). Um sintoma inicial bastante frequente em pacientes com DAP é a dor nas pernas ao caminhar, chamada claudicação intermitente (18, 19). Esse sintoma impede que os indivíduos consigam realizar grandes caminhadas, causando grande morbidade e impactando a qualidade de vida dos mesmos. A evolução desse sintoma ocasiona o quadro "isquemia crítica dos membros inferiores" (ICMI), caracterizado por lesões de pele e dores nas pernas e pés, mesmo em repouso. Esse quadro, se não adequadamente tratado, pode acarretar a necrose dos tecidos e, então, a amputação dos membros (1).

Estudos demonstram que as DAPs são bastante prevalentes na população, acometendo mais de 200 milhões de pessoas no mundo, provenientes tanto de países desenvolvidos como de países em desenvolvimento (20, 21). A prevalência das DAPs aumenta com a idade, sendo inferior a 10% em pessoas com idades entre 55 e 59 anos e chegando a quase 60% em pessoas com idades entre 85 e 89 anos (22). A maioria dos indivíduos sintomáticos para a DAP – que apresentam claudicação intermitente – permanecem com o quadro clínico estável. Contudo, cerca de 20% desses pacientes apresentam progressão da doença, desenvolvendo o quadro de ICMI. Aproximadamente 1 a 2% dos indivíduos com ICMI são submetidos à amputação. Esse risco é ainda maior em diabéticos, atingindo cerca de 5% dos indivíduos (18, 19).

O tratamento do indivíduo diagnosticado com DAP visa à melhora de sua qualidade de vida, focando na redução dos sintomas relatados e na prevenção da amputação de membros (23). Assim, o tratamento é realizado de acordo com a gravidade do quadro clínico apresentado e inclui exercícios físicos, intervenções farmacológicas (Aspirina®, Clopidrogel, Cilostazol) e procedimentos cirúrgicos de revascularização, em quadros mais graves, onde existe a obstrução crônica do vaso (24, 25). Nesse caso, a cirurgia de ponte, ou *by-pass*, é bastante empregada. Nesse procedimento, um enxerto vascular é utilizado para substituir ou contornar o vaso danificado (2, 26).

Atualmente, os vasos autólogos ainda são considerados padrão ouro para uso como enxertos vasculares nos procedimentos cirúrgicos de revascularização e reconstrução vascular. As artérias radial e mamária e a veia safena são os vasos normalmente utilizados como enxertos autólogos nas cirurgias de ponte (26, 27). Contudo, a aplicação de enxertos autólogos é bastante limitada devido às suas desvantagens intrínsecas, tais como trombose no sítio cirúrgico e disponibilidade limitada. Muitos pacientes não possuem vasos disponíveis para "auto-doação" em função de operações prévias ou, ainda, não apresentam vasos de "boa qualidade" para uso como enxerto (28). Além disso, esse método requer dois procedimentos cirúgicos, um para a obtenção do vaso a ser utilizado como enxerto autólogo e outro para sua implantação no local da lesão (29).

Alternativamente, enxertos vasculares sintéticos, produzidos a partir de politetrafluoretileno expandido (e-PTFE) ou poliésteres, como o tereftalato de polietileno (Dacron®), têm sido utilizados satisfatoriamente no reparo de artérias de médio e grande calibre. No entanto, esses enxertos mostram-se inadequados para a aplicação em vasos de menores diâmetros (< 6 mm). Estudos têm demonstrado que os enxertos sintéticos apresentam um grande grau de obstrução quando utilizados em vasos de pequeno calibre, mesmo com pouco tempo após sua aplicação (2). As baixas velocidades de fluxo sanguíneo em vasos com diâmetros inferiores a 6 mm implicam a necessidade de implantes sintéticos com diferentes características daqueles utilizados em veias e artérias de maiores calibres (29, 30). Entre as principais causas de falha desses materiais estão a trombogenicidade e a excessiva hiperplasia da íntima no local do implante. Além disso, em geral, os implantes sintéticos apresentam biocompatibilidade limitada e propriedades mecânicas inadequadas (31). Assim, o uso desses enxertos no reparo de vasos de pequenos diâmetros ainda é insatisfatório. Dessa forma, novas estratégias devem ser

pesquisadas para o estabelecimento do fluxo sanguíneo, quando esses vasos são acometidos.

2.2. Engenharia de tecidos

A engenharia de tecidos é uma ciência multidisciplinar que, através da união de princípios da biologia e das engenharias, visa à criação de substitutos capazes de restaurar, manter ou aumentar a função de um determinado tecido (32). Essa ciência combina o uso de biomateriais, células e moléculas bioativas para regeneração de tecidos e/ou órgãos. Nesse sistema, as células apresentam como função principal a síntese de matriz extracelular, que formará o novo tecido. Os biomateriais, por sua vez, atuam como suportes temporários, denominados matrizes ou *scaffolds*. Essas matrizes proporcionam às células uma estrutura tridimensional onde elas podem se organizar, proliferar e diferenciar (3). Além de orientar o desenvolvimento celular, os biomateriais auxiliam a manter as células no local implantado, favorecendo o recrutamento celular *in situ*, pois minimizam a perda de células pela corrente circulatória, uma vez que as mesmas encontram-se já aderidas a eles (33). Assim, o biomaterial guia as células para que sua proliferação e a secreção de matriz extracelular ocorram no local desejado. Enquanto isso, as moléculas bioativas podem ser fatores de crescimento, fármacos ou outras biomoléculas que auxiliam as células a desempenharem suas funções e estimulam sua proliferação e/ou diferenciação (3, 34).

Devido a essas características, a engenharia de tecidos apresenta-se como uma solução para diferentes quadros envolvendo danos a tecidos ou órgãos e que, ainda, não apresentam uma terapia adequada (7, 35, 36). Ela é uma estratégia bastante atraente para o desenvolvimento de novos enxertos vasculares, principalmente para a aplicação em vasos de pequenos diâmetros. A associação de células autólogas aos biomateriais biodegradáveis possibilitam a construção de estruturas vasculares que, quando implantadas *in vivo*, podem degradar-se ao mesmo tempo em que células vasculares se proliferam, produzem a matriz extracelular e regeneram o vaso lesado. Dessa forma, espera-se que essa combinação entre células e biomateriais possa levar ao reestabelecimento do tecido e à formação de vasos funcionais (37, 38).

Diferentes tipos de biomateriais naturais e sintéticos têm sido desenvolvidos para aplicação na engenharia de tecidos. O mesmo ocorre com as tecnologias para seu processamento (39). A escolha do biomaterial e da tecnologia para sua produção deve ser

baseada nas características do tecido que se deseja reparar (33). O ideal é que, além de biocompatíveis, os biomateriais apresentem características físico-químicas semelhantes ao tecido natural e que consigam integrar-se a esse, quando implantado. Além disso, eles devem suportar o crescimento e as necessidades biológicas da população celular encontrada naquele ambiente (40).

O desenvolvimento de biomateriais vasculares é bastante complexo. Para que haja sucesso em sua aplicação, um biomaterial vascular deve apresentar uma série de características, tais como: baixo risco de infecções, patência de longo prazo, resistência à degradação e ao procedimento de sutura, resistência mecânica e elasticidade, responsividade a estímulos fisiológicos, bem como porosidade adequada para que ocorra a difusão de nutrientes e a migração celular. Por outro lado, ao mesmo tempo, o biomaterial vascular deve impedir o extravasamento de seu conteúdo, bem como apresentar custo/benefício adequado e ser de fácil produção. Além disso, os biomateriais vasculares não devem desencadear resposta imunológica ou liberar produtos tóxicos, devem evitar a formação de trombos em seu lúmen e devem favorecer o desenvolvimento de células vasculares (28, 37). Portanto, um biomaterial vascular deve ser biomimético ao vaso em que será inserido.

Dentre as tecnologias que têm sido empregadas na engenharia de tecidos vasculares, a técnica de eletrofiação, ou *electrospinning*, tem se destacado (30, 41, 42). Essa técnica apresenta a vantagem intrínseca de produzir biomateriais fibrosos que naturalmente conseguem mimetizar fisicamente a matriz extracelular das células (7).

2.3. Desenvolvimento de biomaterial vascular pela técnica de *electrospinning*

O *electrospinning* é uma técnica bastante versátil, pela qual biomateriais poliméricos, formados por fibras de diferentes diâmetros, são produzidos. Apesar de ter sido patenteada ainda em 1934, essa técnica somente despertou interesse comercial a partir da década de 90 (43). A partir desse ponto, um número crescente de trabalhos envolvendo *electrospinning* vem sendo realizado nas mais diferentes áreas, incluindo a engenharia de tecidos (7). O interesse pela aplicação da técnica especificamente na engenharia de tecidos vasculares é mais recente. De acordo com uma busca realizada em março de 2017 na base de dados *Pubmed*, foi possível observar um maior número de trabalhos envolvendo o termo "*electrospinning and vascular scaffolds*", somente a partir do ano de 2010 (Figura 1).

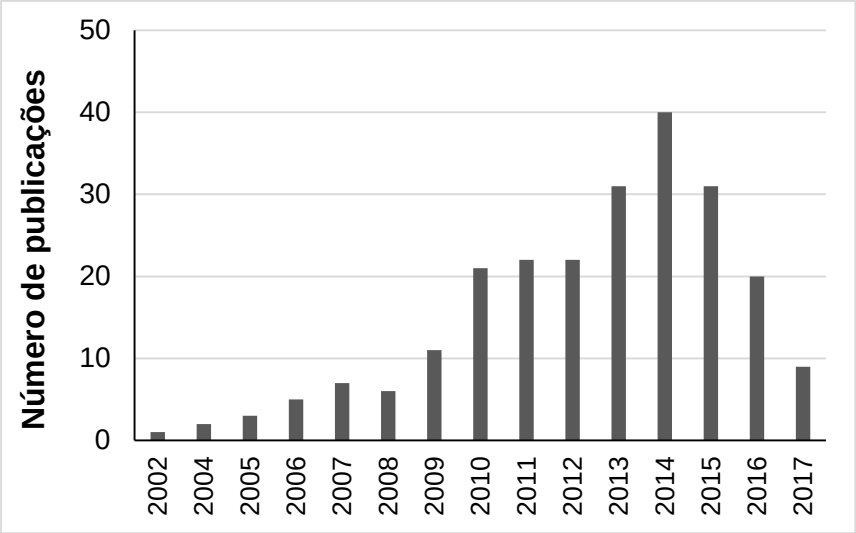

Figura 1. Número anual de publicações envolvendo o termo "*electrospinning and vascular scaffolds*", disponíveis na base de dados *Pubmed*, até março de 2017.

O *electrospinning* tem seu funcionamento baseado no princípio eletrostático, onde uma alta tensão elétrica é aplicada a um polímero, na forma de solução. Portanto, o aparato de *electrospinning* é composto por uma fonte de alta tensão, uma bomba peristáltica, uma seringa acoplada a uma agulha e um "prato coletor", local onde os biomateriais são formados (Figura 2a).

Para a produção dos biomateriais, a fonte de alta tensão é conectada à agulha da seringa e ao prato coletor, através de um eletrodo e um contra eletrodo, respectivamente. A solução polimérica é acondicionada na seringa e, então, é ejetada pela bomba peristáltica. Devido ao campo elétrico gerado, à medida que a solução polimérica é ejetada, ela é carregada eletricamente e, em função disso, se deforma, adquirindo uma forma cônica, chamada de cone de Taylor (Figura 2b). Em consequência à alta densidade de cargas elétricas, o cone colapsa, formando fibras líquidas bastante finas. Essas fibras são atraídas pelo contra eletrodo. Durante esse processo, o solvente da solução polimérica evapora e, então, fibras sólidas são depositadas no prato coletor (44).

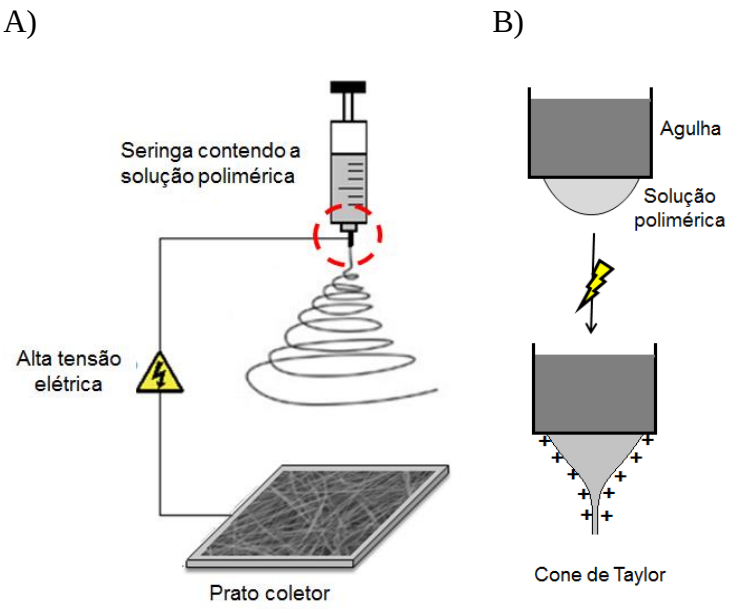

Figura 2. Representação do aparato básico de *electrospinning* (A) e da formação do cone de Taylor (B) (adaptado de Braghirolli et al, 2014).

As fibras formadas por *electrospinning* podem apresentar diâmetros que variam desde 3 nanômetros até alguns micrômetros. A sua superfície pode ser lisa, rugosa ou porosa, como também pode apresentar estruturas em forma de gotas, chamadas contas, mais comumente conhecidas como *beads*. As diferentes morfologias e diâmetros das fibras produzidas são obtidas pela modulação dos parâmetros que coordenam o processo de *electrospinning*. A concentração e a condutividade da solução polimérica, o peso molecular do polímero, a tensão elétrica utilizada, a distância entre a ponta da agulha e o prato coletor e a velocidade da bomba peristáltica são fatores que interferem na formação e na morfologia das fibras (45). Assim, através do ajuste desses fatores, fibras com morfologia e diâmetros semelhantes às fibras de colágeno da matriz extracelular (MEC) natural podem ser obtidas. Além de mimetizarem estruturalmente a MEC, os biomateriais produzidos por *electrospinning* apresentam um grande número de poros interconectados, que favorecem a passagem de nutrientes e oxigênio, a eliminação de metabólitos e a migração celular. Ainda, as fibras constituem um biomaterial com elevada área superficial em relação ao seu volume, o que favorece a adesão, adaptação e proliferação celular (7).

A técnica de *electrospinning* também permite que biomateriais com diferentes formatos possam ser produzidos. Para a engenharia de tecidos vasculares, biomateriais já

com formato tubular podem ser obtidos utilizando um mandril rotatório. Esse mandril atua como prato coletor (Figura 3) (46). Nesse caso, as fibras são homogeneamente depositadas sobre o cilindro em rotação, adquirindo o formato tubular.

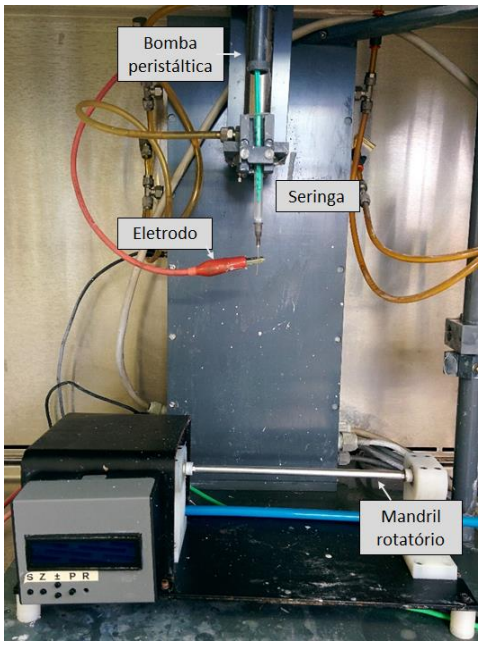

Figura 3. Aparato de *electrospinning* equipado com mandril rotatório. O mandril é utilizado como prato coletor (Laboratório de Hematologia e Células-tronco, Faculdade de Farmácia, UFRGS).

Através do *electrospinning*, os biomateriais podem ser produzidos a partir de uma grande variedade de polímeros naturais ou sintéticos. Como citado anteriormente, esses polímeros devem ser escolhidos de acordo com as características necessárias do tecido que se pretende reparar. Portanto, para a produção de substitutos vasculares, devem ser utilizados polímeros com boa resistência mecânica e também elasticidade. Assim, os biomateriais gerados apresentarão complacência compatível com a dos vasos naturais e capacidade de suportar o estresse hemodinâmico a longo prazo, sem sofrer deformações permanentes (29).

Os polímeros naturais como colágeno, elastina e gelatina são reconhecidos por sua intrínseca bioatividade. Por apresentarem domínios naturais para adesão celular, os biomateriais produzidos com esses polímeros apresentam boa interação com diferentes tipos de células. Contudo, os polímeros naturais são bastante frágeis e não resistem a grandes pressões ou tensões, o que os torna inadequados para o uso na produção de enxertos que exijam resistência mecânica (46, 47).

Enquanto isso, uma gama muito grande de polímeros sintéticos, com diferentes características físico-químicas, é oferecida. Os polímeros sintéticos podem ser adaptados para que exibam as características físico-químicas de interesse. Seu peso molecular e composição podem ser alterados para que apresentem propriedades mecânicas ou taxas de degradação adequadas à finalidade que serão utilizados. Além disso, esses polímeros apresentam uma grande estabilidade, tendo pouca variabilidade entre lotes (47, 48). Assim, esses polímeros têm sido extensivamente utilizados na engenharia de tecidos vasculares.

2.3.1. Poli(caprolactona)

A poli(caprolactona) (PCL) é um polímero sintético, biodegradável e biocompatível, que faz parte da classe dos poliésteres alifáticos. Ela é um polímero já aprovado pelo FDA para algumas aplicações médicas, como sistemas de liberação de fármacos e fios de sutura (Monocryl®) (8, 49). Sua fórmula química é $(C_6H_{10}O_2)_n$ e sua estrutura está demonstrada na figura 4.

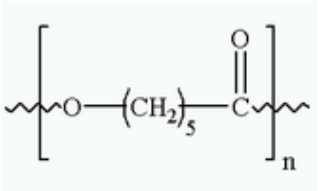

Figura 4. Representação da estrutura química da poli(caprolactona).

A PCL é hidrofóbica e apresenta solubilidade em diferentes solventes orgânicos. Essa propriedade permite que diferentes soluções, com diferentes características, possam ser produzidas com esse polímero e utilizadas na técnica de *electrospinning*. Sua degradação ocorre pela hidrólise das ligações ésteres. Contudo, a taxa de degradação desse polímero é bastante baixa (50). Em função disso, a PCL tem sido investigada para a produção de dispositivos que devam permanecer implantados por longos períodos de tempo, tais como, os biomateriais vasculares. As propriedades mecânicas da PCL, como alta elasticidade e resistência à pressão, também justificam o interesse à PCL para essa aplicação. Já foi mostrado que biomateriais de PCL apresentam uma alta taxa de elongação, podendo apresentar uma deformação superior a 100% em relação ao seu tamanho original, antes da fratura (51). Essas estruturas também são capazes de suportar picos de pressão de até 4000 mmHg, superior aos vasos sanguíneos naturais (em torno de

2000 mmHg) (52). Dessa forma, acredita-se que os biomateriais de PCL sejam resistentes ao estresse hemodinâmico e apresentem elasticidade adequada à aplicação na engenharia de tecidos vasculares.

Assim, como outros polímeros sintéticos, a PCL apresenta uma baixa bioatividade. Embora alguns estudos já tenham mostrado que estruturas produzidas com PCL suportem a adesão de células endoteliais (53, 54), esse polímero não apresenta domínios naturais para a ligação celular em sua estrutura, além de ser bastante hidrofóbico (55). Dessa forma, a adesão e a proliferação de células nos biomateriais de PCL podem ser prejudicadas. Uma estratégia que vem sendo utilizada para minimizar esse problema, é a associação desses biomateriais a fatores de crescimento ou outras moléculas bioativas. A funcionalização dos biomateriais sintéticos, isto é, a ligação de moléculas à sua superfície, agrega propriedades aos mesmos e melhora sua bioatividade, favorecendo a adaptação celular (7, 9).

2.4. Biomoléculas e funcionalização de biomateriais vasculares

Como descrito anteriormente, a engenharia de tecidos é baseada na tríade: biomateriais, células e moléculas bioativas. Não necessariamente os três fatores precisam ser utilizados simultaneamente. Estratégias que recrutam as células do hospedeiro, ou que fazem uso dos próprios fatores liberados no processo de dano tecidual, podem ser utilizadas (3). Entretanto, quando células, biomateriais e biomoléculas já são previamente combinados, o processo de regeneração tecidual é acelerado.

A associação de moléculas bioativas a biomateriais pode ser realizada de diferentes formas, tais como, encapsulação ou ligação química. Esse último processo é conhecido por funcionalização e se dá pela ligação covalente ou pela adsorção eletrostática das biomoléculas à superfície dos biomateriais (9).

A escolha da biomolécula a ser utilizada deve ser baseada nas necessidades do tecido que se quer reparar. Uma das maiores causas da falha de enxertos vasculares em vasos de pequeno calibre é a formação de trombos em seu interior. Portanto, o uso de fatores que previnam a trombose pode evitar esse quadro.

A heparina é um polissacarídeo altamente sulfatado, pertencente à família dos glicosaminoglicanos. Ela é produzida e armazenada principalmente por mastócitos. Sua molécula apresenta uma alta densidade de cargas negativas, maior que qualquer outra macromolécula biológica conhecida (Figura 5). A heparina é utilizada como agente

terapêutico devido a sua capacidade de inibir a coagulação sanguínea, sendo indicada, inclusive, em casos de DAPs (graus 0 a 3) (17). Sua ação se dá pela ligação à antitrombina, o que ocasiona a mudança de conformação dessa proteína e aumento de sua afinidade pela trombina e pelo fator Xa de coagulação. A ligação da antitrombina a esses fatores ocasiona o bloqueio das vias intrínseca e comum da cascata de coagulação (56).

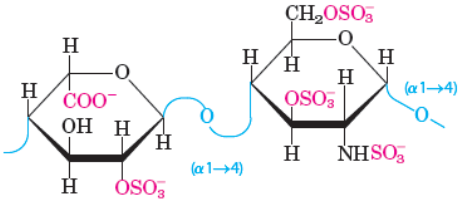

Figura 5. Representação da estrutura química da heparina (retirado de Lehninger, 6ª Ed).

Devido à sua propriedade anticoagulante, a heparina tem sido estudada para funcionalização de biomateriais vasculares. A presença desse polissacarídeo na estrutura desses materiais confere a característica antitrombogênica aos mesmos (10, 57). Além da ação direta sobre a antitrombina, a alta densidade de cargas negativas na estrutura da heparina previne a sua interação com algumas proteínas. O fibrinogênio é uma proteína que também atua na coagulação sanguínea e, no pH fisiológico, é carregado negativamente. Portanto, devido à repulsão eletrostática existente, as estruturas tratadas com heparina não adsorvem fibrinogênio, mecanismo que também contribui para a prevenção da formação de trombos na superfície desses materiais (58).

Um outro efeito conhecido da heparina é sua ação sobre a proliferação de células musculares lisas. O grupo do pesquisador Su demonstrou que a liberação controlada de heparina por nanofibras de poli(lactideo-*co*-caprolactona) ocasionou a redução da proliferação de células musculares lisas sobre a sua superfície. Esse também é um efeito importante para a engenharia de tecidos vasculares, pois a hiperplasia íntima também ocasiona a falha dos enxertos de vasos (59).

Ao mesmo tempo que a superfície "negativa" dos biomateriais tratados com heparina previne a adsorção de certas proteínas, ela favorece a interação com outras. Assim, esse fator pode ser utilizado para a "co-funcionalização" de biomateriais, isto é, a presença da heparina nos biomateriais é aproveitada para que moléculas catiônicas sejam também ligadas à superfície dos mesmos (60, 61). Ye e colaboradores demonstraram que biomateriais produzidos com o conjugado PCL-heparina apresentaram uma maior

eficiência de adsorção do fator de crescimento de fibroblasto-2 (FGF-2) do que biomateriais produzidos com PCL (10). Ainda, a heparina auxilia a preservar a bioatividade das proteínas ligadas a ela, protegendo-as da degradação enzimática e térmica (60).

No âmbito da engenharia de tecidos vasculares, o uso de fatores de crescimento ou proteínas que favoreçam a endotelização dos biomateriais é bastante interessante. O crescimento de células endoteliais na superfície luminal de enxertos vasculares é um fator que contribui para o bom desempenho dos mesmos (62).

O VEGF é um peptídeo que tem papel fundamental nos processos de vasculogênese e angiogênese (63). A vasculogênese é o processo de criação de vasos sanguíneos *de novo*, ou seja, a formação de novos vasos, baseado em angioblastos e células progenitoras endoteliais circulantes. Enquanto isso, a angiogênese refere-se ao processo de brotamento de novos vasos a partir de células endoteliais adultas, provenientes de um vaso pré-existente (64).

A família VEGF é constituída por seis membros, incluindo o VEGF-A, B, C, D, E e o fator de crescimento placentário (PIGF). Desses, O VEGF-A, referido apenas como VEGF, é o membro mais bem caracterizado. Ele apresenta diferentes isoformas, tais como $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ e $VEGF_{206}$. O VEGF é expresso praticamente em todos os tipos de células adultas humanas, mas é secretado principalmente por células estromais e pericitos de sistemas capilares fenestrados (63). Sua ação ocorre principalmente sobre células endoteliais. Essas células apresentam em sua superfície dois tipos de receptores para o VEGF (VEGFR, do inglês *vascular endothelial growth factor receptor*), o VEGFR-1, também chamado de Flt-1, e o VEGFR-2, também chamado KDR (65).

Quando existe um quadro de hipóxia tecidual, a transcrição do gene para VEGF é aumentada. O VEGF, então, é secretado e tem suas ações sobre as células endoteliais, causando o aumento da permeabilidade do endotélio e a promoção da angiogênese (64). O VEGF causa a migração de células endoteliais até o local da hipóxia e também estimula a sua proliferação (61). Em função dessas ações, a associação de VEGF a biomateriais já tem sido bastante estudada na engenharia de tecidos com o objetivo de promover a vascularização do tecido danificado (60, 66). Contudo, o uso de VEGF mostra-se interessante também para a engenharia de tecidos vasculares. O grupo de Anderson e colaboradores aplicou hidrogéis de alginato impregnados com VEGF em ratos, pela via intramuscular. Após 48h da aplicação, uma grande quantidade de células endoteliais foi

encontrada no local (11). Portanto, a presença de VEGF em biomateriais vasculares também pode contribuir para a promoção da sua rápida endotelização luminal.

Tendo como base essas informações, neste trabalho, biomateriais de PCL foram produzidos por *electrospinning* e funcionalizados com heparina e VEGF através de ligação covalente e adsorção eletrostática, respectivamente. A representação do biomaterial desenvolvido é mostrada na figura 6.

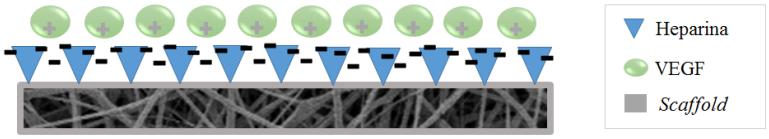

Figura 6. Representação esquemática de biomateriais de PCL funcionalizados com heparina e com VEGF.

2.5. Copolímeros de carbonato de trimetileno

Como já descrito, atualmente, a clínica médica não dispõe de enxertos vasculares adequados à aplicação em vasos de pequeno diâmetro. Dessa forma, novos materiais devem ser pesquisados e criados para que apresentem o máximo das características exigidas para a regeneração vascular e para que, assim, contemplem essa necessidade clínica.

O poli(carbonato de trimetileno) (PTMC) é um elastômero biodegradável. Os elastômeros são também chamados de "polímeros tipo borrachas" e são caracterizados por sua alta elasticidade. O PTMC é dito um polímero de memória, isto é, quando ele é deformado, tem a capacidade intrínseca de retornar completamente à sua forma original (12). Em função dessa propriedade, o PTMC tem sido estudado na engenharia de tecidos, para a construção de materiais que são submetidos a estresses cíclicos, como por exemplo, válvulas cardiovasculares e próteses cartilaginosas (67).

Uma outra característica interessante do PTMC que o torna interessante à engenharia de tecidos, é seu tipo de degradação. Além de apresentar uma baixa taxa de degradação, esse polímero degrada-se por erosão de superfície. Isto é, a redução das dimensões e massa de dispositivos de PTMC ocorre de maneira proporcional à redução de sua área superficial. Com isso, a estrutura e as propriedades mecânicas de tais dispositivos permanecem inalteradas por um longo período de tempo. Enquanto isso, os poliésteres, tais como a PCL, apresentam degradação em bloco. Nesse caso, a massa e as

dimensões dos dispositivos permanecem inalteradas por um período de tempo e após, toda a sua estrutura é rompida (Figura 7). Com isso, ocorre a perda de todas as suas propriedades, o que pode levar à falha do biomaterial (67).

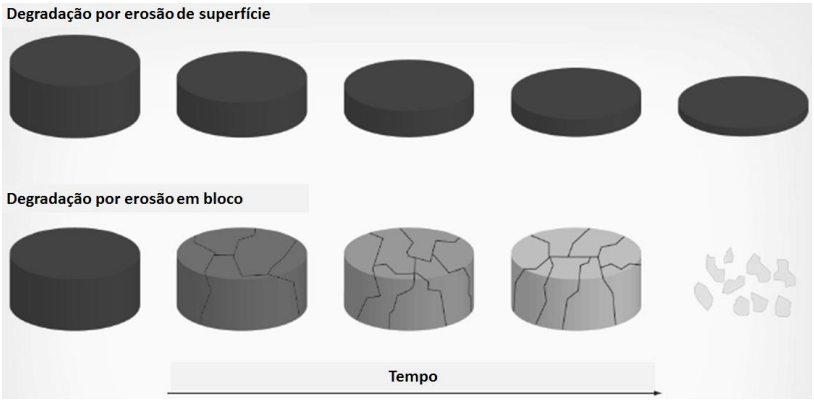

Figura 7. Representação dos processos de degradação por erosão de superfície e por erosão em bloco (adaptado de Bat e colaboradores, 2014) (67).

O uso do PTMC para o desenvolvimento de biomateriais vasculares de pequeno diâmetro é bastante interessante. Esses biomateriais devem suportar o estresse hemodinâmico por um tempo adequado, até que o tecido vascular possa ser gerado, sem perder suas propriedades, como a elasticidade.

A biocompatibilidade desse polímero já foi demonstrada através do estudo da interação de células-tronco mesenquimais e biomateriais que continham PTMC (68). Tais biomateriais não foram citotóxicos para esse tipo celular. A hemocompatibilidade de filmes de PTMC também já foi avaliada. Yang e colaboradores demonstraram que filmes de PTMC apresentam uma baixa taxa de hemólise, demonstrando sua compatibilidade com o sangue humano (69).

Normalmente, o PTMC apresenta um baixo peso molecular, o que dificulta a sua eletrofiação. Essa desvantagem pode ser contornada pela combinação de PTMC a outros polímeros. Já foi demonstrado que polímeros contendo unidades de carbonato de trimetileno e ácido lático foram utilizados com sucesso na técnica de *electrospinning* para produção de biomateriais (70). A copolimerização dessas unidades monoméricas faz com que as propriedades desses polímeros também sejam combinadas. Além de possibilitar a eletrofiação, a combinação de unidades de poliésteres dá maior força mecânica ao carbonato de trimetileno (71). Enquanto isso, o carbonato de trimetileno confere elasticidade e degradação por erosão de superfície aos seus copolímeros.

No presente trabalho, copolímeros contendo unidades de carbonato de trimetileno e ácido lático – poli(carbonato de trimetileno-*co*-ácido lático) – foram sintetizados e investigados para a produção de biomateriais vasculares. A estrutura do poli(carbonato de trimetileno-*co*-ácido lático) (PTMCLA) está demonstrada na figura 8.

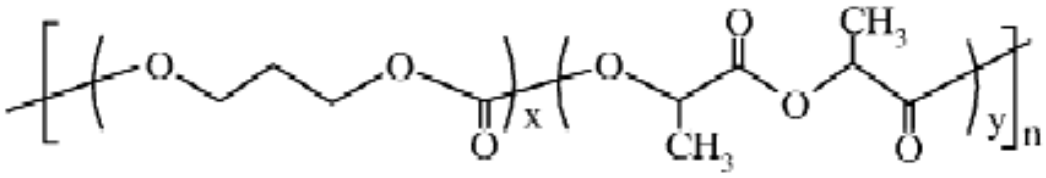

Figura 8. Estrutura química do poli(carbonato de trimetileno-*co*-ácido lático) ("x" representa as unidades de carbonato de trimetileno e "y" as unidades de ácido lático).

