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Utilização de células estaminais mesenquimatosas em Medicina Regenerativa

Sandra Raquel Vieira Gomes
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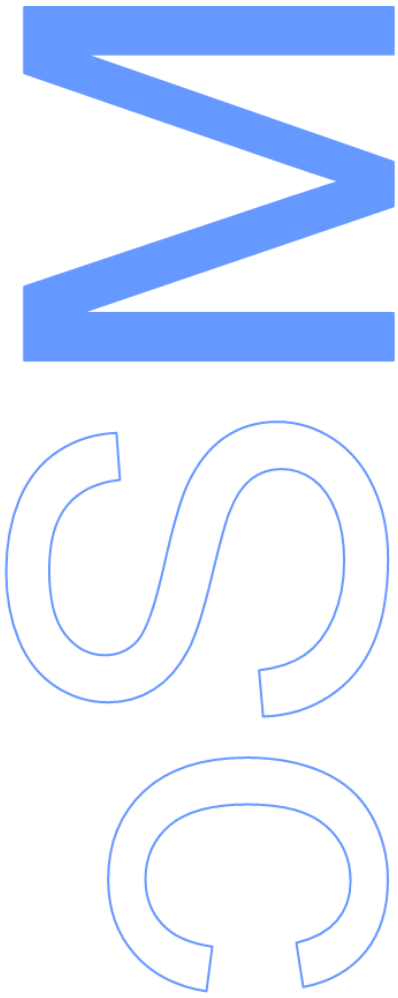

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Resumo

Avanços recentes em Engenharia de Tecidos, nomeadamente do tecido nervoso periférico (SNP) conduziram ao desenvolvimento de tubos-guia, que podem ser implantados vazios ou preenchidos com fatores de crescimento e/ou sistemas celulares. As células estaminais podem ser livremente classificadas em três grandes categorias, baseadas no seu tempo de isolamento durante a ontogénese: embrionárias, fetais e adultas. Nos últimos anos, ocorreu uma explosão no número de populações de células estaminais adultas isoladas e caracterizadas. Enquanto multipotentes, as células estaminais adultas, desde há muito consideradas restritas, dão origem aos tipos celulares dos tecidos de onde são provenientes. Recentemente, estudos atualmente controversos têm desafiado este dogma, o que sugere que as células estaminais adultas podem ser muito mais versáteis do que anteriormente foi descrito. Os tecidos extra embrionários como reservatórios de células estaminais oferecem grandes vantagens comparativamente às outras fontes de células estaminais embrionárias e adultas. Os tecidos extra embrionários são rotineiramente descartados no parto, por isso apresenta pouca controvérsia ética em relação à colheita das populações de células estaminais residentes. Mais significativamente, o volume relativamente grande dos tecidos extra embrionários e a facilidade de manipulação física, teoricamente, aumenta o número de células estaminais que podem ser isoladas. O sangue do cordão umbilical (SCU) representa a típica fonte de células estaminais fetais. As células estaminais hematopoiéticas (HPSC) do SCU têm sido extensivamente estudadas e demonstrado uma grande utilidade clínica, em tratamentos hematopoiéticos e recentemente, em Medicina Regenerativa incluindo a regeneração do sistema do nervoso central e periférico. Por outro lado, as células estaminais mesenquimatosas (MSCs) tornaram-se um dos alvos de maior interesse para a regeneração de tecidos, incluindo o nervo periférico. Tal como as células estaminais mesenquimatosas da medula óssea e as outras MSCs obtidas do sangue periférico por aferése, são aderentes ao plástico, marcadas positivamente para os marcadores de células mesenquimatosas (CD10, CD13, CD29, CD44, CD90, CD105) e negativa para marcadores de linhagem hematopoiética. Estas células são capazes de auto renovação a proliferação sustentada *in vitro* e podem diferenciar-se em várias células mesodérmicas, incluindo células da neuroglia. A plasticidade elevada e a baixa imunogenicidade destas células, transforma-as numa forma desejável de terapia celular para o sistema nervoso lesado sem exigir a utilização de fármacos imunossupressores, durante os tratamentos. As MSCs foram isoladas a partir de

várias outras origens, incluindo a pele, folículos de cabelo, periósteo, líquido amniótico, SCU e no tecido adiposo. A colheita e expansão de MSCs são técnicas atuais, mas que não só são possíveis numa abordagem em laboratório, mas também em relação à sua aplicação clínica. Com estímulos adequados e condições ambientais adequados as MSCs exibem uma plasticidade respeitável. As MSCs suportam a hematopoiese e melhoram o enxerto de células estaminais hematopoiéticas após co-transplante, servindo de co-adjuvante em tratamentos hematopoiéticos. Atualmente, a medula óssea representa a principal fonte de MSCs. No entanto, o número de MSCs da medula óssea diminuiu significativamente com a idade e os dadores HLA compatíveis são muito difíceis de encontrar, o que torna a busca por fontes alternativas adequadas necessárias para o uso autólogo e alogénico. Recentemente o tecido conjuntivo (geleia de Wharton) do cordão umbilical tem sido referido como uma fonte importante destas MSCs, tendo sido testado pelo nosso grupo de investigação em lesões de axonotmese do nervo ciático no modelo experimental do rato. O isolamento destas células a partir da geleia de Wharton, a sua expressão e caracterização foram realizados. Uma grande preocupação tem sido dedicada aos protocolos de criopreservação, recolha e transporte do cordão umbilical do hospital após o parto para o laboratório, a fim de obter amostras com maior número de MSCs viáveis. Uma equipa multidisciplinar tem um papel crucial no desenvolvimento de biomateriais associados a sistemas celulares e em ensaios pré-clínicos. As membranas associadas a MSCs indiferenciadas e diferenciadas a partir da geleia de Wharton do cordão umbilical têm sido testadas no nervo ciático de ratos, em lesões de axonotmese de 3 mm. As MSCs implantadas no nervo lesionado podem produzir fatores de crescimento ou moléculas da matriz extracelular (MEC), ou podem modular o processo inflamatório, para melhorar a regeneração dos nervos. As MSCs desempenham um papel importante, através da modulação do processo inflamatório, crucial para uma adequada degeneração Walleriana, o primeiro passo para o processo de regeneração do nervo periférico após uma lesão. Para a lesão por esmagamento, utiliza-se uma pinça não serrilhada que exerce uma força de 54N e é usada por um período de 30 segundos para criar uma lesão por esmagamento com 3 mm de comprimento. As biomembranas associadas a MSCs diferenciadas *in vitro* em células tipo neuroglial têm sido utilizadas. Noutros grupos experimentais, as células MSCs indiferenciadas foram aplicadas por infiltração local (10^4células/lesão) e a lesão foi envolvida pelo biomaterial em ambas as lesões, axonotmese e neurotmese. A gravação de vídeo da marcha para análise biomecânica, a medição da reação postural de extensão (EPT) e o índice de funcionalidade do ciático (SFI) permitem avaliar a recuperação motora em diferentes abordagens terapêuticas. A recuperação da sensibilidade, por outro lado, é

testada pela avaliação da latência do reflexo flexor (WRL). Os nervos reparados são processados para análises por microscopia ótica e eletrónica, microscopia de confocal, imunohistoquímica e estudos estereológicos, permitindo uma avaliação da recuperação morfológica. Os protocolos de isolamento, expansão e diferenciação das MSCs a partir da geleia de Wharton para uso clínico futuro na reconstrução de nervos periféricos, foram desenvolvidos pelo nosso grupo de pesquisa e outros grupos através de estudos *in vitro*, que incluem a avaliação de citocompatibilidade dos biomateriais usados e caracterização completa das células, por frequência de unidades formadoras de colónias (CFU), a capacidade de diferenciação e caracterização fenotípica da linhagem por imunocitoquímica, citometria de fluxo e RT-PCR. Também durante a expansão e diferenciação das MSCs em células do tipo neuroglial, o meio de cultura foi colhido para análise de ressonância magnética nuclear (RMN), a fim de avaliar o perfil metabólico. Na análise *in vitro* mostrou a diferenciação das MSCs em células do tipo neuroglial, estas foram positivamente coradas para os marcadores específicos de GFAP, GAP-43 e NeuN. A RMN revelou que a expansão das MSCs é glicólise-dependente, mas a sua diferenciação tem uma alteração de perfil metabólico para metabolismo oxidativo. O teste *in vivo* permite uma análise da biocompatibilidade dos biomateriais, avaliação da recuperação funcional e morfológica dos animais submetidos a diferentes tratamentos em lesões de neurotmese e axonotmese. Estes estudos demostraram uma melhoria da função motora e sensorial nos animais tratados com MSCs e células do tipo neuroglial onde se verificou um aumento da bainha de mielina das fibras regeneradas. MSCs isoladas da geleia de Wharton do cordão umbilical aplicadas através de membranas de biomateriais podem ser consideradas um instrumento potencialmente valioso para melhorar o resultado clínico especialmente depois de trauma para os nervos sensoriais, como nervos digitais. Além disso, essas células representam uma fonte não controversa de células primitivas progenitoras mesenquimatosas que podem ser colhidas após o nascimento, criogenicamente armazenadas, descongeladas e expandidas para fins terapêuticos, incluindo lesões de nervos como axonotmese e neurotmese. Além disso, a importância de um estudo longitudinal e completo em relação à Engenharia de Tecidos do nervo periférico, a qual inclui uma equipa multidisciplinar capaz de desenvolver biomateriais, para preparar culturas de células para terapias celulares, e para elaboração de análise *in vitro* e ensaios pré-clínicos relativamente ao bem-estar animal e o modelo animal mais adequado é demonstrada nesta dissertação.

Palavras-chave

Axonotmese, biomateriais, células estaminais, células estaminais mesenquimatosas, cordão umbilical, regeneração do nervo, geleia de Wharton, sistema nervoso, células do tipo neuroglial, Engenharia de Tecidos, Medicina Regenerativa.

Abstract

Recent advances in nerve Tissue Engineering have greatly promoted the generation of nerve conduits, which may be implanted empty, or may be filled with growth factors and/or cellular systems. Stem cells can be loosely classified into 3 broad categories based on their time of isolation during ontogenesis: embryonic, fetal and adult. Recent years have witnessed an explosion in the number of adult stem cells populations isolated and characterized. While still multipotent, adult stem cells have long been considered restricted, giving rise only to progeny of their resident tissues. Recently, and currently controversial studies have challenged this dogma, suggesting that adult stem cells may be far more plastic than previously appreciated. Extra-embryonic tissues as stem cell reservoirs offer many advantages over both embryonic and adult stem cell sources. Extra-embryonic tissues, are routinely discarded at parturition, so little ethical controversy attends the harvest of the resident stem cell populations. Most significantly, the comparatively large volume of extra-embryonic tissues and ease of physical manipulation theoretically increases the number of stem cells that can be isolated. Cord blood represents the prototypical fetal stem cell source. Hematopoietic stem cells isolated from cord blood have been extensively studied and have demonstrated clinical utility including central and peripheral nervous system regeneration. On the other hand, MSCs have become one of the most interesting targets for Tissue Regeneration including the peripheral nerves. Like bone marrow stromal cells and other mesenchymal cells, they are plastic adherent, stain positively for markers of the mesenchymal (CD10, CD13, CD29, CD44, CD90, and CD105) and negatively for markers of the hematopoietic lineage. These cells are capable of self-renewal with sustained proliferation in vitro and can differentiate into multiple mesodermal cells, including neuroglial-like cells. The high plasticity and low immunogenicity of these cells turn them into a desirable form of cell therapy for the injured nervous system without requiring the use of immunosuppressive drugs during the treatments. MSCs have been isolated from various other origins, including skin, hair follicle, periosteum, amniotic fluid, umbilical cord blood and adipose tissue. Harvest and expansion of MSCs are straight forward techniques, which are not only feasible regarding the laboratory approach but also regarding their application with the patient. With appropriate stimuli and environmental conditions MSCs exhibit a respectable plasticity. MSCs support hematopoiesis and enhance the engraftment after co-transplantation. Currently, bone marrow represents the main source of MSCs. However

the number of bone marrow MSCs significantly decreases with age and the HLA compatible donors are very difficult to find, which makes the search for adequate alternative sources necessary for autologous and allogenic use. A recently reported potential alternative tissue source of MSCs is the connective tissue (Wharton's Jelly) of human umbilical cord (UC) which has been tested by our research group in axonotmesis lesions. The isolation of these cells from the Wharton jelly, their expansion and their characterization has been performed. Also great concern has been dedicated to the collection, preservation and transport protocols of the umbilical cord from the Hospital after the parturition to the laboratory in order to obtain samples with higher number of viable MSCs. A multidisciplinary team has a crucial role in the development of these biomaterials associated to cellular systems, and in pre-clinical tests. The membranes associated to undifferentiated and differentiated MSCs from the Wharton's jelly of the umbilical cord have been tested in the rat sciatic nerve in a 3 mm axonotmesis lesion. The MSCs implanted into the injured nerve may produce growth factors or extracellular matrix (ECM) molecules, or may modulate the inflammatory process, to improve nerve regeneration. MSCs play an important role through the modulation of the inflammatory process, crucial for an appropriate Wallerian degeneration, the first step for the regeneration process of the peripheral nerve after injury. For the crush injury, a non-serrated clamp exerting a force of 54N is used for a period of 30 seconds to create a 3 mm long crush injury. The biomembranes covered by MSCs in vitro differentiated into neural cells have been used. In other experimental groups, the undifferentiated MSCs cells were applied by local infiltration (10^4cells/lesion) and the lesion was involved by the biomaterial in both axonotmesis e neurotmesis lesions. Video recording of the gait for biomechanical analysis, measuring extensor postural thrust (EPT), and sciatic functional index (SFI) permit to evaluate the motor recovery in different therapeutic approaches. The sensitive recovery, on the other hand, is tested by the withdrawal reflex latency (WRL). Repaired nerves processed for light, confocal and electronic microscope analysis, imunohistochemistry, and stereological studies permit the morphologic recovery evaluation. The isolation, expansion and differentiation protocols of MSCs from Wharton's jelly to future clinical use in peripheral nerve reconstruction, have been developed by our research group and other groups through in vitro studies that include citocompatibility evaluation of the biomaterials used and full characterization of the cells, by colony-forming units (CFU) frequency, multi-lineage differentiation capacity and phenotype characterization by imunocytochemistry, flow cytometry and RT-PCR. Also during MSCs expansion and differentiation into neuroglial-like cells, the culture medium was collected for nuclear magnetic resonance (NMR) analysis in order to evaluate the metabolic profile. In vitro

analysis showed the differentiation of MSCs into neuroglial-like cells, positively stained for the specific markers GFAP, GAP-43 and NeuN. NMR revealed that MSCs expansion is glycolysis-dependent but their differentiation requires the switch of the metabolic profile to oxidative metabolism. The *in vivo* testing allowed biocompatibility analysis of the biomaterials, evaluation of the functional and morphologic recovery of the animals subjected to different scaffolds treatments in neurotmesis e axonotmesis lesions. These studies showed enhanced recovery of motor and sensory function in animals treated with MSCs and neuroglial-like cells that was accompanied by an increase in myelin sheath of the regenerated fibers. MSCs isolated from the Wharton's jelly of the UC delivered through biomaterials membranes might thus be regarded a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, such as digital nerves. In addition, these cells represent a non controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses, including nerve injuries like axonotmesis and neurotmesis. Also, the importance of a longitudinal and complete study concerning tissue engineering of the peripheral nerve, which includes a multidisciplinary team able to develop biomaterials, to prepare cellular cultures for cell therapies, and to elaborate in vitro analysis and pre-clinical trials concerning animal welfare and the most appropriate animal model is enhanced.

Key words

Axonotmesis, biomaterials, stem cells, mesenchymal stem cells, umbilical cord, nerve regeneration, Wharton's jelly, nervous system, neuroglial-like cells, Tissue Engineering, Regenerative Medicine.