2.6. Tipos celulares utilizadas na engenharia de tecidos vasculares

A parede das artérias sanguíneas é formada por três camadas identificáveis, cada uma composta por células e proteínas específicas (Figura 9). A camada mais interna de todos os vasos sanguíneos, a túnica íntima, é formada por uma monocamada de células endoteliais, o endotélio. Adjacente ao entotélio, é encontrada a membrana basal, formada por colágeno IV e laminina, seguida pela lâmina elástica interna, uma camada de elastina. A segunda camada da parede arteriolar é chamada túnica média. Essa camada é formada por células musculares lisas e por colágenos tipo I e III, principalmente. A túnica média pode apresentar espessura variável de acordo com o tipo de vaso sanguíneo. A artéria aorta e seus ramos principais apresentam paredes rígidas e elásticas. Enquanto isso, artérias menores apresentam uma parede mais muscular. Essa camada é responsável pelo tônus muscular, desempenha vasoconstrição e vasodilatação e é delimitada pela lâmina elástica externa, uma segunda camada de elastina. A túnica adventícia é a camada mais externa da parede das artérias. Ela é constituída por tecido conjuntivo frouxo, contendo fibroblastos, colágeno e fibras elásticas (72, 73).

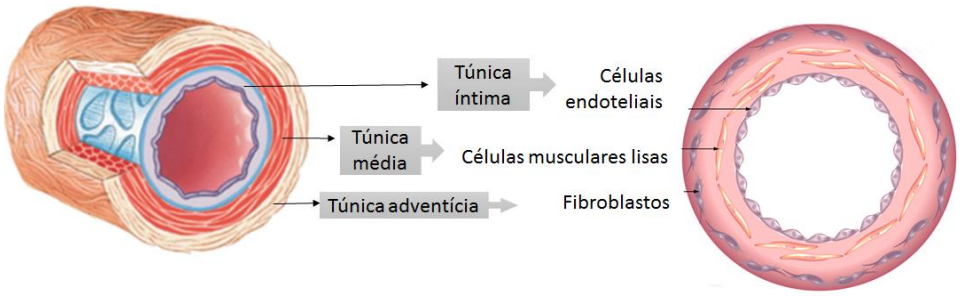

Figura 9. Organização e composição celular da parede arterial (adaptado de Huang e Li, 2008 e Silverthorn, 5ª Ed).

Para que consigam promover uma regeneração tecidual adequada, originando um tecido funcional, os biomateriais devem promover a organização celular e tecidual semelhante àquela presente nos vasos sanguíneos naturais. Portanto, devem ser compatíveis com o crescimento de células endoteliais, células musculares lisas e fibroblastos.

Na engenharia de tecidos de enxertos vasculares, o primeiro foco é estabelecer o endotélio na superfície interna do biomaterial, devido ao papel crucial que essa camada desempenha sobre a homeostasia vascular. As células endoteliais secretam diferentes fatores como fator ativador de plasminogênio, trombomodulina, sulfato de heparana, prostaciclina (PGI2) e óxido nítrico, fazendo com que a camada endotelial apresente propriedades anticoagulantes e antitrombogênicas (4). O endotélio participa da regulação do processo inflamatório, da permeabilidade vascular, da trombose e fibrinólise, controlando as interações do fluxo sanguíneo com a parede do vaso. O endotélio também exerce um importante papel sobre o controle da proliferação e migração das células musculares lisas da túnica média. Dessa forma, a endotelização dos biomateriais é determinante para o sucesso de sua aplicação. Ela previne a formação de trombos e a hiperplasia íntima nos enxertos vasculares sintéticos (74). O estudo de Quint e colaboradores demonstrou o benefício da pré-endotelização de *scaffolds*. No estudo, a semeadura de células endoteliais ou de células progenitoras endoteliais em biomateriais contribuiu para a redução da hiperplasia íntima e favoreceu a permeabilidade ao fluxo sanguíneo quando os mesmos foram testados *in vivo* (62).

Dois grupos principais de células têm sido testados para a formação do endotélio em biomateriais vasculares: as células-tronco e as células vasculares diferenciadas (26). O primeiro grupo inclui células-tronco embrionárias, células-tronco pluripotentes induzidas e células-tronco mesenquimais. Enquanto isso, as células endoteliais e as

células progenitoras endoteliais são células vasculares bastante pesquisadas para endotelização de enxertos (38, 75).

2.6.1. Células-tronco mesenquimais

As células-tronco (CT) são um tipo celular não especializado, com capacidade de autorrenovação e que podem manter-se por longos períodos de tempo com o potencial de diferenciação em uma linhagem celular com funções especializadas. Essas características fazem com que as CT representem uma parcela muito importante da medicina regenerativa e da engenharia de tecidos (76).

Basicamente, as células-tronco são divididas em células-tronco pluripotentes e células-tronco adultas. As células-tronco pluripotentes podem originar qualquer tipo de célula que compõe o organismo humano adulto. São células-tronco pluripotentes, as células-tronco embrionárias (CTEs) e as células-tronco pluripotentes induzidas, mais conhecidas como iPSCs (do inglês "*induced pluripotent stem cells*"). As CTEs são obtidas a partir de embrião de até 5 dias e, devido a sua elevada plasticidade, apresentam um grande potencial para uso na medicina regenerativa. Contudo, seu uso apresenta algumas limitações relacionadas principalmente a sua complexa regulação e implicações éticas (77). Uma alternativa às CTEs, são as iPSCs. As iPSCs são originadas a partir de células adultas, já especializadas, que passam a apresentar características de células-tronco embrionárias após reprogramação genética. Nesse processo, genes de pluripotência, como *Oct-3/4*, *SOX2*, *c-Myc*, and *Klf4* são adicionados às células adultas. A partir disso, elas passam a apresentar autorrenovação e plasticidade, da mesma forma que as CTEs (75). Esse ainda é um procedimento bastante complexo e que apresenta algumas desvantagens como a baixa eficiência de reprogramação e, também, o uso de vetores para a integração dos genes na célula hospedeira (77).

As células-tronco adultas, por sua vez, são células-tronco encontradas em órgãos ou tecidos já formados. As células-tronco mesenquimais (CTMs), ou também comumente chamadas de células estromais mesenquimais, são células-tronco adultas que têm sido alvo de muitas pesquisas devido à sua disponibilidade, plasticidade e facilidade de obtenção e cultivo (76). Além disso, a pesquisa e utilização das CTMs apresentam menores implicações bioéticas que as CTEs e também, menor complexidade de trabalho do que as iPSCs.

Inicialmente, as CTMs foram encontradas na medula óssea. Atualmente, a presença dessas células já foi descrita em vários outros tecidos como tecido adiposo,

cordão umbilical, ligamento periodontal, polpa dentária e pulmões (78). As CTMs representam uma fração de reserva celular para a manutenção e reparo de tecidos. Elas permanecem nos tecidos em um estado quiescente até serem ativadas por mediadores de lesão ou por processos naturais de senescência (79).

De acordo com a Sociedade Internacional de Terapia Celular, podem ser classificadas como células-tronco mesenquimais, as células que apresentam as seguintes características: (a) aderência ao plástico, (b) expressão de marcadores de superfície como CD73, CD90 e CD105, (c) não expressão de marcadores de células hematopoéticas como: CD11b, CD14, CD19, CD34, CD45, CD79 e HLA-DR e (d) capacidade de se diferenciar *in vitro* em osteoblastos, adipócitos e condroblastos (80).

As CTMs apresentam alta plasticidade, sendo caracterizadas como multipotentes. Essas células podem se diferenciar em diferentes tipos celulares como os que originarão ossos, cartilagens, músculos, derme, gordura e estroma de medula óssea. Assim, as CTMs podem ser utilizadas para o reparo de diversos tecidos, incluindo os vasos sanguíneos. Estudos já demonstraram que, se adequadamente estimuladas, as CTMs podem ser diferenciadas em células endoteliais (5, 81). O VEGF é um dos principais fatores utilizados para essa diferenciação e tem sido empregado de diferentes maneiras: solubilizado no meio de cultura, liberado a partir de dispositivos de liberação controlada ou imobilizado em superfícies (77). Em seu trabalho, Wingate e colaboradores demonstraram que a combinação de VEGF solúvel e biomateriais elásticos agiram sinergicamente sobre CTMs, aumentando a expressão de marcadores típicos endoteliais nessas células (81).

Uma outra característica interessante das CTMs são suas ações parácrinas. As CTMs secretam um amplo espectro de moléculas bioativas com funções imunorreguladoras, que auxiliam a estruturação de um microambiente regenerativo (82). Essas moléculas incluem fatores angiogênicos e/ou vasculogênicos como VEGF-A, VEGF-B, bFGF, PIGF, fator de crescimento derivado de plaquetas (PDGF), fator de crescimento de hepatócitos (HGF), Angiopoetina-1, Interleucinas 1 e 6 (IL-1 e IL-6). Assim, CTMs podem contribuir diretamente e indiretamente com a endotelização de biomateriais vasculares. Essas células podem colonizar a estrutura do biomaterial e se diferenciar em células endoteliais, bem como promover o recrutamento e proliferação de células endoteliais do próprio hospedeiro, através da secreção de fatores tróficos (83).

Devido a essas características, esse tipo celular foi avaliado no presente trabalho. Ele foi obtido a partir do tecido pulpar de dentes decíduos. O dente decíduo, popularmente

chamado de “dente de leite”, é uma fonte atraente para obtenção de CTMs visto que é um material que seria naturalmente descartado, sendo assim considerado um processo de obtenção pouco invasivo (84). Além disso, as CTMs de dentes decíduos são facilmente isoladas e cultivadas *ex vivo* e apresentam uma alta taxa de proliferação (85, 86).

2.6.2. Células progenitoras endoteliais

As células endoteliais têm sido amplamente associadas aos biomateriais vasculares para promoção de sua endotelização *in vitro*. Apesar dos bons resultados encontrados, alguns fatores limitam a sua real aplicação clínica. Essas células são isoladas a partir de vasos sanguíneos, sendo esse um processo bastante invasivo, que causa grande morbidade ao doador. Além disso, por tratar-se de células maduras, a sua expansão *in vitro* é bastante limitada (66, 87).

Mais recentemente, as células progenitoras endoteliais (CPEs) têm sido estudadas como fontes de células endoteliais autólogas para diferentes aplicações terapêuticas. As células progenitoras endoteliais foram isoladas pela primeira vez em 1997, por Asahara e colaboradores, a partir de sangue periférico, e foram descritas como uma população celular derivada da medula óssea, envolvida na neovascularização (88).

As CPEs têm sido classificadas por alguns autores como células-tronco adultas devido as suas propriedades de autorrenovação, alta taxa de proliferação, clonogenicidade e capacidade de diferenciação (89). As CPEs estão localizadas principalmente em nichos na medula óssea, mas também existe uma fração circulante dessas células no sangue periférico. Essas células representam 1% das células da medula óssea e menos de 0,01% das células mononucleares presentes no sangue periférico de indivíduos adultos (90). Apesar da baixa frequência, têm-se mostrado que essa fração celular pode ser facilmente isolada a partir da fração de células mononucleares de sangue periférico, sangue de cordão umbilical e medula óssea (6).

Duas populações de CPEs foram identificadas: as células progenitoras endoteliais precoces, chamadas “early EPCs” (*early endothelial progenitor cells*) ou “CACs” (*circulating angiogenic cells*) e as células progenitoras endoteliais tardias, conhecidas como “OECs” (*late outgrowth endothelial cells*). As CACs aparecem entre o 4º e 7º dia de cultivo *in vitro*, apresentam morfologia fusiforme (Figura 10a) e têm proliferação limitada, morrendo aproximadamente após 4 semanas de cultivo. As OECs são chamadas de CPEs verdadeiras, aparecem entre a 2ª e 4ª semana de cultivo *in vitro*, têm forma de paralelepípedo (*cobblestone*) (Figura 10b) e apresentam uma alta taxa de proliferação (91,

92). As CPEs podem ser identificadas pela co-expressão de marcadores de células-tronco hematopoéticas (CD34 e CD133) e marcadores de células endoteliais (CD31, eNOS, fvW e KDR) (11). Ainda, essas células apresentam a capacidade de internalizar lipoproteína de baixa densidade acetilada (Ac-LDL) e ligar-se à lecitina UEA (*Ulex europaeaus agglutinin I*) (93).

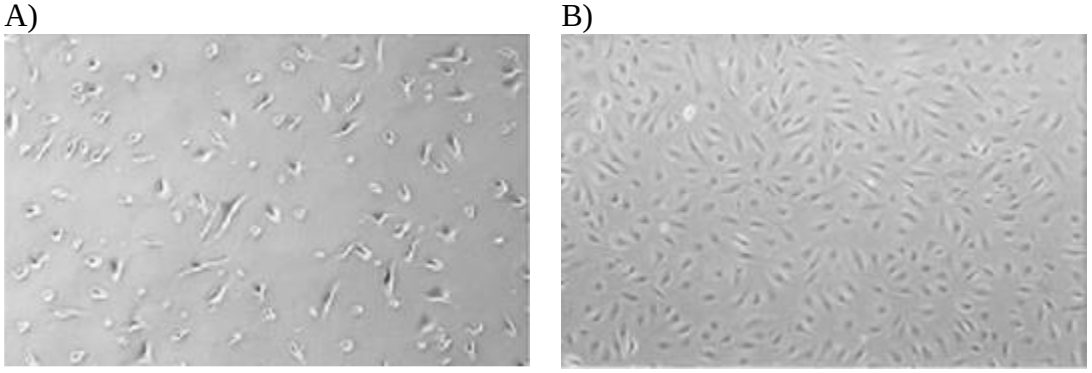

Figura 10. Morfologia de células progenitoras endoteliais precoces (A) e tardias (B) durante seu cultivo *in vitro* (retirado de Smadja e colaboradores, 2007).

A função das CPEs está relacionada à manutenção da hemostasia e reparo vascular. As CPEs são recrutadas a partir da medula óssea em situações de lesões na camada endotelial, quando contribuem diretamente e indiretamente para a formação e reparo do endotélio vascular (94). A ação direta dessas células se dá pela sua proliferação e diferenciação em células endoteliais maduras, que formam o tecido vascular. Além dessa ação, as células progenitoras endoteliais secretam diferentes citocinas pró-angiogênicas, que favorecem a proliferação e a migração de células endoteliais adultas, induzindo a vascularização. Entre as citocinas secretadas, estão o fator de crescimento vascular e endotelial (VEGF), o indutor de óxido nítrico sintase (iNOS), o fator derivado de estroma-1 (SDF-1), o fator estimulante de colônias granulocitárias (G-CSF) e o fator de crescimento semelhante à insulina tipo 1 (IGF-1) (6).

Diferentes grupos de pesquisas já demonstraram que o cultivo de células progenitoras endoteliais sobre biomateriais poliméricos é possível (53, 95). Assim, conforme as características descritas, as CPEs representam uma fonte celular bastante promissora para a engenharia de tecidos vasculares, podendo ser utilizadas para a endotelização de biomateriais nessa área da medicina regenerativa.

2.7. Cultivo dinâmico de células nos biomateriais

A combinação de células e biomateriais, ainda *in vitro*, permite a organização celular previamente à implantação desse sistema *in vivo*, podendo acelerar o processo de remodelamento e reparo tecidual. A incorporação das células aos biomateriais ocorre através do processo chamado de "semeadura". A semeadura estática, ou de superfície, tem sido a técnica utilizada prevalentemente na engenharia de tecidos. Nessa técnica, as células são dispensadas com auxílio de uma micropipeta sobre a superfície do biomaterial (86, 96). Após a semeadura celular, as células são mantidas e expandidas sobre os biomateriais até que elas atinjam uma confluência adequada e/ou tenham produzido matriz extracelular para aplicação *in vivo*. Nessa etapa, o meio de cultivo é colocado sobre os biomateriais e trocado a cada 3 ou 4 dias.

Essas técnicas estáticas de semeadura e cultivo são de fácil execução e não requerem grande habilidade do manipulador. Contudo, essas técnicas não se mostram adequadas para alguns tipos de materiais, como por exemplo, os biomateriais com formato tubular. A semeadura e o cultivo estático não conseguem distribuir uniformemente as células na estrutura desse tipo de biomaterial (97). Além disso, essas técnicas não oferecem os estímulos mecânicos que estão presentes no ambiente *in vivo*. Consequentemente, apresentam dificuldades em criar um tecido funcional *in vitro* (15).

Os biorreatores são dispositivos nos quais processos biológicos e bioquímicos desenvolvem-se em condições ambientais e operacionais controladas (pH, pressão, temperatura). Na engenharia de tecidos, os biorreatores têm sido utilizados como um processo dinâmico de semeadura e cultivo celular (14). Nesses dispositivos, o meio de cultivo é passado continuamente pelas células e pelos biomateriais. Assim, eles conseguem melhor mimetizar o microambiente encontrado *in vivo*, oferecendo estímulos químicos e físicos para o desenvolvimento tecidual.

Os biorreatores são particularmente interessantes à engenharia de tecidos vasculares. Eles conseguem simular alguns estímulos fisiológicos existentes na parede dos vasos sanguíneos, como tensão de cisalhamento, tensão de estiramento e, até mesmo, pressão radial pulsátil (15). Além disso, o movimento do meio de cultura propicia um melhor transporte de nutrientes e de oxigênio (O_2), favorecendo o desenvolvimento uniforme das células por toda a estrutura do biomaterial (14).

Dois tipos principais de biorreatores têm sido utilizados para o cultivo de células em biomateriais vasculares: os biorreatores rotativos e os biorreatores de perfusão. Os

biorreatores rotativos, também chamados biorreatores de parede rotatória, consistem em câmaras onde os biomateriais tubulares são conectados. Essas câmaras giram a baixas velocidades, permitindo a transferência de massas pelas paredes interna e externa dos biomateriais (Figuras 11a e b). Enquanto isso, os biorreatores de perfusão apresentam uma câmara onde os biomateriais são conectados, um reservatório de meio de cultivo e uma bomba de perfusão. Nesse sistema, o meio de cultivo passa pelo lúmen do biomaterial, simulando o fluxo sanguíneo (Figuras 11c e d) (15). Os biorreatores rotativos são bastante utilizados para a semeadura de células na parede de biomateriais tubulares, enquanto que os biorreatores de perfusão são utilizados para a manutenção das células em cultivo (98, 99).

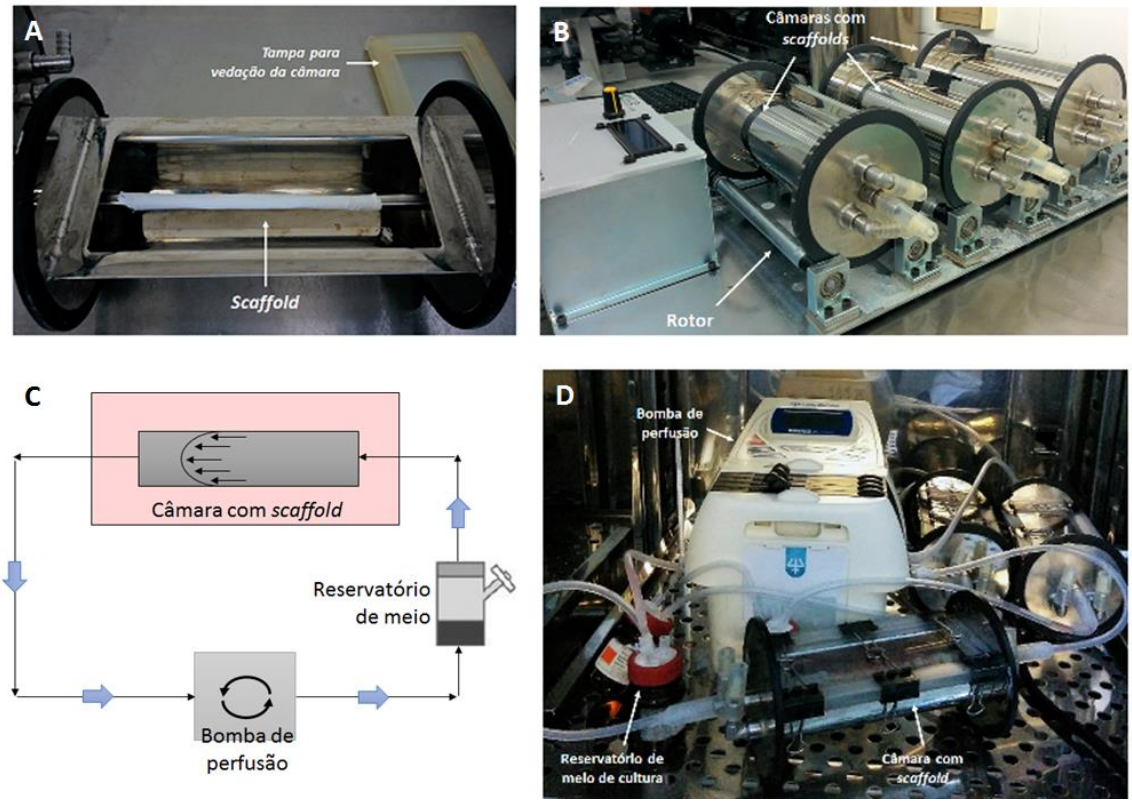

Figura 11. Câmara para biorreator (a), biorreator rotativo (b), esquema ilustrativo de um biorreator de perfusão (c) e biorreator de perfusão (d) (Laboratório de Hematologia e Células-tronco, Faculdade de Farmácia, UFRGS).

As células endoteliais que formam o endotélio vascular natural estão continuamente expostas à tensão de cisalhamento, originada a partir do fluxo sanguíneo.

Essa força mecânica é responsável pelo alinhamento das células na direção do fluxo e modula a sinalização de diferentes vias intracelulares e de mecanorreceptores específicos. Ainda, a tensão de cisalhamento gerada por um fluxo laminar de sangue estimula a produção de óxido nítrico pelas células endoteliais, uma molécula extremamente importante para a homeostase vascular. A expressão do fator KLF-2, envolvido na resposta anti-inflamatória e antitrombótica, das moléculas de adesão CD31 e VE-caderina, dos receptores para VEGF e a secreção da glicoproteína fator de Von Willebrand também são aumentadas em resposta à tensão de cisalhamento. (26). O escoamento do meio de cultura pelo biorreator de perfusão também gera uma tensão de cisalhamento, o que pode contribuir para o desenvolvimento da camada endotelial no lúmen do biomaterial vascular.

Diferentes autores têm demonstrado que as células progenitoras endoteliais também são sensíveis ao fluxo laminar de meio de cultura. Yamamoto e colaboradores demonstraram que a adesão, a proliferação e o potencial vasculogênico de células progenitoras endoteliais são aumentados quando as mesmas são submetidas a um cultivo dinâmico (100, 101). A passagem contínua de meio de cultura também faz com que as CPEs apresentem uma morfologia alongada em torno de seu eixo, na direção do fluxo (101). Ainda, a tensão de cisalhamento gerada pelo fluxo de meio de cultivo tem efeitos sobre diferentes vias de transdução celulares, modulando a expressão de marcadores típicos de células progenitoras endoteliais (102). Em seu trabalho, Obi e colaboradores demonstraram que o cultivo dinâmico de CPEs ocasionou o aumento da expressão dos marcadores KDR, Flt-1 e VE-Caderina nessas células (103).

Com base nisso, acredita-se que o uso de biorreatores favoreça o desenvolvimento de CPEs na estrutura de biomateriais vasculares e também contribua para a “maturação” e, consequentemente, maior funcionalidade desses sistemas.

3. Objetivos

3.1. Objetivo geral

Desenvolver um biomaterial vascular endotelizado, através da associação de biomateriais e células-tronco mesenquimais ou células progenitoras endoteliais, para aplicação como substituto vascular no tratamento de doença arterial periférica grave.

3.2. Objetivos específicos

3.2.1. Produzir biomateriais pela técnica de *electrospinning*, utilizando os polímeros poli(caprolactona) e poli(carbonato de trimetileno-*co*-ácido lático).

3.2.2. Funcionalizar os biomateriais de PCL com heparina e VEGF a fim de evitar a formação de trombos e auxiliar na endotelização de sua superfície;

3.2.3. Avaliar as propriedades físico-químicas dos biomateriais desenvolvidos;

3.2.4. Isolar, cultivar e caracterizar células-tronco mesenquimais obtidas a partir de dentes decíduos e células progenitoras endoteliais obtidas a partir de sangue de cordão umbilical e placentário humano;

3.2.5. Cultivar células-tronco mesenquimais e células progenitoras endoteliais nos biomateriais produzidos e avaliar a interação das células com o biomaterial, através de ensaios biológicos como adesão, proliferação, morfologia celular e citotoxicidade;

3.2.6. Desenvolver um biorreator para o cultivo dinâmico de células no interior de biomateriais vasculares tubulares;

3.2.7. Comparar os métodos de cultivo dinâmico e estático para a endotelização de biomateriais tubulares.

4. Resultados

4.1. Capítulo I

Electrospun scaffolds functionalized with heparin and VEGF increase the proliferation of endothelial progenitor cells

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Electrospun scaffolds functionalized with heparin and vascular endothelial growth factor increase the proliferation of endothelial progenitor cells

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Keywords: endothelial progenitor cells, vascular scaffolds, vascular endothelial growth factor, mesenchymal stem cells, heparin

Supplementary material for this article is available online

Abstract

In severe cases of peripheral arterial disease, tissue loss can occur and the use of vascular grafts can be necessary. However, currently, there are no suitable substitutes for application in small diameter vessels. The aim of this work has been to produce scaffolds with adequate properties for application as vascular substitutes. Polycaprolactone scaffolds were produced by the electrospinning technique. The surface of the scaffolds was functionalized with heparin and vascular endothelial growth factor (VEGF) and their physical–chemical properties were characterized. Human endothelial progenitor cells (EPCs) or mesenchymal stem cells (MSCs) were seeded onto the surface of the scaffolds in order to create an endothelial layer. The electrospun scaffolds exhibited mechanical properties compatible with the native arteries. The presence of heparin prevented blood coagulation on the scaffold surface. The presence of heparin and VEGF favored the adaptation of MSCs and EPCs on the scaffolds in relation to the non functionalized scaffolds. In addition, the EPCs cultivated on the scaffolds maintained the expression of CD31, CD34 and VE-cadherin genes. The results obtained in the present study suggest that electrospun scaffolds functionalized with heparin and VEGF can be applied in vascular tissue engineering. These scaffolds exhibited antithrombogenic properties and favored the development of cells on their surface. The association of heparin and VEGF with electrospun scaffolds increased EPC proliferation, favoring the formation of the endothelial layer and the regeneration of damaged vessels.

1. Introduction

Peripheral arterial disease is a serious public health issue which affects patients of 60 years and over. This condition is characterized by reduced blood flow in peripheral tissue due to the obstruction of the aortic artery and/or its branches. A number of different strategies are in use for treatment according to the clinical severity [1]. The use of by-pass vessel grafts is still an important therapeutic option to restore blood perfusion in ischemic tissue and prevent limb loss. Autologous vessels have remained the material of choice for use as a by-pass. However, about 30% of patients do not have suitable vessels available and need synthetic grafts [2]. Vessel grafts made with expanded polytetrafluoroethylene (e-PTFE) and polyethylene terephthalate have been satisfactorily used in the repair of medium and large caliber arteries. However, they exhibit a high failure rate when applied in small diameter vessels (<6 mm). Thrombus formation in the lumen, intimal hyperplasia and poor endothelization are the principal causes of small synthetic vessel graft failure [3].

Tissue engineering is an emerging science which combines the use of cells, scaffolds and growth factors in order to repair damaged tissue. This tool has been exploited for the development of small vascular substitutes. The combination of cells and biodegradable

scaffolds allows for the creation of a vascular structure. When implanted *in vivo*, the substitute promotes native tissue ingrowth as the cells proliferate and produce extracelular matrix (ECM) and the scaffold degrades. Electrospinning is a method to produce nanostructured scaffolds that are able to mimic the physicochemical properties of the natural ECM, offering a favorable microenvironment for celullar development [4]. Electrospun scaffolds made from polycaprolactone have been extensively studied in vascular tissue engineering due to their mechanical and structural properties and compatibility with the vessel substitute application. In order to prevent failure of the scaffold, its internal surface can be funcionalized with biomolecules that promote endothelium development and avoid thrombogenesis.

Heparin is an anticoagulant that can be linked to the surface of scaffolds. Scaffolds functionalized with heparin exhibit a hydrophilic and negatively charged surface that prevents thrombus formation by hindering the adsorption of proteins such as albumin and fibrinogen [5]. Besides its anticoagulation properties, heparin can be linked to cationic molecules like vascular endothelial growth factor (VEGF) and maintain its slow delivery [6]. VEGF is a potent cytokine that stimulates angiogenesis and vasculogenesis. This growth factor is part of a signaling pathway for restoring oxygen balance in ischemic tissue. It increases the recruitment of endothelial progenitor cells (EPCs) for ischemic tissue, supporting neovascularization. In addition to migration, VEGF also promotes the proliferation and differentiation of endothelial cells [7, 8]. Therefore, the association of VEGF with scaffolds can assist in the formation of a continuous endothelium layer, ensuring the success of the vascular substitutes.

EPCs are a population of cells derived from bone marrow with a high competence level for forming vessels [9]. EPCs can contribute directly or indirectly to the formation of endothelium through their high proliferation rate and ability to secrete vasculogenic cytokines and growth factors, respectively [10]. EPC seeding on scaffold lumen accelerates the formation of a functional mature endothelium. Another powerful cellular alternative to vascular tissue engineering are mesenchymal stem cells (MSCs). MSCs can be obtained from a variety of sources and they also exhibit a high proliferation rate. In addition, MSCs show low immunogenicity and are able to regenerate vascular tissue by transdifferentiation or paracrine mechanisms [11, 12].

In this study, PCL electrospun scaffolds were developed and funcionalized with heparin and VEGF for improving their antithrombogenicity and promoting the development of an endothelial layer. The physico-chemical properties of the scaffolds were characterized and human EPCs or MSCs were then seeded onto their surface. The biological properties and endothelial layer formation of the scaffolds were also investigated.

2. Materials and methods

2.1. Materials

Cell Counting Kit-8 (CCK-8), 4,6-diamidino-2-fenylindole dihydrochloride (DAPI), Dulbecco's Modified Eagle's Medium (DMEM), FITC-labeled Ullex europaeus agglutinin (UEA-1), Hepes, Human VEGF Elisa Kit (RAB0507), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), Polycaprolactone (Mw: 90 000 g mol^{-1} and Mn: 47 000 g mol^{-1}), Rhodamine phalloidin, Toluidine blue and Trypsin-EDTA were purchased from Sigma-Aldrich. Alexa Fluor 488 (A11001), Alexa Fluor 555 (A21422), penicillin and streptomycin, Platinum sybr green QPCR supermix, VEGF recombinant human protein (PHC9391), VEGF antibody (AHG0114), fetal bovine serum (FBS), Tryple Express and the primers used for qPCR were purchased from ThermoFisher Scientific. Activated partial thromboplastin time (APTT) and lactate dehydrogenase (LDH) enzyme dosage assays were purchased from Labtest. Heparin (5000 UI) was obtained from Cristalia. Acetylated Dil lipoprotein from human plasma (Dil-Ac-LDL) was purchased from Biomedical Technologies. Endothelial cell growth médium (EGM-2) was purschased from Lonza.

2.2. Isolation, cultivation and characterization of MSCs

Primary MSCs were isolated from human deciduous teeth pulp, as described by Bernardi and colleagues, after approval by the Ethics Committee of the Federal University of Rio Grande do Sul. The teeth were extracted from children enrolled on the pediatric dentistry program of the Faculty of Dentistry at UFRGS, after informed written consent from the parents. The isolated cells were cultivated in DMEM containing 2.5 g l^{-1} of Hepes (free-acid) supplemented with 10% FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin and maintained in a humid atmosphere of 5% CO_2, at 37 °C. The culture medium was changed every 3 or 4 d. When the cell culture reached 90% confluence, a passage using 0.5% trypsin-EDTA solution was carried out. In the fifth passage, the cells were characterized by their capacity to differentiate into adipogenic, osteogenic and chondrogenic lines, as well as immunophenotyping profile, as previously described [13]. MSCs between the third and sixth passages were used in all the following experiments.

2.3. Isolation and cultivation of EPCs

A primary culture of EPCs was obtained from umbilical cord blood, after the informed written consent from the donors. Mononuclear cells were separated by Ficoll density gradient centrifugation and seeded onto 6-well culture plates. The tissue culture plates were coated with 5 μg cm^{-2} of collagen type I.

The EPCs were cultivated in EGM-2 medium supplemented with FBS at 37 °C in a humidified incubator at 5% CO_2. The medium was changed every 3–4 d and the nonadherent cells were removed by successive changes of the medium. Colonies with cobblestone-like morphology were visualized after 7–15 d in culture. When the cells reached 90% confluence, a passage using TrypLE Express reagente was carried out.

In the second passage, the EPCs were cultivated onto collagen-coated 24-well plates. When 80% confluence was reached, the cells were incubated with 10 mg ml^{-1} Dil-ac-LDL in EGM-2 for 4 h at 37 °C. Subsequently, the cells were fixed with 4% paraformaldehyde for 30 min and incubated for 1 h with FITC-labeled UEA-1, at room temperature. For CD31 staining, the cells were fixed and incubated with 1% bovine serum albumin (BSA) for 30 min. The samples were then washed with phosphate-buffered saline (PBS) and incubated with 10 μg ml^{-1} anti-CD31 antibody overnight. Following this, the samples were washed and incubated with Alexa Fluor 488 secondary antibody (1:200) for 30 min at room temperature. Control without primary antibody was used to exclude nonspecific markers. In all the samples, the nuclei of cells were stained with 0.5 μg ml^{-1} of 4,6-diamidino-2-fenylindole dihydrochloride (DAPI) and analyzed in an inverted fluorescence Nikon microscope.

In the third passage, the cells were analyzed in terms of their immunophenotypic profile by flow cytometer. A total of 1×10^5 cells were incubated with the antibodies against the following antigens: CD31/PE, CD34/FITC, CD45/FITC, CD146/FITC and kinase insert domain receptor (KDR)/PE for 30 min at 4 °C. The cells were then washed and ressuspended in 1 ml of PBS. Nonviable cells were excluded from the analysis by incubation with 7AAD (7-Amino Actinomycin D). Living cells were analyzed using FACSAria III (Becton Dickinson) flow cytometer. Fluorescence adjustment was made with the aid of isotypic controls. Approximately 10 000 events were acquired for each sample. The analysis of the data was performed using the FACSDiva software (Becton Dickinson).

2.4. Scaffold preparation by electrospinning

The scaffolds were composed of PCL and produced by the electrospinning technique. PCL was dissolved in tetrahydrofuran and N,N-dimethylformamide (7:3) so that the final concentration was 10% (w/v). The solution was maintained under magnetic agitation for 24 h for complete homogenization. The polymeric solution was placed in a 1 ml syringe with a 0.7 mm internal diameter needle. The needle was positioned 20 cm from the collector plate and electrospinning was conducted using 0.019 ml min^{-1} flow rate and 19 kV voltage. The electrospun scaffolds were collected onto 15 mm diameter cover slips and sterlized with UV light for 2 h in a laminar flow hood.

2.5. Scaffold functionalization

Before covalent immobilization of heparin, the PCL scaffolds were hydrophilized by alcaline hydrolysis. The scaffolds were exposed to 2 M NaOH for 30 min. Subsequently, the hydrolyzed scaffolds were rinsed with distilled water and immersed in a fresh solution of heparin. Heparin solutions with concentrations varying from 0.5% to 3% (w/v) were prepared in distilled water, containing 5 mg ml^{-1} EDC and 5 mg ml^{-1} NHS. The scaffolds were maintained immersed in these solutions at room temperature overnight. Following this, the samples were rinsed with distiled water to remove excess heparin.

After heparin linkage, the scaffolds were submitted to VEGF adsorption reaction. They were incubated in 1 μg ml^{-1} VEGF solution in PBS at room temperature. After 2 h, they were washed with PBS to remove the excess of the bioproducts.

2.6. Scaffold characterization

Four groups of scaffolds were evaluated: PCL; PCL/NaOH scaffolds submited to NaOH hydrolysis; PCL/Hep scaffolds hydrolyzed with NaOH and linked with heparin; PCL/Hep/VEGF scaffolds hydrolyzed with NaOH and linked with heparin and VEGF.

2.6.1. Morphology and fiber diameter

The morphology of the fibers was analyzed by scanning electron microscopy (SEM; JEOL JSM-6060). The images were obtained using accelerated tension of 10 kV after the samples had been coated with a thin layer of gold using a sputter coater (Bal-Tec SCD 050). The average diameter of the fibers was determined using the software ImageJ 1.383 by measuring 30 fibers from each of the images obtained ($n = 3$).

2.6.2. Water contact angle

The hydrophilicity/hydrophobicity of the scaffolds was determined by water contact angle measurement. This analysis was performed on three samples of each scaffold group using the Krüss goniometer (DSA100, Krüss, Germany). For this, 3 μl of distilled water was dropped onto the surface of the samples. The contact angle between the droplet and the scaffolds was measured at different locations on the samples.

2.6.3. Scaffold degradation

In vitro degradation of the PCL and PCL/NaOH scaffolds was studied. The scaffolds were incubated in 10 ml PBS at 37 °C, stirred at 100 rpm and with solution changes each 4 d. The samples were collected at different times (0, 1, 7, 15, 30 and 60 d), washed with distilled water, and dried at 30 °C for 24 h. The changes in the polymeric molecular weight of the samples were estimated by a size exclusion chromatography module (Viscotek VE 2001) equipped with a refraction index detector. The scaffolds were dissolved

in tetrahydrofuran, filtered and eluted at a flow rate of 1 ml min^{-1} at 45 °C. A polystyrene standard curve was used for calibration.

2.6.4. Mechanical properties

The mechanical properties of the PCL and PCL/NaOH scaffolds were evaluated in terms of Young's modulus and maximum load by dynamic mechanical analysis (DMAQ800, *TA* Instruments) equipped with a tension film clamp and using controlled force mode. The scaffolds were cut into rectangular shapes (5 × 12 mm). The assays were carried out at a constant temperature (37 °C) with ramp force of 0.5 N min^{-1} until 18 N maximum load, under 0.005 N static load ($n = 3$).

2.6.5. Residual solvent content

The residual solvent content of the PCL scaffolds was evaluated by thermogravimetric analysis (TGA) (SDT Q600, TA Instruments) under nitrogen atmosphere. About 10–12 mg scaffold samples were held at 25 °C for 5 min, followed by a heating rate of 20 °C min^{-1} (from 25 °C to 750 °C) and then heating at 70 °C min^{-1} until 800 °C.

2.6.6. Content of immobilized heparin in the scaffolds

The content of heparin linked to the scaffolds was determined by the toluidine blue colorimetric method. A solution of 0.005% toluidine blue was prepared in 0.01 M HCl with 0.2% NaCl (w/v). The scaffolds treated with heparin were immersed in 1 ml toluidine blue solution. After 2 h, 1 ml of hexane was added to each sample and the solutions were vortexed for 10 min for phase separation. The aqueous phase was removed and read at 631 nm on a spectrophotometer. Simultaneously, a standard curve of a known amount of heparin was prepared and read in the same conditions. The content of heparin in the scaffolds was quantified by comparison with the standard curve. As negative control, the PCL scaffolds were submitted to the same functionalization process; however, without heparin (PCL/Hep0).

2.6.7. VEGF distribution, loading and release

To determine the VEGF content in the scaffolds, the supernatants of the VEGF solution used for functionalization were collected. The PBS used in the washes was also collected and added to the supernatants. The amount of VEGF was determined using ELISA kit, in accordance with the manufacturer's instructions. Subsequently, the loading efficiency (LE) of VEGF to the scaffold surface was evaluated by the following equation:

$$\text{LE}\,(\%) = \frac{\text{Wa} - \text{Wb}}{\text{Wa}} \times 100,$$

where Wa and Wb are the amounts of VEGF in solution used for scaffold functionalization and in the supernatant after functionalization, respectively.

To assess the distribution of VEGF on the surface of the scaffolds, they were immersed in a blocking solution (3% BSA in PBS) for 1 h. The samples were then incubated overnight with a monoclonal anti-VEGF antibody (5 μg ml^{-1}). Subsequently, the samples were washed and incubated with AlexaFluor 555 secondary antibody (1:200) for 30 min. Control without primary antibody was used to exclude nonspecific staining. The samples were analyzed in a Nikon fluorescence microscope.

The release of VEGF from the functionalized scaffolds was analyzed by ELISA kit. Herein, samples with a 5 cm diameter were incubated in 10 ml of PBS at 37 °C, under agitation. After 1, 2, 4, 6, 24, 72, 168, 360 and 744 h, an aliquot of 1 ml PBS was removed and 1 ml of fresh PBS was added to the samples. The accumulative release of VEGF from the PCL/Hep/VEGF scaffolds was calculated according to the following equation:

$$\text{Accumulative release}\,(\%) = \left(\frac{m_n}{m_0}\right) \times 100\%,$$

where m_n is the total amount of VEGF released from the scaffolds at a determined time and m_0 is the total amount of VEGF in the scaffolds.

2.6.8. Anticoagulant properties of scaffolds

The anticoagulant properties of the functionalized scaffolds were evaluated by APTT. For this, the scaffolds were incubated in 1 ml of anticlotting human plasma. Subsequently, the plasma was collected and the APTT was measured in accordance with the manufacturer's instructions. As assay, the APTT of the plasma was determined.

2.7. Cell seeding and biological evaluations

The interaction between the MSCs or EPCs and the four groups of scaffolds was evaluated. For this, 5×10^5 viable cells (MSC or EPC) were seeded onto each scaffold. As a control group, the same cell density was also seeded directly onto the culture plate. The cell seeded scaffolds were incubated at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was changed every 3–4 d.

2.7.1. Cell adhesion

Cell adhesion onto the scaffolds was evaluated by nuclei staining. After 3 h of cell seeding, the medium was removed and the samples were fixed with 4% paraformaldehyde for 20 min. The adhered cells were stained with 0.5 μg ml^{-1} DAPI. Nine images were obtained from each sample corresponding to nine different, randomly distributed fields. The number of adhered cells was quantified according to the relation of the total number of counted cells per sample.

2.7.2. Viability and cytotoxicity assays

These parameters were evaluated after 1, 7 and 15 d of cultivation. Cell viability was evaluated by measurement

of the metabolic activity using CCK-8 kit. For this, WST-8 reagent was added to the samples and incubated for 1 h, in accordance with the manufacturer's instructions. Following this, the solution was read at 450 nm.

Cytotoxicity was assessed by measurement of the LDH enzyme activity in the supernatant of the samples. In the predetermined intervals, the supernatants were collected and submitted to colorimetric method for LDH dosage, in accordance with the manufacturer's instructions. A positive control (control of maximum cell death) was used in this assay. For this, cells were cultivated directly onto the culture plate and, in the intervals of analysis, were treated with 1% Triton-X 100 for 30 min.

2.7.3. Morphology and cell distribution on the scaffolds

The integration and distribution of the MSCs and EPCs was observed by SEM. After 1 and 15 d of cultivation, the samples were washed with PBS, fixed with 3% glutaraldehyde and subjected to graded ethanol dehydrations before being coated with gold and imaged.

Cell morphology was observed by confocal microscopy (Olympus SV1000; ×40 lens). After 1 d of cultivation, the samples were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and actin was stained with 50 $\mu g\,ml^{-1}$ rhodamine phalloidin for 40 min and the cell nuclei were stained with 0.5 $\mu g\,ml^{-1}$ DAPI for 1 min.

2.7.4. Gene expression

Total RNA from each sample was extracted using Trizol after 15 d of cultivation. The RNA samples were quantified and their level of purity analyzed by spectrophotometry (NanoDrop, ND-2000). Complementary DNA was synthesized from 2 μg of the total RNA using reverse transcriptase M-MLV kit, in accordance with the manufacturer's instructions. Real time PCR was performed using Platinum Sybr Green Supermix kit. Reactions were submitted to 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. The melting curve was performed for all the reactions to confirme the amplified product. The gene expression was normalized to the housekeeping gene β-actin and the relative quantification of mRNA was determined by $2^{-\Delta\Delta CT}$. The gene expression of the EPCs and MSCs cultivated on the scaffolds was compared to the EPCs cultivated directly on the culture plate. Primer sequences are as follows: CD31 F: GGCTGTTGAATTTCCCACAT, R: TGCTCTTCCAATTTCCAAGG. CD34 F: GCGCTTTGCTTGCTGAGT, R: GGGTAGCAGTACCGTTGTTGT. VON WILLEBRAND FACTOR (VWF) F: AGTGCAGACCCAACTTCACC, R: GTGGGGACACTCTTTTGCAC. VE-CADHERIN F: GAACCAGATGCACATTGATGA, R: CGACTCACGCTTGACTTGAT. β-actin F: AGCACAGAGCCTCGCCTTT, R: CGGCGATATCATCATCCAT.

2.8. Statistical analysis

The symmetry study of the distributions was performed using the Shapiro–Wilk test. One-way ANOVA followed by Tukey's test was applied for fiber diameter analysis and content of immobilized heparin in the scaffolds. The mechanical properties were compared by t-test. One-way ANOVA was used in the adhesion and PCR assay, where the groups were compared to the 2D cultivation (control). Two-way ANOVA followed by Bonferroni's test was used in the viability and cytotoxicity assays. Differences were considered significant when $P < 0.05$.

3. Results

3.1. EPC isolation and characterization

Between 7 and 10 d of cultivation of the mononuclear cells, some colonies were observed. The cultures showed a spindle-shape morphology, characteristic of endothelial cells. After the second passage, the cells were evaluated by their capacity to incorporate Dil-ac-LDL and ability to bind FITC-UEA-1. The cells exhibited positive staining for both (figure 1). CD31 expression was also evaluated by fluorescence and the cells were positive for this marker (figure 1). In the third passage, the cells were evaluated by flow cytometry. The isolated cells showed a high expression of CD31 (99.8% ± 0.1%) and CD146 (97.0% ± 4.7%). CD34 and KDR expression was variable in the isolated cultures. CD34 exhibited a range from 1.5% to 65.8% of expression with a mean of 13.1%. KDR exhibited a range of expression between 2% and 63.8% with a mean of 27.9%. CD45+ cells were virtually absent in all the cultures. The combination of results confirmed the identity of the isolated cells as EPCs.

3.2. Scaffold characterization

3.2.1. Physicochemical properties

The morphology and fiber diameter of the electrospun scaffolds were analyzed by SEM and are exhbited in figure 2. The PCL scaffolds showed a structure formed by continuous and randomly distributed fibers and by an interconnected porous network. The fibers exhibited a smooth surface. The treatment with NaOH did not substantially alter the structure of the scaffolds. The diameter and morphology of the fibers was also maintained after their functionalization with heparin and VEGF ($p > 0.05$). The average diameter for each scaffold group is shown in table 1. In all the scaffold groups, approximately 75% of the fibers had a range of between 200 and 500 nm diameter.

The measurement of the contact angle shows that the PCL scaffolds exhibited a hydrophobic surface (table 1). With the treatment with NaOH, hydrophilicity was clearly enhanced. Heparin functionalization mantained the scaffolds hydrophilic. After VEGF treatment, the contact angle of the surface of the scaffolds showed an increase; however, these scaffolds had

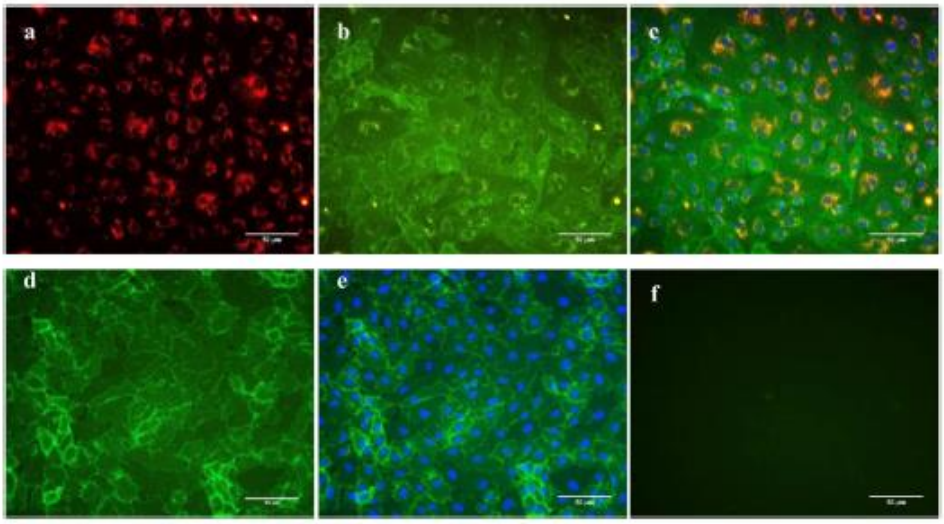

Figure 1. Characterization of progenitor endothelial cells isolated from umbilical cord blood. Immunofluorescence images show the DiI-ac-LDL uptake (red) (a), FITC-UEA-1 binding (green) (b), merged image of DiI-ac-LDL, FITC-UEA-1 and DAPI nuclei staining (blue) (c), anti-CD31 staining (green) (d), merged image of anti-CD31 and DAPI nuclei staining (e) and negative control (f), where first antibody was omitted. The scale bar represents 50 μm (original magnification ×200).

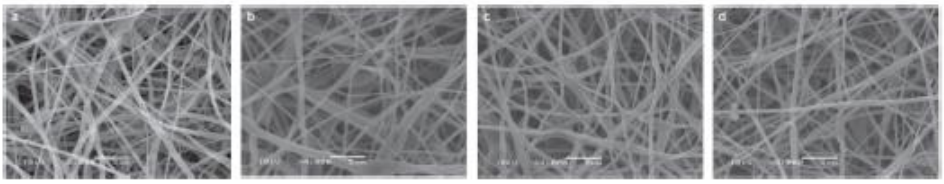

Figure 2. Scanning electron microscopy images of electrospun PCL (a), PCL/NaOH (b), PCL/Hep (c) and PCL/Hep/VEGF (d). The scale bar represents 5 μm (original magnification ×4000).

Table 1. Properties of electrospun scaffolds.

Group	Average diameter (nm)	Contact angle	APTT (s)
PCL	427 ± 195	135.4° ± 3°	45.7 ± 1.5
PCL/NaOH	420 ± 168	80.8° ± 12°	44.7 ± 0.6
PCL/Hep	406 ± 172	67.1° ± 2°	>120
PCL/Hep/VEGF	452 ± 199 nm	107.5° ± 6°	>120
Control plasma			42.3 ± 5.0

a more hydrophilic appearence than the untreated scaffolds.

The *in vitro* degradation of the PCL and PCL/NaOH scaffolds was observed over 60 d by evaluation of the changes in the numeric molecular weight (M_n) of the polymer (figure 3). The time zero corresponds to initial M_n, prior to degradation. The PCL scaffolds showed a slight degradation rate during the experiment period. The group exhibited a reduction of about 17% in M_n on day 1 and maintained this profile throughout the 60 d of analysis. Treatment with NaOH caused an initial reduction of 16.7% in the polymer M_n. The PCL/NaOH group presented a molecular weight of about 39 KDa from the beginning of the assay and was reduced to 30.8 KDa (21%) on day 1. Between days 1 and 60, the molecular weight of this group did not exhibit significant change. The final M_n was similar for the PCL and PCL/NaOH groups.

Figure 4 shows the results of the mechanical properties of the PCL and PCL/NaOH scaffolds, obtained from stress–strain curves. The PCL scaffolds showed a low Young's modulus value and a high elongation capacity. With the hydrolysis treatment, Young's modulus exhibited a slight increase; however, there was no statistical difference. The results show that hydrolysis did not cause significant alterations in the

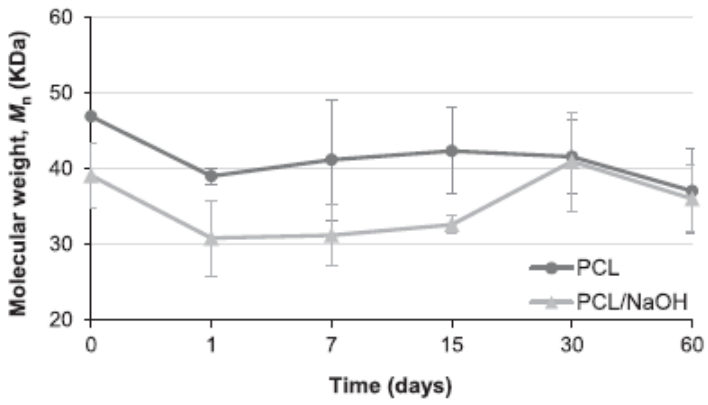

Figure 3. *In vitro* degradation of untreated (PCL) and hydrolyzed scaffolds (PCL/NaOH) over 60 d.

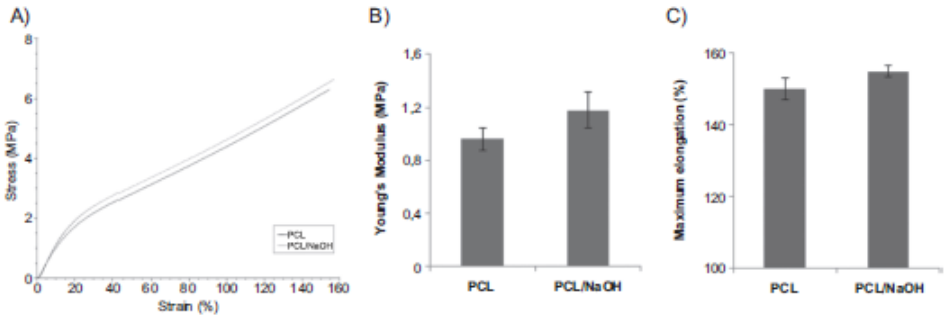

Figure 4. Mechanical properties of the PCL and PCL/NaOH scaffolds: stress–strain curves (A), Young's modulus (B) and maximum elongation (C). Results are shown as mean ± standard deviation. Statistical differences between the groups was not observed in relation to the t-test ($n = 3$).

tensile properties of the scaffolds and they maintained their suitability for use in vascular tissue engineering.

The residual solvent content of the PCL scaffolds was analyzed by TGA (online supplementary figure 1). In this analysis, mass lost at temperatures less than 200 °C corresponds to volatilization of organic solvents or water. The TGA curve did not show loss of mass in this temperature range. It can therefore be supposed that the scaffolds were free from organic solvents or humidity.

Four concentrations of heparin were tested to functionalize the PCL scaffolds (figure 5). As expected, the toluidine blue test shows that the negative controls (PCL and PCL/Hep0 scaffolds) did not contain heparin. The scaffolds functionalized with 0.5% heparin solution also did not show detectable amounts of heparin. The scaffolds functionalized with 1%, 2% and 3% (w/v) heparin solutions had similar amounts of bound heparin to their surfaces. Therefore, the solution containing 1% of heparin was chosen and used for all subsequent assays. This concentration resulted in a density of 24.6 μg of heparin per cm^2 per scaffold.

After heparin functionalization, the surface of the scaffolds was treated with VEGF. The adsorption of VEGF to the scaffold surface had a high LE of nearly 93%. The labeling of the PCL/Hep/VEGF scaffolds with anti-VEGF showed that all the fibers were marked (figure 6). This analysis showed that the VEGF was continuously and uniformly distributed on the scaffold surface and no aggregation or discontinuity of VEGF staining was observed.

The release profile of VEGF from the PCL/Hep/VEGF scaffolds is shown in figure 7. This kinetic study showed that only a small amount of VEGF was released from the scaffolds. However, two stages can be distinguished in the release profile: a peak of VEGF release in the first 2 h of assay, followed by a slow and stable release for up to 30 d. After 30 d, approximately 2.28 ng ml^{-1} of VEGF was released in the medium from the PCL/Hep/VEGF group.

The antithrombogenic properties of the scaffolds were evaluated by APTT test. The results are demonstrated in table 1.

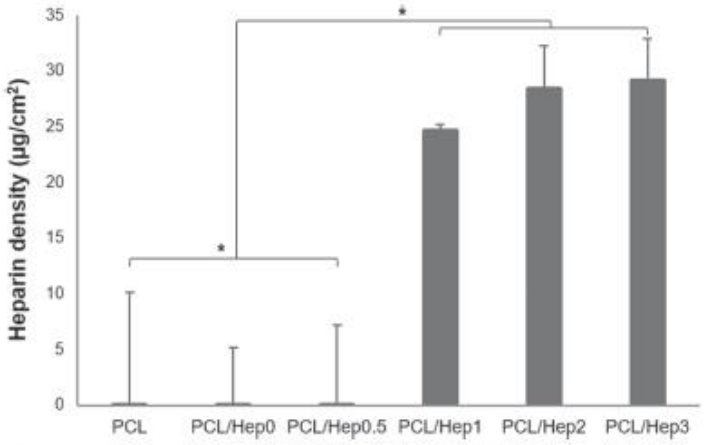

Figure 5. Heparin density on the scaffolds. (PCL: untreated scaffolds, PCL/Hep0: scaffolds treated with solution containing EDC and NHS, PCL/Hep0.5, PCL/Hep1, PCL/Hep2, PCL/Hep3: scaffolds treated with solution containing EDC, NHS and 0.5, 1, 2 and 3% w/v of heparin, respectively). *Shows statistical difference in comparison with the other groups (one way ANOVA followed by Tukey's test, $p < 0.05$).

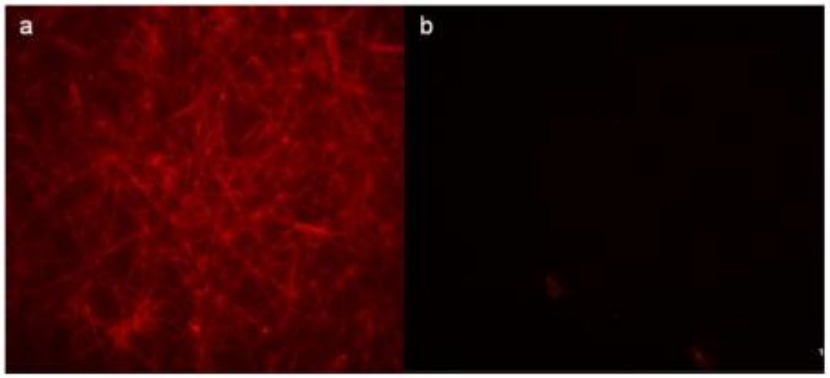

Figure 6. Verification of the VEGF adsorption in the scaffolds. Immunofluorescence images of PCL/Hep/VEGF (a) marked with anti-VEGF antibody followed by staining with conjugated secondary antibody and negative control (b) where first antibody was omitted. Scale bar represents 10 μm (original magnification ×200).

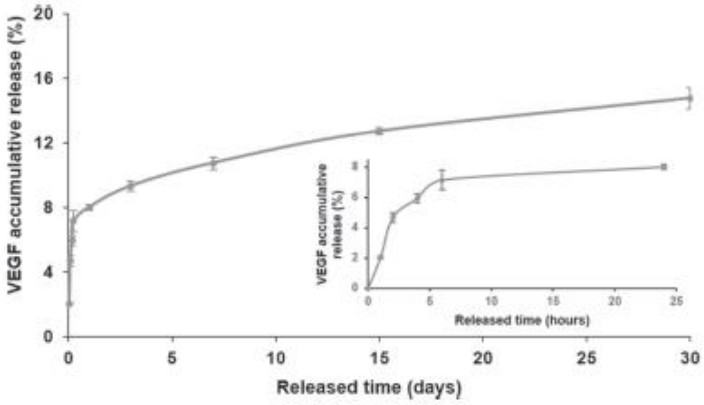

Figure 7. Release profile of VEGF from the PCL/Hep/VEGF scaffolds.

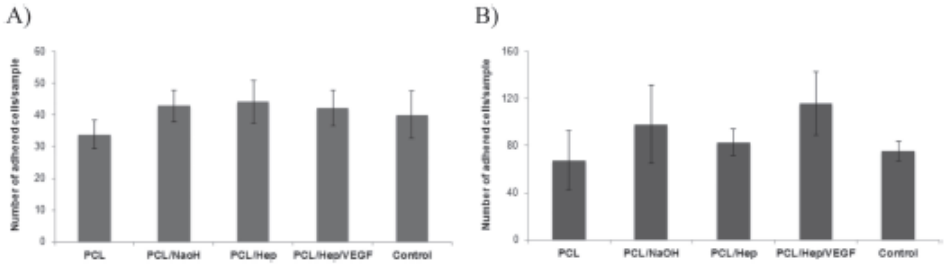

Figure 8. Adhesion of mesenchymal stem cells (A) and endothelial progenitor cells (B) on the different groups of scaffolds. Results are shown as mean ± standard error. No statistically significant differences were observed between the groups for either cell types analyzed (One way ANOVA, $p < 0.05, n = 5$).

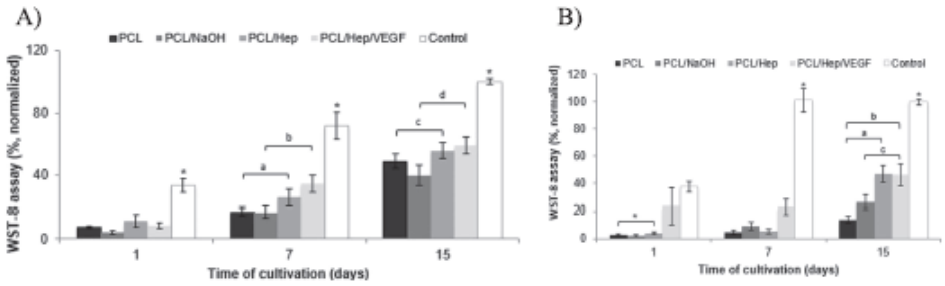

Figure 9. WST-8 assay of mesenchymal stem cells (A) and endothelial progenitor cells (B) cultivated for 1, 7 and 15 d on the different groups of scaffolds and tissue culture plate (control). *,a,b,c Shows statistical difference in comparison with the other groups (two-way ANOVA followed by Bonferroni's test, $p < 0.05, n = 5$).

3.2.2. Biological properties

The biological properties of the different groups of scaffolds were evaluated through their interaction with the MSCs and EPCs.

To examine the capacity of the scaffolds for supporting cell adhesion, the MSCs and EPCs were seeded onto the scaffold surface and cultivated for 3 h. By DAPI nuclei staining, it was possible to verify that both the MSCs and EPCs were able to adhere to all the scaffold groups (figure 8). Comparatively, in 3 h of cultivation, a greater number of EPCs than MSCs were adhered to the scaffold surface. For the MSCs, a similar number of cells attached to the different groups of scaffolds and the culture plate. Meanwhile, a greater number of adhered EPCs was observed in the PCL/Hep/VEGF scaffolds. However, there was also no statistical difference in EPC adhesion between the different groups of scaffolds and control.

To determine the effect of scaffold treatment in supporting cell growth, MSCs and EPCs were cultivated on scaffolds and evaluated by CCK-8 assay at set intervals (1, 5 and 15 d). As seen in figure 9(A), MSC proliferation was higher on the culture plate compared to the other groups. However, these cells were also able to proliferate on the different scaffolds. The number of viable MSCs increased significantly between the 1st and 15th days of cultivation in all the scaffold groups. In addition, the scaffolds treated with heparin or with heparin and VEGF enhanced the proliferation of the MSCs, demonstrated by the largest number of viable cells in the PCL/Hep and PCL/Hep/VEGF groups on the 7th and 15th days of cultivation.

When the EPCs were analyzed, it was found that the culture plate also showed a higher proliferation rate than the scaffold groups (figure 9(B)). However, the PCL, PCL/NaOH, PCL/Hep and PCL/Hep/VEGF groups were also able to support proliferation and a greater number of EPCs were demonstrated on the 15th day of cultivation. It was also observed that the PCL/Hep/VEGF group showed a greater number of viable EPCs on the first day of cultivation than the other groups ($p < 0.05$) and it was statistically similar to the control group. On the 15th day, a large number of viable cells were detected in the PCL/Hep and PCL/Hep/VEGF groups. These two groups were significantly different from the PCL and PCL/NaOH groups in terms of supporting the growth of EPCs.

The results obtained by CCK-8 assay are corroborated by SEM and confocal analysis. As can be seen in figure 10, on the first day of cultivation, the MSCs were able to adhere to all the scaffold groups. The MSCs exhibited a rounded morphology on the PCL and PCL/NaOH scaffolds and had a more spread cytoskeleton on the PCL/Hep and PCL/Hep/VEGF group (figure 12). After 15 d of cultivation, a high number of MSCs were present on the scaffolds. At this time, all

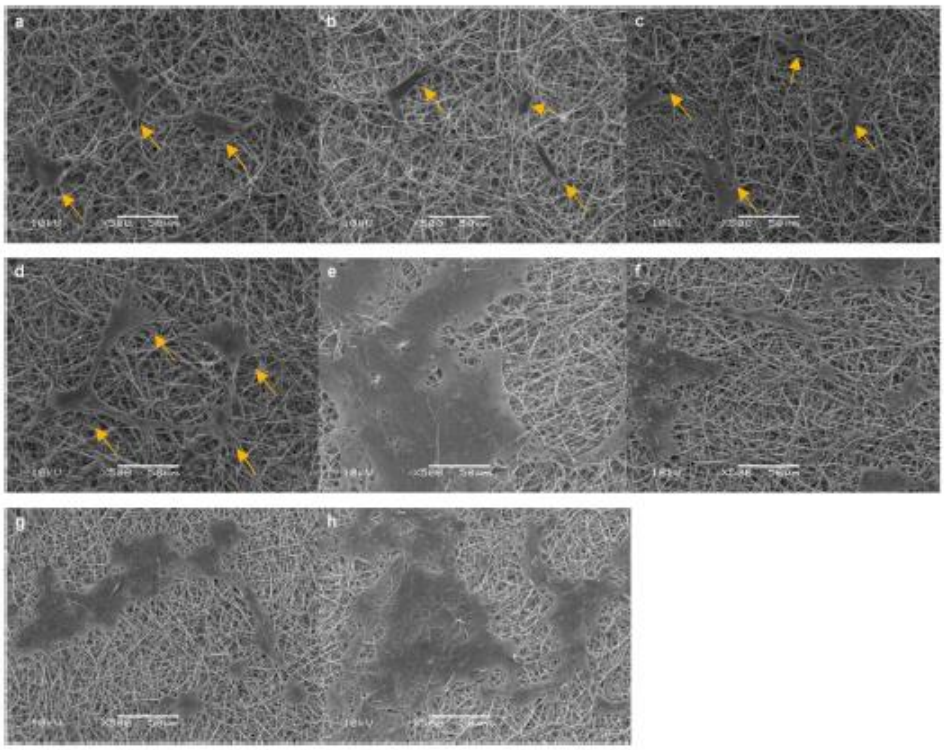

Figure 10. Scanning electron microscopy images of MSCs cultivated for 1 (a)–(d) and 15 (e)–(f) days on the scaffolds. PCL (a), (e), PCL/NaOH (b), (f), PCL/Hep (c), (g) and PCL/Hep/VEGF (d), (h) scaffolds. Yellow arrows demonstrate the presence of cells. Scale bar represents 50 μm (original magnification ×500).

the scaffold groups had more than 50% of their surface covered by spread MSCs. When the EPCs were evaluated, it was observed that a small number of cells had adhered to the scaffolds (figure 11). The cells exhibited few points of adhesion on the fibers of the PCL, PCL/NaOH and PCL/Hep scaffolds, demonstrated by the very rounded cell morphology. Meanwhile, in the PCL/Hep/VEGF group, the EPCs had a spread morphology (figure 12). After 15 d of cultivation, an increase in the number of EPCs in all the scaffold groups was observed. However, the PCL/Hep and PCL/Hep/VEGF scaffolds had a large percentage of their surface covered by EPCs (figure 11).