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Lista de abreviaturas

%	percentagem
°	Graus
°C	graus Celsius
µm	Micrómetro
3D	três dimensões
ASST	Autoridade para os Serviços de Sangue e da Transplantação
ATP	Trifosfato de Adenosina
ATPase	Adenosina Trifosfatase
AVC	acidente vascular cerebral
BDNF	Fator Neurotrófico Derivado do Cérebro
Ca^{2+}	iões de cálcio
cAMP	adenosina monofosfato cíclico
CAMs	Moléculas de Adesão Celular
CD	Complexo de Diferenciação
CFU	unidades formadoras de colónias
cm	centímetro
cm^3	centímetro cúbico
CO_2	forma molecular do dióxido de carbono
DGV	Direção Geral de Veterinária
DMSO	Dimetilsulfóxido
DPBS	*Dulbecco's Phosphate-Buffered Saline*
EPT	Reação postural de extensão
ERPE	Reação Postural de Extensão do Membro Experimental

FACT	Fundação para a Acreditação de Terapia Celular
FCUP	Faculdade de Ciências da Universidade do Porto
FDA	*Food and Drug Administration*
FEUP	Faculdade de Engenharia da Universidade do Porto
g	Grama
GAP-43	*Growth Associated Protein 43*
GDNF	Doença do Transplante Contra o Hospedeiro
GFAP	*Glial fibrillary acidic protein*
GVHD	*Graft-versus-host disease*
h	hora
H&E	hematoxilina-eosina
HBSS	*Hank's Balanced Salt Solution*
HLA	*human leukocyte antigen*
HPSC	Células Estaminais e Progenitoras Hematopoiéticas
I&D	Investigação e desenvolvimento
ICAM-1	Molécula-1 de Adesão Celular
ICBAS-UP	Instituto de Ciências Biomédicas Abel Salazar - Universidade do Porto
IGF	Fator de Crescimento *Insulin-Like I*
ISCT	Sociedade Internacional de Terapia Celular
ITS	Distância do segundo ao quarto dedo
K^+	iao de potássio
LFA-1	Antigeno-1 Linfocitário Associado a Função
LRF	Avaliação da latência do reflexo flexor
MAC-1	Antígeno de Macrófago 1

MAC-3	Recetor de complemento-3
MAO	Enzima Mitocondrial Monoamina Oxidase
MEC	moléculas da matriz extra celular
MHC	Complexo Maior de Histocompatibilidade
min	minuto
mL	mililitro
mm	milímetro
mm/24h	milímetro por 24 horas
mm/dia	milímetro por dia
mmHg	Milímetro de Mercúrio
ms	milissegundos
MSC	Células Estaminais Mesenquimatosas
mV	*Milivolt*
Na^+	ião de sódio
NaCl	Cloreto de sódio
NeuN	*neuronal nuclear antigen*
NGF	Fator de Crescimento do Nervo
nm	Nanómetro
NRPE	Reação Postural de Extensão do Membro Normal
NT	Neurotrofina
OECs	Células da mucosa olfativa
PL	Distância do calcanhar ao terceiro dedo, comprimento da pegada
PLC	poli(DL-lactide-ε-caprolactona)
PLGA	Copolímero de Ácido Láctico e Ácido Glicólico

PVA	ácido poli-vinílico
REFR	refrigerado
RMN	ressonância magnética nuclear
SBF	soro bovino fetal
SCU	sangue do cordão umbilical
SFI	Índice de Funcionalidade do Ciático
SNC	sistema nervoso central
SNP	sistema nervoso periférico
SSI	Índice de Funcionalidade do Ciático em Condições Estáticas
T3	Triodotironina
TA	temperatura ambiente
TCU	Tecido do cordão umbilical
TS	Distância do primeiro ao quinto dedo
WRL	Avaliação da Latência do Reflexo Flexor

Capítulo I

Revisão Bibliográfica

1. Introdução à Medicina Regenerativa

A evolução recente da Medicina Regenerativa tem sido bastante relevante provindo daí o aparecimento de novos conceitos e descobertas. Antes deste aumento de conhecimento, os tecidos biológicos eram considerados como incapazes de regeneração extensa, no entanto hoje em dia os órgãos e tecidos (como o cérebro, medula espinal ou músculos cardíacos) aparecem como capazes de regenerar baseado em *know-how* da Engenharia de Tecidos e das Terapias celulares (Triffitt, 2002).

Vários estudos com células estaminais têm sido realizados a nível internacional, relativamente a procedimentos clínicos para o tratamento de doenças e numa tentativa de melhorar a saúde e a expectativa de vida. Ao longo dos anos verifica-se uma evolução até na própria definição de célula estaminal. Em 1967, Lajtha afirmava que as células estaminais adultas podem ser encontradas somente em órgãos regenerativos, tais como sangue, intestino, osso, cartilagem e pele. Atualmente, considera-se que estas células existem mesmo em tecidos sem compromisso de regeneração, como no sistema nervoso central (SNC) (Lajtha, 1967; Triffitt, 2002).

2. Sistema Nervoso

No corpo humano existe uma grande variedade de células e para coordenar as suas funções temos dois grandes sistemas de controlo: o sistema endócrino e o sistema nervoso. O primeiro pode ser descrito como sendo um conjunto de mensageiros denominados glândulas (segregam hormonas) que são transportadas pelo sangue e que atuam de forma lenta. O sistema nervoso ao contrário do anterior é um sistema de controlo rápido (Widmaier *et al*., 2006).

No fim do século XIX, surgiu a primeira geração de neurobiólogos que reconheceram a natureza unitária da célula nervosa, com a ajuda dos microscópios e técnicas de coloração disponíveis. O trabalho pioneiro realizado pelo ilustre médico e biólogo italiano Camilo Golgi, no qual eram realizados estudos sobre o sistema nervoso humano através do reconhecimento do primeiro organelo celular que atualmente possui o nome de Complexo de Golgi, defendia que as células nervosas se encontravam conectadas com as células vizinhas através de uniões protoplasmáticas, formando uma malha contínua de neurónios, chamada retículo (Purves *et al*., 2005). Contudo esta teoria desenvolvida por Camilo Golgi, foi rapidamente contrariada pelas

investigações feitas pelo neuroanatomista espanhol Santiago Ramón y Cajal que defendia que os neurónios comunicavam entre si através de contactos especializados, os quais viriam a ser chamados de sinapses. Baseados nas teorias contraditórias destes dois investigadores surgem os primeiros debates das neurociências modernas. Este trabalho, iniciado por Golgi e Ramón y Cajal, marcou a neurociência, e por isso foram ambos os investigadores reconhecidos, em 1906, pela entrega do Prémio Nobel de Fisiologia e Medicina. Nesta altura, juntamente com a colaboração de outros investigadores tornou-se consensual que o sistema nervoso era composto por duas classes de células: células nervosas ou neurónios e células de apoio, de suporte ou neuroglia (Purves *et al*., 2005).

O sistema nervoso é dividido funcional e estruturalmente em Sistema Nervoso Central e Sistema Nervoso Periférico. O SNC é formado pelo encéfalo e medula espinal representando a maior parte de todo o sistema nervoso. Já o SNP é formado pelos nervos e por pequenos agregados de células nervosas denominados gânglios nervosos (Figura 1) (Junqueira & Carneiro, 2004). As células neurogliais do SNC são os astrócitos, oligodendritos e as células microgliais, enquanto que as células do SNP são as células de Schwann (Purves *et al*., 2005). O SNP tem como função a comunicação entre o SNC e o ambiente (Seeley *et al*., 1995).

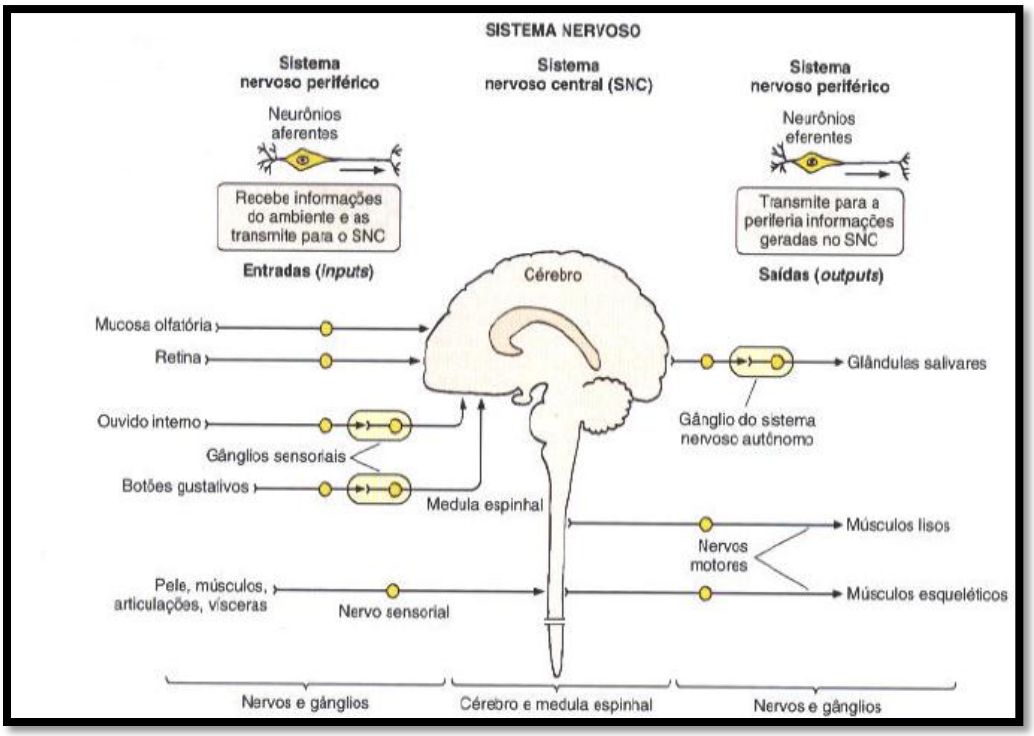

Figura 1 - Desenho esquemático e simplificado mostrando a organização funcional do sistema nervoso central e periférico (adaptada de Junqueira & Carneiro, 2004).

O SNP recebe estímulos e depois faz a tradução para informações úteis, na forma de potenciais de ação, transmitindo estas informações para o SNC (Hiatt & Gartner, 1997). Esta transmissão é feita de forma rápida através de finas fibras nervosas e por isso o sistema nervoso é capaz de integrar funções motoras, sensitivas e cognitivas dos seres humanos e animais (Trapp, 2004). O tecido nervoso é composto por dois componentes principais: os neurónios (células geralmente com dois prolongamentos) e vários tipos de células da glia ou neuroglia em que uma das suas principais funções é sustentarem os neurónios. Os neurónios são denominados de células "excitáveis", devido à sua capacidade de responder a alterações do meio em que se encontram (estímulos) com modificações da diferença de potencial elétrico existente entre as superfícies externa e interna da membrana celular (Junqueira & Carneiro, 2004). A modificação do potencial pode restringir-se ao local do estímulo ou propagar-se ao resto da célula, através da membrana e esta propagação constitui o que se denomina impulso nervoso, cuja função é transmitir informações a outros neurónios, a músculos ou a glândulas. Os prolongamentos dos neurónios normalmente longos e numerosos formam circuitos neuronais que podem ser de diversos tamanhos e ter vários graus de complexidade. Em vez de ser simples, um circuito neuronal geralmente é a combinação de dois ou mais circuitos que interagem para executar uma função. Muitos circuitos elementares (simples) comunicam entre si em grau crescente de complexidade para executar funções cada vez mais complexas (Junqueira & Carneiro, 2004).

Os neurónios são formados por um corpo celular ou pericário, que contém o núcleo e do qual saem prolongamentos. Normalmente, o volume total dos prolongamentos de um neurónio é maior do que o volume do corpo celular (Junqueira & Carneiro, 2004).

O neurónio é formado pelos seguintes componentes principais (Figura 2):

- Axónio - prolongamento único, especializado na condução de impulsos que transmitem informações do neurónio para outras células (nervosas, musculares, glandulares);
- Corpo celular ou pericário - é o centro trófico da célula, contém o núcleo e o citoplasma e pode apresentar formas do tipo esférico, piriforme ou anguloso e é capaz de receber estímulos;
- Dendritos - são prolongamentos citoplasmáticos especializados em receber os estímulos do meio ambiente, de células epiteliais sensoriais ou de outros neurónios e aumentam muito a superfície recetora dos neurónios, proporcionando a captação de uma grande variedade de impulsos (Junqueira & Carneiro, 2004);

- Telodendritos - são as porções terminais dos axónios nos quais existem umas pequenas dilatações designadas por botões terminais ou sinápticos através dos quais o axónio estabelece comunicação com dendritos, axónios ou corpo celular de outros neurónios - sinapse (Varejão, 2003).

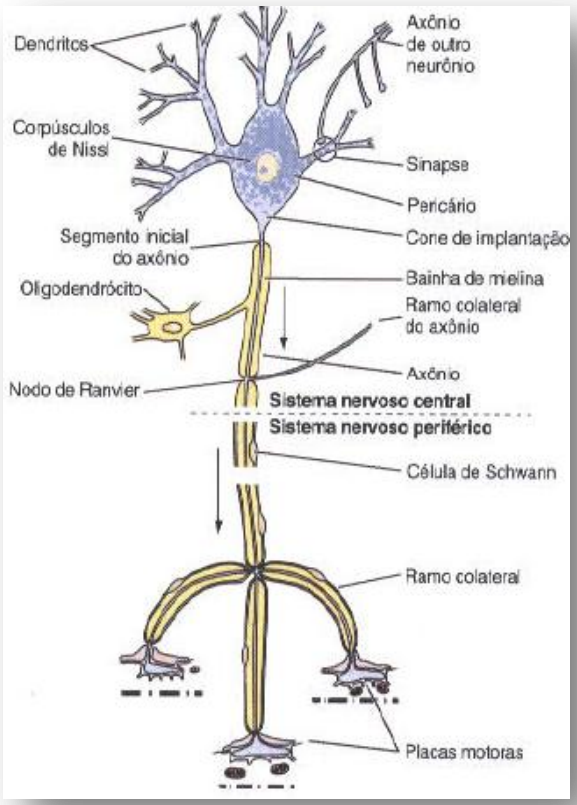

Figura 2 - Neurónio motor. A mielina que envolve o axónio no SNC é produzida pelos oligodendrócitos e no SNP pelas células de Schwann. O corpo celular do neurónio contém um núcleo grande, com um nucléolo bem visível e é de cor clara. O pericário contém corpúsculos de Nissl encontrados também nos dendritos mais grossos. Na parte superior direita é mostrado um axónio de outro neurónio, com três botões terminais, um dos quais faz sinapse com o neurónio principal. O axónio deste neurónio termina em três placas motoras que transmitem o impulso nervoso para as fibras musculares estriadas esqueléticas. As setas indicam a direção do impulso nervoso (adaptada de Junqueira & Carneiro, 2004).

Os neurónios e os seus prolongamentos variam muito em termos de tamanho e forma. Normalmente, as células nervosas são grandes, em que o corpo celular pode medir até 150µm. Uma célula com esta dimensão, quando isolada, é visível sem necessidade de recorrer a ampliação microscópica ou de uma lupa. Contudo existem neurónios muito pequenos como é o caso dos neurónios denominados células granulosas do cerebelo que são das menores células que os mamíferos têm, em que o seu corpo celular mede apenas de 4 a 5µm de diâmetro (Junqueira & Carneiro, 2004).

Quanto à sua morfologia, isto é, em número de processos que emergem do seu corpo celular, os neurónios podem ser assim classificados:

- Neurónios bipolares - com dois prolongamentos, um dendrito e um axónio e são típicos do sistema visual, auditivo e vestibular;
- Neurónios multipolares - com mais de dois prolongamentos e que são os mais abundantes no SNC;
- Neurónios pseudo-unipolares - que apresentam, próximo do corpo celular um prolongamento único que se divide em dois, em que um ramo se dirige à periferia e outro ao SNC (Junqueira & Carneiro, 2004; Kierszenbaum, 2004).

Os neurónios podem ainda ser classificados segundo a sua função:

- Neurónios motores - controlam órgãos efetores (glândulas exócrinas e endócrinas e fibras musculares) e que se encontram localizados na substância cinzenta ventral da medula espinal;
- Neurónios sensoriais - recebem estímulos sensoriais do meio ambiente e do próprio organismo e encontram-se localizados nos gânglios da raiz dorsal;
- Interneurónios - estabelecem conexões entre outros neurónios, formando circuitos complexos (Junqueira & Carneiro, 2004).

2.1. Sistema nervoso periférico

O SNP é composto por nervos, gânglios e terminações nervosas. Os nervos são feixes de fibras nervosas envolvidas por tecido conjuntivo (Junqueira & Carneiro, 2004). No SNP, um conjunto de neurónios forma um gânglio, o qual pode ser sensitivo (gânglio de raiz dorsal e gânglio do trigémio) ou motor (gânglio visceromotor ou autónomo). Os axónios derivados de um gânglio organizam-se em nervos, ramos ou raízes (Kierszenbaum, 2004).

Este sistema abrange todos os neurónios externos ao cérebro e à medula espinal. Os nervos periféricos são os nervos cranianos e espinais (Kierszenbaum, 2004). Por exemplo, no Homem, os nervos periféricos são 12 pares de nervos cranianos (do encéfalo) e 31 pares de nervos raquidianos (da medula espinal) (Seeley *et al*., 1995).

2.2. Estrutura do nervo periférico

No SNP as fibras nervosas estão agrupadas em feixes, originando assim os nervos. A cor esbranquiçada dos nervos deve-se ao facto de o seu conteúdo ser de mielina e colagénio, exceto nos raros nervos muito finos formados somente por fibras amielínicas. Os nervos são os responsáveis por estabelecerem a comunicação entre os centros nervosos e os órgãos sensitivos e efetores (músculos e glândulas) e estes podem possuir fibras aferentes, fibras eferentes ou as duas. Os que possuem apenas fibras de sensibilidade (aferentes), que são aquelas que levam para os centros as informações obtidas no interior do corpo e no meio ambiente, são chamados de nervos sensitivos. Os denominados de nervos motores são aqueles que são formados apenas por fibras que levam a mensagem dos centros para os efetores. A maioria dos nervos possui fibras dos dois tipos, sendo, portanto, nervos mistos, tendo fibras mielínicas e amielínicas (Junqueira & Carneiro, 2004).

Os nervos possuem três invólucros de tecido conjuntivo (Figura 3): o epineuro, o perineuro e o endoneuro.

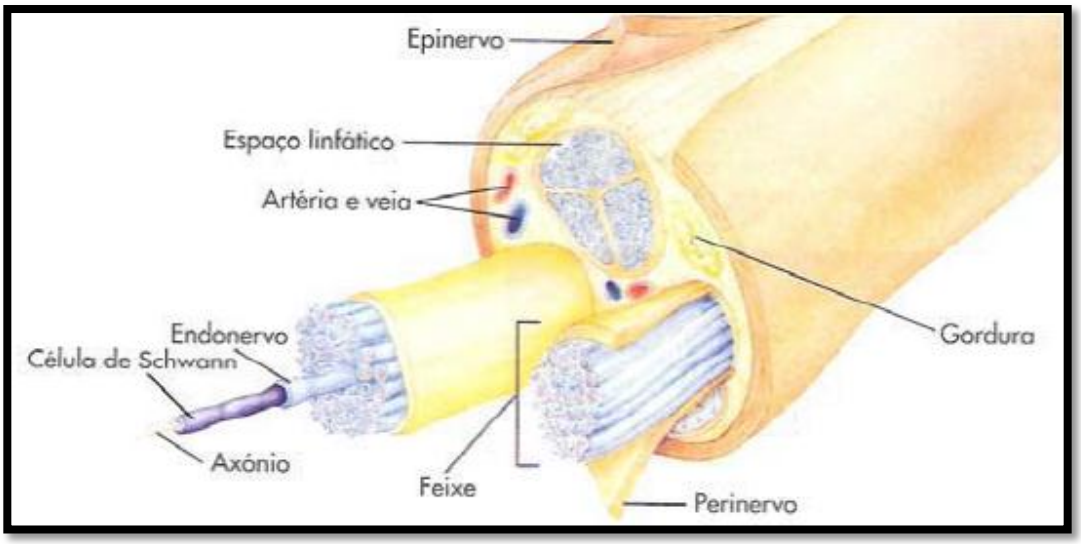

Figura 3 - Representação da estrutura do nervo periférico (adaptada de Seeley *et al*., 1997).