Regarding the LDH dosage, no significant differences were observed between the scaffold groups and the control group during the 15 d of cultivation for both types of cells (figure 13) except for the Triton X-100-treated group, which was used as positive control of the assay. The high dosage of LDH obtained in the positive control demonstrated the funcionality of the assay.

The gene expression profile of the EPCs and MSCs cultivated onto the four groups of scaffolds was evaluated and compared to the EPCs cultivated on the culture plate (reference group) (figure 14). The MSCs did not exhibit changes in expression of CD31, CD34, fvW and VE-Chaderin genes. The results of gene expression in the EPCs cultivated on the scaffolds showed a high variation between the samples and did not present a significant variation in relation to the 2D-culture. Although not significant, the EPCs cultivated on all the scaffold groups showed an increase in VE-cadherin expression (figure 13). The expression of CD31 increased in the PCL (220%), PCL/NaOH (150%) and PCL/Hep (230%) groups in relation to the 2D-culture. Meanwhile, CD34 expression was slightly reduced. Similarly, even if not statistically significant, the fvW expression in the EPCs was reduced in the scaffold groups in comparison with the control group.

4. Discussion

Peripheral vascular disease is a prevalent disorder in the world which affects millions of people each year. In severe cases, the implantation of a vascular graft is needed in order to restore the blood flow in peripheral tissue. Unfortunately, there is still not an adequate synthetic graft for use in small vessels, which fail, mainly due to the thrombus formation in their lumen. The present study has evaluated the use of tissue engineering for the development of vascular

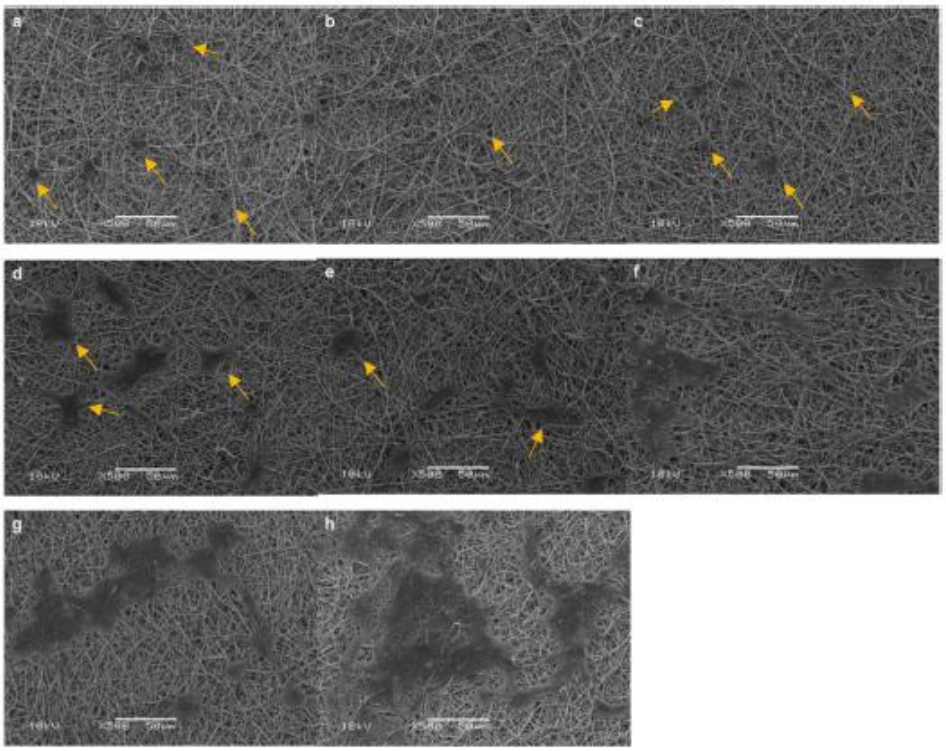

Figure 11. Scanning electron microscopy images of EPCs cultivated for 1 (a)–(d) and 15 (e)–(h) days on the scaffolds. PCL (a), (e), PCL/NaOH (b), (f), PCL/Hep (c), (g) and PCL/Hep/VEGF (d), (h) scaffolds. Yellow arrows demonstrate the presence of cells. Scale bar represents 50 μm (original magnification ×500).

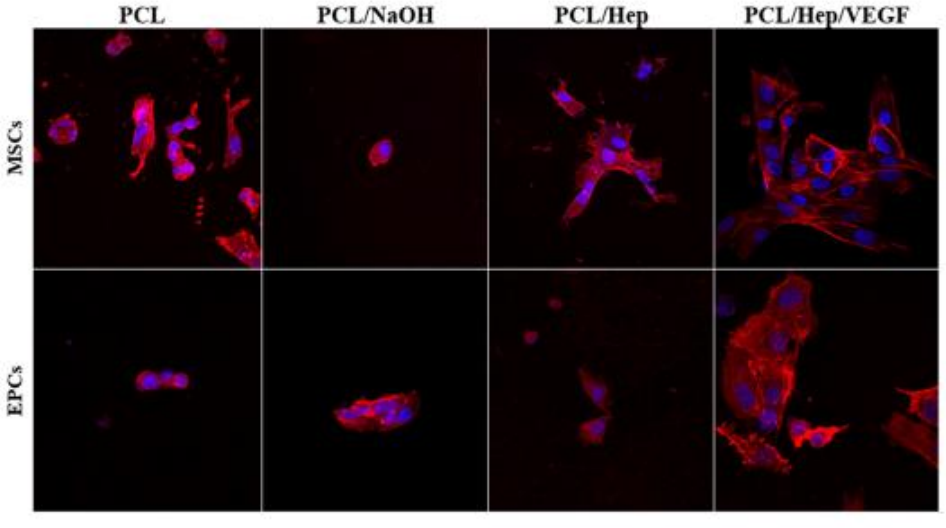

Figure 12. Confocal images of MSCs and EPCs cultivated on PCL, PCL/NaOH, PCL/Hep and PCL/Hep/VEGF for 1 d. The cells were stained with DAPI for nuclei (blue) and rhodamine-phalloidin for cytoskeleton (red). (Original magnification ×60).

substitutes. The results demonstrate that functionalizated electrospun scaffolds can prevent blood coagulation and favor cellular development on their surface.

The scaffolds were produced by electrospinning and exhibited fibers with diameters distributed in a nanometric range and a high number of interconected

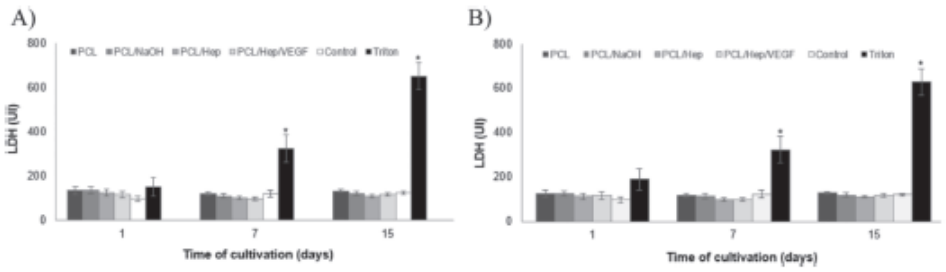

Figure 13. LDH dosage from mesenchymal stem cells (A) and endothelial progenitor cells (B) cultivated onto different groups of scaffolds and tissue culture plate (control) for 1, 7 and 15 d. The triton group represents the positive control. *Shows statistical difference between Triton and the other groups (two-way ANOVA followed by Bonferroni's test, $p < 0.05$, $n = 5$).

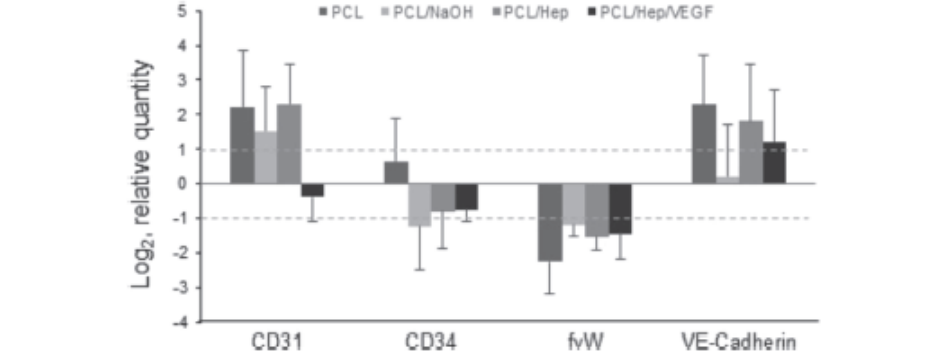

Figure 14. Expression of CD31 (A), CD34 (B), fvW (C) and VE-Cadherin (D) in EPCs cultivated on PCL, PCL/NaOH, PCL/Hep and PCL/Hep/VEGF scaffolds for 15 d, compared to the culture plate (control group).

pores. These characteristics are very important for tissue engineering as nanometric fibers form a raised superficial area, which provide several points for cellular adhesion, while the presence of pores ensures the nutrients and O_2 diffusion necessary for cell growth.

As can be seen in the SEM images, the alkaline hydrolysis did not cause visible damage to the structure of the PCL scaffolds. The surface characteristics and the average diameter of the fibers in the PCL/NaOH group were maintained. At the same time, the contact angle analysis showed that treatment with NaOH was effective and caused an increase in the density of the hydrophilic groups on the scaffold surface. The exposure of these functional groups is very important for the functionalization process. These groups are required for covalent binding between the heparin and the polymer. It is important to note that the superficial area of scaffolds influences the measurement of the contact angle. Usually, porous materials have a measured value of a contact angle higher than the real value [14]. One of the causes for this variation is the presence of air in the structure of porous scaffolds, which is hydrophobic [15]. However, it is possible to state that the hydrolyzed and functionalized scaffolds obtained in this study presented a greater facility of humidification than the untreated scaffolds. The PCL/Hep and PCL/Hep/VEGF also showed no alterations in morphology and diameter of fibers, indicating that the functionalization process is not detrimental to the scaffold structure.

PCL was chosen to produce the scaffolds in the current study due its established biocompatibility and its physicochemical properties, which are suitable for vascular tissue engineering. Vascular grafts must have appropriate mechanical properties, which include the ability to resist hemodynamic stress and have physiological compliance [3]. DMA analysis confirmed the elasticity and a high elongation capacity of the produced scaffolds. The PCL scaffolds exhibted Young's moduli similar to human healthy arteries, which have values comprised of between 0.1 and 1 Mpa [16]. Alkaline hydrolysis causes the exposure of –OH and –COOH groups by breaking the ester bonds in the polymeric chain. This effect can cause some changes in the physicochemical properties of the scaffolds, such as their mechanical properties. Because of this, the PCL/NaOH group was also evaluated by DMA assay. The PCL/NaOH scaffolds showed no significant reduction of their elasticity or elongation capacity. These results show that hydrolysis treatment did not cause changes in the structure of the scaffolds. It is believed that the PCL/Hep and PCL/Hep/VEGF

groups can also maintain the same profile once the functionalization process occurs from the hydrophilic groups already exposed in the polymer chain.

For vascular applications, the polymeric scaffold should exhibit slow degradation in *in vitro* cultivation or after *in vivo* implantation so that the cells have the opportunity to synthetize sufficient amounts of extracellular matrix and are able to organize and construct the new tissue [3, 17, 18]. The analysis of the polymer molecular weight demonstrated that in 60 d the polymeric degradation was very low even after hydrolysis of the PCL. The hydrolysed PCL scaffolds showed a slight increase of M_n during the assay. However, this increase is attributed to intrinsic variations in the size exclusion chromatography technique and was not significant. The stability of the PCL and PCL/NaOH scaffolds during the 60 day period ensures that the endothelial tissue can be organized [19, 20], a characteristic essential for the success of the vascular substitutes.

As previously described, for the production of scaffolds by electrospinning, a polymeric solution was prepared using organic solvents. During the formation of the fibers, the solvents evaporate. However, a small amount of the organic solvents may be retained in the fibers and affect cell growth [21, 22]. The TGA did not detect the presence of humidity or residual solvent on the scaffolds, ensuring their low toxicity for the cells and their biological performance.

Heparin is a glycosaminoglycan anticoagulant with consolidated safety and efficacy. This potent anticoagulant is in use for covering the surface of medical devices with vessel applications, thereby improving the patients' prognosis [17, 23]. In the current study, heparin was successfully bound to the scaffold surface. The anticoagulant activity of heparin was not altered by its covalent bonding to the PCL, as evidenced in the ATTP test, in which the PCL/Hep scaffolds prevented blood coagulation on their surface. The PCL/Hep/VEGF groups were also able to inhibit coagulation on their surface, showing that the heparin remained active even with adsorption of the growth factor. The production of scaffolds with an antithrombogenic surface is very important because thrombosis is one of the major causes of failure of synthetic vascular grafts [24, 25]. Moreover, it has been shown that heparin affects the growth of smooth muscle cells (SMCs) [26]. Su *et al* demonstrated that the heparin delivered from poly(L-lactide-*co*-caprolactone) nanofibers inhibited the proliferation of SMCs [27]. Therefore, the functionalization of the internal surface of scaffolds with heparin may also reduce the occurrence of neointimal hyperplasia, the major cause of stenosis, another frequent cause of vascular graft failure.

VEGF adsorption sussesfully occurred on the entire surface of the scaffolds. Only a small proportion of adsorbed VEGF was released from the scaffolds. The high retention of VEGF in the scaffolds was probably caused by the presence of heparin. Heparin has negatively charged functional groups, which may bind positively charged aminoacids in proteins, such as VEGF. Thus, the growth factor is electrostatically arrested in the anticoagulant network. This effect was previously reported by Singh and co-authors, who demonstrated that scaffolds functionalized with heparin had higher retention of VEGF compared to non functionalized scaffolds. Furthermore, the study showed that the growth factor retention is proportional to the heparin content [28]. The interaction with heparin was important to reduce the initial burst release and to mantain a controlled release of VEGF. In addition, this interaction contributes to maintaining the activity of the factor by protecting it from thermal and enzymatic degradation [28].

VEGF has an important role in stimulating angiogenesis and vasculogenesis, where recruiting angioblast precursors or EPCs are in action [7]. Anderson and co-authors demonstrated that VEGF is a potent chemo-attractant for EPCs. In an *in vivo* study, the authors showed that alginate gels containing VEGF accumulated a large number of EPCs in the place where they were injected. Furthermore, the exposure to VEGF contributed to blood vessel formation by EPCs in an *in vitro* assay and to restoring blood perfusion in an *in vivo* model of ischemia [8]. Thus, the presence of VEGF in scaffolds can contribute to their more efficient and faster endothelization.

Despite having released only a small rate of VEGF from the scaffolds produced in this study, the PCL/Hep/VEGF group presented interesting results for biological tests for both types of cells. The growth and homongeneous distribution of the cells onto the surface of the nanofibers and subsequent formation of the endothelial layer is crucial to the success of vascular scaffolds. The endothelium is the inner layer of natural vessels which has the function of controlling blood flow interactions with the vessel wall and also participates in the regulation of inflammation, vascular permeability, thrombosis and fibrinolysis. The endothelial layer plays a crucial role in controlling the proliferation and migration of SMCs of the tunica media (Melchiorri *et al* 2013). Quint and co-authors demonstrated that when endothelial cell-seeded scaffolds where implanted *in vivo*, the neointimal hyperplasia was reduced and the permeability to blood flow was favored [10]. Thus, the establishment of an active and uniform endothelial layer in the lumen of scaffolds ensures functionality and also contributes to reducing the failure of these substitutes.

Mature endothelial cells and EPCs are currently used in vascular tissue engineering [20, 29]. EPCs are derived from bone-marrow and present a high proliferation rate, self-renewal and vasculature formation capacity, greater than mature endothelial cells. These cells contribute directly and indirectly to the repair of the vascular endothelium. EPCs proliferate and differentiate into mature endothelial cells, which form the endothelial tissue. In addition, EPCs induce the

formation of the endothelial layer by secretion of different proangiogenic factors. Due to their characteristics, EPCs have been widely applied for endothelization of vascular scaffolds [30, 31]. Adhesion is the first step of interaction between cells and scaffolds and is crucial for further cell development in the structures. As shown in the adhesion assay, after 3 h of seeding, the EPCs were able to adhere on all the scaffold groups. However, when analyzed by CCK-8 assay between 1 and 15 d of cultivation, the cells cultivated on the PCL/Hep/VEGF group showed the best results. Through evaluation of mitochondrial activity, the CCK-8 test allows for an estimation of cell proliferation. It was observed that in the first day of cultivation there was already a large number of EPCs in the scaffolds functionalized with VEGF, which was larger than in the other types of scaffolds. The number of metabolically active cells in this group increased until the 15th day of cultivation and was also greater than in the other scaffold groups. EPCs exhibit in their cellular membranes the vascular endothelial growth factor receptor-2 (VEGFR-2), also known as KDR, a receptor for VEGF. The binding of the growth factor to the receptor forms the complex VEGF-VEGFR-2, which causes a signal transduction inside the cell [32]. It might explain the better adaptation of EPCs observed in the PCL/Hep/VEGF scaffolds. These results are supported by other works that also demonstrated the positive effect of VEGF on EPC proliferation [28, 33]. Chen and collaborators show that scaffolds containing VEGF cause a high proliferation of EPC in a dose and time dependent manner. Their work demonstrates that EPCs grow faster in VEGF-loaded scaffolds than in scaffolds without the growth factor [34]. The analysis of morphology also demonstrated the better and faster integration of EPCs on the surface of the scaffolds containing VEGF. On first day of analysis, the cells showed a spread morphology, with several points of adhesion between their cytoskeleton and the fibers. This effect is possibly due to chemotaxis exerted by VEGF on this cell type. On the 15th day of cultivation, the number of EPCs increased in the PCL/Hep group, remaining similar to the PCL/Hep/VEGF group. Ye and collaborators also demonstrated the combined effect of heparin and VEGF on EPC growth. Their study showed that decellularized heart valves treated with heparin and VEGF resulted in greater adhesion, proliferation and migration of EPCs than untreated valves [33]. In addition to the anticoagulant property, heparin has been indentified as a co-receptor of the VEGF-VEGFR system [35]. Heparin increases the affinity of VEGF to its receptor by stabilizing ligand-receptor complex. Thus, it is suggested that the synergic effect of heparin loaded scaffolds promotes a greater response of EPCs to VEGF present in the culture medium.

In the cell cultivation conditions tested on the scaffolds, no statistically significant variation was detected in the gene expression of the EPCs compared to the 2D culture. However, the cultivation in the different groups of scaffolds showed a tendency to increase the gene expression of VE-cadherin compared to the culture plate. It is believed that the three-dimensional structure of the scaffolds provided greater contact between the cells, causing this increase of VE-cadherin, a component of endothelial adherens junctions. In addition, the EPCs cultivated on the PCL, PCL/NaOH and PCL/Hep scaffolds exhibited a tendency to increase CD31 expression, relative to the 2D cultivation. At the same time, these groups caused a tendency to decrease CD34 expression. The PCL/Hep/VEGF group did not exhibit this behavior. The increase in CD31 expression is related to maturation and differentiation of EPCs, whereas CD34 is downregulated as progenitor cells mature [31]. Thus, the results suggest that the presence of VEGF on the scaffold surface is able to maintain the progenitor characteristics of EPCs. However, a study with longer culture time should be performed to elucidate the expression of these genes in the cultivation conditions proposed in this work.

MSCs represent another cell type that has been investigated for the formation of an endothelial layer in scaffold luminal surface. MSCs can be isolated from different sources and be easily expanded, showing multipotency and low immunogenicity [36]. These cells have the ability to differentiate into endothelial cells if properly stimulated [11, 37]. In the present study, MSCs were able to adhere on all the scaffold groups in a similar way. However, on the PCL/Hep group and mainly on the PCL/Hep/VEGF groups, the cells exhibited a stretched cytoskeleton after 1 d of cultivation. This morphology indicates that these scaffolds show a more favorable environment for cell attachment and integration, where MSCs could accommodate and spread quickly. After 15 d, the four groups promoted better cell integration, which showed a spread morphology on all the scaffolds. This data is corroborated by CCK8 assay, which demonstrated the increase of metabolically active cells on all the groups during the time of cultivation. The PCL/Hep and PCL/Hep/VEGF groups exhibited a greater number of cells on the last day of analysis compared to the other scaffolds types. The supplementation of the culture medium with heparin has been previously reported to increase proliferation of human MSCs, corroborating with the results obtained in this study [38]. The extracellular matrix consists mainly of proteins and glycosaminoglycans. Thus, heparin also provides natural domains on the scaffold surface, which contributes to recognition and development of cells [39].

Despite the fact that the MSCs did not express VEGF receptor, studies have been shown that the growth factor can promote proliferation and migration by activating other receptors in MSCs, such as the PDGF receptor [12]. Thus, the presence of VEGF must also have contributed to the better adaptation of the MSCs on the scaffolds. Wingate *et al* demonstrated

that there is a synergic effect between matrix stiffness and VEGF on endothelial differentiation of MSCs. When they were cultivated on three-dimensional matrices with VEGF, the MSCs showed an upregulation of endothelial markers and eliminated the expression of smooth muscle marker [11]. In this study, the cultivation on PCL/Hep/VEGF scaffolds did not cause changes in expression of the endothelial markers in the MSCs, compared to the 2D cultivation. The amount of VEGF released from the scaffolds was probably not sufficient to cause changes in cellular gene expression. Although not present in *in vitro* differentiation, MSCs produce proangiogenic factors, such as VEGF [12]. Thus, *in vivo* MSCs may stimulate proliferation and migration of host endothelial cells to the scaffold, favoring the creation of endothelium in its lumen.

5. Conclusion

The scaffolds produced in this study exhibited structural and physico-chemical properties compatible with the application of vascular grafts. The functionalization of scaffolds with heparin prevented blood coagulation on their surface. The presence of heparin or heparin and VEGF in the scaffolds favored the growth of EPCs and MSCs in relation to the non-functionalized scaffolds. In addition, the presence of VEGF in the scaffolds did not alter the gene expression profile in the EPCs compared with the 2D cultivation and they maintained their progenitor characteristic. It can be concluded, therefore, that the scaffolds developed in this work have appropriate properties for application in tissue engineering and vessel regeneration.

Acknowledgments

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4.2. Capítulo II

Dynamic endothelialization of electrospun scaffolds functionalized with heparin and VEGF with endothelial progenitor cells combining rotating and perfusion bioreactors

Daikelly Iglesias Braghirolli e Patricia Pranke

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Dynamic endothelialization of electrospun scaffolds functionalized with heparin and VEGF with endothelial progenitor cells combining rotating and perfusion bioreactors

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Abstract

Vascular tissue engineering has been extensively investigated for the production of small diameter vascular grafts by combining vascular cells and scaffolds. This group previously developed an electrospun polycaprolactone scaffold functionalized with heparin and vascular endothelial growth factor, which promotes proliferation of endothelial progenitor cells (EPCs). The present study evaluated the use of bioreactors for the endothelialization of these vascular scaffolds. EPCs were seeded on scaffold lumen by a rotating bioreactor. Following this, the scaffolds were maintained in a perfusion bioreactor. Cellular viability, morphology and metabolism were evaluated and compared to static cultivation. The rotating bioreactor promotes a homogeneous cell distribution in the luminal wall of the scaffold, requiring less time for cell adhesion (3 hours). The perfusion bioreactor favors better development of EPCs than static cultivation. The number of EPCs significantly increased during the 5 days of cultivation ($P<0.05$) in the scaffolds under dynamic conditions. These cells exhibited an elongated shape and an initial alignment with the medium flow. The metabolic activity of the EPCs demonstrated that the perfusion bioreactor optimized the use of glucose by the cells. The results demonstrate that the combination of rotating and perfusion bioreactors is an important approach for the endothelialization of scaffolds and development of vascular substitutes.

Key-words: vascular tissue engineering, tubular scaffolds, bioreactor, endothelial progenitor cells

Introduction

Peripheral arterial disease (PAD) is a prevalent vascular disease that represents a major challenge for patients and government healthcare systems. PAD is characterized by the obstruction or narrowing of the aortic artery and/ or its branches, causing the reduction of blood volume flow in peripheral tissues (1). The resulting lack of oxygenation and nutrients causes symptoms such as claudication, ulcers, tissue gangrene and critical limb ischemia (2). In these cases, surgical replacement of a damaged vessel is performed in order to restore blood flow (3, 4).

Currently, autologous arteries or veins have been substitutes widely used in vascular reconstruction surgeries. However, a large number of patients do not have suitable vessels for self-donation as result of a previous surgery or lack of a good quality vessel (5, 6). Synthetic grafts such as ePTFE (expanded polytetrafluorethylene) and Dacron (polyethylene terephthalate fibre) represent an alternative to autologous vessels. These grafts show positive results in medium and large caliber arteries but not for application in vessels with inner diameter < than 6 mm (7, 8). Trombogenicity, intimal hyperplasia and infections are frequent causes of small synthetic grafts failure (9). Due to the great medical need for suitable vascular grafts, several studies have been researching into new materials that can be used as vascular substitutes.

Through the combination of cells, biomaterials and biomolecules, tissue engineering has been shown as an important tool for the development of vascular substitutes, especially those of small diameter (10, 11). Recently, this study group developed electrospun scaffolds of poly(caprolactone) functionalized with heparin and vascular endothelial growth factor (VEGF) for use in vascular tissue engineering (12). It was shown that the scaffolds had mechanical properties similar to natural vessels and prevented blood coagulation on their surfaces. The scaffolds also supported the adaptation and high proliferation of endothelial progenitor cells on their surfaces. Therefore, the developed scaffolds have proved to be suitable candidates for use as vascular substitutes.

The development of an endothelial layer on a luminal surface of scaffolds is crucial to their success (13, 14). The endothelium has an important role in vascular homeostasis by regulating permeability, inflammation, thrombosis, fibrinolysis and preventing intimal hyperplasia (15). Several studies have reported that both mechanical and biochemical stimuli have key roles in the maturation and functionality of endothelialized scaffolds (13, 16, 17). The use of bioreactors in vascular tissue

engineering provides a dynamic culture system that mimicks the natural physiological conditions. The bioreactors promote faster and more homogeneous cell colonisation of tubular scaffolds and guide cellular organization by simulating the mechanical forces found *in vivo* (18). Thus, it is assumed that bioreactors can optimize the endothelialization of vascular scaffolds, favoring their clinical application.

Therefore, in the present study, the endothelialization of functionalized PCL scaffolds under dynamic conditions were evaluated. PCL scaffolds were produced by the electrospinning technique and functionalized with heparin and VEGF. Endothelial progenitor cells were seeded and cultivated in the luminal wall of the scaffolds under dynamic conditions, through the use of bioreactors. The effect of dynamic culture on cell proliferation, morphology and energetic metabolism was evaluated by comparison with the static cultivation controls.

Materials and methods

Isolation and cultivation of endothelial progenitor cells

Endothelial progenitor cells (EPCs) were isolated from umbilical cord blood after approval by the Ethics Committee of the Universidade Federal do Rio Grande do Sul and the Ethics Committee of Moinhos de Vento Hospital. The EPCs were isolated and characterized, as previously described (12). The cells were cultivated in EGM-2 medium (Lonza), supplemented with 15% fetal bovine serum (ThermoFisher Scientific) and 1% penicillin and streptomycin (ThermoFisher Scientific). When the cells reached 90% confluence, a passage using Tryple Express (ThermoFisher Scientific) reagent was performed.

Bioreactor design

Two types of bioreactors were constructed to cultivate the endothelial progenitor cells in tubular scaffolds: a rotating bioreactor and a perfusion bioreactor.The rotating bioreactor was employed with the purpose of seeding the cells in the luminal wall of the scaffolds. Meanwhile, a perfusion bioreactor was used to maintain the culture of the adhered cell-scaffolds. Cultivation chambers were designed so that they could be used in both bioreactors, avoiding extensive manipulation of the scaffolds. Each chamber accommodated a single scaffold and was composed of a stainless steel cylinder and a

glass cover sealed by a silicon membrane (Figure 1A). These materials provided isolation and sterility for the system and allowed for the visualization of the scaffolds during the cultivation period. Orifices were created on the side walls of the chambers for conection to the perfusion systems. These orifices could be sealed with silicone caps. For the cell seed, the chambers were placed in rotating bioreactors. The rotating bioreactor was composed of a rotor with a speed of 1 rpm (Figure 1B). The perfusion bioreactor was composed of the culture chamber, a culture medium reservatoir (volume of 20 mL) and a perfusion pump (Minipuls 3 Peristaltic Pump, Gilson) (Figure 1C). Each chamber was individually connected to a medium reservatoir through silicone tubes and the culture medium was recirculated in this circuit at a controlled and constant speed.

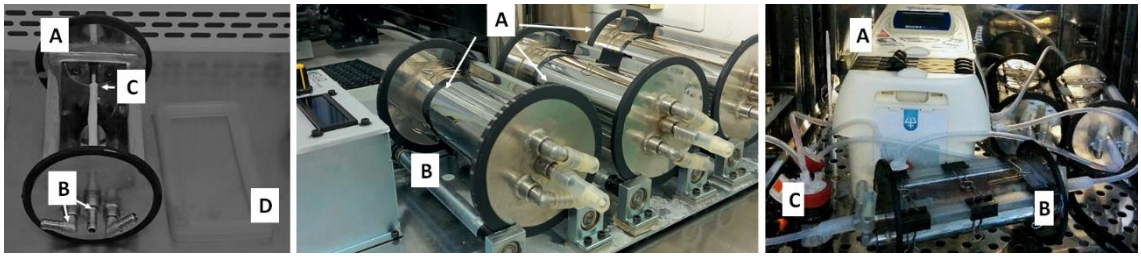

Figure 1. (A) Culture chamber used in rotating and perfusion bioreactor: stainless steel cylinder (a), connectors for the perfusion system (b) shafts for insertion of the scaffold (c) and glass cover (d). (B) Rotating bioreactor composed of the culture chambers (a) and rotor (b). (C) Perfusion bioreactor composed of a perfusion pump (a), culture chambers (b) and culture medium reservoir (c).

Tubular scaffold production and characterization

PCL scaffolds were produced by the electrospinning technique. The PCL (molecular weight 90,000 Da, Sigma-Aldrich) was dissolved in tetrahydrofuran (THF) (Sigma-Aldrich) and *N*,*N*-dimethylformamide (DMF) (Sigma-Aldrich) (7:3) at 10% (w/v) concentration. For the electrospinning, the solution was loaded into a 1 mL syringed fitted with a 22 gauge needle. The solution was injected at a constant flow rate of 0.019 mL/min. A voltage of 19 kV was applied. The fibers were collected on a grounded mandril with a 6 mm diameter, placed approximately 15 cm from the tip of the needle, rotating at 1 rpm. The tubular scaffold was obtained by carefully sliding it off the mandril. After removal from mandril, the inner diameter of the scaffold was 5 mm. About 2.4 mL of polymeric solution was used for the production of each scaffold. After their production, the scaffolds

were sterilized by exposure to UV-light in a laminar flow hood. Every 30 minutes, the scaffolds were rotated so that their entire surface was exposed to UV light.

The fiber scaffolds were characterized with a scanning electron microscope (SEM) (Zeiss Evo 50). The samples were sputter-coated with a thin layer of platinum and analyzed with an acceleration of 7 kV. The average fiber diameter was determined from the SEM images using ImageJ software by 30 measurements for each sample (n=3). The pore size was determined in accordance with Hussain and colleages, who determined that, on average, the ratio of the geometric pore size to the fiber diameter amounts to about six. (20) The thickness of scaffolds was measured by a micrometer (Digimatic Micrometer MDC-25MY—Mitutoyo).