O epineuro (Figura 3) é composto por colagénio do tipo I e por fibroblastos e é caracterizado por ser uma camada fibrosa, de tecido conjuntivo denso, que reveste externamente todo o nervo, preenchendo os espaços entre os feixes de fibras nervosas. Também contém tecido adiposo e vasos sanguíneos – os *vasa nervorum*, através dos quais se dão as trocas de nutrientes que caracterizam qualquer tecido vivo. Estes formam um plexo vascular bem desenvolvido com numerosos vasos

longitudinais, cuja função é suprimir os capilares do endoneuro (Sunderland, 1978; Junqueira & Carneiro, 2004; Kierszenbaum, 2004).

O perineuro (Figura 3) é uma bainha de várias camadas de células achatadas e justapostas que revestem cada um dos feixes de fibras nervosas (Junqueira & Carneiro, 2004). Este separa os axónios em fascículos e é composto por camadas concêntricas de fibroblastos, que se encontram unidos entre si por junções de oclusão para formar uma barreira de proteção à passagem de macromoléculas – barreira hemato-neural. As células do perineuro criam um isolamento, mas que não é completo a nível estrutural, pois existem pequenos vasos sanguíneos que o penetram, acompanhados por algumas fibras de colagénio, dispostas longitudinalmente, que contribuem para a resistência do perineuro. Para ocorrer a rotura do nervo é necessária uma pressão intrafascicular de cerca de 750mmHg, mostrando assim a elevada força mecânica do perineuro (Selander & Sjöstrand, 1978). Assim, o perineuro tem como responsabilidade responder em termos de resistência elástica aos possíveis acidentes de estiramento (Sunderland & Bradley., 1961).

O endoneuro (Figura 3) envolve os axónios individuais e as células de Schwann associadas e é composto por colagénio de tipo II e alguns fibroblastos contendo ainda os capilares. Estes capilares do endoneuro derivam dos *vasa nervorum* e são revestidos por células endoteliais unidas por junções de oclusão e fazendo parte integrante da barreira hemato-neural (Junqueira & Carneiro, 2004; Kierszenbaum, 2004).

2.2.1. Fibras nervosas

Fibra Nervosa é a designação dada ao conjunto formado pelo axónio, bainha de mielina e células de Schwann que se unem em diferentes dimensões, número e padrão, dando origem a fascículos, que por sua vez se organizam em nervos (Sunderland, 1978; Junqueira & Carneiro, 2004). As fibras nervosas do SNP transmitem sinais entre o SNC, recetores e efetores em todas as partes do corpo (Widmaier *et al*., 2004). As fibras nervosas individuais do SNP são envolvidas pelas células de Schwann formando as fibras mielínicas e amielínicas. No caso das primeiras, uma célula de Schwann envolve apenas um axónio e um axónio é envolvido por mais do que uma célula de Schwann. As células de Schwann são responsáveis pela formação de um conjunto de camadas concêntricas em torno do axónio que é designada por bainha de mielina. No caso das fibras amielínicas, uma célula de Schwann é capaz de envolver mais do que um axónio, que por norma são de menor

diâmetro que os anteriores e são envolvidos apenas por uma dobra de célula circundante (Junqueira & Carneiro, 2004). A bainha de mielina é um complexo lipoproteico que é geralmente removido pelas técnicas histológicas de rotina, mas caso seja esse o objetivo pode ser demonstrada quando fixada e embebida pelo tetróxido de ósmio, conferindo-lhe uma coloração negra. É constituída por lípidos (fosfolípidos, glicolípidos e colesterol) que representam cerca de 70% dos seus constituintes e também por proteínas em que cerca de 60% destas pertencem ao grupo das glicoproteínas (Wolman, 1992). A sua formação é da responsabilidade do axónio, pois é este que envia o sinal à célula de Schwann, no sentido de produção de uma quantidade detetável de glicolípidos e glicoproteínas que formam a bainha de mielina (Mirsky *et al.*, 1980).

Os nódulos de Ranvier são os espaços entre secções adjacentes da bainha de mielina, em que a membrana do axónio fica exposta ao fluido extracelular (Widmaier *et al*., 2004). O intervalo entre dois nódulos de Ranvier é designado de internódulo e é recoberto por uma única célula de Schwann. Como é de esperar, nas fibras amielínicas não existem nódulos de Ranvier uma vez que nelas as células de Schwann formam uma bainha contínua, sem interrupções (Junqueira & Carneiro, 2004).

2.2.2. Sinapses e Potencial da membrana

As sinapses são estruturas extremamente especializadas, onde a transmissão do impulso nervoso de um neurónio para outro depende destas. A terminação sináptica é especializada na transmissão química em resposta a um potencial de ação. Uma sinapse pode assim ser caracterizada como sendo a junção entre a terminação pré-sináptica de um axónio e a superfície recetora da membrana pós-sináptica que normalmente é de um dendrito (sinapses axodendríticas). Contudo existem outros tipos de sinapses menos frequentes: axoaxónicas (entre neurónios), dendrodendríticas (entre dendritos) e axossomáticas (entre axónio e corpo celular). Uma fenda sináptica é um espaço com cerca de 20 a 30nm que se situa entre as membranas de dois neurónios que estabelecem uma sinapse (Junqueira & Carneiro, 2004).

Em vesículas sinápticas são armazenados os neurotransmissores (acetilcolina, glutamato, ácido δ-aminobutírico – GABA, entre outros) que são os mensageiros químicos dos neurónios, e são transportados até à terminação sináptica por transporte anterógrado, mediado pela cinesina. A membrana da vesícula sináptica possui proteínas de ancoragem vesicular que se ligam às proteínas de ancoragem de

membrana, da membrana pré-sináptica. A condução axonal do impulso é proporcionada devido às modificações nos canais iónicos da membrana do axónio, o que causa a entrada de Na^+ e saída de K^+ com gasto de energia (ATP). Surge uma acumulação de iões positivos na superfície interna, devido ao facto da entrada de Na^+ ser maior que a saída de K^+, fazendo com que a membrana externa se torne carregada negativamente. O potencial de repouso é quando um axónio está, como o nome indica, em repouso e existe neste caso uma diferença de potencial de -90 mV entre o interior e o exterior da membrana. Quando um neurónio é excitado, ocorre o chamado potencial de ação ou impulso nervoso, onde o interior da membrana se torna positiva (Shumway-Cook & Woollacott, 2001), com uma voltagem intracelular de +35 mV (despolarização) (Junqueira & Carneiro, 2004). Os potenciais de ação têm uma duração muito curta de cerca de 1ms e ocorrendo repolarização imediatamente depois (Shumway-Cook & Woollacott, 2001). O potencial de ação é uma sequência de uma despolarização e uma repolarização da membrana celular do neurónio (Correia *et al*., 2003).

A despolarização da terminação do axónio advém de uma alta concentração de Ca^{2+}, transportado através de canais de Ca^{2+} sensíveis à voltagem, para dentro da terminação do axónio. Este aumento da concentração de Ca^{2+} leva à exocitose da vesícula sináptica. O mensageiro químico é assim libertado na fenda sináptica e vai ligar-se a um recetor (colinérgico ou adrenérgico) na membrana pós-sináptica de forma a transmitir a informação. O mensageiro químico pode ser degradado enzimaticamente na fenda sináptica (acetilcolina pela acetilcolonesterase) ou então ser captado por endocitose mediada pelo recetor (norepinefrina) e mais tarde degradado pela enzima mitocondrial monoamina oxidase (MAO) (Kierszenbaum, 2004). Caso a união entre o neurotransmissor e o recetor tenha efeitos de excitação são chamadas de sinapses excitatórias mas se pelo contrário tiver efeitos de inibição são sinapses inibitórias (Junqueira & Carneiro, 2004).

2.2.3. Transporte axonal

Cada neurónio tem um único axónio, que é um cilindro de comprimento e diâmetros variáveis conforme o tipo de neurónio. Normalmente o axónio é mais comprido que os dendritos e por exemplo no Homem os axónios das células motoras da medula espinal que enervam o pé, podem ter cerca de um metro de comprimento (Junqueira & Carneiro, 2004).

O axónio tem um citoplasma muito rico em neurofilamentos, porém é muito pobre em mitocôndrias, reticulo endoplasmático liso e microtúbulos. O centro de produção de proteínas é o corpo celular ou pericário. Transporte axonal é um movimento bidirecional muito ativo de proteínas, neurotransmissores, lípidos, mitocôndrias e outros organelos ao longo dos axónios (Varejão, 2003; Junqueira & Carneiro, 2004; Kierszenbaum, 2004). É um transporte ativo que implica gasto de energia, mediado pelas ATPases (cinesina e dineína) que quebram uma ligação da molécula de adenosina trifosfato (ATP), libertando energia (Junqueira & Carneiro, 2004).

O transporte anterógrado é caracterizado pelo movimento de substâncias no sentido do corpo celular em direção às terminações axonais e é mediado pela cinesina (Kierszenbaum, 2004). Este tipo de transporte existe em duas correntes principais:

i. Rápida: inclui vesiculas sinápticas, enzimas de síntese e precursores de neurotransmissores (20-410mm/dia);
ii. Lenta: transporta uma maior variedade e quantidade de elementos, nomeadamente proteínas (0.1-30 mm/dia) (Grafstein & Forman, 1980; Wang & Brown, 2002).

O transporte retrógrado é o movimento de substâncias no sentido do axónio em direção ao corpo celular e é mediado pela dineína (Kierszenbaum, 2004), tendo como principal função a transmissão de informação do estado de funcionamento do axónio e das suas células alvo para o corpo celular do neurónio (Grafstein, 1975). São transportadas diversas moléculas (fatores de crescimento) para serem recicladas pelo corpo celular e também material captado por endocitose (vírus e toxinas). Em neurofisiologia este tipo de transporte é utilizado para estudar o trajeto das fibras nervosas, utilizando para tal marcadores que se injetam nas regiões terminais dos axónios (peroxidase, por exemplo) (Junqueira & Carneiro, 2004; Kierszenbaum, 2004).

2.3. Fisiopatologia das lesões do nervo periférico

2.3.1. Classificação das lesões do nervo periférico

As lesões a que o SNP está sujeito são provenientes de acidentes quer de origem externa (acidentes de viação, queimaduras, etc…) quer de origem interna (neoplasias, compressões, causas iatrogénicas, etc…). É necessário que haja um bom conhecimento acerca das estruturas anatómicas que compõem o nervo, devido à sua função ativa nos processos fisiológicos, moleculares e celulares, beneficiando a caracterização do tipo de lesão e a sua gravidade (Burnett & Zager, 2004).

Anatomicamente, o SNP encontra-se menos protegido que o SNC, daí ocorrerem mais frequentemente lesões no nervo periférico. Várias classificações têm sido propostas para estes tipos de lesão, nomeadamente por Seddon e Sunderland, dois investigadores muito importantes na área do estudo da cirurgia do nervo periférico (Seddon, 1943; Sunderland & Bradley, 1961; Seddon, 1972; Sunderland, 1978; Sunderland, 1990) (Figura 4).

Para Seddon a classificação era realizada segundo três categorias: Neuropraxia, Axonotmese e Neurotmese (Seddon, 1943; Seddon, 1972). Contudo para Sunderland a classificação incluía cinco categorias: Tipo I até Tipo V de acordo com níveis superiores de gravidade de lesão (Sunderland & Bradley, 1961; Sunderland, 1978; Sunderland, 1990).

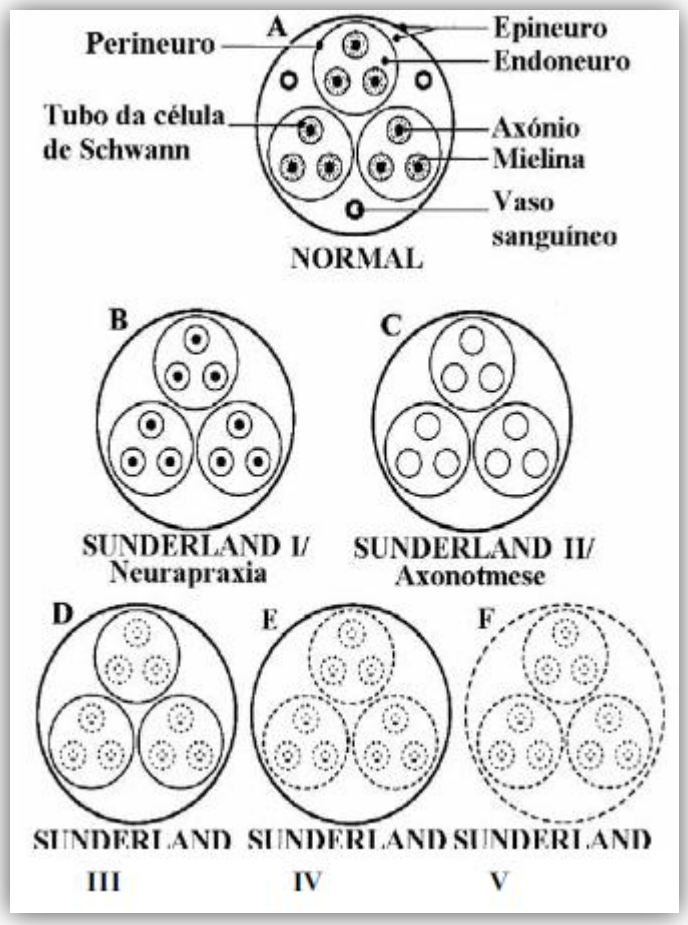

Figura 4 - Classificação das lesões do nervo periférico: **(A)** Nervo Normal; **(B)** Lesão de Sunderland tipo I ou Neuropraxia (Seddon) - em que os fascículos ainda se mantêm intactos e apenas desaparece a bainha de mielina; **(C)** Lesão de Sunderland tipo II ou Axonotmese (Seddon) – em que apenas são preservados os tubos do endoneuro, desaparecem os axónios e a bainha de mielina; **(D)** Lesão de Sunderland tipo III – desaparece o endoneuro e é preservado o perineuro; **(E)** Lesão de Sunderland tipo IV – desaparece o perineuro e é preservado o epineuro; **(F)** Lesão de Sunderland tipo V ou Neurotmese (Seddon) – em que há secção completa de todo o nervo. (adaptada de Lundborg *et al*., 1991 e gentilmente cedida por Prof. Artur Varejão, 2003)

2.3.1.1. Neuropraxia ou lesão de Sunderland Tipo I

Neste tipo de lesão existe apenas uma compressão ou estiramento da fibra nervosa, onde a continuidade axonal é mantida preservando o ótimo alinhamento dos axónios e ocorrendo normalmente danificação ao nível das bainhas de mielina (Figura 4B). Clinicamente, esta lesão traduz-se numa paralisia motora e em que geralmente as fibras sensitivas são poupadas, fazendo com que a recuperação da condução nervosa ocorra geralmente de forma espontânea ao fim de algumas semanas. Logo é considerado o tipo de lesão do nervo periférico menos grave (Spiegel *et al*., 1993).

Temos como exemplo desta lesão em Medicina Humana as mononeuropatias compressivas agudas como é o caso da paralisia de *Sábado à Noite* e da paralisia da *Perna Cruzada*. No caso da primeira ocorre uma paralisia radial no sulco espiral do úmero enquanto na segunda ocorre uma paralisia fibular na cabeça da fíbula. Ambas têm uma recuperação de apenas algumas semanas e o prognóstico é extremamente favorável (Luís, 2008).

2.3.1.2. Axonotmese ou lesão de Sunderland Tipo II

A axonotmese é um tipo de lesão muito semelhante à anterior, havendo a diferença da compressão ou tração da fibra nervosa causar uma perda de continuidade axonal e portanto degenerescência Walleriana, permanecendo ainda intacto o tubo do endoneuro (Figura 4C). A recuperação desta lesão é geralmente mais demorada quando em comparação com a anterior, no entanto é ainda de prognóstico bastante favorável. Como o endoneuro não é danificado, este mantém toda a estrutura da fibra nervosa perfeitamente orientada, possibilitando que os axónios se direcionem de forma correta até aos órgãos, daí não ser necessária a implicação de uma intervenção cirúrgica. Porém é possível que haja danos clínicos irreversíveis tendo em conta várias razões: uma possível lesão do órgão alvo (atrofia muscular neurogénica) durante um período mais extenso de regeneração axonal ou por défices causados pela morte de corpos celulares que poderão ocorrer após a lesão axonal (Luís, 2008).

2.3.1.3. Neurotmese ou lesão de Sunderland Tipo III-IV

Este tipo de lesão é a que pode ser considerada como sendo o grau mais grave de lesão do nervo periférico. Estamos perante esta lesão sempre que que ocorre a rotura ou a transecção do axónio, mielina e componentes do tecido conjuntivo (Greenfield, 1997; Schwartz, 1999; Ristic *et al*., 2000). Em certas lesões deste grau a continuidade externa pode eventualmente ser preservada, contudo ocorre fibrose intraneural com bloqueio da regeneração axonal (Grant *et al*., 1999; Schwartz, 1999). Na neurotmese apesar de ocorrer regeneração, a função raramente é recuperada na totalidade (Greenfield, 1997).

Segundo Seddon (1943, 1972), uma lesão de neurotmese acarreta a perda da continuidade de todas as estruturas da fibra nervosa (axónios e elementos periféricos constituintes do nervo) (Figura 4D, 4E e 4F). Uma intervenção cirúrgica de reparação é sempre necessária nestas lesões pois permite a aproximação dos topos nervosos e a orientação dos axónios do topo proximal e topo distal. Esta aproximação e orientação são fundamentais para promover a recuperação funcional dos órgãos alvo. É importante que a cirurgia seja feita o mais rapidamente possível de forma a evitar a formação não só de neuromas no local da lesão mas também lesões irreversíveis dos órgãos alvo (em especial, dos músculos), por falta prolongada de funcionamento (atrofia). Por outro lado Sunderland classifica a lesão de neurotmese em três grupos de acordo com as estruturas lesadas (Sunderland, 1978):

i) Lesão de Sunderland tipo III – a única estrutura preservada é o perineuro.

ii) Lesão de Sunderland tipo IV – a única estrutura que permanece intacta é o epineuro.

iii) Lesão de Sunderland tipo V – nenhuma estrutura do nervo é preservada. O nervo é completamente seccionado resultando daí maiores ou menores graus de separação dos topos do nervo. Segundo esta classificação este último tipo é a lesão mais grave da fibra nervosa (Sunderland, 1978) e é aquela em que uma intervenção cirúrgica se torna sempre imprescindível (Luís, 2008).

2.4. Degenerescência e regeneração das fibras nervosas

Nos mamíferos a destruição de um neurónio representa uma grande perda, pois estes não têm capacidade de se dividirem. Contudo e dentro de certos limites os seus prolongamentos podem regenerar-se devido à atividade sintética dos respetivos pericários. Esta recuperação apenas é possível apenas quando o corpo celular ou

pericário são preservados. Quando uma célula nervosa é destruída, normalmente todas as outras células que estão inter-ligadas a esta, são preservadas, exceto nos raros casos em que um neurónio recebe impulsos unicamente de outro. Neste caso, o neurónio que estava dependente dos impulsos nervosos enviados pelo neurónio que foi destruído ou degenerou, sofre a chamada degeneração transneuronal. Por outro lado, as células da glia do SNC e as do SNP, nomeadamente as células de Schwann e as células satélites dos gânglios, são dotadas de grande capacidade de proliferação. Quando células ou fibras nervosas do SNC são destruídas por acidente ou doença, os espaços deixados são preenchidos por células da neuroglia (Junqueira & Carneiro, 2004).

Ao contrário do verificado com SNP, a regeneração do SNC em mamíferos adultos, é extremamente limitada e não espontânea após lesão, verificando-se persistência de défices funcionais aquando de uma lesão da medula espinal, traumatismo crânio-encefálico, acidente vascular cerebral (AVC) e condições relacionadas que envolvem a desconexão axonal. No caso do SNP, pode ocorrer mesmo após lesões de neurotmese, a regeneração axonal e recuperação funcional em adultos. Aguayo e seus colaboradores demonstraram que pelo menos alguns neurónios do SNC retêm a capacidade de regenerar quando implantados como um enxerto num nervo periférico (Richardson *et al*. 1980; David & Aguayo, 1981; Benfey & Aguayo, 1982; Richardson *et al*., 1984). Este trabalho sugere que o ambiente do SNP é estimulador e/ou que o ambiente do SNC é inibidor para o crescimento axonal e por isso, para a sua regeneração. Estudos posteriores identificaram fatores de promoção de regeneração no SNP e fatores de inibição da regeneração no SNC. Estes últimos incluem proteínas específicas da mielina do SNC e moléculas associadas com a cicatriz astroglial. Além disso, a remoção mais lenta de detritos celulares após lesão ou traumatismo no SNC em relação ao SNP podem impedir o re-crescimento axonal. Uma compreensão dos fatores que influenciam o crescimento axonal é crítico para o desenvolvimento de terapêuticas para promover a regeneração do SNC (Huebner & Strittmatter, 2009).

As lesões dos nervos não são raras devido ao facto de estarem distribuídas ao longo de todo o corpo. Quando um nervo é seccionado, ocorrem alterações degenerativas, seguidas de uma fase de reparação e deve-se distinguir a porção da fibra que, pela lesão, se desligou do seu neurónio - porção distal, e a porção que continua unida ao neurónio - porção proximal. O segmento proximal, por manter contacto com o pericário, que é o centro trófico, frequentemente é regenerado, enquanto o segmento distal degenera totalmente e acaba por ser reabsorvido (Junqueira & Carneiro, 2004). Este fenómeno que acontece no segmento distal é

chamado de degenerescência Walleriana e é algo similar à degenerescência que ocorre em determinadas neuropatias do nervo periférico de humanos (Glass, 2004).

Em 1816-1870, o fisiologista inglês Augustus Volney Waller descreveu a degenerescência Walleriana (Waller, 1850; Stoll *et al*., 2002; Koeppen, 2004), como sendo um conjunto de fenómenos que se seguem após uma lesão grave do axónio e que se iniciam pela degradação do axoplasma, acompanhados pela destruição da mielina que é removida pelas células de Schwann e por macrófagos que invadem o local. Nos dias de hoje, define-se degenerescência Walleriana como um processo intrínseco ativo do axónio associado a alguns princípios de apoptose (Beirowski *et al*., 2005) sendo fundamental para que ocorra posteriormente a regeneração axonal. Este fenómeno ocorre nos axónios do SNP e no SNC, sendo que no SNC é mais lenta que no SNP e aqui a idade do animal tem uma grande influência (Koeppen, 2004). Temos como exemplo o caso de um mamífero neonatal, a degenerescência Walleriana é tão rápida no SNC como no SNP (Koeppen, 2004). Desde a investigação pioneira de Waller sobre este fenómeno, muito se tem investigado sobre degenerescência nervosa ao longo de dois topos nervosos separados nomeadamente em que direção esta degenerescência ocorre (anterógrada, retrógrada ou em ambas as direções simultaneamente) e os fatores que a influenciam (Chaudhry *et al*., 1992; Rodríguez *et al*., 2004; Beirowski *et al*., 2005). A velocidade da degenerescência Walleriana depende da espessura da fibra nervosa e foi calculada em cerca de 45.6mm/24h para as fibras mais grossas e de cerca de 252mm/24h para as fibras mais finas (Koeppen, 2004). A temperatura é um dos fatores que influencia a velocidade, em que sob temperaturas mais altas verificam-se velocidades de degenerescência Walleriana mais rápidas. Merzbacher demonstrou que não foi encontrada degenerescência Walleriana no SNC em animais que foram mantidos a temperaturas baixas (Koeppen, 2004). A direção da degenerescência é determinada pelo tipo de lesão, em lesões de transecção é no sentido de proximal para distal enquanto em lesões de esmagamento é no sentido inverso (Beirowski *et al*., 2005). A capacidade regenerativa do nervo encontra-se cerca de 66% mais baixa numa axonotomia de longo termo quando comparada com lesões de transecção reparada de imediato (Gordon & Fu, 1997; Dhillon *et al*., 2004). Há estudos que sugerem, que um atraso na sutura de um nervo lesado pode ser benéfico para a regeneração axonal, no entanto, tal atraso é simultaneamente prejudicial devido ao prolongamento do período de desinervação muscular (Gordon *et al*., 2003).

A regeneração de um nervo periférico ocorre na taxa de aproximadamente 1mm/dia (Colohan *et al*., 1996; Greenfield, 1997; Grant *et al*., 1999; Ristic *et al*., 2000).

Nas lesões mais proximais, a recuperação pode não ser óbvia por muitos meses (Greenfield, 1997; Grant *et al*., 1999).

As figuras 5 e 6 explicam de modo esquemático as modificações que ocorrem nas fibras nervosas lesadas e nos respetivos pericários (Junqueira & Carneiro, 2004).

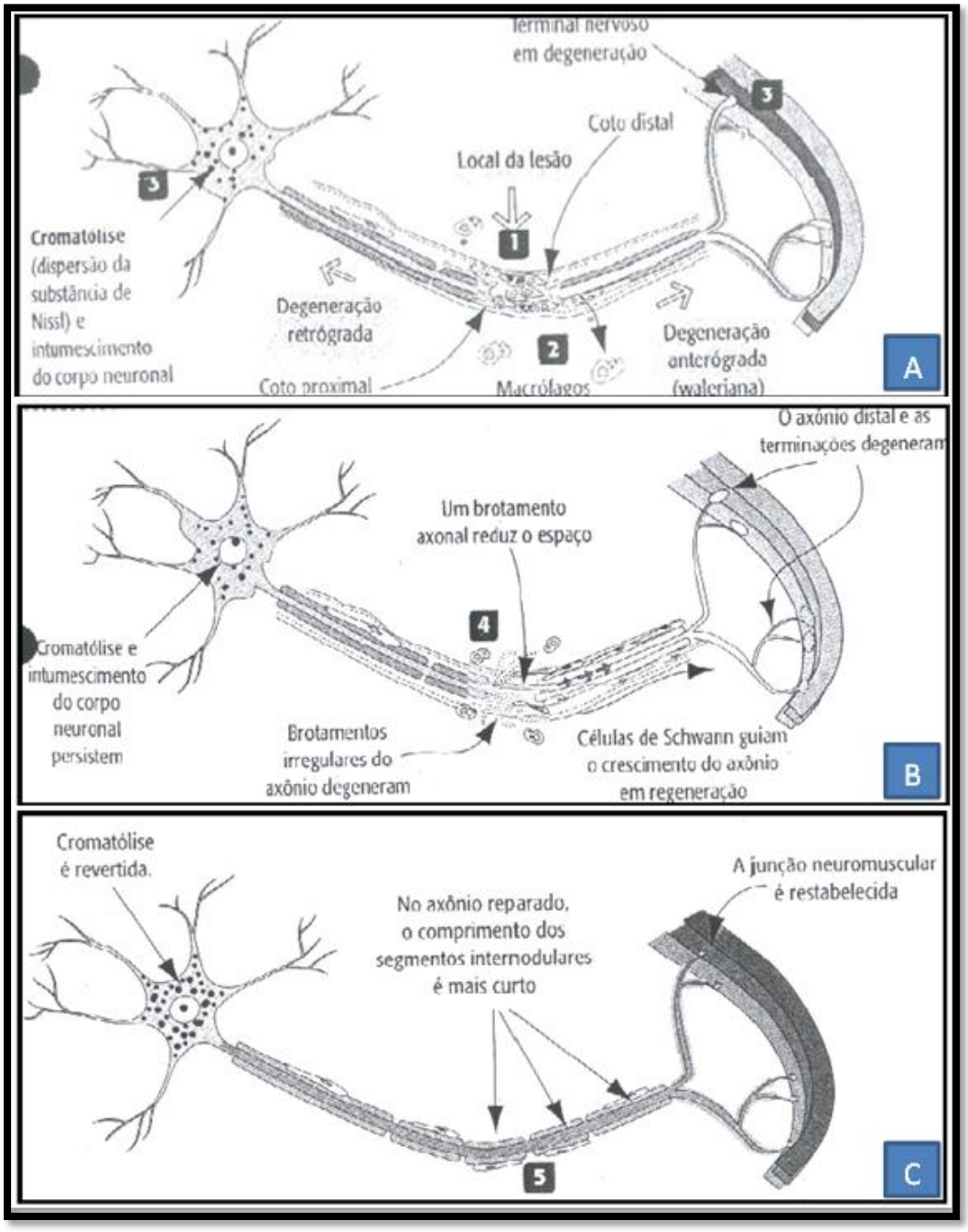

Figura 5 – (A) Lesão danifica a fibra nervosa. As células de Schwann sofrem mitose e preenchem o espaço entre o topo proximal e distal (seta 1). As células de Schwann fagocitam a mielina, que é excretada e fagocitada de seguida pelos macrófagos tecidulares (seta 2). Cromatólise acontece no corpo celular (seta 3) e degenerescência das terminações dos axónios e nos segmentos proximal e distal (seta 3). **(B)** Do segmento proximal surgem múltiplos brotamentos que avançam por entre as células de Schwann. O processo de regeneração inicia-se, mas é suspenso pela ausência do endoneuro. **(C)** Uma vez que o axónio regenerado atinge o órgão alvo, as células de Schwann começam a produzir mielina (Adaptado de Kierszenbaum, 2004).

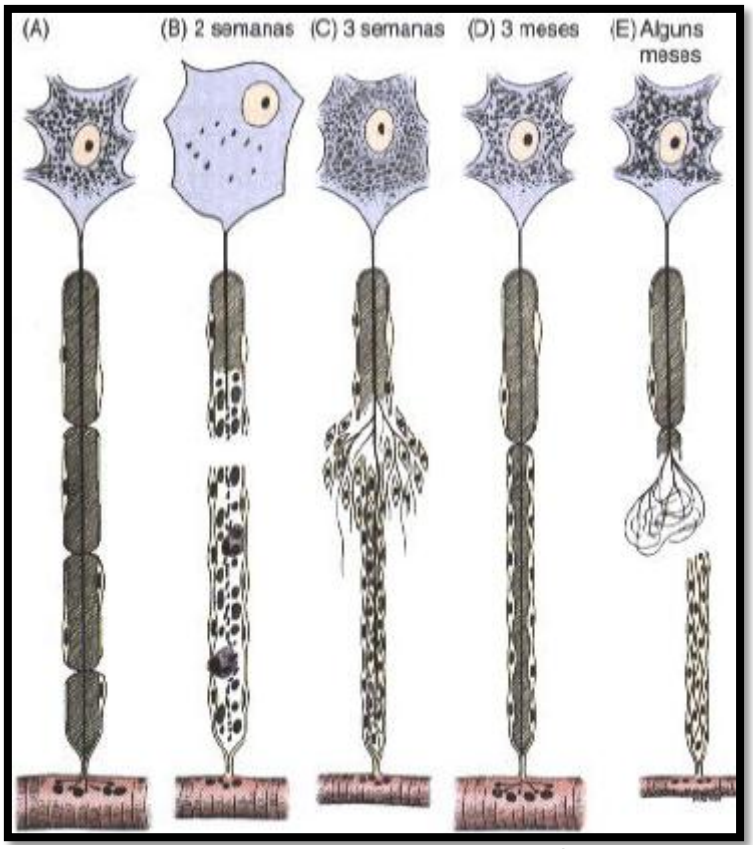

Figura 6 - Modificações que podem ocorrer quando a fibra nervosa é seccionada. **(A)** Fibra nervosa motora normal; **(B)** Fibra nervosa com 2 semanas de lesão, o núcleo do neurónio desloca-se para a periferia e diminui a quantidade de substância de Nissl; **(C)** Fibra nervosa com 3 semanas de lesão, devido à falta de uso, a fibra muscular estriada atrofia; **(D)** Fibra nervosa após 3 meses, com regeneração bem sucedida; **(E)** Fibra nervosa com alguns meses, em que o axónio não encontrou um cilindro de células de Schwann, o seu crescimento é desordenado, formando muitas vezes os "neuromas de amputação" (adaptada de Junqueira & Carneiro, 2004).

2.5. Fatores que influenciam a degenerescência e regeneração do nervo periférico

A técnica de microcirurgia, assim como a experiência do cirurgião, incluindo materiais utilizados são fatores importantes a ter em atenção (Robinson & Madison, 2004). Uma vez esgotados os aperfeiçoamentos das técnicas de microcirurgia, bem como o conhecimento do neurologista, o melhoramento dos resultados clínicos que têm sido obtidos em lesões do nervo periférico, parecem ser à custa do maior conhecimento da biologia celular e molecular da regeneração do nervo (Dubový, 2004). Quanto maior for o conhecimento sobre estes fatores que modulam o processo após a lesão de um nervo periférico, mais estratégias e terapias podem ser desenvolvidas com o objetivo de minimizar as lesões e promover a sua recuperação (morfológica e funcional). A degenerescência Walleriana, o grande número de

substâncias produzidas nesta fase e a importância dos fatores de crescimento e citoquinas em todo o processo, têm sido muito investigados através de métodos de biologia molecular. O SNP tem uma grande capacidade de regeneração, o que lhe confere um prognóstico favorável em algumas neuropatias axonais, quando comparado com o SNC (Amado, 2007).

Os mecanismos celulares e moleculares responsáveis pelo recrutamento de monócitos/macrófagos durante a fase de degenerescência, ainda não são muito conhecidos. Existe um tipo de células denominadas por CAMs (moléculas de adesão celular) que são importantes em fenómenos de adesão entre axónio-axónio ou axónio-célula de Schwann-axónio da membrana basal, e regulam assim o processo regenerativo do nervo periférico (Varejão, 2003). As CAMs que têm um papel mais importante são: ICAM-1 (Molécula-1 de Adesão Celular), moléculas MAC-3 (Recetor de Complemento-3) e LFA-1 (Antigeno-1 Linfocitário Associado à Função) (Avelino *et al*., 2004). Foi demonstrado que após transecção do nervo ciático a expressão de ICAM-1 é máxima ao 3º dia e decresce até aos níveis basais até ao 14º dia, no entanto, há um aumento de MAC-1 e LFA-1 após o 2º dia aumentando até ao 14º dia (Avelino *et al*., 2004).