Scaffolds functionalization

After sterilization in autoclave, the bioreactor chambers were mounted into a laminar flow cabinet. The sterile scaffolds were carefully inserted in the chambers and the functionalization of their lumen was performed, as previously described (12). The scaffold lumen were filled with 2 mL of 2 M NaOH for 30 minutes. Subsequently, the scaffolds were extensively rinsed with distilled water and then left in contact with 2 mL of a fresh solution of 1% (w/v) heparin, 5 mg/mL N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC, Sigma-Aldrich) and 5 mg/mL N-hydroxysuccinimide (NHS, Sigma-Aldrich) (overnight). Following this, the scaffolds were rinsed again and submitted to VEGF adsorption reaction. The lumen of the heparinized scaffolds were filled with 2 mL of 1 µg/mL VEGF (ThermoFisher Scientific) solution. After 2 hours, the same rinsing procedures were realized. For all functionalization steps, the chambers containing the scaffolds were accommodated in the rotating bioreactor and rotated at 1 rpm.

Cell seeding and cultivation in tubular scaffolds

Confluent endothelial progenitor cells at passages 4-6 were detached from the culture flasks and resuspensed in EGM-2 culture medium at a concentration of $1x10^6$ cells/mL. The scaffolds were seeded by injecting 2 mL of the EPC suspension into their luminal space. The chambers were filled with the same culture medium and placed in the rotating bioreactors within the incubator (37ºC and 5% CO_2). They were rotated at 1 rpm for 3 or

24 hours. After seeding, the chambers were connected to the perfusion circuit. The cell seeded scaffolds were perfused with EGM-2 medium at 1.7 mL/min for 1-5 days.

The dynamic culture of the scaffolds in the perfusion bioreactor was compared to the static culturing. For static control, the scaffolds were seeded in the same way as previously described. After adhesion under the rotating bioreactor, the scaffolds were removed from the chambers, cut in fragments of 1 cm^2 and placed in a 24-well plate. The EGM-2 medium was added and the cell seeded scaffolds were maintained for 1-5 days under static conditions at 37ºC and 5% CO_2.

Cell adhesion

The time required for complete adhesion of the EPCs to the scaffold luminal wall was investigated. After seeding, the scaffolds were maintained for 3 or 24 hours in the rotating bioreactor. Following this, the scaffolds were removed from the bioreactor chambers, cut in fragments of 1 cm^2 and placed in 24-well plates. The samples were rinsed with phosphate-buffered saline (PBS) and submitted to adhesion assays. The number of adhered cells was estimed by a colorimetric method using Cell Coutting Kit-8 (CCK-8, Sigma-Aldrich) and their viability by Live/Dead assay (ThermoFisher Scientific).

For CCK-8 assay, the scaffolds were rinsed with PBS and incubated with WST-8 reagent for 1 hour, in accordance with the manufacturer´s instructions. Following this, the absorbance was read at 450 nm on a spectrophotometer (Multiscan FC, Thermo Scientific) and compared with a standard absorbance curve for EPCs of a known concentration.

The cell viability after 3 or 24 hours adhesion also was evaluated by Live/Dead assay. For this, the scaffolds were rinsed with PBS and incubated in PBS with 1 µM calcein and 2 µM ethidium homodimer-1 for 20 minutes. The samples were then rinsed and evaluated using a fluorescence inverted microscope (Nikon).

Physical conditions of the dynamic culture

The flow profile in the perfused scaffolds was determined by calculating the Reynolds number, according to the following equation:

$$\mathrm{Re} = \frac{\rho \mathrm{U} \mathrm{D}}{\mu}$$

where ρ is the density of the culture medium, U is the velocity of the fluid, μ is the dynamic viscosity of the culture medium, and D is the inner diameter of the scaffold.

The mean wall shear stress, τ_{mean} (dyn/cm^2), to which the seeded EPCs were exposed was calculated by the Hagen-Poiseuille equation (21), described below:

$$\tau_{mean} = \frac{4\mu \mathrm{Q}}{\pi R_o^3}$$

where μ is the dynamic viscosity of the culture medium (cP), Q is the flow rate (mL/min) and R_o is the scaffold internal radius.

The viscosity (0.88 cP) and density (1.01 g/cm^3) of the culture medium were measured by Ostwald viscometer and pycnometer, respectively, in accordance with the Brazilian Pharmacopeia.

Biological analysis of cultivated scaffolds

After 1 and 5 days of cultivation in the perfusion bioreactor or static conditions, the scaffolds were analyzed in terms of cell viability, morphology and metabolic activity (n=3).

For all these assays on the days of analysis, the scaffolds cultivated in the bioreactor were removed from the culture chambers and cut in samples of 1 cm^2. The fragments were placed in 24 well-plates and rinsed with PBS. Meanwhile, in the scaffolds maintained under static cultivation, the culture medium was removed and the samples were washed with PBS.

Cell viability

Cell viability was also evaluated by CCK-8 assay, as described above for the adhesion assay.

Cell metabolism

The metabolic state of the EPCs cultivated on the scaffolds was evaluated by glucose consumption and lactate production measurements. For this, culture medium samples were collected from the bioreactor medium reservoir and static wells after 1 and 5 days of cultivation. A sample of fresh medium was also collected. Glucose and lactate concentrations were measured using commercially available kits (Glucose GOD-PAP K082 and Lactate Enzimatic Method K084, Bioclin/Quibasa), in accordance with the manufacturer´s instructions.

Cell morphology

The morphology of the EPCs on the scaffolds was observed by confocal microscopy. The samples were fixed with 4% paraformaldehyde for 30 minutes, washed with PBS and incubated with 0.1% triton X-100, for 30 minutes at room temperature. The cells were stained with 50 μg/mL rhodamine phalloidin (40 min) (Sigma-Aldrich) for the actin and 0.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) (1 minute) for the nuclei. Further images were acquired with a Leica TCS SP5 laser confocal microscope, with a 63x lens.

Statistical analysis

The symmetry study of the distributions was performed using the Shapiro–Wilk test. The groups were compared by Student´s t-test. Differences were considered significant when $P < 0.05$. The statistical analysis was performed with GraphPad.Prism software.

Results and discussion

The great need for suitable vascular grafts, mainly for application in small diameter vessels, has generated a significant amount of research for new materials that can be employed in replacement vessels surgeries (22). The great challenges related to the development of vascular grafts are associated with endothelialization and the prevention of thrombosis on their luminal surface (9, 23). In a previous study, this research group developed a vascular substitute through the principles of tissue engineering. The scaffolds were produced using the PCL polymer by the electrospinning

technique and functionalized with heparin and VEGF. They exhibited antitrombogenic properties and prevented thrombi formation on their surface. In addition, the scaffolds favor the adaptation and proliferation of endothelial progenitor cells in their structure (12).

A number of studies in the literature have reported that the use of bioreactors in vascular tissue engineering provides the mechanical forces found *in vivo*, improving the maturation and funcionality of the developed scaffolds (16). In this study, it was hypothesized that the dynamic seeding and cultivation of endothelial progenitor cells in developed scaffolds could optimize *in vitro* cultivation, favoring a homogeneous adhesion and development of the cells on the scaffold lumen. The EPCs were cultivated with the scaffolds by rotaing and perfusion bioreactors and compared to the static controls. The cultivation of scaffolds in the bioreactor favored the growth of endothelial progenitor cells and optimized their energetic metabolism.

Characterization of tubular electrospun scaffolds

Tubular scaffolds were successfully produced by the electrospinning technique (Figure 2A). The parameters of the electrospinning and polymeric solution used for their production were previously standardized (12). In this study, the produced scaffolds exhibited well-shaped and randomly distributed fibers, with an average diameter of 797 ± 442 nm (Figure 2B). The fibrous scaffolds showed a porous structure with pores of an approximate diameter of 4,782 nm. The average thickness of the scaffolds was 189 ± 16 µm.

The structural feature of the electrospun scaffolds mimics the extracelular matrix of natural tissue. The morphology of the fibers showed a great physical similarity with the natural collagen fibers, which may favor cellular adaptation and subsequent formation of tissue (24). Meanwhile, the presence of a large number of interconnected pores in the produced scaffolds ensures a bidirectional metabolic exchange. However, these scaffolds exhbited a small pore size, which may limit cell migration in direction towards the inner regions of the scaffolds (25). For this study, this charactheristic does not present a problem as the aim was to coat the luminal surface of the vascular scaffolds with a monolayer of endothelial progenitor cells.

As demonstrated in the previous study, the functionalization of the scaffolds does not alter their structure and morphology. Moreover, this process generates antithrombogenic characteristics and facilitates their endotheliazation.

A) **B)**

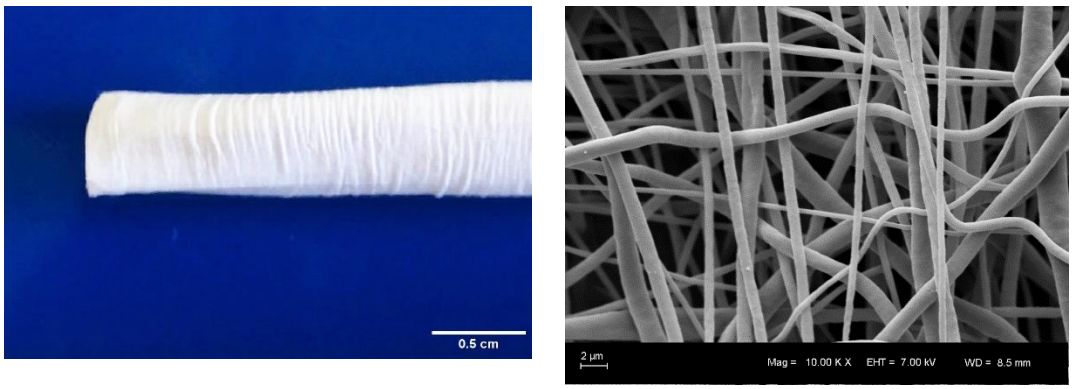

Figure 2. Tubular scaffolds produced by the electrospinning technique: gross appereance (A) and SEM image of fibers (B). Scale bars of 0.5 cm.

Cell adhesion in the luminal wall of the tubular scaffolds

The homogeneous initial distribution of the cells on the scaffold surface is very important for the development of regenerated tissue (26). Due to their tubular geometry, uniform cell seeding in vascular scaffolds still represents a challenge for tissue engineering (10). In this work, this limitation was addressed by the use of a rotating bioreactor. The rotating bioreactor ensured that the cells were homogeneously distributed in the entire inner wall of the tubular scaffold (Figure 3A and B).

In the literature, there is no consensus regarding the necessary time for complete adhesion of cells to scaffold surfaces. Different time periods ranging from 3 to 24 hours have been used for evaluation of cell adhesion, involving dynamic seeding techniques (27-30). As a result, this work has investigated the required time for adhesion of EPCs to the inner wall of the produced scaffolds. After seeding, the cells were incubated for 3 or 24 hours in the rotating bioreactor in order to promote their adhesion to the luminal wall of the scaffolds.

The number of adhered cells was estimated by the CCK-8 colorimetric method. The scaffolds incubated for 3 hours showed a larger number of adhered cells than the scaffolds incubated for 24 hours ($P<0.05$) (Figure 3C). The live/dead assay confirmed this result. A larger number of labeled cells/field was observed after 3 hours and 24 hours incubation (Figure 3A and B). Several dead cells were observed after these periods. The ratio of dead cells to total cells was similar for both adhesion times. The rotational movement of the fluid inside the bioreactor chamber exposed the cells to mechanical stress. The results suggest that the exposure of endothelial progenitor cells to rotational

stress for a long period of time impairs their adhesion and / or causes their detachment from the scaffold surface. Meanwhile, a shorter period of time does not appear to cause this effect and is sufficient to ensure a homogeneous cell distribution in the inner wall of the tubular scaffolds. Thus, the adhesion time of 3 hours was standardized for future assays. By comparison with the standard curve, it was possible to estimate that the scaffolds maintained for 3 hours in the rotating bioreactor had a density of $1.43x10^5 \pm 3.7$ adhered cells/cm^2.

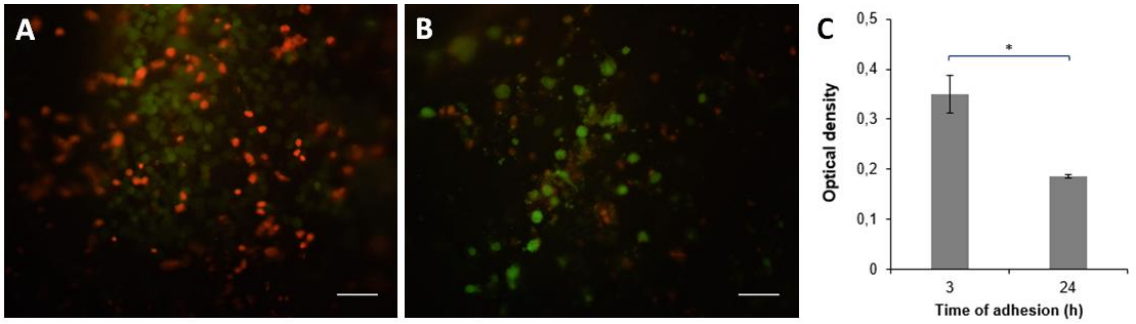

Figure 3. Adhered endothelial progenitor cells in the lumen of functionalized tubular scaffolds after 3 or 24 hours incubation. Live/Dead staining of cells after 3 hours (B) and 24 hours (C) of incubation.CCK-8 assay (C). *Shows $P<0.05$ according t-test. Scale bars of 30 µm (x 200 magnification).

Culture of seeded scaffolds in the perfusion bioreactor

After 3 hours adhesion, the chambers containing the seeded scaffolds were connected to the perfusion bioreactor and perfused with the culture medium. The cultivation of the cell-scaffolds in the perfusion bioreactor provides a better simulation of the microenvironment found in natural vessels (16). The perfusion of cellularized scaffolds with culture medium mimicks the blood flow and exposes the endothelial cells to the mechanical forces existing *in vivo*. Studies have shown that the preconditioning of endothelial cell-seeded scaffolds in bioreactors is important for cell adaptation, avoiding their detachment when subjected to high laminar shear stress after their implantation in vivo (6, 28).

The laminar flow of the culture medium is necessary for homogeneous diffusion of nutrients and oxygen and metabolic waste products to and from the scaffolds (27, 30). In this study, the average Reynolds number was 26.2. This is an indication that the perfusion bioreactor employed ensured the development of a laminar flow profile inside the tubular scaffolds. The laminar flow imparts constant shear stress against the luminal

wall of the vascular scaffolds (31). The flow of medium created a wall shear stress of 3.8 dyn/cm^2. The vascular endothelium is normally exposed to physiological shear stress ranging from 5 to 20 dyn/cm^2 (6). As shown in the shear stress equation, this force is dependent on the velocity of the flow. In this study, when larger flows were applied to the system, a leakage of the culture medium through the scaffolds was observed. This fact can be attributed to the recent cellularization of the scaffolds. The short time of the cell-scaffold cultivation may not have been sufficient for the EPCs to secrete the extracelular matrix and cover the pores present on the surface of the scaffolds, faciliting the passage of the medium through them. Although it is less than the physiological value, the wall shear stress used in this study is in agreement with several other studies available in the literature which report the success of dynamic cultures of vascular cells using even lower shear stresses (1-3.2 dyn/cm^2) (16, 21, 32). The endothelial progenitor cells seeded on the scaffolds were maintained up to 5 days under these dynamic conditions and were compared to the scaffolds maintained in static conditions. This cultivation period was used, as proposed by Uzarski and collaborators. According to the authors, a period of 5 days of maturation, under shear stress, is ideal for the optimization of cell-scaffold binding and prevention of early cell detachment of scaffolds (28).

After 1 day cultivation, the number of viable EPCs was similar under static and dynamic perfusion conditions (Figure 4). The number of viable cells increased significantly between days 1 and 5 for both culture conditions. Although not significant, a larger number of cells was observed in the dynamic perfusion in relation to the static group on the 5th day of cultivation. This small difference may have been caused by the shear stress found in the dynamic culture. Yamamoto and collaborators reported that laminar shear stress stimulates the proliferation of endothelial progenitor cells. The authors demonstrated that the shear stress increases the percentage of EPCs found in the S, G2 and M phases of the cell cycle (32). It is believed that if the cells had been maintained for a longer time period under cultivation, a significant difference between the dynamic and static conditions would have been observed.

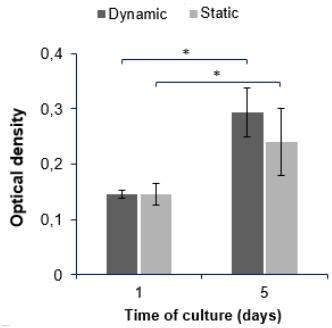

Figure 4. Viability of EPCs cultivated on the luminal wall of vascular scaffolds under perfusion bioreactor (dynamic) and static conditions of cultivation. *Shows statistical difference between the groups, according to Student's t-test.

The consumption of glucose and the formation of lactate were measured in the culture medium in order to analyze the metabolic activity of the EPCs in the scaffolds cultivated under dynamic and static conditions. Under aerobic conditions, the cells produced energy preferentially by oxidative phosphorylation, forming CO_2 and water. Meanwhile, under anaerobic conditions, the glycolytic route was used and lactate was formed (33). Glucose consumption was higher in the cell-scaffolds cultivated in the perfusion bioreactor than in the static conditions (1.4 and 2.0 in days 1 and 5 of cultivation, respectively) (Figure 5A). The higher consumption of glucose, mainly on the 5^{th} day of dynamic cultivation, is related to the greater number of cells present in this system. In addition, endothelial cells maintained in dynamic cultures seem to require more energy. Ciechanowaka et al suggest that endothelial cells continuously exposed to shear stress need to continually reorganize adhesive proteins within cellular processes, thereby demanding more energy. This state of cells would be more similar to that found in situ (29).

Lactate production was lower in the cell-scaffolds maintained in the perfusion bioreactor than in the static conditions (Figure 5B). The calculated production of lactate per cell on the 5^{th} day of cultivation was 9.2 and 48 pmol/mL under dynamic and static conditions, respectively. The lower formation of lactate indicates that the cell-scaffolds under dynamic culture used the glucose preferentially by aerobic metabolism. The movement of the medium inside the scaffold caused by the perfusion bioreactor should have favored the access of the EPCs to dissolve oxygen and nutrients, supporting oxidative phosphorylation. This result is in agreement with other studies demonstrating that bioreactors improve mass transfer in scaffolds (27, 33).

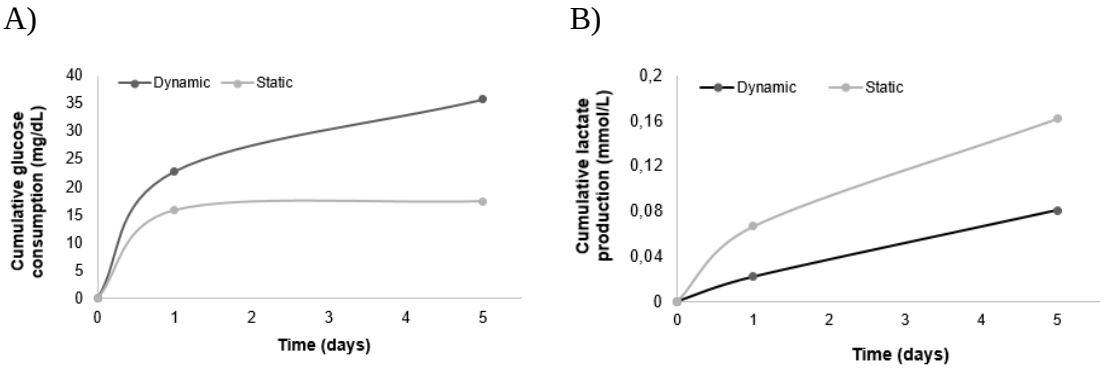

Figure 5. Cumulative glucose consumption (A) and lactate formation (B) in culture medium of EPC seeded-scaffolds cultivation under dynamic (perfusion bioreactor) and static conditions for 5 days.

The morphology of the cultivated EPCs on the lumen of the scaffolds was analyzed by confocal microscopy after 1 and 5 days of cultivation (Figure 6). On the first day, the EPCs cultivated under bioreactor and static conditions showed a similar morphology (Figure 6A and B). The cells exhibited a rounded nuclei and a still undeveloped cytoplasm. After 5 days, the EPCs subjected to dynamic culture showed an increase of their surface area and acquired an elongated shape. In addition, the cells were in close contact one to the other, forming a cellular layer, which appears to be in alignment with the medium flow direction (Figure 6C). The EPCs maintained in static cultivation also showed a more extended cytoplasm. However, these cells did not present a preferred direction of spreading and had few contact points one with the other (Figure 6D).

The results observed in this study suggest that the laminar flow and the wall shear stress promote the development of the endothelial layer in the lumen of vascular scaffolds. The shear stress created by the perfusion bioreactor initiated the orientation of the EPCs around their longitudinal axis. This process is important for their maturation and differentiation into endothelial cells (34). The shear stress modulates the activation of mechanoreceptors and intracellular signaling pathways, contributing to the functionality of the endothelial layer developed on the surface of the scaffolds (6). Quint and collaborators demonstrated that shear stress pre-condiotionaded endothelialized scaffolds showed a high success rate when applied in vivo, resisting to intimal hyperplasia and to thrombotic occlusion (14).

Biochemical and biomechanical factors are present in natural vessels and act synergistically, promoting vascular homeostasis. This study successfully combined the two stimuli to develop a vascular graft. As previously shown, the presence of heparin and VEGF in vascular scaffolds prevents the formation of thrombi and promotes the adhesion and proliferation of endothelial progenitor cells on their surface. This study have also demonstrated that bioreactors can promote fast cellular adhesion and an efficient endothelialization of vascular grafts. A future study should be performed to evaluate the functionality of the endothelial layer created.

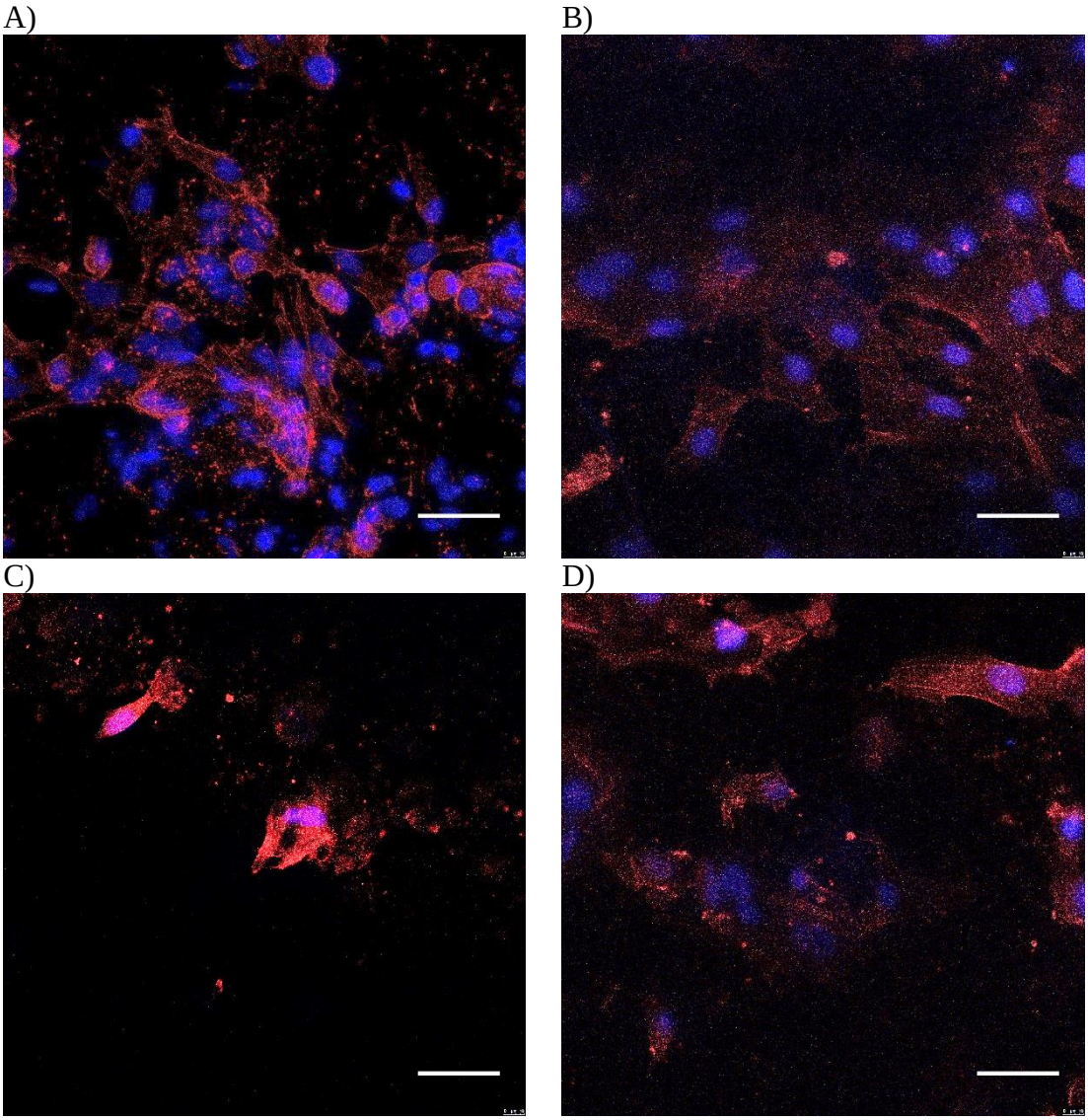

Figure 6. Representative confocal images of EPCs cultivated under dynamic (A, B) and static conditions (C, D) for 1 (A, C) and 5 days (B, D). The cells were stained with DAPI for nuclei (blue) and rhodamine-phalloidin for cytoskeleton (red). Scale bar represents 20 µm (original magnification x63).

Conclusions

This study demonstrates that the use of bioreactors in vascular tissue engineering can promote fast endothelialization of the lumen of heparin and VEGF functionalized electrospun scaffolds. The rotating bireactor was able to promote a homogenous seeding of endothelial progenitor cells in the luminal wall of the scaffolds, requiring little time for their adhesion. Meanwhile, the use of the perfusion bioreactor promoted the proliferation and organization of the cells. The EPCs exhibited an elongated shape and an initial orientation around their longitudinal axis. In addition, the perfusion bioreactor ensured better diffusion of nutrients and oxygen in relation to the static conditions of cultivation, optimizing the energetic metabolism of the cells. This study combined biochemical and biomechanical stimuli to developed vascular substitutes. It can be concluded, therefore, that rotating and perfusion bioreactors can efficiently endothelialize electrospun scaffolds functionalized with heparin and VEGF. This tool offers an intersting alternative for the development of vascular grafts.

Acknowledgments

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4.3. Capítulo III

Poly(trimethylene carbonate-*co*-lactide) electrospun scaffolds for use as vascular grafts

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Poly(trimethylene carbonate-*co*-lactide) electrospun scaffolds for use as vascular grafts

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Abstract

Currently, there is great clinical need for suitable vascular grafts, mainly for application in small caliber vessels. In this study, poly(trimethylene carbonate-*co*-L-lactide) (PTMCLLA) electrospun scaffolds were investigated for use as vascular substitutes. Copolymers with varying trimethylene carbonate/lactide ratios were evaluated in terms of their physical-chemical and biological properties. PTMCLLA 20/80, 30/70 and 40/60 scaffolds were produced by electrospinning and showed smooth fibers with an average diameter of 771 ± 273, 606 ± 242 and 697 ± 232 nm, respectively. When the degradation ratio was evaluated, the three scaffold groups had a similar molecular weight (M_w) on the final day of analysis. PTMCLLA 30/70 and 40/60 scaffolds exhibited greater flexibility than the PTMCLLA 20/80. However, the PTMCLLA 40/60 scaffolds showed a large wrinkling and their biological properties were not evaluated. The PTMCLLA 30/70 scaffolds supported the adhesion and growth of mesenchymal stem cells (MSCs), endothelial progenitor cells and smooth muscle cells (SMCs). In addition, they provided a spreading of the MSCs and SMCs. Given the results, the electrospun scaffolds produced with PTMCLLA 30/70 copolymer can be considered promising candidates for future applications in vascular tissue engineering.

Key-words: poly(trimethylene carbonate-*co*-lactide), vascular scaffolds, mesenchymal stem cells, endothelial progenitor cells, smooth muscle cells

1. Introduction

Cardiovascular disease (CVD) affects a large number of people worldwide, causing about 30 % of all global deaths. The treatment of CVD involving vessel lesions can include surgical intervention, where a vascular graft is implanted (1). Synthetic vascular grafts have been widely used in by-pass surgeries of large and medium caliber vessels. However, when vessels with an inner diameter less than 6 mm are affected, these grafts present a high failure rate (2). Thrombosis, intimal hyperplasia and/or infection are the most common causes of failure of these vascular grafts (3). Therefore, vascular tissue engineering (VTE) can be considered an area of growing importance given the great need for vascular grafts, mainly for small vessels (1). In VTE, scaffolds populated with vascular cells are used as temporary grafts and favour the regeneration of the damaged tissue.

Scaffolds for use as vascular grafts should exhibit adequate mechanical properties and good interaction with the host tissue, in addition to supporting the development of endothelial and smooth muscle cells (4). Electrospun scaffolds produced from aliphatic polyester have been widely used in tissue engineering. However, polyesters such as poly(L- ou DL-lactide) (PLA) and polyglycolide (PGA) are stiff materials and do not show an elasticity compatible for vascular applications (5). Moreover, the degradation of these polymers by bulk erosion causes a large loss in scaffold strength (6).

Poly(trimethylene carbonate) (PTMC) is a biodegradable elastomer that can be applied for the production of scaffolds in soft tissue engineering. This polymer shows high extensibility and shape recovery capability, besides degrading in nonacid and nontoxic residues (5, 6). Various studies have demonstrated that the association between polyesters and PTMC allows for the creation of materials with mechanical characteristics and a degradation profile appropiate for vascular reconstruction (7, 8). The physicochemical properties of the copolymer poly(trimethylene carbonate-*co*-L- lactide) (PTMCLLA) has be ajusted by varying the molar ratio of lactide (LA) and trimethylene carbonate (TMC) monomers (9).

In the present work, PTMCLLA scaffolds were produced by the electrospinning technique. A number of molar ratios of LA and TMC were evaluated in terms of their physicochemical and biological properties in order to obtain a scaffold for application as a vascular graft. The mechanical properties and degradation rate of the PTMCLLA

scaffolds were studied. Their biological properties were analyzed for their ability to promote adaption and growth of endothelial progenitor cells, smooth muscle cells and mesenchymal stem cells on their surface.

2. Materials and methods

2.1. Synthesis and chacterization of TMC-LA polymers

L-lactide and 1,3-trimethylene carbonate in different TMC/LLA feed ratio (20/80, 30/70 or 40/60 mol %) were introduced in a schlenk flask previously dried in an oven at 100 ^{o}C for 24 hours. The flask was charged with the desired amount of the initiator tin octoate to obtain a monomer/initiator molar ratio = 2,500 and then immersed in an oil bath at a selected temperature (180°C) under magnetic stirring. The copolymerization was carried out for 2 hours. At the end of the reaction time, the product was fast cooled to interrupt the reaction. The solid was then dissolved in chloroform and precipitated in cold ethanol, filtered and dried in an oven at 50^{o}C for 24 hours.

The number-average (M_n) and weight-average (M_w) molecular weight of the copolymers were determined by means of Gel Permeation Chromatography (GPC) in a Shimadzu LC 20 equipped with a set of two Phenogel columns and a RID-20A differential index detector. Monodisperse polystyrene standards were used for calibration and chloroform ($CHCl_3$) as the solvent. Analyses were carried out at 30 ^{o}C with a flow rate of 1.0 mL/min. The molecular weights were determined by Shimadzu software.

2.2 Scaffolds production

Polymeric scaffolds were prepared by the electrospinning technique. Initially, different combinations of solvents, concentrations of polymer solutions and electrospinning parameters were evaluated. The parameters were tested until homogeneous and smooth fibers were obtained. The parameters chosen to produce the scaffolds are presented in table 1. The fibers were collected on 15 mm diameter cover slips and sterilized by UV radiation for 2 hours.

Table 1. Parameters tested for scaffold production

Copolymer	*Polymeric solution parameters*		*Electrospinning parameters*		
	Concentration	Solvent	Flow rate (mL/h)	Voltage (kV)	Distance between needle and plate collector (cm)
PTMCLLA 20/80	16%	DCM:DMF (7:3)	1.74	19	15
PTMCLLA 30/70	14%	DCM:DMF (7:3)	1.74	21	15
PTMCLLA 40/60	14%	HFIP	1.14	14	18

*Dichloromethane (DCM), Dimethylformamide (DMF) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP).

2.3. Scaffold characterization

Morphology and fiber diameter

The morphology of the electrospun fibers was analyzed by scanning electron microscopy (Zeiss Evo 50) with an accelerating voltage of 10 kV after sputter coating with platinum. The average diameter was determined in SEM images by the measurement of 30 fibers from each sample (n=3) using ImageJ 1.46r software.