Os fatores neurotróficos incluem as citoquinas e os fatores de crescimento e estes são sintetizados pelo tecido alvo, pelas células da glia (no caso do SNP pelas células de Schwann) e também pelo neurónio (Yuen, 2001). Os diversos fatores neurotróficos cumprem um papel essencial na sobrevivência do neurónio, após lesão axonal, como se tem verificado nos mais diversos trabalhos experimentais. Todos eles promovem o crescimento e a regeneração axonal (Terenghi, 1999) e são sintetizados ao nível do segmento distal pelas células de Schwann e transportados em direção ao soma via transporte retrógrado (Lindsay, 1996). Incluem o fator de crescimento do nervo (NGF), a neurotrofina 4/5 (NT-4/5), o fator de crescimento do nervo derivado do cérebro (BDNF), o fator neurotrófico derivado da linha celular da glia (GDNF), diversos fatores de crescimento similares à insulina (IGFs), N- cadherina, integrinas, bem como moléculas da matriz extracelular como a laminina e a fibronectina (Steuer *et al.*, 1999; Hudson *et al*., 2000). As células de Schwann são também responsáveis pelo aumento do número de recetores para as hormonas da tiroide, especialmente a triodotironina (T3), que é conhecida também por favorecer a regeneração nervosa (Barakat-Walter, 1999), funcionando a sua membrana como substrato disponível para o crescimento axonal (Steuer *et al*., 1999).

No quadro 1 é possível ver alguns fatores implicados na regulação das células de Schwann durante o processo de regeneração do nervo periférico.

Quadro 1 - Resumo de vários fatores implicados na regulação das células de Schwann durante o processo de regeneração do nervo periférico (tabela adaptada de Chen *et al*., 2007).

Fatores	Efeitos nas células de Schwann	Referências
Moléculas de Matriz Extracelular		
Laminina	Efeito durante a remielinização	Chen & Strickland 2003, Masaki *et al.*, 2003; Chen *et al.*, 2007
Distroglicano	Manutenção das bainhas de mielina e regulação da espessura de mielina	Masaki *et al.*, 2003
L-periaxina	Manutenção das bainhas de mielina e regulação da espessura de mielina	Williams & Brophy, 2002
tPA/plasminogénio	Absorve a fibrina e promove a remielinização	Akassoglou *et al.*, 2002
Fibrina	Inibe a remielinização	Akassoglou *et al.*, 2002
Fatores Neurotróficos e Receptores		
BDNF (*Brain Derived Neurothrofic Factor*)	Promotor da remielinização	Zhang *et al.*, 2000
p75 NTR (*p75 Neurotrophin receptor*)	Mediador do efeito mielinopromotor do BDNF	Song *et al.*, 2006
FGF-2 (*Fibroblastic growth factor)*	Promotor da proliferação das células de Schwann mas inibe a remielinização durante a regeneração	Jungnickel *et al.*, 2004a; Jungnickel *et al.,* 2006
TGF-β (*Transforming Growth Factor- beta*)	Aumenta o efeito trófico das células de Schwann sobre o crescimento do axónios	Rogister *et al.*, 1993; Sulaiman & Gordon, 2002
Reguladores intracelulares		
Mensageiro *PI 3-Kinase/Akt*	Promotor da remielinização	Ogata *et al.*, 2004
Ciclina D1	Necessário para a regeneração e proliferação das células de Schwann	Atanasoski *et al.*, 2001; Kim *et al.*, 2000
Hormonas		
Progesterona	Promotor da remielinização	Koenig *et al.*, 1995
Hormona da Tiróide (T3/T4)	Promotor da remielinização	Voinesco *et al.*, 1998
Eritropoietina	Estimula a regeneração das células de Schwann e diminui a expressão de TGF-β nas células de Schwann	Campana *et al.*, 2006; Li *et al.*, 2005

Existem diversos fatores que influenciam a regeneração do nervo periférico e que estão associados ao tipo de lesão do nervo que temos em causa, isto é, de estiramento (neuropraxia) (Spiegel *et al*., 1993), de esmagamento (axonotmese) (Witzel *et al*., 2005) e secção ou secção com perda de substância (neurotmese). O local da lesão também é importante, ou seja, a maior ou menor proximidade com o corpo celular (Madison *et al*., 1996; Mears *et al*., 2003; Franz *et al*., 2005; Pfister *et al*., 2007), sendo que, quanto mais distal pior é o prognóstico (Hudson *et al*., 2000). A distância existente entre o local da lesão e a medula espinal parece também ter

importância, uma vez que se consegue um melhor prognóstico quanto maior for esta distância, podendo este ponto dever-se ao facto de quanto mais distal mais delineada está a divisão entre nervo motor e nervo sensitivo (Chalfoun *et al*., 2006). A distância entre os dois topos da lesão é um fator essencial para a determinação da capacidade de regeneração do nervo em causa e posteriormente ajudar na seleção do prognóstico clínico a adotar (Pfister *et al*., 2007). O período que decorre entre a lesão e a cirurgia também parece ser um fator importante, apesar de ainda estar pouco estudado, bem como determinar o período crítico durante o qual não ocorram alterações irreversíveis capazes de comprometer a reparação cirúrgica (Peker *et al*., 2005). É de conhecimento geral que quanto mais precoce for a reparação cirúrgica melhores são os resultados, infelizmente na prática clínica nem sempre é possível atuar imediatamente (Peker *et al*., 2005). Para a obtenção de uma melhor recuperação funcional é necessário que a reparação cirúrgica ocorra dentro das primeiras 4 semanas (Sulaiman & Gordon, 2000; Sulaiman *et al*., 2002). Um tema ainda pouco documentado, é a influência que o pós-operatório instituído durante o período de recuperação, nomeadamente técnicas de fisioterapia e/ou de exercício físico, têm na recuperação funcional do nervo, contrariando assim os efeitos negativos que um longo tempo de inatividade do músculo desinervado causa na recuperação funcional (Luís, 2008).

3. Reparação cirúrgica do nervo periférico

A reparação cirúrgica do nervo periférico é uma situação muito comum mas nem por isso deixa de ser um autêntico desafio para os cirurgiões no sentido de uma recuperação funcional aceitável (Chen *et al*., 2006b; Li *et al*., 2006). Muitas destas lesões resultam em perda da função do nervo podendo mesmo na sua maioria levar à amputação do membro (Brancher & Torres, 2005).

Uma das técnicas usadas para a reparação do nervo periférico quando este sofre transecção é a aplicação de suturas epineurais que consiste na sutura do epineuro. Esta técnica apesar de ser meticulosa, geralmente origina pequenas lesões nervosas provocadas pela manipulação e com frequente recuperação insatisfatória da função (Lauto & Trickett, 1997). A aplicação das suturas epineurais e perineurais tem sido objeto de discussão, devido a dois aspetos menos positivos que são observados no exame histopatológico: o mau direcionamento do tecido endoneural e a compressão feita pelo fio de sutura. Em contrapartida, a aproximação das extremidades nervosas e o seu envolvimento com uma bainha (membrana) favorecem

a regeneração axonal por intermédio do espaço existente no seu interior, pois evita a invasão externa de fibroblastos e a aderência de tecido cicatricial (Suematsu, 1989; Torres & Graça, 2003).

Daí ser extremamente necessário que haja um avanço na técnica de reparação nervosa, possivelmente tornando-a mais simples, facilitando assim a sua aplicação tanto em Medicina Veterinária como Medicina Humana, devido à alta frequência de lesões de nervos periféricos (Brancher & Torres, 2005).

3.1. Engenharia de Tecidos

Segundo Langer & Vacanti (1993) a Engenharia de Tecidos é "um campo interdisciplinar de investigação que aplica os princípios da engenharia e de ciências da vida para o desenvolvimento de substitutos biológicos que restauram, mantêm ou melhoram a função dos tecidos".

A Engenharia de Tecidos surge de forma a se tornar uma possível alternativa ao uso de transplantes de órgãos e tecidos. Consistindo em três principais estratégias:

a) Implantação de células cultivadas *in vitro* (Terapia Celular), na presença ou não de uma matriz tridimensional, com o potencial de serem integradas e de formar tecido funcional após a implantação;
b) Implantação de tecidos funcionais formados em laboratório pela junção de células, provenientes do paciente ou de um dador e uma matriz (por exemplo: pele artificial);
c) Regeneração dos tecidos "*in situ*", utilizando uma matriz que promove a migração e adesão de populações específicas de células, as quais levarão à reparação ou substituição do tecido danificado (Langer, 1999).

Podemos assim afirmar que precisamos, essencialmente, de células, matriz extracelular, comunicação intercelular, interações células-matriz e fatores de crescimento, que têm de ser combinados de forma coordenada para a obtenção de um bom resultado final (Mano *et al*., 2004).

A vantagem da Engenharia de Tecidos é o número reduzido de cirurgias, resultando num curto período de tempo de recuperação do paciente quando se recorre a células autólogas, eliminando totalmente a rejeição do enxerto e a possível transmissão de doenças víricas e priónicas (desvantagem da utilização de enxertos heterólogos e de xenoenxertos). No entanto, as exigências dos materiais para a Engenharia de Tecidos são múltiplas assim como é extremamente complexa a

preparação, isolamento, expansão e a diferenciação dos sistemas celulares envolvidos (Langer, 1999).

Qualquer tecido consiste numa matriz com um ou, normalmente, vários tipos de células. A matriz é um *scaffold* tridimensional (3D) para células, que lhes fornece um tecido envolvente específico e uma arquitetura (Huang & Huang, 2006). Além disso, serve de reservatório de água, nutrientes e fatores de crescimento. Nesta perspetiva, e para restaurar a função ou regenerar os tecidos, a matriz atuará como uma estrutura temporária para a proliferação celular e deposição da matriz extracelular (MEC), com consequente crescimento do tecido até que o novo tecido seja totalmente restaurado ou regenerado. Pode ainda atuar como suporte para a vascularização desse novo tecido, a angiogénese do novo tecido e o restabelecimento da vascularização local são fundamentais para o sucesso terapêutico (Agrawal & Ray, 2001). É então lógico dizer que uma matriz tridimensional (3D) apropriada é um componente essencial para a Engenharia de Tecidos. Entretanto, é importante entender que a matriz deve ter uma série de propriedades que a tornem satisfatória para o crescimento celular e para a sua integração nas estruturas envolventes. Além da escolha do material adequado, as propriedades macroestruturais e microestruturais dos materiais são de extrema importância. Estas podem afetar propriedades de sobrevivência das células, tais como, crescimento, propagação e reorganização (Boccaccini & Blaker, 2005). Assim, resumem-se as seguintes exigências de uma matriz para a Engenharia de Tecidos: i) biocompatibilidade e biodegradabilidade; ii) elevada porosidade e rede 3D de poros interconectados; iii) bioatividade satisfatória para explorar o processo de reparação natural do corpo; iv) superfície química ótima para a adesão e diferenciação das células; v) integridade mecânica; e vi) facilidade do processo de fabricação (Leong *et al.*, 2003). Além disso, têm sido feitos esforços no sentido de desenvolver matrizes com incorporação de moléculas, tais como fatores de crescimento ou fármacos. Estas matrizes podem libertar de forma controlada, localmente os fatores de crescimento ou fármaco (como um antibiótico ou um anti-inflamatório) e potenciar a regeneração do tecido lesado (Boccaccini & Blaker, 2005; Yao *et al*., 2005).

3.2. Conduto neuronal

Numa lesão do nervo periférico se não houver uma perda muito significativa de tecido nervoso, recorre-se por norma à aproximação e sutura entre os seus topos (sutura topo-a-topo) para efetuar a reparação cirúrgica. Mas se é verificada uma perda

significativa de tecido nervoso então recorre-se à interposição de enxerto autólogo. A aplicação de enxertos autólogos apresenta várias desvantagens, nomeadamente:

a) Necessidade de uma segunda intervenção cirúrgica;
b) Diâmetro do enxerto de nervo autólogo ser pequeno devido à opção de normalmente ser utilizado um nervo sensorial cutâneo;
c) Existir o risco de necrose do enxerto autólogo ao longo da sua extensão;
d) Poderem surgir alterações de natureza sensorial;
e) Poder ocorrer a formação de neuromas e de tecido fibroso junto à segunda linha de sutura (Millesi, 1990; Madison *et al*., 1992; Dahlin & Lundborg, 1998; Flores *et al*., 2000; Varejão, 2003).

De forma a tentar ultrapassar todas estas limitações associadas aos enxertos autólogos surge a técnica de tubulação. Desde o século XIX, que esta possibilidade de ser utilizado um conduto não nervoso para colmatar um defeito do nervo, guiando as fibras nervosas do topo proximal até ao topo distal, tem vindo a ser largamente estudado (Brunelli *et al*., 1994; Meek & Coert., 2002; Lungborg, 2003; Ijkema-Paassen *et al*., 2004). Esta técnica cirúrgica fundamenta-se nos conhecimentos divulgados pelos considerados *pais* da neurobiologia moderna, A.V. Waller e L. Ranvier e que afirma que a regeneração do nervo ocorre por crescimento do axónio no local seccionado, do topo proximal para a lesão e não regeneração do topo distal (Stoll *et al*., 2002).

A técnica de tubulação consiste na utilização de um canal oco tubiforme de material natural ou sintético, que é interposto nos segmentos lesionados com o objetivo de orientar a regeneração das fibras nervosas (fenómenos de quimiotropismo) levando a que as fibras do topo proximal se encontrem com as do topo distal, através de sinais químicos originados do topo distal (Hudson *et al*., 2000). A utilização dos tubos-guia em alternativa aos enxertos autólogos na reparação do nervo periférico é cada vez mais utilizada pelas suas relevantes vantagens: inexistência das complicações associadas ao uso do enxerto autólogo; o fenómeno regenerativo é direcionado fisicamente pela presença do tubo-guia; verifica-se uma diminuição da tensão no local da sutura; o risco de formação de neuromas é muito menor; diminuição da invasão dos fibroblastos; aumento da concentração dos fatores neurotróficos e possibilidade de manipular o meio interno (Lundborg *et al*., 1982; Gibson *et al*., 1991; Madison *et al*., 1992; Meyer *et al*., 1997; Hudson *et al*., 2000; Lee & Wolfe, 2000; Heijke *et al*., 2001; Varejão, 2003). Existem algumas propriedades básicas que deverão ser tidas em conta aquando do fabrico dos tubos-guia para que a sua utilização seja um êxito: i) diâmetro e espessura de acordo com lesão a colmatarem; ii) resistentes e que mantenham a sua força de tensão durante o tempo necessário à

regeneração; iii) fácil implantação por técnicas de microcirurgia; iv) esterilizáveis; v) biodegradáveis e vi) possuir porosidade adequada (Hudson *et al*., 2000). Outras características também podem ser levadas em consideração com o objetivo de incitar a regeneração do nervo periférico: i) a capacidade de incorporação e suporte de células (células estaminais, por exemplo); ii) a capacidade de controlar a libertação de fatores neurotróficos; iii) fornecer um substrato capaz de orientar o nervo (como, fibras de colagénio); iv) conter canais intraluminais e v) capacidade de atividade elétrica através da criação de campos elétricos com um potencial impacto na regeneração de tecidos (Huang & Huang, 2006).

A implantação dos tubos-guia por microcirurgia tem de ser facilitada, daí que a utilização de um tubo-guia transparente seja sempre o ideal e o recomendado de forma a melhorar a visualização e monitorização de todo o processo de implantação e de suturas. Os estudos realizados têm demonstrado que também devem ser semipermeáveis (porosos), possibilitando quer a incorporação de matrizes com manutenção do microambiente, quer ainda a possibilidade de eliminação de produtos resultantes do metabolismo local, promovendo ainda a revascularização local, a entrada de nutrientes e a oxigenação (Lundborg *et al*., 1982a; Gibson *et al*., 1991, Madison *et al*., 1992, Meyer *et al*., 1997, Steuer *et al*., 1999; Hudson *et al*., 2000; Lee & Wolfe, 2000; Heijke *et al*., 2001). Com todos estes requisitos, os engenheiros de biomateriais têm um árduo trabalho para conseguir que um só material tenha todas estas propriedades (Luís, 2008).

A presença de fatores de crescimento no local da lesão cumpre um importante papel no controlo de sobrevivência, migração, proliferação e diferenciação de vários tipos de células envolvidas na regeneração do nervo (Fu & Gordon, 1997; Amado *et al.*, 2008). Um dos principais problemas destes fatores de crescimento é a sua curta semi-vida que vai de minutos a horas e que para ser ultrapassado é necessário aumentar o seu período de libertação quando introduzidos no próprio biomaterial do tubo-guia (Tria *et al*., 1994). Os fatores de crescimento atingem o local da lesão, numa concentração adequada e durante o período de degenerescência Walleriana / regeneração, de modo a apresentarem um efeito terapêutico adequado sem reações adversas (Amado *et al*., 2008).

A distribuição dos fatores de crescimento pode ser feita por 3 vias diferentes: i) distribuição de proteínas no lúmen ou parede do tubo-guia diretamente na área lesada do nervo; ii) sistemas celulares (no interior) do lúmen do tubo-guia que produzam fatores de crescimento; iii) uso de terapia genética para transferir células residentes para expressar determinada proteína (Amado *et al*., 2008). Em 2003, Dekker e seus colaboradores conseguiram resultados promissores do seu trabalho experimental,

especialmente na recuperação funcional, com a utilização da tubos-guia e fatores de crescimento (Dekker *et al*., 2003).

Uma das vantagens na utilização de tubos-guia, tal como já foi referido, é o facto de permitir a formação de um microambiente capaz de ser manipulado. E sobre este tema têm sido investigados os mais variados materiais e/ou componentes, sempre com o mesmo objetivo – promover a regeneração do nervo periférico. Começou por se preencher este espaço do tubo guia com solução salina, gel de colagénio (Labrador *et al.*, 1998; Ceballos *et al*., 1999), fibrina (Williams & Varon, 1985; Williams *et al.*, 1987), laminima (Madison *et al.*, 1985; Madison *et al*., 1988; Tong *et al.*, 1994), fibronectina (Bailey *et al.*, 1993), músculo fresco (Varejão, 2003), células de Shwann (Guenard *et al.*, 1992; Levi *et al.*, 1997) e fatores tróficos (Rich *et al.*, 1989). O objetivo é que seja criado em substrato para a migração de células não neuronais (fibroblastos, células endoteliais, células de Schwann) de forma a criar uma matriz de ligação entre os dois topos (Labrador *et al.*, 1998) e permitir a formação de uma ponte que sirva de suporte para o crescimento dos axónios (Figura 7).