In vitro degradation

The PTMCLLA scaffolds (12-20 mg) were immersed in 10 mL phosphate buffer saline (PBS) in individual falcon tubes and maintained at 37º in an orbital shaker (100 rpm). The PBS solution was changed each 4 days. The scaffolds were removed from the PBS at regular time intervals and dried at 30º for 24 hours. The changes in the polymeric molecular weight of the samples were estimated by a size exclusion chromatography module (Viscotek VE 2001) equipped with a refraction index detector. The samples were dissolved in tetrahydrofuran, filtered and eluted at a flow rate of 1 mL/min at 45 ºC. A calibration curve made with polystyrene standard was used for molecular weight determination.

Mechanical testing

Tensile stress-strain measurements were evaluated in a DMA Q800 (*TA* Instruments) equipped with a tension film clamp and using controlled force mode. Young´s modulus, stress at break and elongation at break were measured. The analyses were performed using rectangular strips (5 x 20 x 0.06 mm) cut from the scaffolds. The assays were carried out at a constant temperature (37°C) with ramp force of 0.5 N/min until 18 N maximum load, under 0.005 N static load (n=4).

2.4. Mesenchymal stem cell isolation and culture

Mesenchymal stem cells (MSCs) were isolated from deciduous teeth pulp and characterized in accordance with Werle and collaborators, after approval by the Ethics Committee of the Universidade Federal do Rio Grande do Sul (10). MSCs were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) containning 2.5 g/L Hepes (Sigma-Aldrich), supplemmented with 10% fetal bovine serum (FBS) (ThermoFisher Scientific) and 1% penicillin and streptomycin (ThermoFisher Scientific), at 37ºC, in 5% CO_2. The culture medium was changed each 3 – 4 days. When a confluence of 90% was reached, the cells were detached with 0.5% trypsin-EDTA solution (Sigma-Aldrich).

2.5 Endothelial progenitor cells isolation and culture

Endothelial progenitor cells (EPCs) were isolated from umbilical blood cord after approval by the Ethics Committee of the Universidade Federal do Rio Grande do Sul and Ethics Committe of Moinhos de Vento Hospital. The EPCs were isolated and characterized, as previously described (11). The cells were cultivated in EGM-2 medium (Lonza) and supplemented with 15% FBS. When the cells reached 90% confluence, a passage using Tryple Express (ThermoFisher Scientific) reagent was carried out.

2.6. Smooth muscle cell culture

Human primary aortic smooth muscle cells (SMCs) were ceded by the Heart Institute (InCor - University of Sao Paulo Medical School - HCFMUSP). The cells were cultivated in DMEM supplemented with 20% FBS and 1% penicillin and streptomycin at 37ºC, in 5% CO_2. The culture medium was changed each 3 – 4 days. When 90% confluence was reached, the cells were detached with 0.5% trypsin-EDTA solution.

2.7. Biological properties of scaffolds

The interaction between the PTMCLLA scaffolds and MSCs, EPCs and SMCs was evaluated in terms of cellular adhesion, morphology and viability during the 14 days of cultivation. For all analyses, the scaffolds were fixed in 24-well plates using a silicone o-

ring and sterilized by UV irradiation for 2 hours. Following this, trypsinised MSCs, EPCs or SMCs were seeded onto the scaffolds at a density of 5 x 10^4 cells/sample and incubated at 37ºC, in 5% CO_2 humidified atmosphere. Control cells were seeded and cultivated in wells without scaffolds.

Cell adhesion

Cell adhesion was evaluated by a colorimetric method using Cell Coutting Kit-8 (CCK-8, Sigma-Aldrich) assay. After 3 hours of cell seeding, the medium was removed and the samples were submitted to CCK-8 assay, in accordance with the manufacture´s instructions. The absorbance was read at 450 nm on a spectrophotometer (Multiscan FC, Thermo Scientific) and compared with a standard absorbance curve for each type of cell in a known concentration.

Cell viability

Cell viability was also evaluated by CCK-8 assay. After 1, 7 and 14 days cultivation, the medium was removed and the cell viability was evaluated with CCK-8 kit, as described above. The cell growth rate (GR) was estimated by the following equation:

$$GR = \frac{OP\ 14}{OP\ 1}$$

where *OP14* and *OP1* are the optical density obtained by CCK-8 assay after 14 and 1 day of cell cultivation.

Cell morphology

After 24 hours cultivation, the samples were fixed with 4% paraformaldehyde for 30 minutes, washed with PBS and incubated with 0.1% triton X-100, for 30 minutes, at room temperature. The cells were stained with 50 µg/mL rhodamine phalloidin (40 minutes) (Sigma-Aldrich) for actin and 0.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI) (1 minute) for nuclei. The images were analyzed by confocal microscopy (Leica TCS SP5; ×63 lens).

2.8. Statistical analysis

The results were analyzed and are presented as means ± standard deviations. The symmetry study of the distributions was performed using the Shapiro–Wilk test. The diameter of the fibers and the mechanical properties of the scaffolds were compared using Kruskal-Wallis followed by Dunn´s post test. The cellular adhesion and viability results were compared using ANOVA followed by Tukey post test. Differences were considered significant when $P<0.05$.

3. Results

3.1. Synthesis of copolymers

The copolymers of TMC and LA were obtained in three different ratios: PTMCLLA 20/80, PTMCLLA 30/70 and PTMCLLA 40/60. The molecular weight of each copolymer is presented in table 2.

Table 2. Molecular weight of PTMCLLA copolymers

Ratio of TMC and LA in copolymers	Numeric molecular weight (M_n)	Average molecular weight (M_w)	M_w/M_n
20:80	69,700	132,000	1.89
30:70	56,500	92,000	1.63
40:60	22,800	36,800	1.61

Physicochemical properties of scaffolds

The scaffolds were successfully produced by the electrospinning technique. Various solvents and concentrations of copolymers were tested to produce the fiber scaffolds and the electrospinnig parametes were varied. Using the parameters presented in table 1, uniform fibers were obtained for the three types of copolymers. All the groups exhibited interconnected porous and smooth fibers, which distributed randomly in the scaffoldss structure (Figure 1a, b and c). The PTMCLLA 20/80 scaffolds showed fibers with an average diameter of 771 ± 273 nm and 55% of the fibers had diameters between 500 and 799 nm (Figure 1d). Meanwhile, the PTMCLLA 30/70 exhibited fibers with an average diameter of 606 ± 242 nm, distributed mainly in the range of 300 to 799 nm (61%). The PTMCLLA 40/60 had fibers with an average diameter of 697 ± 232 nm, with 76% of the

fibers showing diameter between 400 and 899 nm. The PTMCLLA 30/70 fibers were significantly smaller than the other groups ($p<0.05$).

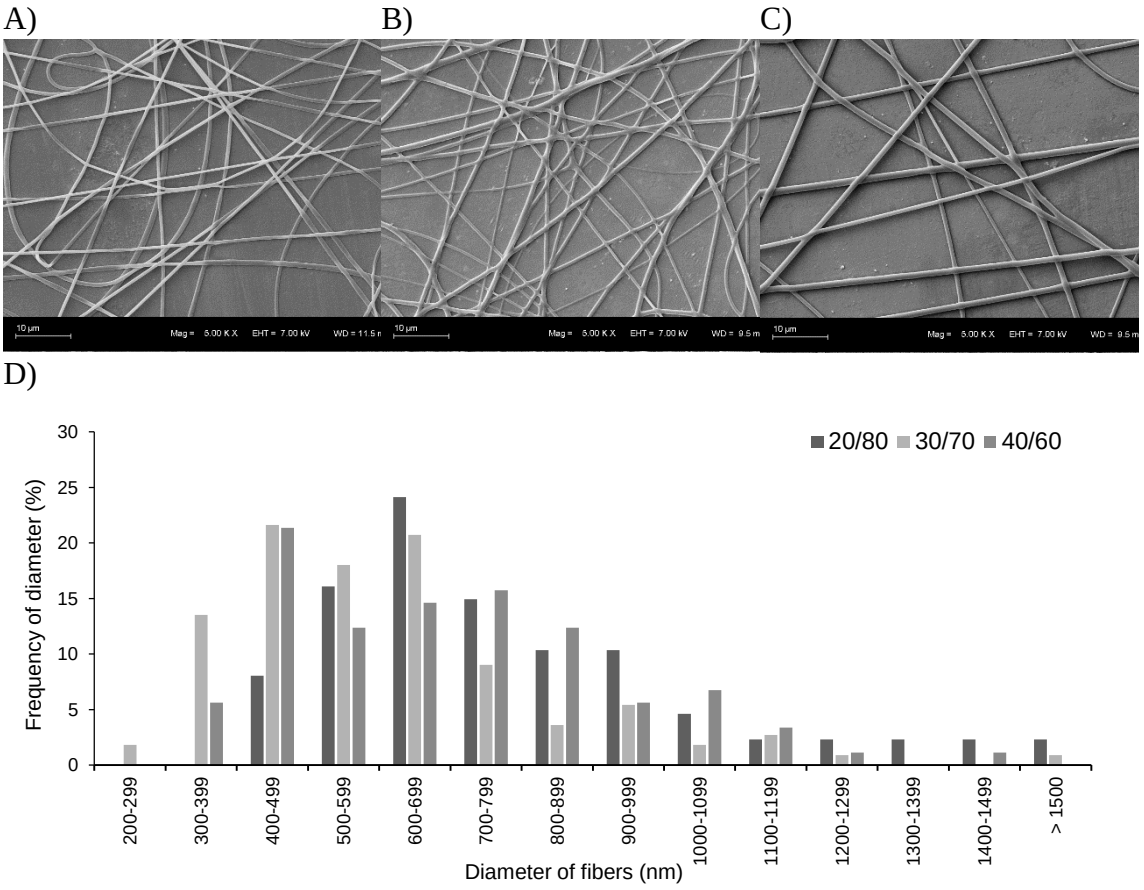

Figure 1. SEM images of PTMCLLA 20/80 (A), 30/70 (B) and 40/60 (C) (magnification x 5000, 10 µm scale bars). Distribution of diameter of fibers in the PLLATMC 20/80, 30/70 and 40/60 scaffolds (D).

The degradation profile of the three scaffold groups was evaluated for 60 days, through the evaluation of the polymer molecular weight. On the 60th day of analysis, the molecular average weight (M_w) of the three copolymer scaffolds was similar. However, the degradation profile was different between some groups (Figure 2). The copolymer PTMCLLA 20/80 and 30/70 scaffolds presented a similar reduction of M_w over the period analyzed. The PTMCLLA 20/80 and the PTMCLLA 30/70 scaffolds showed a 48% and 43% decrease in M_w, respectively. Meanwhile, the scaffolds produced with PTMCLLA 40/60 presented a greater reduction of M_w in the first 7 days (about 42%) of the assay. After this time, this group showed a gradual reduction of molecular average weight, reducing by about 70% their initial *Mw*.

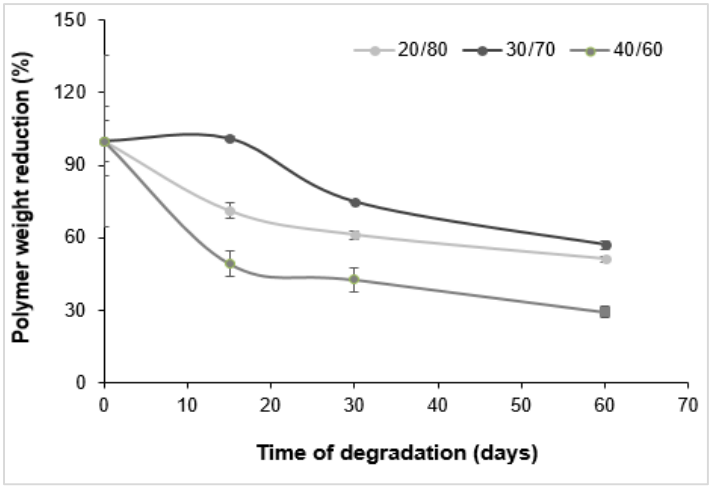

Figure 2. Average molecular weight (*Mw*) of electrospun PTMCLLA 20/80, 30/70 and 40/60 scaffolds, during the 60 days of degradation assay.

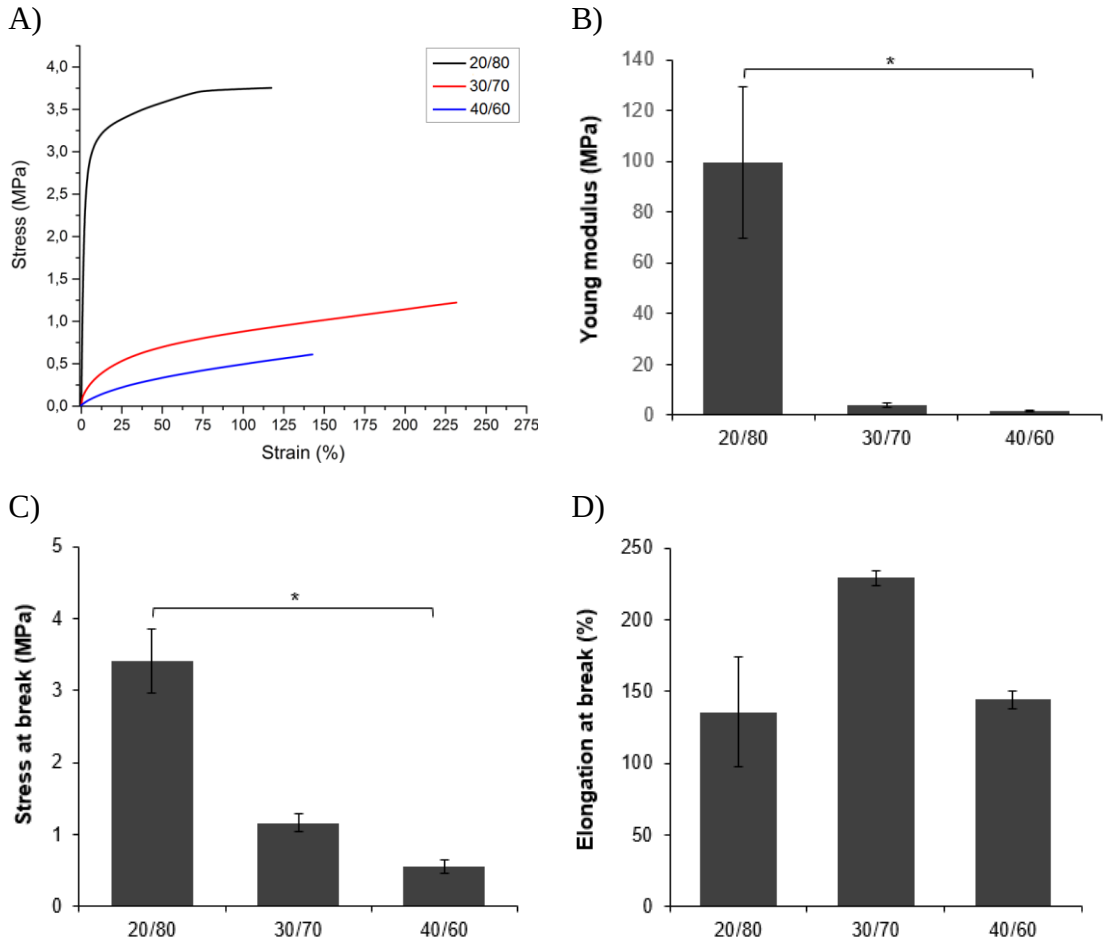

Figure 3. Mechanical properties of PTMCLLA 20/80, 30/70 and 40/60 scaffolds. Stress-strain representative curve (a), Young modulus (b), stress at break (c) and elongation at break (d). *Shows statistical difference between groups, in accordance with Kruskal-Wallis followed by Dunn´s post test.

Figure 3 presents stress-strain curves and derived tensile properties of the PTMCLLA electrospun scaffolds. The Young modulus and the stress at break varied in accordance with the ratio of the TMC and LA in copolymers. The Young modulus of the scaffolds reduced with the increase of TMC proportion in the copolymer: 99.5±30, 4.2±0.8 and 1.6±0.3 MPa for PTMCLLA 20/80, 30/70 and 40/60, respectively. This mechanical parameter was significantly different between the scaffolds produced with PTMCLLA 20/80 and PTMCLLA 40/60. Similar behavior was also observed for the stress at break. Meanwhile, the elongation at break did not appear to depend on the composition of the copolymers. There was no significant difference between the three scaffolds groups and they all showed an elongation at break higher than 130%.

Biological properties

The mesenchymal stem cells, endothelial progenitor cells and smooth muscle cells were seeded onto the three scaffold groups and culture plate. The biological compatibility of the scaffolds was evaluated in terms of cell adhesion, morphology and viability.

When the seeded PTMCLLA 40/60 scaffolds were incubated at 37^{o}, they showed a large wrinkling already within the first three hours of cultivation. The area of this scaffold was greatly reduced, making it difficult to carry out the biological assays in this group. Thus, the PTMCLLA 40/60 scaffold was removed from the assays.

The adhesion of cells to the scaffold surface was estimated by CCK-8 test, using a calibration curve. In all the groups, a great number of adhered cells was observed in the control group (culture plate) (Figure 4 a, b and c). However, the different types of cells: MSCs, EPCs and SMCs also successfully adhered to the scaffold surface. All the types of cells showed a similar initial adhesion on the PTMCLLA 20/80 and PTMCLLA 30/70 scaffolds ($P>0.05$).

Cell viability was estimated during a period of 14 days by evaluating the mitochondrial activity by CCK-8. The increase rate of viable cells over 14 days was highest in the control group, for all the cellular types (Figure 4 b, d and f) ($p<0.05$). Meanwhile, the different cell types presented different behavior in relation to the two scaffold groups. A greater number of viable mesenchymal stem cells was observed in the PTMCLLA 20/80 than the PTMCLLA 30/70 scaffolds. Although the number of viable cells was higher in the PLLATMC 20/80 scaffold, the growth rate of MSCs between the

first and last day of cultivation was similar between the two groups: 1.6 for PTMCLLA 20/80 and 1.4 for PTMCLLA 30/70. The control had a growth rate of 2.3.

The number of viable EPCs in both types of scaffolds was similar during the 14 days of cultivation ($p>0.05$). The growth rate of the EPCs between days 1 and 14 was 2.3 for PTMCLLA 20/80 and 1.6 for PTMCLLA 30/70. Meanwhile, the control group showed a growth rate of 2.5 for the EPCs.

A similar number of SMCs was observed for the PTMCLLA 20/80 compared to the PTMCLLA 30/70 scaffolds. This cellular type did not show an expressive increase in the number of cells on the scaffolds and on the culture plate. The growth rate, between days 1 and 14 for both scaffold groups was about 1.0 and 1.3 for the control group.

The morphology of adhered cells to the scaffold surface was analysed by confocal microscopy after 24 hours of cultivation (Figure 5). The MSCs exhibited a very rounded morphology on the PTMCLLA 20/80 scaffolds. These cells had a slightly more developed cytoskeleton when cultivated on the PTMCLLA 30/70 scaffolds. The EPCs presented a similar morphology on the PTMCLLA 20/80 and 30/70 scaffolds. They showed a rounded cytoskeleton in both groups. Meanwhile, the SMCs exhibited a higher spreading rate on the scaffolds than the other cell types, mainly on the PTMCLLA 30/70 surface. The morphology of the SMCs indicates a better adaptation of these cells to biomaterials.

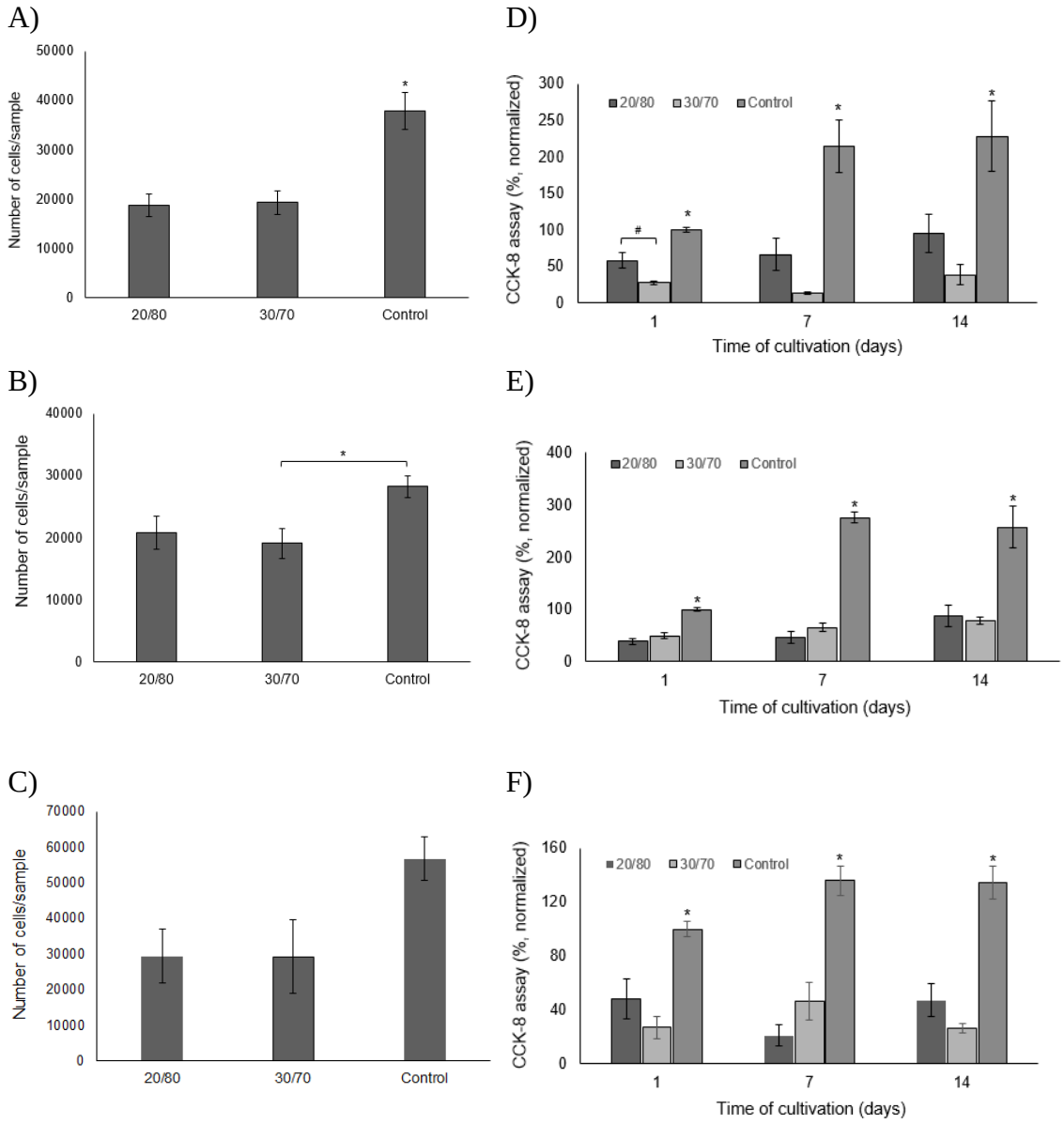

Figure 4. Adhesion of mesenchymal stem cells (A), endothelial progenitor cells (B) and smooth muscle cells (C) in the electrospun PTMCLLA 20/80 and 30/70 scaffolds and in the culture plate (control group) after 3 hours of incubation. Viability of MSCs (D), EPCs (E) and SMCs (F) during 14 days of cultivation in the electrospun PTMCLLA 20/80 and 30/70 scaffolds and in the culture plate. *Shows statistical difference between the control and other groups and # shows statistical difference between the PTMCLLA 20/80 and 30/70 groups in accordance with ANOVA followed by Tukey post test.

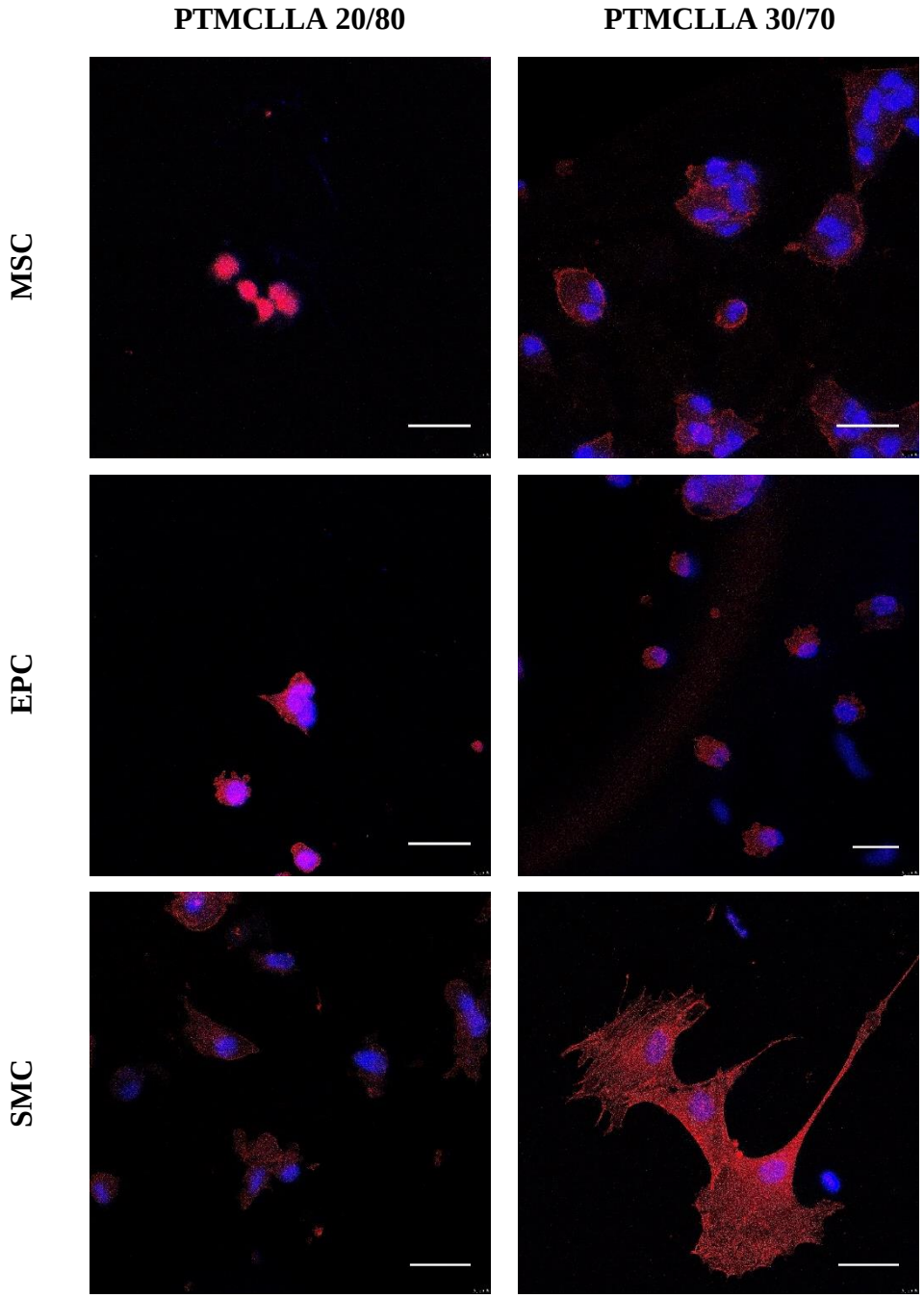

Figure 5. Confocal images of MSCs, EPCs and SMCs cultivated on the PTMCLLA 20/80 and 30/70 scaffolds for 24 hours. The cells were stained with DAPI for nuclei (blue) and rhodamine-phalloidin for cytoskeleton (red). Scale bar represents 30 µm (original magnification x63).

Discussion

The great clinical need for vascular grafts and the lack of adequate substitutes for vessels of small diameters has driven researchs in the area of vascular tissue engineering. The aim of VTE is to create biocompatible vascular scaffolds that favor cell development and,

when implanted, transform into autologous tissue, improving its functions (4). However, the development of a suitable vascular scaffold is a very challenging task. A vascular substitute should exhibit suitable physical-chemical and biological characteristics in order to successfully fulfill all the vessel functions after implantation.

A functional vascular scaffold must be biocompatible and be able to support cellularization, both integrating and being indistinguible from the host tissue. In addition to the biological properties, the scaffolds must be sufficiently elastic to withstand hemodynamic stress, without permanent changes in their structure (3). Synthetic polymers, mainly polyesters, have been employed in different areas of tissue engineering (12, 13). However for vascular tissue, polyesters such as PGA and PLA are rather stiff and fragile, requiring combination with more elastic materials (14). Trimethylene carbonate is a part of the shape memory polymers class. This elastomer polymer has the intrinsic ability to recover its original shape, after physical modifications (9). In addition, TMC copolymers show good haemocompatibility. Yang and coworkers showed that copolymers composed of TMC and polyesters such as L-lactide (LLA), DL-lactide (DLLA) or caprolactone (CL) present very low haemolytic ratios, indicating their good compatibility with human blood (15).

Based on this, the use of copolymers containing TMC and LLA to produce electrospun scaffolds that could be applied as vascular substitutes was evaluated in this study. The physical-chemical and biological properties of the scaffolds were investigated.

Three copolymers with different ratios of TMC and LLA were produced. The electrospinning process was successfully carried out with each copolymer, also forming three scaffold groups . All the scaffolds exhibited interconnected pores in their structure. This feature ensures cellular migration, as well as the passage of nutrients and O_2 through the scaffold structure, enabling tissue development (3). The three scaffold groups showed fibers with a smooth surface. However, the PTMCLLA 30/70 scaffolds showed smaller diameter fibers than the other groups. In their work, Bao and collaborators found that the lower molar ratio of TMC in the PTMCLLA copolymer resulted in a smaller diameter of the electrospun fibers (9). In the present study, the variation in fiber diameter was not dependent on the TMC molar rate in the copolymers. Unlike the paper of Bao and collaborators, in this study, each copolymer was processed under different parameters for the production of the scaffolds. The concentration of the polymer in solution, the molecular weight of the polymer and conductivity of the solution are some of the

parameters that affect the diameter of electrospun fibers (16). The PTMCLLA 30/70 solution presented a lower concentration than the solution of PTMCLLA 20/80. Meanwhile, the different solvent systems used for solubilizing the PTMCLLLA 30/70 and PTMCLLA 40/60 copolymers could have altered the conductivity of their solutions and, consequently, their electrospinnability. These factors have possibly contributed to the differences in the fiber diameters between the PLLATMC 30/70 and the other scaffold groups. Despite the difference in fiber diameter, the morphology of the three groups of scaffolds was similar.

Different studies have shown that the TMC homopolymer exhibits a slowly rate of *in vitro* degradation (17, 18). In addition, the materials made with TMC show degradation by erosion, losing the mass and dimensions proportionally to the surface area. Therefore, these materials are able to preserve their structural integrity and mechanical properties for a long time, supporting complete tissue formation (6). Yang and coworkers showed that when the TMC is associated with polyesters, this characteristic is maintained. In their study, the mass of scaffolds made with poly(trimethylene carbonate-*co*-D,L-lactide) did not show great variation during the 20 weeks under *in vitro* degradation assay. However, when GPC analysis was perfomed, changes in polymer molecular weight were observed. In the present study, the polymer average molecular weight also changed during the assay for all the scaffold groups, corroborating to the data of Yang and collaborators. The scaffolds of PTMCLLA 20/80 and 30/70 showed a similar degradation profile and the reduction of polymer M_w was graded over the course of the assay. Meanwhile, the PTMCLLA 40/60 scaffolds exhbited a great rate of M_w reduction during the first 7 days, followed by a proportional degradation until 60 days. It is already well established that the ester bonds of lactyl units in PTMCLLA copolymers are more susceptible to hydrolytic cleavage than the carbonate bonds (5, 18, 19). Thus, it was expected that the PTMCLLA 40/60 scaffolds presented a higher resistance to degradation than the other groups, since they have a higher proportion of TMC in their composition. The observed behavior may have occurred because of the low molecular weight of this polymer and because of the presence of smaller polymeric chains.

The total reduction of the M_w for the scaffolds produced in this study can be considered elevated (20). The *in vitro* pre-cellularized vascular scaffolds are expected to show small rates of degradation. These scaffolds need to support the tissue organization, mantaining their physico-chemical integrity until their *in vivo* application (19, 20). In

order to reduce the rate of M_w loss in the produced scaffolds, the PTMCLLA can be cross-linked. This process makes the polymer chains more resistant to degradation, besides optimizing the mechanical properties of the scaffolds (5).

The study of mechanical properties of scaffolds is essential to ensure that they can be used as support for the regeneration of specific tissue. For vascular applications, the scaffolds should exhibit elasticity able to withstand hemodynamic stress. The DMA analysis showed that the PTMCLLA 20/80 scaffolds had a stress-strain behavior typical of polyesters (21). The high modulus and stress at break exhibited by this group reflected their stiffness. As expected, the increase in the TMC content in the copolymers made the scaffolds more flexible. The increase of the TMC ratio reduced the Young modulus and the stress at break of the scaffolds. These results are consistent with other studies and demonstrate that the scaffolds with higher TMC content show a behavior more similar to rubbery materials (9, 22). Despite the reduction of strength, the PTMCLLA 30/70 and 40/60 scaffolds presented stress at break closer to the value found in the literature for native vessels (1.55 ± 0.4 MPa) (8).

The wall of blood vessels is organized in three concentric layers, each being formed by a different cellular type. The innermost, in contact with the blood, is the endothelial layer and is formed by endothelial cells. The middle layer is formed by smooth muscle cells and the outermost layer, called the adventitia layer, is formed by fibroblasts (23). To mimick the strutucture and functions of native vessels, the scaffolds must be compatible with the development of these three types of cells. In this study, the biocompatibility of PTMCLLA scaffolds was demonstrated throught the interaction of endothelial progenitor cells, smooth mucle cells and mesenchymal stem cells with their structure.

Endothelial progenitor cells are derived from bone marrow and play a crucial role in repairing damaged blood vessels. EPCs exhibit a high proliferation rate and may contribute to the formation of the endothelial layer in vascular scaffolds through adhesion and differentiation in mature endothelial cells. EPCs also contribute to endothelialization by the secretion of biomolecules that cause the migration of host endothelial cells towards the implanted scaffold (24). In addition, these cells can be isolated from peripheral blood, a source less invasive than the endothelial cells that are obtained from vascular wall (2). Therefore, the EPCs represent a very interesting cell type for vascular tissue engineering.

Mesenchymal stem cells are multipotent stem cells that can be isolated from different sources and show ease of *in vitro* expansion (1). These cells were used in this

study because of their great plasticity. MSCs are able to differentiate into vascular cells, including endothelial cells and smooth vascular cells (25-27). MSCs have an intrinsic ability to differentiate into mesodermal cell lineages and may also give rise to fibroblasts (28). Another important feature of MSCs is their low immunogenicity and their capacity to secrete soluble factors that facilite tissue regeneration (29, 30).

The biological characterization was conducted with all the scaffold groups. However, during the cultivation period, the PTMCLLA 40/60 presented a large shrinkage in size and was excluded from these assays. Ji and coworkers also observed this behavior for PTMCLLA electrospun scaffolds. The authors were only able to evaluate the biocompatibility of scaffolds produced with PTMCLLA 15/85 (31).

Cellular adhesion is the first step of cell-scaffold interaction. This step is crucial for the success of biological scaffolds (32). The scaffolds should favor the adhesion of the cells so that they can proliferate and secrete extracelular matrix, organizing the tissue. In a similar way, the scaffolds made with PTMCLLA 20/80 and 30/70 copolymers support the adhesion of MSC, EPC and SMC. The number of adhered cells in the scaffolds was lower than in the culture dish for the three cellular lines. These results were expected as the commercial culture plates represent a gold standard suface for cell culture. Stefani and collaborators also observed this finding for MSCs and PCL/PTMCLLA electrospun scaffolds. The authors reported that in relation to the culture plates, only ¾ of the MSCs seeded on the PCL/PTMCLLA scaffolds remained adherent to their surface (8).

Once adhered to the scaffolds, the MSCs and EPCs showed an increase in absorbance of the CCK-8 assay, suggesting that proliferation of these cells occurred. The PTMCLLA 20/80 exhibited a great number of viable MSCs on the first day. This group also showed a larger number of viable MSCs than the PTMCLLA 30/70 on days 7 and 14. However, the diferences were not significants. When the EPCs were evaluated, no statistical diferences were observed between the scaffolds groups. These results suggest that the variation of TMC from 20 to 30% in the PTMCLLA composition did not have an impact on the proliferation of cells and that both electrospun scaffolds types can support the growth of MSCs and EPCs. These results are corroborated by Dargaville and co-workers. These authors evaluated the development of MSCs on electrospun scaffolds produced with copolymers containing from 30 to 70% TMC and no diferences were found among the groups (5). Meanwhile, the SMCs did not exhibite a significative proliferation

on the two types of scaffolds. The SMCs are already differentiated cells, which have a lower proliferation rate than immature cells like MSCs and EPCs. This characteristic is likely to have contributed to the observed result.

The good adaptation of the cell to scaffolds is crucial for tissue organization. In order to better analyze the interaction of the cells to the scaffolds, their morphology was analyzed by confocal microscopy after 24 hours of cultivation. Cytoskeleton staining showed that the EPCs had a similar behavior in both the scaffold groups. However, the MSCs, and mainly the SMCs, had a more spreading morphology when cultivated on the PTMCLLA 30/70 scaffolds. Although the PTMCLLA 20/80 and 30/70 groups were similar in terms of cell proliferation, the difference in TMC content affected the adaptation of the MSCs and SMCs on the scaffolds. Ji and collaborators also found differences in the adaptation of fibroblasts cultivated on the PLLA and PTMCLLA 15/85 electrospun scaffolds. After 24 hours, the fibroblasts cultivated on the scaffolds containing TMC exhibited a larger size than the fibroblasts cultivated on the PLLA scaffolds (31). This difference found in the present study is probably associated to scaffold flexibility. The more flexible substrate seems to be more favorable for adaptation of MSCs and SMCs than the stiff surfaces.

The PTMCLLA 30/70 copolymer allows for the fabrication of flexible and resitant scaffolds which favour the adaptation of vascular and stem cells. Altough *in vivo* analysis is required, this study has demonstrated that the electrospun PTMCLLA 30/70 scaffolds are potential biomaterials for use as vessel substitutes in surgical treatment of cardiovascular disease.

Conclusions

In this study, a series of PTMCLLA copolymers was evaluated to produce scaffolds for application as vascular grafts. Electrospun scaffolds were successfully produced from the three PTMCLLA ratios: 20/80, 30/70 and 40/60. The PTMCLLA 30/70 and 40/60 scaffolds showed higher flexibility, showing more appropriateness for application as vascular scaffolds. However, the 40/60 scaffolds presented a high shrinkage rate, which made their biological analysis difficult. The PTMCLLA 20/80 and 30/70 scaffolds supported the adhesion and growth of mesenchymal stem cells, endothelial progenitor cells and smooth muscle cells. However, the MSCs and SMCs showed a greater spread

morphology when cultivated on the PTMCLLA 30/70 scaffolds. Given the physical-chemical and biological results, the electrospun scaffolds produced with the PTMCLLA 30/70 ratio are promising candidates for future application in vascular tissue engineering.

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5. Discussão

O presente trabalho teve por objetivo desenvolver biomateriais que pudessem ser utilizados como substitutos vasculares no tratamento cirúrgico das doenças arteriais periféricas. As DAPs constituem uma das desordens cardiovasculares mais prevalentes na população de países desenvolvidos e em desenvolvimento, afetando, principalmente, indivíduos com idade acima de 60 anos (21). Esse quadro clínico é caracterizado pela obstrução de vasos sanguíneos periféricos e, consequente oxigenação inadequada dos tecidos irrigados por ele (16). Em casos graves, onde ocorre a lesão/necrose do vaso, a intervenção cirúrgica pode ser necessária (25). Nesse caso, o vaso obstruído é substituído por um enxerto vascular (26, 28). Atualmente, a clínica médica apresenta uma grande necessidade por enxertos vasculares adequados, principalmente para a aplicação em vasos de pequeno diâmetro (29, 30). Devido às diferentes características apresentadas por esses vasos, quando os enxertos vasculares, principalmente sintéticos, são aplicados, apresentam um curto período de patência, precisando ser trocados em até 5 anos (74). Nesse contexto, novas estratégias têm sido utilizadas para o desenvolvimento de enxertos vasculares.

A engenharia de tecidos tem se mostrado uma abordagem bastante interessante para o desenvolvimento de novos substitutos vasculares (35). Nessa ciência, biomateriais, células e moléculas bioativas são combinadas para a construção de estruturas que auxiliam a regeneração do tecido danificado (32, 36). Para a recuperação dos vasos lesados, biomateriais com características físico-químicas semelhantes às dos vasos naturais devem ser utilizados. Esses biomateriais são, então, associados a células vasculares a fim de se estabelecer uma organização celular e tecidual semelhante àquela existente *in situ* (37, 38, 73). Vários trabalhos disponíveis na literatura têm relacionado o sucesso da engenharia de tecidos vasculares à endotelização da superfície dos biomateriais (62, 74). O desenvolvimento de uma camada endotelial contínua e funcional no lúmen dos enxertos vasculares é crucial para a homeostase sanguínea. O endotélio vascular controla a interação do fluxo de sangue com a parede dos vasos, regulando os processos inflamatórios, a permeabilidade vascular, a cascata de coagulação e, até mesmo, a proliferação de células musculares lisas (4, 74). Assim, o endotélio contribui para a prevenção da formação de trombos e da hiperplasia intimal (74). Portanto, o

estabelecimento de uma camada endotelial confluente na superfície de biomateriais representa o primeiro desafio da engenharia de tecidos vasculares.

Neste trabalho, dois tipos de biomateriais foram desenvolvidos para uso como substitutos vasculares, baseados nos princípios da engenharia de tecidos. No capítulo I foi mostrado que matrizes de policaprolactona, funcionalizadas com heparina e VEGF, apresentam propriedades antitrombogênicas e propiciam o desenvolvimento de células-tronco mesenquimais e de células progenitoras endoteliais em sua superfície. Esse trabalho teve como foco principal a caracterização desses biomateriais e a avaliação da sua interação com células que pudessem ser utilizadas para a formação de uma camada endotelial em sua superfície. No estudo, quadro grupos de biomateriais foram desenvolvidos pela técnica de *electrospinning*: biomateriais de PCL, biomateriais de PCL tratados com NaOH (PCL/NaOH), biomateriais de PCL tratados com NaOH e funcionalizados com heparina (PCL/Hep) e biomateriais tratados com NaOH e funcionalizados com heparina e VEGF (PCL/Hep/VEGF). Os dois primeiros grupos foram utilizados como controles da funcionalização.

A funcionalização da superfície dos biomateriais foi realizada com o intuito de aumentar a taxa de sucesso de sua aplicação. Sabe-se que uma das principais causas de falha dos enxertos vasculares é a oclusão de seu lúmen pela formação de trombos (29, 104). A heparina, ligada covalente à PCL, conferiu a propriedade antitrombogênica aos biomateriais (Capítulo I, Tabela I). Além disso, a presença dessa molécula aniônica na estrutura tridimensional favoreceu a adsorção do VEGF (Capítulo I, Figura 6). Esse fator de crescimento, por sua vez, desempenha importante papel sobre a vasculogênese, exercendo quimiotaxia e estimulando a proliferação de células endoteliais (63). O perfil de liberação do VEGF demonstrou que houve um pico de liberação nas primeiras 6 horas de avaliação, seguido por uma liberação sustentada do fator ao longo de 30 dias (Capítulo I, Figura 7). Esse perfil de liberação favorece a regeneração tecidual. A liberação de maiores quantidades de VEGF nas primeiras horas de aplicação *in vivo* ou cultivo *in vitro* favorece a adesão de células à superfície do biomaterial, enquanto que a sua liberação sustentada ao longo do tempo estimula a proliferação celular e auxilia na organização do tecido que está sendo formado (105).

Todos os biomateriais desenvolvidos apresentaram propriedades físico-químicas compatíveis com a aplicação vascular. Os biomateriais apresentaram fibras distribuídas homogeneamente por sua estrutura e que se assemelharam fisicamente às fibras de

colágeno da MEC (Capítulo I, Figura 2). Essa característica intrínseca das matrizes produzidas por *electrospinning*, associada à elevada área superficial dessas estruturas, contribuem para a adesão e crescimento celular (7).

A cinética de degradação dos biomateriais está intimamente relacionada à sua estabilidade *in vivo*, devendo ser avaliada na área da medicina regenerativa (106). O biomaterial deve apresentar uma taxa de degradação proporcional à taxa de regeneração do tecido ao qual é empregado. Assim, o biomaterial poderá suportar o desenvolvimento do tecido até que o mesmo esteja completamente formado, sem atrasar, ou até mesmo prejudicar, a sua regeneração (34). A biodegradação da PCL ocorre através da hidrólise de suas ligações ésteres, originando produtos na forma de oligômeros ou monômeros (50). Como mostrado no capítulo I, os biomateriais de PCL apresentaram uma baixa taxa de degradação *in vitro*, mesmo após a hidrólise com NaOH (Capítulo I, Figura 3). Essa é uma característica bastante importante para a aplicação vascular dos biomateriais. Esse tipo de biomaterial deve apresentar estabilidade por maiores períodos de tempo para que as células tenham tempo hábil para se organizar, secretar quantidades suficientes de matriz extracelular e construir o tecido vascular (2, 14, 15).

Além disso, para que possam ser aplicados como substitutos vasculares, os biomateriais devem apresentar complacência e resistência à fratura (29). As propriedades mecânicas dos biomateriais são determinadas tanto pela sua macro e microestrutura física, como também pelas propriedades do polímero a partir do qual são produzidos (7). A PCL é caracterizada como um polímero de alta elasticidade e resistência à pressão (51, 52). A caracterização mecânica dos biomateriais produzidos demonstraram que essas características foram mantidas (Capítulo I, Figura 4). Os biomateriais de PCL apresentaram uma grande flexibilidade e uma alta capacidade de elongação até a fratura. Além disso, esses biomateriais apresentaram módulo de elasticidade semelhante ao dos vasos naturais. Esses resultados sugerem que os biomateriais desenvolvidos são capazes de resistir à deformação plástica, isto é, à deformação permanente, quando expostos às forças hemodinâmicas.

As células-tronco mesenquimais são células multipotentes, podendo se diferenciar em diferentes tipos celulares, incluindo células endoteliais (5, 76, 81). Devido a essa característica e ao fato de poderem ser obtidas a partir de fontes pouco invasivas (84, 86), essas células são bastante interessantes para o desenvolvimento da camada endotelial em

matrizes vasculares (83). As CTMs foram capazes de se aderir e proliferar sobre os biomateriais desenvolvidos, sendo que os biomateriais de PCL/Hep e PCL/Hep/VEGF apresentaram um maior número dessas células no 15º dia de análise (Capítulo I, Figuras 8a e 9a). Além disso, a presença de heparina e de VEGF no biomaterial ocasionou o aumento do citoplasma celular, indicando uma boa adaptação das CTMs ao substrato (Capítulo I, Figuras 10 e 11).

Além de sua propriedade anticoagulante, a heparina tem a capacidade de se ligar a diferentes fatores não relacionados à cascata de coagulação sanguínea. Alguns estudos têm relatado que a heparina, presente em meios de cultura, potencializa a ação ou promove a sinalização de algumas biomoléculas, como FGF e Wnt, promovendo a proliferação celular *in vitro* (107, 108). Esse efeito foi demonstrado por Ling e colaboradores em CTMs. Os autores mostraram que a suplementação do meio de cultura com baixas doses de heparina (< 200 ng/mL) ocasiona a proliferação de CTMs humanas, provenientes de medula óssea (109). Tais resultados corroboram com os encontrados neste estudo. O VEGF também contribuiu sinergicamente para a proliferação desse tipo celular e ocasionou a sua melhor adaptação no biomaterial PCL/Hep/VEGF (Capítulo I, Figura 12). Atribui-se esse resultado à interação do VEGF com alguns receptores presentes em CTMs, como o receptor para PDGF, que, quando ativado, promove a migração celular (83).

Apesar de terem conseguido se organizar e adaptar sobre os biomateriais, a análise de expressão gênica demonstrou que, nas condições de cultivo utilizadas, as CTMs não se diferenciaram em células endoteliais. Diferentes estudos demonstram que o VEGF é fundamental para o processo de diferenciação de CTMs em células endoteliais (5, 77). Wingate e colaboradores também demonstraram que o uso de substratos macios e elásticos para o cultivo de CTMs agem sinergicamente junto ao VEGF, nesse processo (81). Os meios de cultura utilizados para a diferenciação endotelial são suplementados com VEGF, em concentrações que variam de 10 a 50 ng/mL (5, 81). Nesse trabalho, o VEGF foi fornecido a partir do próprio biomaterial em que as células estavam sendo cultivadas. Assim, acredita-se que apesar da presença de um substrato elástico, a concentração de VEGF liberada no meio de cultura não foi suficiente para desencadear o processo de diferenciação celular. Apesar de não contribuírem diretamente para a endotelização, quando aplicadas *in vivo*, as CTMs podem propiciar a migração de células endoteliais do próprio hospedeiro em direção ao biomaterial, através de sua extensa ação

parácrina (83). Porém, essa ação demandaria um maior período de tempo para que a superfície luminal dos biomateriais fosse endotelizada, o que poderia limitar o seu sucesso de aplicação.

As células progenitoras endoteliais são uma população celular derivada da medula óssea e que apresentam uma ampla capacidade vasculogênica, superior às das células endoteliais maduras (74, 88). As CPEs podem se diferenciar em células endoteliais maduras, contribuindo diretamente com a endotelização de biomateriais, como também, podem causar a quimiotaxia de células endoteliais do hospedeiro em direção ao biomaterial implantado, através da secreção de diferentes fatores pró-angiogênicos e vasculogênicos (6, 94). Além disso, esse tipo celular exibe uma alta taxa de proliferação e de autorrenovação (89). Dessa forma, vários estudos têm investigado as CPEs como fontes celulares para a endotelização de enxertos vasculares (62, 74, 102, 110).

No estudo realizado, as CPEs humanas apresentaram uma boa adesão e um grande espalhamento do citoesqueleto sobre os biomateriais de PCL/Hep e, principalmente, sobre os biomateriais de PCL/Hep/VEGF (Capítulo I, Figuras 8b, 11 e 12). Ainda, os biomateriais funcionalizados com heparina e VEGF propiciaram o aumento significativo do número de EPCs durante o cultivo *in vitro*, superior aos demais grupos de biomateriais (1º e 7º dia de análise) (Capítulo I, Figura 9b). As CPEs apresentam receptores para VEGF (VEGFR-2) em sua membrana plasmática. A ativação desses receptores a partir da ligação com VEGF induz uma cascata de sinalização intracelular que propicia eventos angiogênicos e vasculogênicos (65). Portanto, acredita-se que a ligação das CPEs com o VEGF presente nos biomateriais ocasionou a ativação de mecanismos intracelulares que contribuíram para a melhor adaptação celular sobre essas estruturas. No 15º dia de cultivo, foi observado que o grupo de biomateriais funcionalizado apenas com heparina também propiciou um aumento da proliferação celular (Capítulo I, Figura 9b). Nesse ponto de análise, o número de CPEs viáveis foi semelhante entre os dois grupos de biomateriais funcionalizados: PCL/Hep e PCL/Hep/VEGF. Além de modular fatores que contribuem para a proliferação celular, a heparina é um glicosaminoglicano natural (111); logo, exibe domínios que podem ser reconhecidos pelas células, favorecendo a adaptação das mesmas. Ainda, Teran e Nugent demonstraram que a heparina age como um co-receptor para o VEGF, modulando a sinalização celular induzida por esse fator de crescimento (112). Esse efeito pode, portanto, ter contribuído para a ação do VEGF (presente no meio de cultura utilizado) sobre as células progenitoras endoteliais.

Apesar da presença de heparina e de VEGF ter melhorado as propriedades biológicas dos biomateriais desenvolvidos e estimulado a proliferação das EPCs, as placas de cultivo tratadas com colágeno (grupo controle) propiciaram um maior aumento do número de células do que esses biomateriais (Capítulo I, Figura 9b). Esse resultado demonstra que o contato desse tipo celular com proteínas da MEC aumenta sua proliferação. Embora a proliferação das EPCs tenha sido inferior nos biomateriais, os mesmos ocasionaram, nessas células, um aumento da expressão de VE-Caderina, em relação ao grupo controle (Capítulo I, Figura 14). A VE-caderina é uma proteína específica de células endoteliais e é responsável pelas junções aderentes entre células vizinhas. Essa proteína desempenha funções relacionadas à integridade do endotélio vascular, tendo papel importante na regulação de sua permeabilidade (113). O resultado obtido demonstrou que a estrutura tridimensional dos biomateriais desenvolvidos favoreceu o contato célula-célula em relação ao cultivo bidimensional (placa de cultura). Essa é uma característica bastante importante para a engenharia de tecidos, visto que a presença de VE-caderina deve contribuir para o desenvolvimento da camada endotelial na superfície dos biomateriais. A análise de expressão gênica também demonstrou que a presença de VEGF nos biomateriais manteve a expressão de CD31 e CD34 das CPEs (Capítulo I, Figura 14). Normalmente, quando CPEs são mantidas por maior tempo em cultivo *in vitro* elas se diferenciam em células endoteliais maduras, aumentando a expressão de CD31 e reduzindo a expressão de CD34 (101, 102). O estudo demonstrou que o contato com o VEGF, aderido à superfície dos biomateriais, fez com que a imaturidade das CPEs fosse conservada. Assim, as CPEs podem ser mantidas por maiores períodos de cultivo *in vitro*, se necessário, sem que percam suas características.

Apesar de a grande maioria dos trabalhos científicos da área da medicina regenerativa utilizar o método estático de cultivo, sabe-se que esse sistema apresenta grande limitação em termos de distribuição e crescimento não homogêneo das células na estrutura do biomaterial (97). Os biorreatores são dispositivos utilizados para o cultivo dinâmico *in vitro* de células em biomateriais e têm sido bastante empregados na engenharia de tecidos vasculares (14, 15). Esses dispositivos proporcionam uma distribuição e organização celular homogênea nos biomateriais e, ainda, melhor mimetizam as condições encontradas no microambiente natural dos vasos sanguíneos (114). O fluxo de meio de cultura, provocado pelos biorreatores, cria forças mecânicas, como a tensão de cisalhamento e de estiramento, contra a parede do biomaterial,

simulando alguns dos estímulos fisiológicos presentes no endotélio vascular (115, 116). Diferentes estudos disponíveis na literatura têm demonstrado que esses estímulos mecânicos são cruciais para a funcionalidade de substitutos vasculares (15, 26). A tensão de cisalhamento, por exemplo, aumenta a divisão de células endoteliais e contribui para a formação de uma camada endotelial homogênea na superfície luminal de enxertos vasculares (100, 101). Com base nisso, no capítulo II do presente trabalho, foi buscada uma maior eficiência de endotelização dos biomateriais de PCL/Hep/VEGF, através do uso de biorreatores. Para isso, os biomateriais de PCL funcionalizados com heparina e VEGF foram produzidos utilizando os mesmos parâmetros empregados no capítulo I, no entanto, foram construídos em formato tubular. As células progenitoras endoteliais foram associadas e mantidas nesses biomateriais através de biorreatores de parede rotatória e de perfusão, respectivamente.

Os biorreatores utilizados no trabalho foram desenvolvidos em parceria com a empresa DUO Engenharia. No projeto, foram buscadas características como: (a) fácil manuseio dos biomateriais nos biorreatores; (b) câmaras de cultivo que possibilitassem o cultivo isolado de cada biomaterial, evitando propagação de possíveis contaminações microbianas; (c) esterilização das câmaras de cultivo em autoclave; (d) capacidade do biorreator de parede rotatória gerar uma distribuição celular uniforme na parede luminal dos biomateriais; (e) capacidade do biorreator de perfusão gerar um fluxo laminar de meio de cultura no interior dos biomateriais; (f) possibilidade de cultivar células na parede externa dos biomateriais, em futuros experimentos e (g) manutenção da esterilidade do sistema durante todo o período de experimentação. As câmaras de cultivo desenvolvidas puderam ser utilizadas nos dois tipos biorreatores, evitando a manipulação dos biomateriais durante o cultivo dinâmico (Capítulo II, Figura 1a). Ainda, os biorreatores fabricados puderam ser totalmente colocados no interior de incubadoras, garantindo as condições de temperatura e umidade necessárias para o cultivo celular.

Os biorreatores de parede rotatória consistiram nas câmaras de cultivo dos biomateriais e em um rotor com velocidade de 1 rpm (Capítulo II, Figura 1b). O movimento rotacional gerado por esse sistema faz com que as células sejam jogadas contra a parede interna do biomaterial, propiciando uma distribuição celular uniforme nessa estrutura (15). Um estudo prévio desse grupo de pesquisa demonstrou que o tempo de 3 h de adesão é suficiente para que as células possam se aderir à superfície de biomateriais planos, produzidos pela técnica de *electrospinning* (117). Esse foi o tempo

utilizado para a adesão celular no trabalho exposto no capítulo I. Contudo, sob condições dinâmicas de semeadura, um maior intervalo de tempo pode ser requerido para que se estabeleça a adesão das células ao biomaterial. Na literatura, diferentes períodos de tempo (de 3 até 24 h) são empregados para o processo de adesão celular em biomateriais tubulares, sob o uso de biorreatores (99, 116, 118, 119). Em função disso, a adesão das CPEs foi avaliada após 3 e 24 h de semeadura e cultivo em biorreator de parede rotatória. Surpreendentemente, os biomateriais mantidos por 3 h nos biorreatores de parede rotatória apresentaram um maior número de células do que aqueles mantidos por 24 h nos dispositivos. A viabilidade celular foi semelhante para os dois períodos avaliados (Capítulo II, Figura 3). Acredita-se que o maior tempo de exposição à tensão rotacional dificultou a interação das CPEs ou ocasionou o seu destacamento da parede dos biomateriais. De acordo com esse resultado, assumiu-se que o período de tempo normalmente utilizado para a adesão celular estática pode ser mantido para as condições dinâmicas de semeadura, em biomateriais produzidos por *electrospinning*.

Após 3 h de incubação, os biomateriais já celularizados foram submetidos ao cultivo em biorreatores de perfusão. Para isso, as câmaras de cultivo, que continham os biomateriais, foram facilmente retiradas dos biorreatores de parede rotatória e conectadas a frascos com meio de cultura, através de mangueiras de silicone. Uma bomba de perfusão garantiu a passagem de meio de cultura pelo interior dos biomateriais, a uma velocidade de 1,7 mL/min (Capítulo II, Figura 1c). O cultivo dinâmico em biorreator de perfusão foi comparado ao cultivo estático. Através do cálculo do número de Reynolds, foi possível verificar que as condições utilizadas para o cultivo dinâmico geraram um fluxo laminar de meio de cultura no lúmen dos biomateriais (Número de Reynolds < 2.100). Nos vasos sanguíneos, o fluxo laminar é caracterizado pelo movimento do sangue em linhas de fluxo, sem a mistura das camadas de sangue e, idealmente, forma um perfil de fluxo parabólico. O fluxo laminar favorece a difusão homogênea de nutrientes, de O_2 e de produtos do metabolismo, no interior dos biomaterias (119, 120). Além disso, esse perfil de fluxo gera uma tensão de cisalhamento constante contra a parede dos biomateriais (121).

A tensão de cisalhamento constante é importante para que a formação da camada endotelial luminal não seja prejudicada e para que a parede dos biomateriais não apresente variações de espessura ao longo de seu comprimento, o que poderia acarretar a falha do substituto vascular (122). No presente trabalho, a tensão de cisalhamento foi de 3,8

dyn/cm^2. As células endoteliais encontradas *in situ* são expostas a tensões que variam de 5 a 20 dyn/cm^2 (26). A tensão de cisalhamento poderia ter sido aumentada através do aumento da velocidade de perfusão do meio de cultura. Contudo, quando essa medida foi adotada, houve escape de meio de cultura do lúmen do biomaterial para a câmara de cultivo do biorreator. Esse vazamento foi atribuído à recente celularização dos biomateriais desenvolvidos e a sua extensa rede de poros interconectados. Apesar do pequeno tamanho, esses poros possibilitam a chegada de meio de cultura por toda a espessura do biomaterial. Essa é uma característica bastante importante, pois possibilita as trocas metabólicas no sistema (123), porém, especificamente para enxertos vasculares, a camada endotelial deve cobrir toda superfície interna dos biomaterias a fim de regular a permeabilidade dos mesmos. Devido ao pouco tempo de interação das células com os biomateriais, não houve grande secreção e desenvolvimento de matriz extracelular sobre sua superfície luminal e os poros existentes na estrutura permaneceram totalmente abertos. Assim, quando velocidades de perfusão superiores a 1,7 mL/min foram utilizadas, a tensão do fluido contra a parede do biomaterial foi aumentada de tal forma que o meio de cultura acabou ultrapassando toda a sua espessura e se depositando na câmara de cultivo. Apesar de a tensão de cisalhamento presente no interior dos biomateriais ser inferior à encontrada *in situ*, ela foi suficiente para favorecer o desenvolvimento de células progenitoras endoteliais em sua parede luminal (Capítulo II, Figuras 4 e 6). Ainda, esse valor de tensão está de acordo com outros trabalhos disponíveis na literatura, que empregam tensões de cisalhamento ainda menores do que a utilizada no presente estudo e reportam sucesso no cultivo de células vasculares (15, 100, 115).

O biorreator de perfusão ocasionou o crescimento e a organização de CPEs na parede luminal dos biomateriais de PCL/Hep/VEGF. Apesar de não ter sido observada diferença significativa, os biomateriais cultivados em biorreator apresentaram um maior número de células viáveis do que os biomateriais cultivados estaticamente (Capítulo II, Figura 4). Esses efeitos são atribuídos à tensão de cisalhamento criada com o cultivo dinâmico de perfusão. Essa força mecânica atua sobre mecanorreceptores de células endoteliais maduras e de células progenitoras endoteliais, modulando seu comportamento (26). Yamamoto e colaboradores demonstraram que a tensão de cisalhamento (0,1 – 2,5 dyn/cm^2) aumenta a proliferação de CPEs (100). Os autores mostraram que a exposição a essa força mecânica reduz o número de CPEs nas fases G_0 e G_1 do ciclo celular e aumenta o número de células nas fases G_2-M. Além disso, o mesmo trabalho demonstrou

que a tensão de cisalhamento também aumenta a expressão de receptores para VEGF e da molécula VE-caderina nessas células. Esses efeitos contribuem para organização celular e formação da camada endotelial, o que foi observado na análise de microscopia confocal desse estudo (Capítulo II, Figura 6). Essa análise demonstrou que as CPEs cultivadas dinamicamente apresentavam um maior contato entre elas. Ainda, elas já exibiam uma discreta orientação do seu citoesqueleto na direção do fluxo de meio de cultura, no 5º dia de análise. A elongação das células endoteliais em torno de seu próprio eixo contribui para a sua funcionalidade e previne seu destacamento da superfície dos biomateriais (26, 101). O trabalho de Quint e colaboradores demonstrou que biomateriais pré-cultivados dinamicamente com células progenitoras endoteliais apresentaram uma alta taxa de sucesso quando foram implantados na artéria carótida de porcos. Esses biomateriais apresentaram elevada taxa de patência e preveniram tanto a formação de trombos, como também, a hiperplasia intimal no local em que foram implantados (62).

O fluxo laminar existente no interior dos biomateriais celularizados favoreceu a difusão de glicose e de O_2 por essas estruturas. As CPEs cultivadas nos biomateriais através do biorreator de perfusão apresentaram um maior consumo de glicose e uma menor produção de lactato do que as células mantidas em cultivo estático (Capítulo II, Figura 5). Dois fatores podem estar relacionados ao maior consumo de glicose pelas CPEs cultivadas dinamicamente: (a) maior número de células presentes no biomaterial e (b) remodelamento de suas proteínas de adesão. O aumento do consumo de glicose pelas CPEs, durante o cultivo dinâmico, provavelmente acompanhou o aumento da densidade celular nos biomateriais. Além disso, como sugerido por Ciechanowska e colaboradores, as células mantidas em condições dinâmicas de cultivo precisam reorganizar constantemente suas proteínas de adesão, o que gera um maior consumo de energia (116).

O uso de glicose pelas células pode se dar pela via glicolítica aeróbica e pela via glicolítica anaeróbica. No metabolismo aeróbico, a glicose é totalmente degradada até H_2O e CO_2 e gera uma grande quantidade de ATP (30 – 32 moléculas), através da fosforilação oxidativa. Enquanto isso, na privação de O_2, a glicose é convertida em lactato e uma menor quantidade de ATP é gerada (2 moléculas) (111). A baixa produção de lactato pelas CPEs cultivadas dinamicamente sugere que a glicose foi utilizada principalmente pela via glicolítica aeróbica, diferente das células mantidas em cultivo estático, que produziram uma alta quantidade de lactato. Dessa forma, é possível inferir que o uso do biorreator garantiu uma melhor distribuição de O_2 pelo biomaterial,

possibilitando que a fosforilação oxidativa da glicose pudesse acontecer. Esse resultado corrobora com outros estudos da literatura que demonstram que o fluxo laminar gerado por biorreatores de perfusão favorecem o transporte de massa pelos biomateriais (118, 120). O uso do biorreator de perfusão, portanto, otimiza o metabolismo energético das CPEs cultivadas em biomateriais.

Com base nos resultados obtidos, é possível afirmar que os biomaterias de PCL funcionalizados com heparina e com VEGF são candidatos bastante promissores para uso como substitutos vasculares, em quadros graves de doenças arteriais periféricas. Ainda, o estudo demonstrou que o uso de biorreatores pode promover o crescimento mais rápido de células progenitoras endoteliais no parede luminal desses biomateriais, contribuindo para a sua rápida endotelização.

Apesar dos resultados bastante positivos encontrados para os biomateriais de PCL/Hep/VEGF, outros substitutos vasculares devem ser desenvolvidos a fim de atender a grande necessidade médica por enxertos que possam ser empregados adequadamente, principalmente, em vasos de pequeno diâmetro. Com base nisso, este grupo de pesquisa buscou desenvolver e avaliar um segundo tipo de biomaterial vascular. No capítulo III, biomateriais produzidos a partir de copolímeros de carbonato de trimetileno e ácido lático foram investigados quanto a sua aplicação vascular.