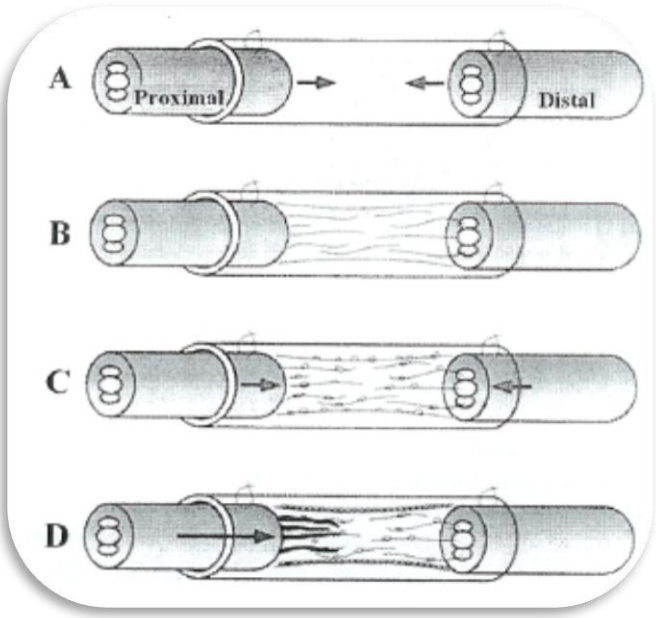

Figura 7 - Desenvolvimento do meio interno dentro de um tubo-guia de silicone, para uma solução de continuidade de 10 mm no nervo ciático do rato. **(A)** Acumulação de fluído de origem plasmática logo nas primeiras horas pós-secção. **(B)** Formação da matriz de fibrina entre os segmentos do nervo, no decorrer da primeira semana. **(C)** Durante a segunda semana, a fase celular corresponde à migração de fibroblastos, de células de Schwann e de células endoteliais, a partir de ambos os segmentos. **(D)** A fase axonal é definida pela migração dos axónios a partir do segmento proximal (Adaptado de Varejão, 2003).

3.2.1. Biomateriais

Biomateriais tal como o nome indica são materiais que entram em contacto com fluidos corporais seja de forma contínua ou descontínua, mesmo quando localizados fora do corpo entrando na categoria dos dispositivos médicos. Este tipo de material é feito com o objetivo de reparar e/ou reconstituir partes ou restaurar as funções do corpo humano sem que haja reações adversas ou de "corpo estranho" (Legeros, 2002). Vários tipos de biomateriais têm sido usados como tubos-guia pelo nosso grupo de investigação e inúmeros grupos em todo o mundo, e podem ser classificados em duas grandes categorias: materiais naturais ou materiais sintéticos. Os materiais sintéticos podem ainda classificar-se em biodegradáveis e não biodegradáveis (Luís, 2008). Os biomateriais, em especial, os sintéticos biodegradáveis, são utilizados como materiais ideais para a produção de matrizes para a Engenharia de Tecidos (Agrawal & Ray, 2001; Leong *et al*., 2003). Pode-se afirmar que os tubos-guia biodegradáveis são preferencialmente usados em relação aos não biodegradáveis, devido ao facto destes últimos ao permanecerem no local aumentam o risco de infeção, podem ser uma fonte de compressão do nervo durante o processo de regeneração e para a sua remoção é necessário uma segunda intervenção cirúrgica (Luís, 2008).

Os polímeros biodegradáveis naturais são obtidos de fontes naturais como animais ou vegetais e temos como exemplo: o colagénio (Ignatius *et al*., 2005), o fibrinogénio (Hojo *et al*., 2003), o quitosano e derivados da quitina (Zhao *et al*., 2002), o ácido hialurónico (Liu *et al*., 1999) e poli-hidroxibutirato (Chen & Wang, 2002). Este tipo de material tem várias vantagens: o baixo potencial imunogénico, o comportamento bioativo, a capacidade de interagir com o tecido recetor, a versatilidade química e o facto de serem provenientes de fontes renováveis e abundantes (Schmidt & Leach, 2003).

Por outro lado existem os polímeros biodegradáveis sintéticos que geralmente são os mais utilizados em Engenharia Biomédica e que são os poli (- hidroxiácidos) (Mooney *et al*., 1996; Ishaugh-Riley *et al*., 1997), a poli (ε-caprolactona) (Washburn *et al.*, 2002), os poli (carbonatos) e os poli (anidridos) (Uhrich *et al*., 1998; Levenberg & Langer, 2004). Como exemplo de polímeros não biodegradáveis sintéticos destaca-se o silicone (Dahlin & Lundborg, 2001). A utilização de tubos-guia de silicone na tubulização em nervos lesionados, ganhou grande realce em 1980, sendo considerado o modelo experimental *standard* (Lundborg *et al*., 1982). A única desvantagem associada é a necessidade do tubo-guia ter de ser retirado para aliviar o desconforto

provocado pela contração cicatricial sempre que se usam materiais não reabsorvíveis como o caso de silicone (Hudson *et al*., 2000).

Outro exemplo é o colagénio, um material natural, sendo tipo de proteína mais abundante do organismo, representando 30% do seu peso seco. Os diversos tipos de colagénio dos vertebrados constituem uma família de proteínas produzidas por diversos tipos de células e distinguem-se pela sua composição química, características morfológicas, distribuição, funções e patologias (Junqueira & Carneiro, 2004). A síntese de colagénio foi inicialmente associada a um grupo restrito de células do tecido conjuntivo como os fibroblastos, osteoblastos e condroblastos. Atualmente existem evidências que mostram que vários outros tipos de células produzem esta proteína. Os principais aminoácidos que o constituem são a glicina, a prolina e a hidroxiprolina. Alguns aminoácidos, como a hidroxiprolina e a hidroxilosina, são característicos do colagénio (Junqueira & Carneiro, 2004). O colagénio é um biomaterial amplamente usado em Medicina e faz parte integrante da fibra nervosa (especialmente colagénio tipo III). Juntamente com outros componentes (como a laminina, fibronectina) faz parte integrante da MEC do nervo e tem um papel ativo e importante na regeneração do nervo periférico (Luís, 2008). Vários estudos em regeneração nervosa têm utilizado o colagénio para o fabrico de tubos-guia (Mackinnon & Dellon, 1990b; Navarro *et al*., 1996; Tyner *et al*., 2007). Tal como também várias formas de tratamento das fibras de colagénio que produzem o tubo-guia têm sido já estudadas (Ahmed *et al*., 2004; Ahmed *et al*., 2005). Isto é, a forma como as fibras de colagénio se encontram entrelaçadas (*crosslinking*) pode de alguma forma intervir não só na capacidade de crescimento do nervo regenerado como também na capacidade e velocidade de degradação do biomaterial (Navarro et al., 1996; Ceballos *et al*., 1999).

O quitosano é outro exemplo de biomaterial usado e é um polissacarídeo derivado da quitina com propriedades antitumorais e antimicrobianas que tem sido demonstrado como promotor da migração e proliferação das células de Schwann e portanto, com grande interesse na utilização na regeneração nervosa (Yuan *et al*., 2004). Entre os vários biomateriais propostos para o fabrico de tubos-guia para regeneração do nervo, o quitosano tem recentemente atraído muita atenção pela sua biocompatibilidade, biodegradabilidade, baixa toxicidade, baixo custo, aumento da cicatrização e efeitos antibacterianos (Chandy & Sharma, 1990). Além disso, a potencial utilidade do quitosano na regeneração nervosa tem sido demonstrada *in vitro* e *in vivo* (Chandy & Sharma, 1990; Yamaguchi *et al*., 2003; Freier *et al*., 2005; Amado *et al*., 2008).

Também é preciso não esquecer que todos os biomateriais para aplicação *in vivo* são previamente aprovados pela Food and Drug Administration (FDA) e pelas autoridades nacionais como o INFARMED. Infelizmente em regeneração do nervo periférico são poucos os biomateriais aprovados pela FDA (Archibald *et al*., 1995). Muitos deles são ainda limitados a soluções de continuidade muito pequenas não podendo ser utilizados em grandes defeitos (Schmidt & Leach, 2003). Os materiais sintéticos bioreabsorviveis que têm vindo a ser testados pelo nosso grupo de investigação foram vários: o copolímero de poli (-l-ácido láctico): poli (-ácido glicólico) (PLGA) na proporção 90:10, a poli (-ε-caprolactona) (PLC) (Neurolac®), o quitosano e mais recentemente o ácido poli-vinílico (PVA) (Luís *et al*., 2007; Luís *et al*., 2008; Luís *et al*., 2008a).

3.3. Sistemas celulares utilizados em Medicina Regenerativa

Vários estudos têm surgido sobre a inclusão de um suporte celular na proliferação axonal, em que são testados vários tipos de células bem como a sua associação a diversos biomateriais (Luís, 2008). Entre as células mais estudadas estão: as células de Schwann (Hadlock *et al*., 2001), os macrófagos (Lundborg, 2000), os fibroblastos (Patrick *et al*., 2001), as células HEK-293 (McConnell *et al*., 2004; Chalfoun *et al*., 2006), as células da mucosa olfativa (OEC's) (Möllers *et al*., 2009), as células da medula óssea (Zhang *et al*., 2005), as células N1E-115 (neuroblastomas de ratinho diferenciados *in vitro*) (Rodrigues *et al*., 2005a; Rodrigues *et al*., 2005b; Amado *et al*., 2008; Luís *et al*., 2008), as HPSCs e as MSCs (Maurício *et al*., 2011) Estas últimas têm sido alvo de muitos estudos e de resultados muito promissores, em especial aquelas obtidas a partir do SCU e do próprio cordão umbilical, devido ao crescente número de criopreservações realizadas por Bancos públicos e Bancos privados de SCU a nível mundial.

3.3.1. Células N1E-115

A linha celular de N1E-115 estabelecida a partir de neuroblastomas de ratinho (Amano & Nirenberg, 1972) tem sido usada como sistema celular por diversos investigadores (Rodrigues *et al*., 2005a; Rodrigues *et al*., 2005b; Amado *et al*., 2008; Luís *et al*., 2008; Luís *et al*., 2008a) para produzir e distribuir localmente fatores neurotróficos em lesões do nervo periférico experimentais. *In vitro*, as células N1E-115

suportam a diferenciação neuronal em resposta ao dimetilsulfóxido (DMSO), 3', 5' adenosina monofosfato cíclico (cAMP), ou redução da percentagem de soro bovino fetal adicionado ao meio de cultura. Por indução da diferenciação, a proliferação das células N1E-115 cessa, é observado o crescimento extensivo da neurite e as membranas tornam-se altamente excitáveis (Rodrigues *et al*., 2005a; Rodrigues *et al*., 2005b; Amado *et al*., 2008; Luís *et al*., 2008a; Luís *et al*., 2008). O intervalo de tempo ideal de diferenciação de 48h foi previamente determinado por medição da concentração intracelular de cálcio ($[Ca^{2+}]_i$), quando as células N1E-115 apresentavam já características morfológicas e funcionais de células neuronais e ainda não tinham iniciado o processo de morte celular (apoptose) (Figura 8) (Rodrigues *et al.*, 2005a; Rodrigues *et al*., 2005b; Amado *et al*., 2008; Luís *et al*., 2008a; Luís *et al*., 2008).

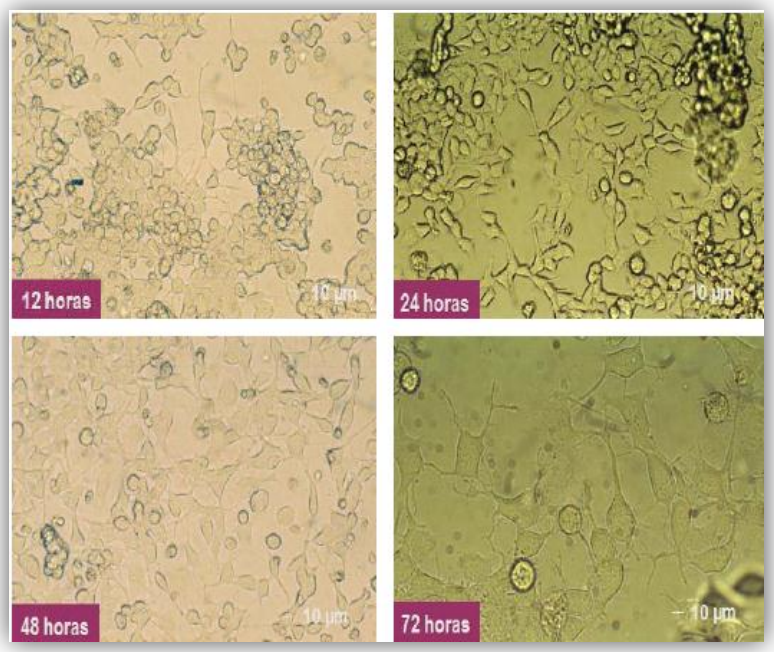

Figura 8 - Células neuronais N1E-115 cultivadas em vários períodos de diferenciação entre 12 e 72h. Após 48h de diferenciação e na presença de DMSO 1.5% a proliferação das células N1E-115 é interrompida, surgem longos prolongamentos citoplasmáticos e as suas membranas plasmáticas tornam-se altamente excitáveis. Imagens obtidas por microscópio invertido (Zeiss, Germany) com ampliação 100x (adaptada de Rodrigues *et al*., 2005a; Rodrigues *et al*., 2005b).

3.3.2. Células estaminais provenientes do cordão umbilical

As células estaminais são células capazes de se auto-renovarem, dividindo-se indefinidamente e que se podem diferenciar em diversas linhagens celulares. Estas podem ser classificadas em dois tipos principais em função da sua origem e/ou da sua capacidade de diferenciação em: embrionárias e não embrionárias. As células estaminais embrionárias são pluripotentes e podem diferenciar-se em qualquer uma

das três camadas germinativas embrionárias (endoderme, mesoderme e ectoderme) e ainda dar origem a todos os tipos de células. Já as células estaminais não embrionárias podem ser encontradas em muitos tecidos do organismo adulto sendo por isso também conhecidas por células estaminais adultas e são geralmente multipotentes (Figura 9). É possível isolar estas células a partir de tecidos como a medula óssea, o sangue, o tecido adiposo, bem como a partir da pele, do fígado, do pâncreas e de outros órgãos. Podem ainda ser encontradas em alguns tecidos neo-natais como é o caso da placenta ou o SCU (pois são obtidas após o nascimento) e estas em particular têm um elevado potencial proliferativo. Em regra, as células estaminais adultas dão origem aos tipos celulares dos tecidos de onde são provenientes, apesar que quando na presença de determinados microambientes e de fatores de crescimento, podem originar outro tipo de células (Bajada *et al*., 2008).

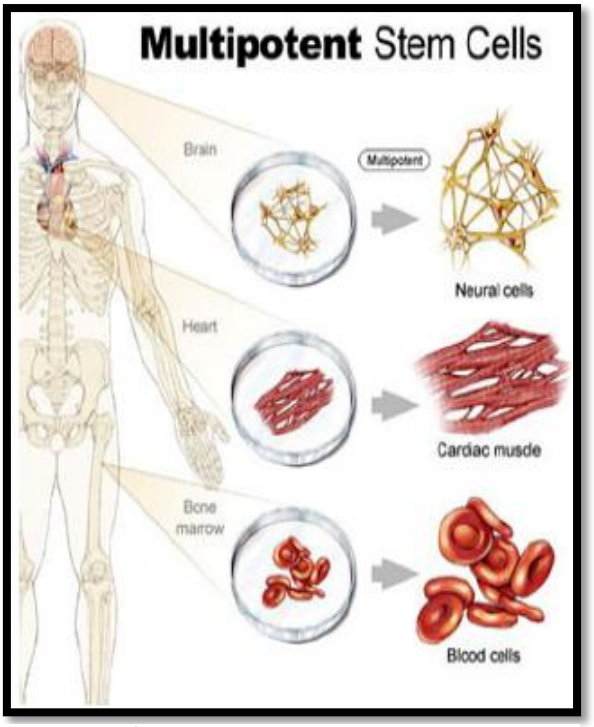

Figura 9 - Células estaminais multipotentes localizadas em diversos locais do corpo humano (adaptada de Stem Cells: Scientific Progress and Future Research Directions, 2001)

O cordão umbilical é uma estrutura que permite a comunicação entre o feto e a mãe durante a gravidez. É composto por vasos umbilicais e um epitélio amniótico e entre estes encontra-se um tecido embrionário gelatinoso (tecido conjuntivo rico em proteoglicano) designado por geleia de Wharton (Figura 10,11,12 e 13). A Geleia de Wharton tem como principal função proteger os vasos e evitar o seu colapso (Can *et al.*, 2007; Karahuseyinoglu *et al*., 2007). No cordão umbilical podem ser encontrados

dois tipos de células: as Células Estaminais e Progenitoras Hematopoiéticas e as Células Estaminais Mesenquimatosas (Weiss *et al*., 2006).

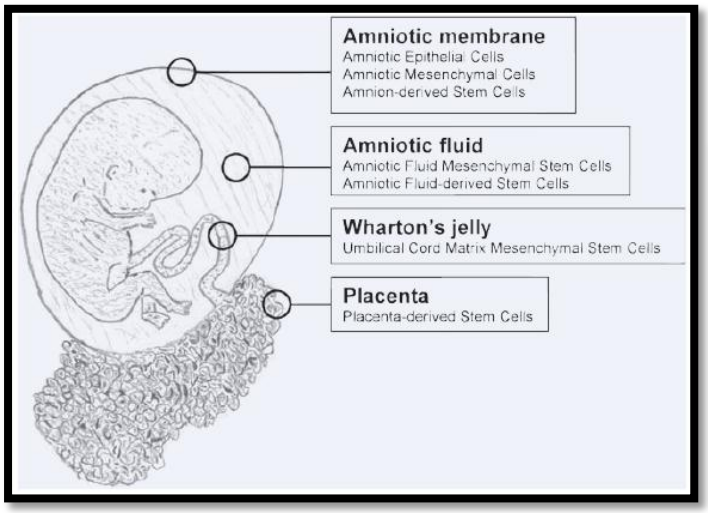

Figura 10 - Várias fontes extra-embrionários de onde têm sido isoladas células estaminais: membrana amniótica, líquido amniótico, cordão umbilical (Geleia de Wharton) e placenta (Marcus *et al.*, 2008).

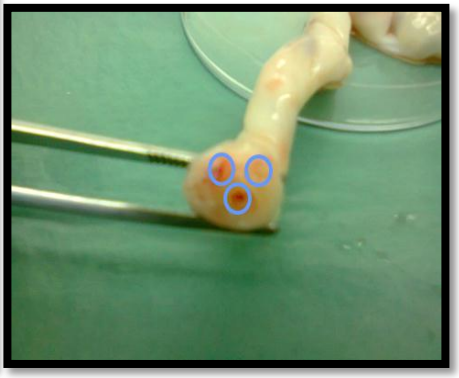

Figura 11 – Corte transversal do cordão umbilical, evidenciando os 3 vasos que contém (2 artérias e 1 veia).

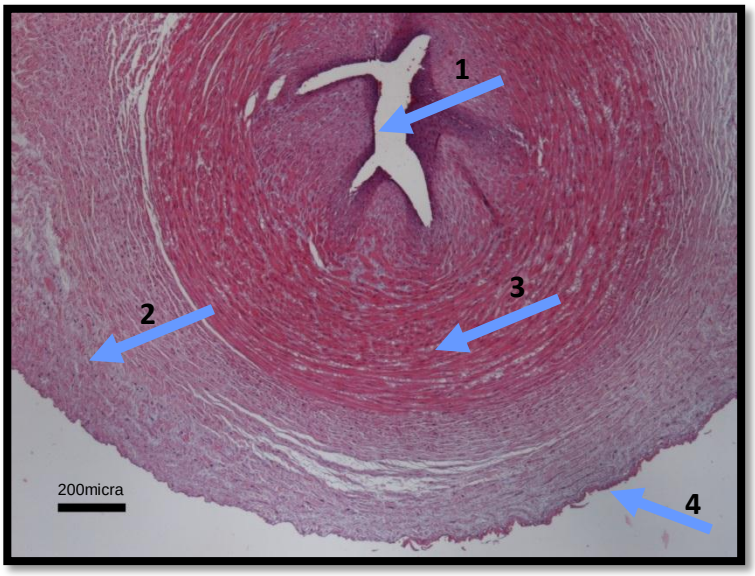

Figura 12 – Corte histológico de um cordão umbilical. **(1)** endotélio; **(2)** tecido conjuntivo laxo; **(3)** vaso umbilical; **(4)** mesotélio. (ampliação 40x)

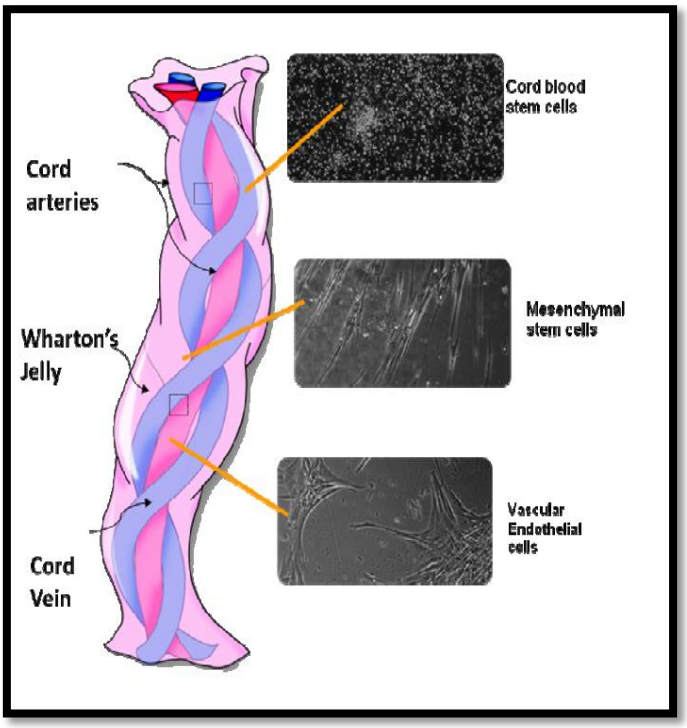

Figura 13 - Constituição do cordão umbilical e os diferentes tipos de células dos vários constituintes (Grossi *et al.*, acedido em 06/07/2011).

As HPSC podem ser encontradas no SCU e podem diferenciar-se em todas as células da linhagem sanguínea. Em comparação com as células estaminais adultas estas são mais primitivas e têm uma capacidade maior de re-população *in vivo* (Gluckman & Rocha, 2005).

O conceito de MSCs foi proposta pela primeira vez em 1991 por Caplan (Caplan, 1991) e baseava-se principalmente na sua capacidade de se diferenciarem numa grande variedade de tecidos mesodérmicos, mais tarde foi validado por uma pesquisa adicional em 1999 (Pittenger, 1999). Devido aos muitos métodos e abordagens diferentes que são utilizados para cultura de MSCs, o comité para as células e tecido estaminal da Sociedade Internacional de Terapia Celular (ISCT), sugeriu três critérios para definir estas células: i) população celular aderente ao plástico, ii) tem marcadores de superfície específicos (ex: CD14-, CD34-, CD45-, HLA classe II negativas, CD73+, CD29-, CD105+, CD90+) e iii) capacidade de se auto-renovarem e diferenciar *in vitro* em várias linhagens celulares incluindo, tecido ósseo, cartilagíneo, adiposo (Domicini *et al.*, 2006).

Na Terapia Celular é dada uma grande importância à utilização de células estaminais devido à sua capacidade de se diferenciarem em vários tipos de células (Figura 14), podendo assim, por exemplo, substituir células lesadas ou destruídas e regenerar tecidos irreversivelmente danificados. As que têm sido mais utilizadas são as HPSC particularmente em doenças hematológicas/oncológicas nas quais é

necessário regenerar o sistema sanguíneo e imunológico do doente (Copelan, 2006), uma vez que conseguem originar todas as células que constituem o sangue e sistema imunitário, podendo diferenciar-se em linfócitos (B e T), eritrócitos, plaquetas, neutrófilos, monócitos, eosinófilos, basófilos e macrófagos (Rocha & Gluckman, 2006). No indivíduo adulto estas células estão na sua maioria localizadas na medula óssea mas também é possível serem obtidas no SCU, daí este ter-se tornado, nos últimos anos, numa alternativa à medula óssea nos transplantes de HPSC (Rocha & Gluckman, 2006). As vantagens de ser escolhido um transplante de HPSC do SCU, em vez da medula óssea são: disponibilidade imediata das células para transplantação, menor risco de infeção com agentes infeciosos, maior tolerância na histocompatibilidade HLA (de 1 a 3 discrepâncias) e reduz ainda o risco de doença do transplante contra o hospedeiro (GVHD) (Barker & Wagner, 2002; Frassoni *et al*., 2003). O volume de sangue recolhido do cordão umbilical não é muito elevado, o que implica que a quantidade de células obtidas a partir do SCU seja menor mesmo tendo uma concentração de células estaminais elevada. Este facto faz com que transplantes de células estaminais do SCU seja maioritariamente feito em crianças ou adultos de baixo peso, uma vez que é o peso do doente que determina o número de células estaminais necessárias para o transplante. Sendo o número limitado de células estaminais uma das limitações do SCU existem atualmente grupos de investigação a desenvolverem protocolos de expansão que permitam aumentar o número de HPSC (Tanavde et al., 2002).

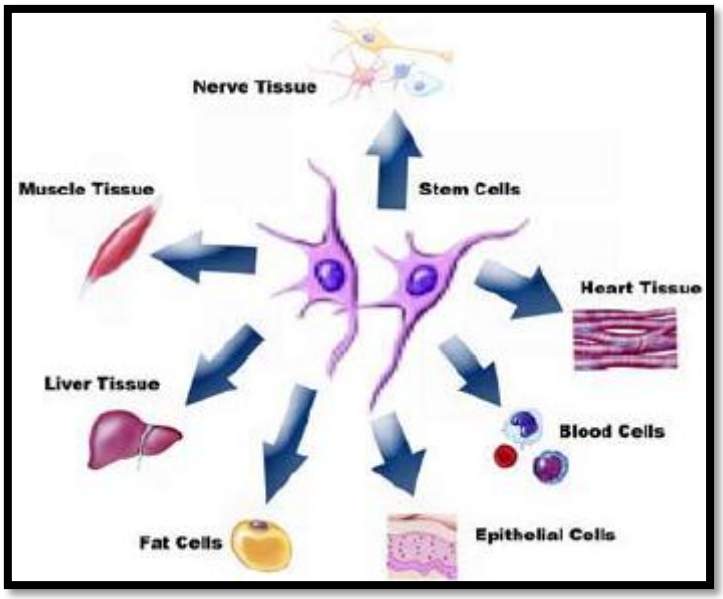

Figura 14 - Esquema ilustrativo da capacidade de diferenciação das células estaminais em vários tipos de células (adaptada de Stem Cells: Scientific Progress and Future Research Directions, 2001)

As MSCs, que são isoladas a partir da geleia de Wharton do cordão umbilical e em comparação com as embrionárias ou da medula óssea são mais facilmente obtidas. Têm a capacidade de se diferenciar *in vitro*, em células osteogénicas, condrogénicas, adiposas e miogénicas e uma das suas vantagens mais importantes é serem MHC Classe II negativas e os seus níveis de expressão MHC Classe I poderem ser manipulados (Baksh *et al.*, 2007; Yang *et al.*, 2008).

3.3.3. Identificação, contagem e classificação das células estaminais

Para identificar qualquer um dos tipos de células estaminais, o modo de identificação mais usado é através dos marcadores que são moléculas que existem à superfície das células e são específicos de cada linhagem celular. Estes conjuntos de marcadores (proteínas) que permitem a distinção dos vários tipos de células (linhagens celulares), são denominados de Complexos de Diferenciação (CD) e são numerados à medida que vão sendo descobertos e descritos (Wiley & Sons, 1997). Como alguns marcadores só são expressos em determinadas fases do ciclo celular possibilita além da identificação do tipo de célula, também nos informa em que fase do seu ciclo se encontra. Como dito anteriormente, cada linhagem celular tem à sua superfície marcadores específicos, logo para a sua identificação é necessário usar uma molécula complementar a esses marcadores e que tem agregado a si um corante fluorescente, para que a sua cor seja detetada usando a citometria de fluxo (Figura 15) (Vieira, 2008).

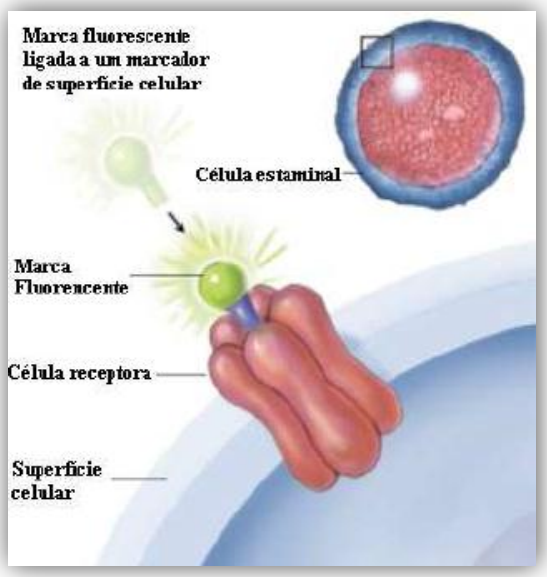

Figura 15 - Identificação de marcadores da superfície celular através de fluorescência (adaptada de Stem Cells: Scientific Progress and Future Research Directions, 2001).

A citometria de fluxo tem sido bastante utilizada para contagem diferentes sub-populações de células estaminais e também é um indicador de grande utilidade para determinar a capacidade hematopoiética reconstitutiva das células estaminais do sangue periférico em transplantes. Ao longo dos últimos anos, esta técnica tem sido aperfeiçoada, principalmente, através do melhoramento dos aparelhos para que mais parâmetros possam ser medidos (a nível da fisiologia e morfologia celular, por exemplo) e que mais áreas científicas possam desfrutar deste método (Wiley & Sons, 1997).

É um sistema que identifica células ou partículas que se encontram em suspensão num líquido que é atravessado por um feixe laser e em que a avaliação dos parâmetros é baseada na dispersão da luz bem como na cor dada pelas partículas fluorescentes (fluorocromos ligados a anticorpos ou corantes) (Macey, 2007).

A citometria permite identificar uma subpopulação homogénea dentro de uma sub-população heterogénea de células (Valete, 2005) e ainda avaliar a viabilidade celular (por exemplo através do marcador 7AAD) ajudando no diagnóstico médico em casos de patologias de foro hematológico e imunológico, por exemplo.

O anticorpo CD34 é um dos melhores marcadores para as HPSC. Este anticorpo pode ser utilizado para a identificação e contagem das células CD34+ do sangue periférico, medula óssea, SCU e também para produtos de aférese (Sutherland *et al*, 1996; Chin-Yee *et al*., 1997). Através da técnica de citometria de fluxo é possível determinar o número de células que expressam o marcador de superfície CD34 numa amostra sanguínea. É detetada a fluorescência emitida pelo fluorocromo que fica ligado à molécula complementar da CD34 que se encontra à superfície da célula, podendo ser assim determinado o número total de células presentes numa amostra (Kriete, 2005).

Através da figura 16 confirmamos que uma HPSC e uma célula progenitora megacariócitica apenas expressam a molécula CD34 na fase de diferenciação e não durante a fase de maturação. Contudo é necessário saber que esta proteína (CD34) não é um indicador exclusivo da presença das HPSC (Silva *et al*., 2003).

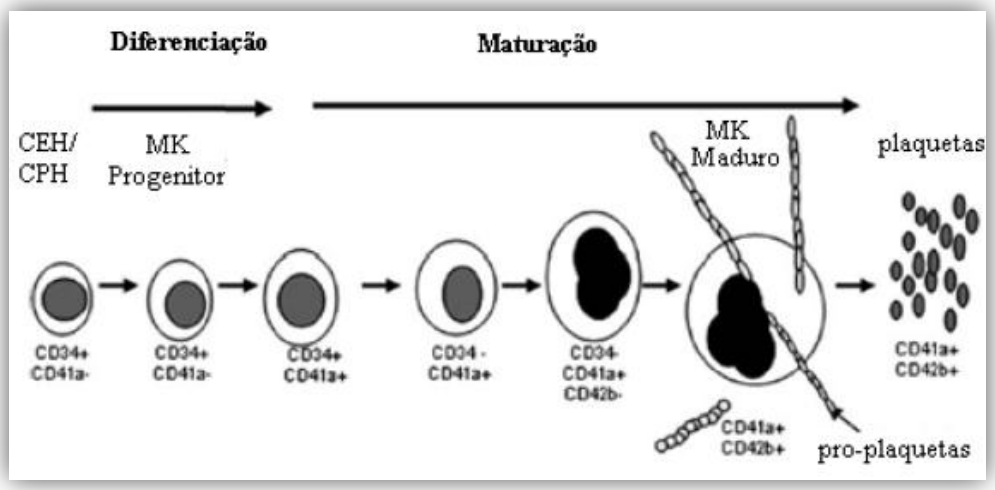

Figura 16 - Expressão dos CD34 nas células progenitoras que irão originar plaquetas sanguíneas (adaptada de Audet & Stanford, 2009).

Sabe-se que a linhagem mieloide expressa principalmente as CD15 e CD33 e que a linhagem linfoide as CD3 e CD19. As MSCs expressam as proteínas CD105, CD73 e CD90 e não podem apresentar níveis elevados dos marcadores hematopoiéticos CD34 e CD45. O marcador CD45 é típico da superfície das células progenitoras dos glóbulos brancos (Stem Cells: Scientific Progress and Future Research Directions, 2001).

A citometria de fluxo tem um papel importante no diagnóstico médico e no crescente conhecimento a nível da biologia celular como o desenvolvimento de novos métodos de diagnóstico rápido de algumas doenças através da quantificação e identificação das proteínas que lhes estão associadas (Kriete, 2005).

É necessário haver um bom conhecimento acerca dos diferentes marcadores específicos de cada linhagem celular para que haja um aumento de trabalho laboratorial desenvolvido e de melhor qualidade. Este trabalho é de extrema importância pois pode promover grandes avanços na área da saúde, beneficiando assim o ser humano. Devido a todas as discussões que surgem sobre o uso e a origem de certas células, como por exemplo as provenientes de embriões, tem de ser levado em conta as questões éticas e legislação associadas ao tema de forma a esclarecer o processo para beneficiar o progresso científico (Lopes, 2009).

A figura 17 demonstra um resultado de citometria de fluxo obtido no laboratório Biosckin para uma amostra de SCU, utilizando os marcadores CD34, CD45 e 7AAD, com o objetivo de saber quantas células estaminais hematopoiéticas viáveis tem o SCU que é criopreservado.