Os copolímeros de carbonato de trimetileno são caracterizados por apresentarem uma elevada elasticidade, sendo chamados elastômeros ou "materiais tipo-borracha" (12). Esses polímeros apresentam a capacidade de se deformar mediante uma tensão mecânica e retornar, completamente e rapidamente, à sua forma original após a retirada do estímulo (67). Os copolímeros constituídos por carbonato de trimetileno e ácido lático apresentam propriedades combinadas dos dois monômeros. Já foi demonstrado que o copolímero PTMCLLA apresenta alta elasticidade, devido à presença dos meros de TMC, e resistência à ruptura, fornecida pelos meros de ácido lático (13, 70). Em função dessa característica, biomateriais produzidos a partir de copolímero de PTMCLLA têm sido pesquisados para o desenvolvimento de substitutos vasculares.

No capítulo III, biomateriais foram fabricados por *electrospinning* a partir de três copolímeros de PTMCLLA. Tais copolímeros apresentavam diferentes razões de TMC e ácido lático em sua cadeia: 20/80, 30/70 e 40/60. Todos os biomateriais produzidos apresentaram morfologia semelhante, sendo constituídos por fibras lisas e uniformemente

distribuídas em sua estrutura (Capítulo III, Figura 1). Os biomateriais de PTMCLLA 30/70 apresentaram um diâmetro de fibra menor do que os demais. O diâmetro e a morfologia das fibras produzidas por *electrospinning* é um resultado da combinação das propriedades da solução polimérica e dos parâmetros utilizados para a execução da técnica (45). A concentração da solução apresenta uma relação direta com o diâmetro das fibras produzidas (124). As soluções poliméricas mais concentradas apresentam alta viscosidade. Essa propriedade faz com que a solução não sofra um grande estiramento durante o processo de *electrospinning*, originando fibras mais grossas (43). Ainda, o tipo de solvente utilizado para solubilização do polímero afeta a condutividade da solução final, o que altera a sua eletrofiação (43). Acredita-se que esses fatores tenham ocasionado a diferença de diâmetro encontrada entre as fibras de PTMCLLA 30/70 e as demais. Mesmo com fibras mais finas, esses biomateriais não apresentaram comprometimento de suas propriedades mecânicas ou degradabilidade aumentada (Capítulo III, Figuras 2 e 3).

A análise de degradação demonstrou que os biomateriais fabricados a partir de PTMCLLA apresentaram grande redução do peso molecular polimérico ao longo de 60 dias de análise (Capítulo III, Figura 2). As ligações ésteres, presentes nos resíduos de ácido lático, são mais suscetíveis à hidrólise do que as ligações de carbonato, existentes nos resíduos de TMC. Dessa forma, polímeros com maiores taxas de TMC devem apresentar maior resistência à degradação do que aqueles constituídos por maiores quantidades de ácido lático. Contudo, esse não foi o perfil observado no presente trabalho. Os biomateriais de PTMCLLA 20/80 e 30/70 apresentaram menor degradabilidade *in vitro* do que os biomateriais de PTMCLLA 40/60 (Capítulo III, Figura 2). Esse perfil foi atribuído ao fato do copolímero PTMCLLA 40/60 ter um peso molecular bastante inferior aos outros dois (Capítulo III, Tabela 2). Assim, essas cadeias poliméricas acabam sendo mais suscetíveis ao processo de hidrólise (125).

Como descrito anteriormente, os biomateriais utilizados como substitutos vasculares devem permanecer por um maior período no local do implante para que as células consigam se organizar e restaurar e/ou construir o tecido. Assim, esses biomateriais devem fornecer uma estrutura íntegra até que o tecido vascular seja completamente organizado, um processo que pode se estender por mais de 40 semanas (126). Os biomateriais de PTMCLLA apresentam degradação por erosão, isto é, eles apresentam perda de sua superfície proporcionalmente à redução de sua massa e dimensões. Esse perfil é muito importante para a medicina regenerativa, já que, esses

biomateriais mantêm sua integridade e propriedades mecânicas por um longo período de tempo (67). Contudo, os três biomateriais de PTMCLLA produzidos no trabalho exposto no capítulo III apresentaram uma redução do peso polimérico considerada elevada para a aplicação vascular. Essa característica poderia ser melhorada através da reticulação dos copolímeros. A formação de ligações cruzadas entre as cadeias poliméricas torna o polímero mais resistente à hidrólise e, ainda, pode otimizar suas propriedades mecânicas (13, 68).

A análise dinâmico-mecânica demonstrou que a resistência e a elasticidade dos biomateriais de PTMCLLA são dependentes da proporção entre TMC e ácido lático no copolímero (Capítulo III, Figura 3). Os biomateriais de PTMCLLA 20/80 apresentaram um perfil mecânico semelhante ao dos poliésteres (97). Eles tiveram uma grande resistência à fratura; contudo, apresentaram uma baixa flexibilidade. Enquanto isso, os biomateriais de PTMCLLA 30/70 e 40/60 apresentaram uma menor resistência à fratura e uma alta flexibilidade, perfil semelhante ao dos elastômeros (12, 127). A elasticidade é uma característica essencial para um enxerto vascular, já que ele deve ter a capacidade de resistir aos estresses hemodinâmicos presentes *in situ* (29). Apesar de serem menos resistentes do que o PTMCLLA 20/80, os biomateriais fabricados com PTMCLLA 30/70 e 40/60 exibiram uma resistência semelhante a dos vasos naturais, que apresentam valor médio de tensão à fratura de 1.55 ± 0.4 MPa (70). Portanto, esses dois biomateriais apresentaram-se mais adequados à aplicação como substitutos vasculares, em termos de propriedades mecânicas. Contudo, em função da alta elasticidade, os biomateriais de PTMCLLA 40/60 apresentaram uma grande taxa de enrugamento quando expostos ao meio líquido. Essa característica poderia ocasionar a falha clínica do implante, pois provocaria a redução de seu diâmetro inicial e, consequentemente, a oclusão de seu lúmen. Dessa forma, esse grupo foi removido dos ensaios biológicos.

Apesar de alguns grupos de pesquisa já terem examinado o PTMCLLA para a fabricação de substitutos vasculares (68, 70, 128), ainda existem poucos estudos na literatura avaliando as propriedades biológicas desses materiais. Assim, o presente trabalho buscou avaliar a interação dos biomateriais de PTMCLLA com CTMs, CPEs e células musculares lisas. Esses tipos celulares estão presentes na parede dos vasos naturais ou, então, podem se diferenciar nas células existentes em tal (73, 74, 81).

A adesão celular foi o primeiro ensaio biológico realizado devido a sua grande importância para o sucesso do biomaterial (123). Somente após a adesão, as células

conseguem se proliferar e organizar as proteínas da matriz extracelular para formação tecidual (105). A adesão celular é mediada pela adsorção de proteínas da matriz extracelular à superfície do biomaterial e pela ligação via receptores de integrina, presentes na membrana celular (129). Logo, esse processo é bastante afetado pela topografia e superfície química do biomaterial (130). Tanto os biomateriais de PTMCLLA 20/80 como os de PTMCLLA 30/70 conseguiram, de maneira semelhante, promover a adesão dos três tipos celulares (Figura 4, Capítulo III). Contudo, a placa de cultivo, utilizada como grupo controle, apresentou um maior número de células aderidas, para as três linhagens utilizadas. Esse resultado já foi observado por outros autores, como Stefani e colaboradores. Em seu trabalho, eles demonstraram que CTMs apresentaram uma maior adesão sobre placas de cultivo do que em biomateriais produzidos a partir de blendas de PCL e PTMCLLA (70). As placas de cultivo comerciais são tratadas quimicamente para promoverem a adaptação celular e representam, hoje, o padrão ouro para o cultivo de células (70, 114, 130).

Após a adesão, tanto as CTMs, como as CPEs foram capazes de se proliferar sobre os biomateriais de PTMCLLA (Figura 4 d, e; Capítulo III). Para essa análise, também não foram encontradas diferenças significativas entre os dois grupos. Esse resultado sugere que a variação da razão TMC/ácido lático não interfere na proliferação desses tipos celulares. O estudo de Dargaville e colaboradores corrobora com esse achado. Os autores demonstraram que a variação de 30 a 70% no conteúdo de TMC no copolímero PTMCLLA não acarreta diferença no crescimento de CTMs (13). Messias e coautores também não observaram variação no número de osteoblastos cultivados em biomateriais de PTMCLLA e biomateriais de PLLA, evidenciando que a presença de TMC nessas estruturas não afeta a proliferação celular (131). As células musculares lisas também não apresentaram diferença significativa em sua viabilidade, em função do conteúdo de TMC nos biomateriais (Capítulo III, Figura 4 f). Porém, os biomateriais não proporcionaram o aumento do número dessas células, ao longo do período de cultivo. Acredita-se que esse comportamento ocorreu pelo fato de as células musculares lisas já serem células adultas, isto é, diferenciadas. Sabe-se que células já diferenciadas apresentam um menor potencial de proliferação em comparação a células-tronco ou progenitoras (77).

Apesar do conteúdo de TMC no copolímero PTMCLLA não ter alterado a proliferação celular, ele afetou o processo de adaptação das CTMs e das células musculares lisas. Quando cultivadas sobre os biomateriais de PTMCLLA 30/70, essas

células exibiram um aumento da sua área superficial (Capítulo III, Figura 5). Os dois tipos celulares exibiram um citoesqueleto mais espalhado sobre a superfície dos biomateriais de PTMCLLA 30/70 do que sobre os biomateriais de PTMCLLA 20/80. Ji e colaboradores também encontraram resultados semelhantes quando avaliaram a adaptação de fibroblastos sobre filmes de PTMCLLA e de poli(L-ácido lático) (PLLA) (132). Os autores mostraram que, sobre PLLA, os fibroblastos apresentam uma morfologia arredondada, pouco espalhada. Enquanto isso, sobre os filmes de PTMCLLA, os fibroblastos têm o seu citoesqueleto espalhado, demonstrando uma maior interação com o substrato. Essa diferença foi atribuída a maior flexibilidade dos filmes que continham TMC. Com base nessa avaliação, sugere-se que as CTMs e as células musculares lisas também conseguem melhor adaptar-se sobre superfícies mais flexíveis do que rígidas.

As CPEs não apresentaram grandes diferenças em sua morfologia quando cultivadas sobre os biomateriais de PTMCLLA 20/80 e 30/70. Essas células apresentaram-se arredondadas sobre as duas superfícies. Isso indica que as CPEs não conseguiram estabelecer uma grande interação com esses materiais. Como relatado anteriormente, esse tipo celular apresenta uma maior dependência do contato com proteínas da matriz extracelular para seu desenvolvimento (94, 133, 134). Diferente dos biomateriais de PCL/Hep/VEGF, apresentados no capítulo I, os biomateriais de PTMCLLA não apresentam nenhum domínio natural para o ancoramento celular. Acredita-se que esse fator tenha contribuído para a menor adaptação das CPEs nos biomateriais de PTMCLLA do que nos de PCL/Hep/VEGF. Uma alternativa para melhorar essa propriedade e promover a endotelização, seria também realizar a funcionalização da superfície do biomaterial de PTMCLLA 30/70. Assim, se teria um biomaterial extremamente flexível e com características biológicas mais adequadas à manutenção de células progenitoras endoteliais.

De acordo com as características apresentadas, biomateriais de PTMCLLA 30/70 apresentam propriedades físico-químicas compatíveis para a aplicação como substitutos vasculares. Ainda, suportam a adesão de células-tronco mesenquimais, células progenitoras endoteliais e células musculares lisas. Portanto, também são candidatos bastante interessantes para o uso na engenharia de tecidos vasculares.

6. Conclusões

Gerais:

- A funcionalização de biomateriais de PCL com heparina e VEGF previne a formação de trombos e favorece a proliferação e a adaptação de células progenitoras endoteliais sobre a sua superfície;
- O uso de biorreatores de parede rotatória e de perfusão favorece a endotelização do lúmen de biomateriais tubulares de PCL, funcionalizados com heparina e com VEGF;
- Os biomateriais produzidos a partir de PTMCLLA 30/70 apresentam propriedades físico-químicas e biológicas compatíveis com a aplicação na engenharia de tecidos vasculares.

Específicas

- Biomateriais fibrosos de PCL e de PTMCLLA foram produzidos adequadamente pela ténica de *electrospinning;*
- Os biomateriais de PCL apresentam taxa de degradação, propriedades mecânicas e morfologia adequadas à aplicação na engenharia de tecidos vasculares;
- A funcionalização dos biomateriais de PCL com heparina e VEGF previne a formação de trombos e favorece o crescimento de CTMs e de CPEs em relação aos biomateriais não funcionalizados;
- A expressão de VE-caderina é aumentada nas CPEs cultivadas sobre biomateriais de PCL;
- A quantidade de VEGF liberada pelos biomateriais de PCL funcionalizados com esse fator não é suficiente para promover a diferenciação de CTMs em células endoteliais. No entanto, o VEGF presente nos biomateriais mantém a expressão de CD34 e de CD31 nas CPEs;
- Os biomateriais produzidos com PTMCLLA nas razões 30/70 e 40/60 apresentam alta flexibilidade. Porém, os biomateriais de PTMCLLA 40/60 apresentam uma alta taxa de enrugamento quando colocados em contato com meio de cultivo, impossibilitando sua avaliação biológica e aplicabilidade clínica;

- Os biomateriais de PTMCLLA 30/70 e 20/80 suportaram a adesão e/ou crescimento de células-tronco mesenquimais, células progenitoras endoteliais e células musculares lisas. As células cultivadas sobre os biomateriais de PTMCLLA 30/70 exibiram um citoesqueleto mais espalhado, indicando uma melhor adaptação sobre esse copolímero;
- Os biorreatores de parede rotatória favorecem a semeadura uniforme de CPEs no lúmen de biomateriais de PCL funcionalizados com heparina e VEGF;
- Os biorreatores de perfusão promovem a proliferação de CPEs e propiciam seu alongamento na direção do fluxo do meio de cultura. Além disso, esses biorreatores otimizam a difusão de nutrientes e de oxigênio pelos biomateriais, favorecendo o metabolismo energético aeróbico das células.

Os resultados obtidos demonstram que biomateriais de PCL funcionalizados com heparina e VEGF e biomateriais de PTMCLLA 30/70 são interessantes candidatos para aplicação como substitutos vasculares em quadros graves de doença arterial periférica. Ainda, que a endotelização de biomateriais vasculares pode ser otimizada através do uso de biorreatores de parede rotatória e de perfusão.

7. Perspectivas

I. Promover a formação da camada muscular nos biomateriais de PCL funcionalizados com heparina e VEGF;

II. Realizar o co-cultivo de células progenitoras endoteliais e de células musculares lisas nos biomateriais de PCL funcionalizados com heparina e com VEGF;

III. Avaliar os biomaterias de PCL funcionalizados com heparina e VEGF em modelo animal de doença arterial periférica;

IV. Construir biomateriais de PTMCLLA 30/70 em formato tubular e associá-los a células progenitoras endoteliais e células musculares lisas através do uso de biorreatores.

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9. Anexo

Outras publicações realizadas durante o período de doutorado

Drug Discovery Today • Volume 00, Number 00 • April 2014 REVIEWS

ELSEVIER

Electrospinning for regenerative medicine: a review of the main topics

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Electrospun fibers are promising tissue engineering scaffolds that offer the cells an environment that mimics the native extracellular matrix. Fibers with different characteristics can be produced by the electrospinning technique according to the needs of the tissue to be repaired. In this review, the process of electrospinning was examined, providing a description of the common techniques used for the physicochemical and biological characterization of electrospun fibers. The review also discusses the potential applications of electrospun scaffolds for tissue engineering, based on scientific literature.

Introduction

Failure of organ and/or tissue function as a result of injury, disease or aging has a high impact on quality of life and also incurs a large social and economic cost. Current treatments vary with the organ affected, but all of them have their limitations [1]. Frequently, organ transplantation is the indicated therapy for these clinical cases; however, there are several disadvantages in using autologous or allogenic grafts, including shortage of appropriate donor organs, risk of disease transmission and immune rejection [2]. Tissue engineering (TE) is an exciting area that involves engineering and biological knowledge to create or restore tissue and organs. It utilizes three basic tools: cells, biomaterials and biomolecules. Electrospinning (ES) is a simple and versatile technique that produces scaffolds formed by nano- and micro-fibers, which offer a favorable microenvironment for cellular development by mimicking the native extracellular matrix [3]. The aim of this review paper is to describe the production of electrospun scaffolds and examine the most commonly used techniques for their physicochemical and biological characterizations. It also discusses the main biomedical applications of electrospun fibers and the cell sources used in TE.

The electrospinning process

From the techniques used to construct biomaterials to be cultivated with cells, ES is the most widely studied and it has also been demonstrated as giving the most promising results in terms of TE applications. The first recorded citation detailing the application of high electrical potentials to generate aerosols from drops of fluids was in 1745; but not until 1929 was the application of an electrical field to produce artificial silk described. Until the 1990s, there was no commercial interest in this technique, although advances and patents in the field were achieved [4]. A search in February 2014 for the keyword 'electrospinning' in PubMed showed an increased interest for this technique after 2000.

The electrospinning process works by the electrostatic principle. As can be seen in Fig. 1, the machine is basically composed of a syringe with a nozzle, a counter electrode (normally a metal plate), a source of electrical field and a pump. The solution to be electrospun is applied to the system via the nozzle of the syringe and is pulsed by the pump. It is then subjected to a difference in an electrical voltage present between the nozzle and the counter electrode. This electrical voltage generated by the source causes a cone-shaped deformation of the drop of polymer solution. The solvent in the solution evaporates on its way to the counter electrode and, at the end of the process, solid continuous filaments are yielded [5]. It is important to note that the gravitational forces do not interfere in the process because the acceleration of the fiber formation is up to 600 m/s^2, which is close to two orders of magnitude greater than the acceleration of gravitational forces. Because of this, it is possible to form fibers from top-down, bottom-up or other types of arrangements [6].

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Novel Chemically Modified Bacterial Cellulose Nanocomposite as Potential Biomaterial for Stem Cell Therapy Applications

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Abstract: Bacterial cellulose (BC) has become established as a remarkably versatile biomaterial and can be used in a wide variety of applied scientific applications, especially for medical devices. In this work, the bacterial cellulose fermentation process is modified by the addition of hyaluronic acid and gelatin (1% w/w) to the culture medium before the bacteria is inoculated. Hyaluronic acid and gelatin influence in bacterial cellulose was analyzed using Transmission Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). Adhesion and viability studies with human dental pulp stem cells using natural bacterial cellulose/hyaluronic acid as scaffolds for regenerative medicine are presented for the first time in this work. MTT viability assays show higher cell adhesion in bacterial cellulose/gelatin and bacterial cellulose/hyaluronic acid scaffolds over time with differences due to fiber agglomeration in bacterial cellulose/gelatin. Confocal microscopy images showed that the cell were adhered and well distributed within the fibers in both types of scaffolds.

Keywords: Bacterial cellulose, cell viability study, Nanoskin®, natural nanocomposites, regenerative medicine, stem cells.

1. INTRODUCTION

Gluconacetobacter xylinus (bacterial cellulose, BC) is an emerging biomaterial with great potential in several applications due its high purity, ultra-fine network structure and high mechanical properties in dry state [1]. These features allow its application as scaffolds for tissue regeneration, medical applications and nanocomposites. Some studies have used bacterial cellulose mats to reinforce polymeric matrices and scaffolds with wound healing properties. BC is a natural cellulose produced by bacterial synthesis by biochemical steps and self-assembling of the secreted cellulose fibrils on the medium. Shaping of BC materials in the culture medium can be controlled by the type of cultivation that changes chain size, origin of strains which produces different proportions of crystalline phase of BC and the kind of bioreactor. BC hydrogel or BC in dry state is then obtained by methods, such as freeze-drying [2]. Although chemically identical to plant cellulose, the cellulose synthesized by the bacteria has a fibrillar nanostructure, which determines its physical and mechanical properties, necessary characteristics for modern medicine and biomedical research [3]. The structural features of microbial cellulose, its properties and compatibility as a biomaterial for regenerative medicine can be changed by modifying its culture medium [4] or surface modification by physical [5, 6]; chemical methods [7] and genetic modifications [8] to obtain a biomaterial with less rejection when in contact to the cell and cell interaction.

Different gelatin formulations have been studied to evaluate the drug loading capacity and release rate. Like other hydrogels, drug release profiles obtained from gelatin hydrogels can be readily adjusted by changing the network cross-linking density. Because gelatin has a sol-gel transition temperature around 30°C, it should be cross-linked chemically to avoid dissolution at body temperature [9]. Gelatin nanofibers play a dominant role in maintaining the biological and structural integrity of various tissues and organs, including bone, skin, tendon, blood vessels and cartilage. There are several commercially available gelatin based carriers for drug delivery that are being applied in tissue engineering [10]. Physical and chemical permeation enhancers can be used in conjunction with cellulose bacterial membrane (Nanoskin®) to affect the desired level of delivery. Early results also showed that hyaluronic acid (HA) was effective in protecting retinal damage during ophthalmic surgery, reducing scarring, preventing post-operative adhesions and reducing pain while increasing mobility in arthritic joints [11]. In addition, HA also provides important structural sup-

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Nanofiber Scaffolds Support Bone Regeneration Associated with Pulp Stem Cells

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Abstract: Currently, there are a number of alternatives for bone grafting, though when used correctly they present physical, chemical or biological limitations, which justifies the pursuit for new alternatives for bone regeneration. This study gives a report on the potential for bone regeneration in the use of biodegradable nanofibers from poly (lactic-co-glycolic acid) (PLGA) in association with human mesenchymal stem cells from dental pulp of deciduous teeth (SCDT). Five samples of SCDT were seeded with scaffolds (test) or without scaffolds (control) for cell adhesion and viability assay. To evaluate the ability of the association in promoting bone formation, critical defects were made in the calvarium of rats (n=20), which were then divided into the following groups: I – sham group; II – implant of scaffolds; III – scaffolds/SCDT; and IV – scaffolds/SCDT. They were kept for 13 days in osteogenic media. After 60 days, the histomorphometric analysis was performed. It was observed that the adherence and viability of SCDT in the control and test group were similar throughout the experiment ($p>0.05$). The association of scaffolds/SCDT maintained in osteogenic media, showed greater bone formation than the other groups ($p<0.05$). The study demonstrated that the association of SCDT seeded in biodegradable PLGA scaffolds has the ability to promote bone regeneration in rats, which is a promising alternative for application in regenerative medicine.

Keywords: Bone regeneration, deciduous teeth, human mesenchymal stem cells, nanotechnology, scaffolds, tissue engineering.

1. INTRODUCTION

Bone loss represents a challenge to reconstructive surgery, as in many cases it is necessary to select a source for bone grafting for treatment. In the search for bone sources, bone banks can be accessed, for the use of tissue from patients with inherent morbidity [1-2]. Other sources of bone can be used from xenografts which are marketed as inert and free of antigens, but which have poor osseointegration [3]. Another possibility is to use hydroxyapatite biomaterials that are biocompatible, but they often have poor ossification in more internal grafts [4]. Lyophilized bone is another choice, which has osteoinductive capacity but which does not provide optimal biomechanical properties when used as structural implants [5]. The autologous donor sites are considered the gold standard in bone grafting; however, the availability of donor sites may become restricted in some patients and their systemic conditions may represent a limiting factor [6]. Thus, despite the availability of different methods for bone grafting, the existence of limiting factors (physical, chemical or biological) associated with such options justifies the pursuit for new alternatives for bone regeneration [7]. Bioengineering, therefore, involves the use of biomaterials and cellular therapy, providing an adequate and viable solution for tissue regeneration [8]. Scaffolds are among the available biomaterials used in bioenineering, that can physically simulate an extracellular matrix (ECM), functioning as a framework for cell adhesion and proliferation when associated with cellular therapy [9-10]. Poli (D,L-lactic-co-glycolic acid) (PLGA) is a polymer used for the fabrication of scaffolds which presents no toxicity, has biocompatibility and has a controllable rate of degradation because of the lactic acid and glycolic acid ratios [11, 12]. The biodegradation of PLGA involves the hydrolysis of ester linkages in their backbone chains, generating lactic acid and glycolic acid [13, 14]. These products of reaction are metabolized by the Krebs cycle and eliminated through urine and breath as CO_2 and H_2O. When produced by the electrospinning technique, the PLGA scaffolds are a highly porous structure, which is of great importance because it provides structural space to accommodate the cells and promotes an efficient exchange of nutrients and metabolic residues between the cell and the environment [9]. Aside from the use of biomaterials, another important branch of bioengineering is cellular therapy with the use of stem cells. Human beings possess different niches of adult stem cells, amongst which are included stem cells from dental pulp of decidious teeth (SCDT) [15, 16]. These cells are obtained from an easily accessible

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The Laryngoscope

Mesenchymal Stem Cells and Nanofibers as Scaffolds for the Regeneration of Thyroid Cartilage

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Objectives/Hypothesis: The aim of this study has been to establish an alternative approach in the form of regeneration of the thyroid cartilage.

Study Design: Four 1-month old pigs (Sus scrofa) were used (divided into 3 groups) and submitted to general anesthetic to perform cervictomy with exposure of the thyroid cartilage in a total of 12 (twelve) samples.

Method: A resection of 4.0 cm^2 of cartilage was carried out in the right upper region and in the left upper and lower left region of the cartilage, where a scaffold with or without stem cells was implanted. In the left lower region, no biomaterial was implanted and the defect was left open (lesion control [L]).

Results: The average extension of the cartilaginous neoformation of L group was 136.3 μm ($\pm$ 9.6) and 387.7 μm ($\pm$ 43.2) in the scaffold (SCA) group, presenting a significant statistical difference ($P < 0.01$). The analysis carried out on the lesion site sections of the cartilage of the larynx of the animals from the SCA group + mesenchymal stem cells (SCA+MSC) showed an average of the extension of neocartilage of 825.4 μm ($\pm$ 122.1), showing a more extensive area of neocartilage when compared to the other groups. These results demonstrated a high significantly statistical difference ($P < 0.001$) when compared with the L and SCA groups.

Conclusion: In 100% of the cases for which SCA+MSCs were used, a significant success in the cartilage growth and closing of the lesion in the thyroid cartilage was obtained compared to the other two groups for which MSCs were not used.

Key Words: Stem cells, thyroid cartilage, regeneration, scaffolds..

Level of Evidence: N/A.

***Laryngoscope*, 124:E455–E460, 2014**

INTRODUCTION

Cancer of the larynx is diagnosed annually in approximately 10,000 men and women in the United States and is among the most common types of cancer of the upper aerodigestive tract.[1]

Reconstruction of the airways, mainly those with cartilaginous formation in their structure, continue to challenge medical science. A very large variety of techniques for cartilaginous reconstruction have been described in laryngotracheal reconstruction surgeries—not always with very promising results in milder cases (level II of Cotton) and those of benign origin.[2,3]

These different therapeutic approaches have been developed around the world with the aim of offering an improvement in the life quality of this group of patients.

The main types of tracheobronchial substitutes that have been used in airway transplantation are synthetic prostheses, bioprostheses, allografts, autografts, and bioengineered conduits. According to a recent review by Martinod et al.,[4] despite the fact that research has been carried out in this area for more than 50 years, airway transplantation is still one of the biggest challenges for thoracic surgery and regenerative medicine.[4]

In the last decade, research using stem cells (SCs) has attracted a great deal of attention from the academic and scientific worlds because it shows enormous potential for modifying the concepts of traditional therapies, with a wide impact on genetic therapy, carcinogenesis, tissue damage, and regeneration, among others. The identification, isolation, and differentiation of embryonic stem cells have broadened the spectrum of potentials for cellular therapy. Recent findings[5] have demonstrated the possibility of isolating skeletal muscular cells derived from SCs of embryoid bodies. The intramuscular and systemic transplantation of these cells in dystrophic

From the Department of Morphological Sciences (G.P.J., P.R.D.L.S., S.O.K., R.F.F.L.); the Hematology and Stem Cells Laboratory, Pharmacy School (D.S., D.I.B., P.P.); the Post Graduate Program in Physiology (D.S., D.I.B, P.P.); the Medicine Veterinary School (C.A.B, M.M.); the Medicine School; Federal University of Grande do Sul (B.M.); the Federal University of Health Sciences of Porto Alegre (C.G.Z.); and the Stem Cell Research Institute (P.P.), Porto Alegre, RS, Brazil.

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Evaluation of semi-automated cells counting in peritoneal fluid

Avaliação de semiautomação para contagem de células em líquido peritoneal

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ABSTRACT

Introduction: Currently, the cytological analysis of biological fluids, such as peritoneal fluid, is performed by manually cells counting in Fuchs-Rosenthal chamber. However, this method has a number of limitations. Because of these limitations, automatic counters have been evaluated for cell counting in this type of sample in order to make it faster and more reliable test. **Objective:** The aim of this study is to compare the manual and semi-automated leukocytes and erythrocytes counting in peritoneal fluid. **Materials and methods:** The samples were analyzed manually and using the Countess™ (Invitrogen). **Results:** The results showed that although there is a correlation between the two counting methods, the correlation is relatively low, for both leukocytes and erythrocytes analysis. **Conclusion:** The results suggest that peritoneal fluid should continue to be analyzed in Fuchs-Rosenthal chamber. However, further studies should be conducted with a greater number of samples to investigate the possibility of using automated cells counting in serous fluids and, thus, provide greater speed and quality of results.

Key words: peritoneal fluid; ascites; cell count; body fluids.

INTRODUCTION

Ascites is a condition characterized by the accumulation of serous fluid between peritoneal membranes. The most frequent cause of this clinical condition is liver disease, such as cirrhosis, peritonitis and cancer[1,2]. The study of serous fluid, also referred to as peritoneal ascites fluid, provides important information about differential diagnosis of stroke and disease status monitoring. Routine laboratory investigations of peritoneal fluid include physical, cytological, biochemical and bacteriological analyses[3]. This may also be complemented by other techniques, such as cytochemistry, immunocytology, cytogenetics and molecular biology. These analyses contribute to differentiate the fluid between exudate and transudate, evaluate the presence of tumor markers and detection of bacterial infections. They are, therefore, very important for clinical and therapeutic decisions-making[3,4].

Cytological analysis is the microscopic examination of the fluid, where total count and differential cell is performed[3]. Currently, most clinical laboratories perform cell count in serous fluid and cerebrospinal fluid (CSF) by manual microscopy using counting chambers, such as Fuchs-Rosenthal[5,6]. However, this method has several limitations. Besides being a time-consuming technique, low accurate and has considerable inter and intra-operator variability, requiring highly skilled and experienced technicians[9,10]. Based on these facts, different automated devices have been evaluated for cell counts in different biological fluids[9, 11, 12]. It is believed that the use of automation in this examination would help in obtaining more reliable results, better organization and manipulation of the samples, and optimization of time, human resources, space and material[7,10]. The semi-automated Countess™ (Invitrogen) is used for cell count, mainly in research laboratories. The present study has aimed to evaluate the semi-automated method for cells counting

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Effect of feeder free poly(lactide-co-glycolide) scaffolds on morphology, proliferation and pluripotency of mouse embryonic stem cells

No benefit of any kind will be received either directly or indirectly by the author(s).

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7
Electrospun Scaffolds of Biodegradable Polyesters: Manufacturing and Biomedical Application

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7.1
Introduction

Polymeric materials have been applied successfully in many different fields, such as in membrane and thin-film technology, sensors, adhesion, protective coatings, microelectronic devices, composites, and other applications. Indeed, in the field of medical and related applications, polymers have been used extensively for decades and in particular in the last three decades a paradigm shift from bio-stable biomaterials to biodegradable (hydrolytically and enzymatically degradable) biomaterials has been observed [1]. It is expected that in the near future many of the permanent prosthetic devices used nowadays for temporary therapeutic applications will be replaced by biodegradable devices. These new medical tools could help the body to repair and regenerate damaged tissue in a natural way. The emergence of novel biomedical technologies, including tissue engineering, gene therapy, controlled drug delivery, and bionanotechnology are the main driving force for the aforementioned movement.

Current biomaterial research has contributed to major advances in regenerative medicine. In tissue engineering, cell therapy is complemented with biomaterials to be applied to damaged tissue and to assist in its repair. Biomaterials act as scaffolds, providing an initial supportive environment in which seeded cells can organize themselves and produce extracellular matrix (ECM) for subsequent regeneration of the damaged tissue [2]. An ideal scaffold should exhibit certain characteristics for successful application, such as (i) adequate architecture for cell attachment and proliferation, (ii) high number of interconnected pores for cell growth and transport of nutrients and metabolic waste, and (iii) mechanical properties suitable for its manipulation at the implantation site [3]. For these reasons, the selection of biomaterial types and their processing for the creation of the scaffolds are the most important factors for successful tissue reconstruction using tissue engineering principles.

Scaffolds can be produced from a variety of materials, including metals, ceramics, and polymers. In dental and bone implants, metallic alloys are preferred [4, 5], while ceramics with good osteoconductivity have been used for bone tissue

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