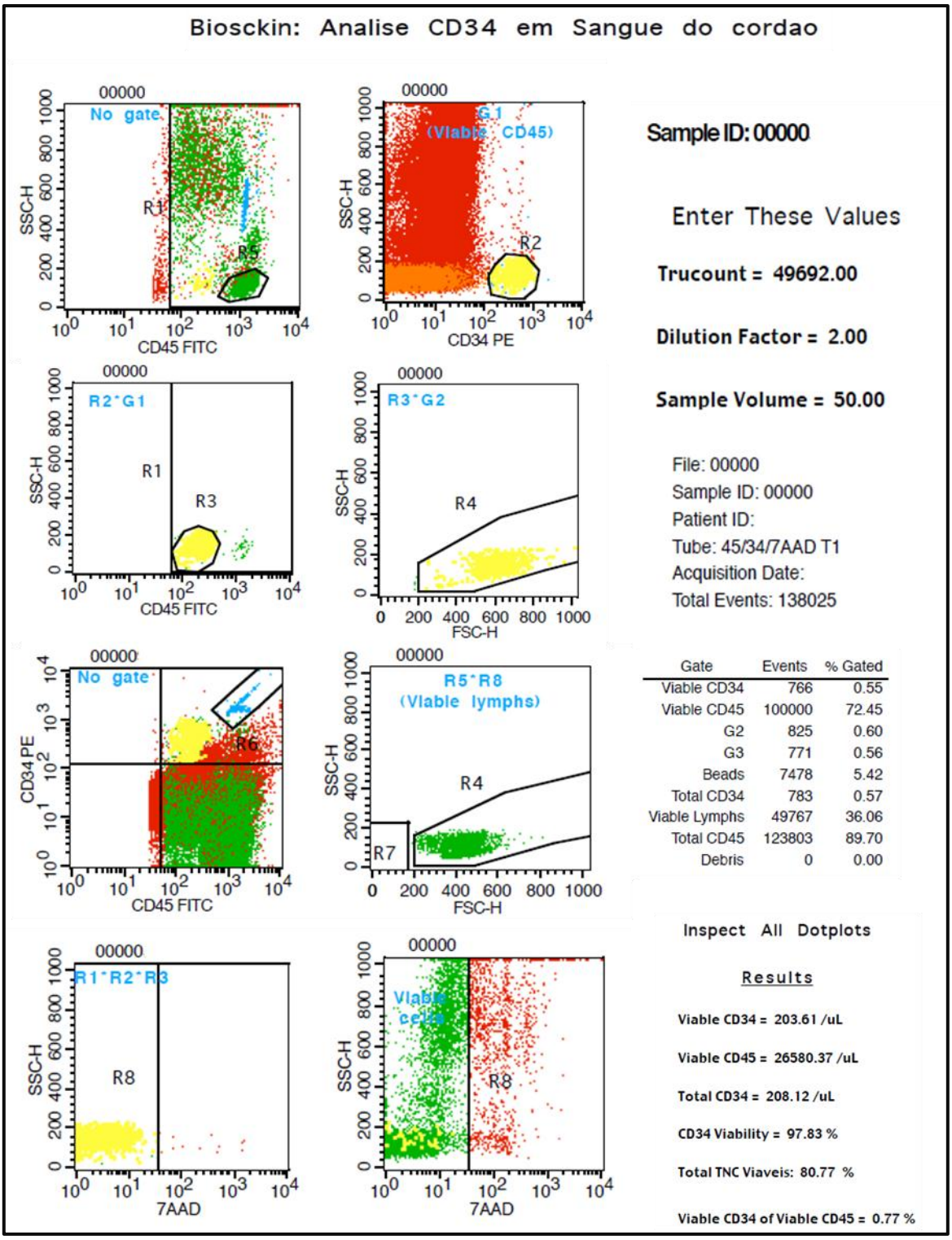

Figura 17 - Resultado de citometria de fluxo obtido no laboratório Biosckin para uma amostra de SCU, denominada 00000, utilizando os marcadores CD34, CD45 e 7AAD. (gentilmente cedida pelo Laboratório Biosckin)

4. Vascularização do nervo periférico

O nervo periférico possui um sistema microvascular bem desenvolvido no epineuro, perineuro e endoneuro. Os seus vasos encontram-se distribuídos em várias camadas no nervo, e são interligados através de numerosas anastomoses (Figura 18) (Bell & Weddell, 1984).

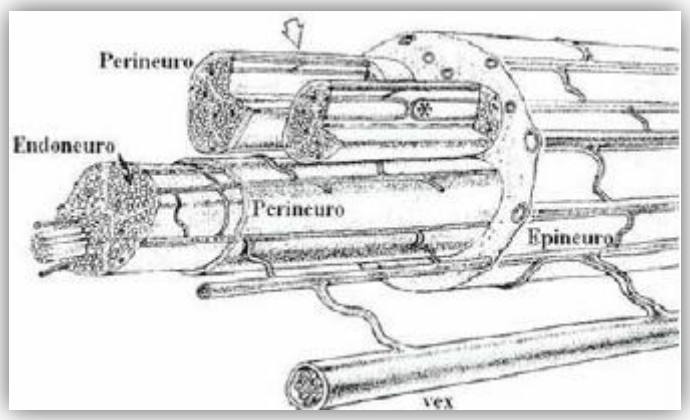

Figura 18 - Esquema da vascularização do nervo periférico (gentilmente cedido por Prof. Artur Varejão).

O sistema microvascular extrínseco abrange todos os vasos da região anatómica próxima ao tronco nervoso, cuja função é suprir a microcirculação intraneural. Essa estrutura intraneural funciona como um sistema de reserva, de forma a manter o fluxo sanguíneo, mesmo quando o nervo sofre uma lesão mais grave (Lundborg, 1987; Lundy-Ekman, 2004; Pachioni & Stellutti, 2006).

A transmissão do impulso nervoso, bem como o transporte axonal, requer um suprimento de energia contínuo proporcionado pelos microvasos intraneurais. O sistema microvascular possui uma grande capacidade de reserva para compensar a mobilização ou lesão dos vasos regionais locais. No epineuro, os vasos orientados longitudinalmente exibem um padrão característico, ou seja, os vasos estão presentes superficialmente em todas as camadas do epineuro assim como, entre os feixes fasciculares nas camadas profundas do nervo (Lundborg & Dahlin, 1996; Rempel *et al*., 1999).

A importância da vascularização dos nervos periféricos deve-se ao facto dos axónios dos nervos periféricos serem vulneráveis à isquémia devido à grande distância que existe entre o corpo neuronal e a extensão do axónio (Reina *et al*., 2000).

O nervo ciático do rato é o ideal para o estudo dos vasos intraneurais devido à sua semelhança com os nervos periféricos humanos. É facilmente acessível, tem vários fascículos, é um nervo misto e apresenta densa irrigação sanguínea (Pachioni & Stellutti, 2006). Alguns investigadores demonstraram um aumento no espaço vascular bem como um aumento da permeabilidade dos capilares endoneurais após esmagamento de nervo ciático de sapo, concluindo que a lesão altera a permeabilidade perineural e vascular endoneural e inicia o edema (Lundborg *et al*., 1983; Weerasuriya, 1990). O edema endoneural pode afetar seriamente as diversas funções das fibras nervosas, mesmo com diferenças no animal, método e duração do esmagamento e tempo de colheita (Chen *et al*., 1992).

No trabalho realizado por Pachioni e colaboradores, a análise microscópica dos vasos do nervo ciático mostrou regiões de hematoma endoneural e epineural nas diferentes cargas utilizadas indicando que as forças de esmagamento utilizadas foram suficientes para lesar os vasos intraneurais, especialmente os capilares endoneurais, porém os valores críticos de pressão e duração com respeito à lesão nervosa são desconhecidos. O hematoma é o resultado do aumento da permeabilidade vascular pela lesão direta da parede dos capilares, proporcionada pela força do esmagamento (Pachioni & Stellutti, 2006).

Ainda com o mesmo estudo, os mesmos autores mostraram claramente que existe um comprometimento vascular importante nas lesões por esmagamento, especialmente nos grupos com cargas elevadas. A destruição dos vasos deverá levar à isquémia e lentificação dos processos de regeneração. A formação do hematoma endoneural certamente criará um microambiente desfavorável para a regeneração das fibras nervosas que também foram lesadas nesse modelo. Estes investigadores concluem que o esmagamento causa lesão direta dos vasos endoneurais, com a formação de hematoma endoneural de gravidade diretamente proporcional à carga (força) de esmagamento. A lesão dos vasos intraneurais e a formação de um hematoma endoneural são fatores que podem prejudicar a regeneração nervosa e interferir com a função nervosa (Pachioni & Stellutti, 2006).

Alterações inflamatórias tais como edema intraneural, são alterações que acompanham a compressão do nervo, ocorrem não só no local da compressão, mas também em áreas afetadas pela degenerescência Walleriana após compressão, aparecendo em simultâneo numerosas células inflamatórias no interior do nervo (Scholz & Woolf, 2007).

5. Métodos de avaliação da recuperação neurológica

A espécie mais utilizada como modelo na investigação de doenças neurológicas quer do sistema nervoso periférico e central, quer ainda em doenças neurovegetativas (ex: Alzheimar e Parkinson) são os ratos (Cenci *et al*., 2002). Os investigadores que se dedicam ao estudo de lesões do nervo periférico têm como uma das principais dificuldades a avaliação da recuperação funcional. Para ultrapassar este problema foram desenvolvidos ao longo do tempo vários métodos e técnicas, nos quais não existe um que seja perfeito na sua avaliação, logo todos eles se utilizados em simultâneo, ajudam a minimizar as imperfeições de cada um e portanto, a validar as conclusões finais. Também têm de ser levadas em consideração as diferenças que existem no tipo de locomoção quadrúpede dos animais e bípede dos humanos, tendo sido no entanto demonstradas algumas semelhanças básicas entre ambos (Figura 19) (Duysens *et al*, 2002).

No caso do Homem, o nervo ciático bifurca-se em dois importantes ramos: o ramo ciático poplíteo externo e o ramo ciático poplíteo interno. O ramo ciático poplíteo externo ou peroneal divide-se nos nervos músculo-cutâneo e tibial anterior e é responsável pela inervação dos músculos anteriores e laterais da perna e pé. O nervo ciático poplíteo interno ou tibial inerva grande parte dos músculos posteriores da coxa e perna, os músculos plantares do pé e a pele que reveste a planta do pé, através das ramificações que formam os nervos plantares interno e externo. Outras ramificações fornecem parte da inervação cutânea da face posterior da perna e superfície plantar do pé, e constituem o nervo safeno externo (ramo terminal do nervo femoral) (Figura 19) (Seeley *et al*., 1995).

Desta forma, é compreensível que por exemplo numa lesão do nervo ciático, dependendo das raízes afetadas, possa levar a uma paralisia muscular e/ou alterações da sensibilidade de determinados músculos do membro inferior, prejudicando gravemente a locomoção (Amado, 2007).

Frequentemente, para testar as várias estratégias terapêuticas utiliza-se o nervo ciático do rato, nomeadamente para o exercício (Varejão *et al*., 2003b), os efeitos do laser (Menovsky & Beek, 2001) e dos ultra-sons (Crisci, 2001), sendo também o mais usado na investigação em neurobiologia (Metz *et al*., 2000; Varejão, 2003; Nichols *et al*., 2005). Inicialmente, o investigador deve-se inteirar sobre qual o modelo animal a ser escolhido, que melhor se adeque à experiência. Também tem que ter em atenção se os desenhos experimentais seguem os princípios científicos válidos e se retratam as condições clínicas específicas a estudar, de modo a que este possibilite a

replicação do procedimento/intervenção e reflitam resultados semelhantes aos encontrados nos humanos (Lubischer & Tompson, 1999). Não deve ainda ser esquecido o bem-estar animal nem a legislação em vigor relativa à experimentação.

As metodologias de avaliação tradicionais de recuperação do nervo após lesão, como é o caso da histomorfometria e eletrofisiologia não revelam verdadeiramente se existe um retorno da função motora e sensitiva (Strasberg et al., 1996), como é o caso da extrapolação dos parâmetros eletrofisiológicos e que por isso pode levar a interpretações incorretas (Varejão et al., 2001). Contudo os estudos histológicos revelam-se úteis na avaliação dos efeitos fisiológicos de determinadas técnicas terapêuticas usadas na regeneração do nervo periférico, como também é o caso da avaliação funcional, após lesão do nervo periférico em estudos experimentais que utilizam o modelo do rato, que pode ser quantificada através de vários métodos. Pode-se afirmar assim, que como modelo ideal para avaliar a relação entre os dados da biologia celular e a avaliação funcional, ao nível do SNP, é utilizado o modelo experimental do nervo ciático do rato (Amado, 2007).

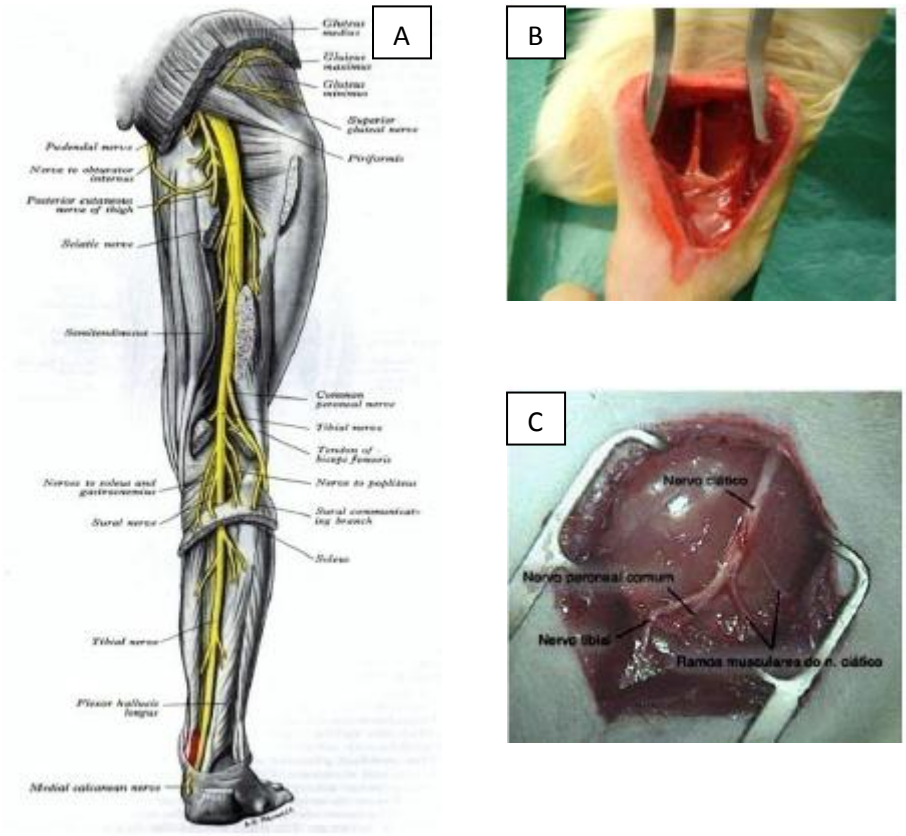

Figura 19 - (A) Esquema da localização do nervo ciático no Homem, no membro inferior direito (Warwick & Williams, 1973); **(B)** Fotografia do nervo ciático direito de rato (gentilmente cedida por Prof. Ana Lúcia Luís); **(C)** Acesso cirúrgico ao nervo ciático do rato, mostrando a união dos seus ramos musculares e a sua bifurcação em nervo peroneal e nervo tibial (gentilmente cedida por Prof. Artur Varejão).

5.1. Testes de avaliação funcional

5.1.1. Reação postural de extensão

Para a avaliação da função motora dos membros pélvicos do rato foi proposta a EPT, no ano de 1995 (Thalhammer *et al*., 1995). Em Medicina Veterinária, esta reação postural está inserida no exame neurológico que é utilizada na clínica de animais de companhia. Para se iniciar a realização deste teste, é necessário que todo o corpo do animal esteja envolvido num pano, com exceção dos membros pélvicos, sendo suspenso pelo tórax numa posição vertical e de seguida transportado para uma plataforma de uma balança digital. O operador segura no membro que não vai ser testado, enquanto o membro a testar realiza um movimento de extensão a antecipar o contacto com a plataforma (Figura 20). Deve ser utilizada uma balança digital com uma escala compreendida entre os 0 e 500g, para registar a força em gramas produzida pelo membro a testar (Luís, 2008).

Figura 20 - Execução do teste motor de EPT (imagem gentilmente cedida por Prof. Ana Lúcia Luís, 2008).

Este teste é realizado tanto no membro normal (NRPE) como no membro de experiência (ERPE) e os valores obtidos são posteriormente introduzidos na Equação1, de forma a traduzirem um valor que multiplicado por 100, traduz a percentagem de défice motor do membro experimental em relação ao membro normal:

$$\% \text{ DE DÉFICE MOTOR} = (NRPE - ERPE) / NRPE$$

(Equação 1)

Uma das vantagens deste teste é de ter uma fácil execução, dependendo apenas de alguma sensibilidade do operador. É um teste de grande importância para avaliação da função motora, mesmo quando temos dificuldade na realização do estudo das pegadas do rato para dedução do SFI e do SSI, como por exemplo, em casos de autotomia. Tem vindo a ser bastante usado por ter mostrado resultados comparáveis aos obtidos com o estudo das pegadas do rato (Hadlock *et al*., 1999; Koka & Hadlock, 2001).

5.1.2. Avaliação da latência do reflexo flexor

A avaliação da latência do reflexo flexor foi proposta por Sherrington em 1910 e baseia-se na quantificação do reflexo flexor através do qual o animal exibe um reflexo de retirada do membro quando exposto a um estímulo térmico, mecânico ou elétrico. A avaliação nociceptiva é então testada através da observação do reflexo de retirada do membro pélvico como resposta a uma estimulação nociceptiva (Schouenborg & Kalliomaki, 1990). A função nociceptiva é avaliada utilizando um estímulo térmico, utilizando uma placa térmica aquecida a 56ºC, tendo como base uma técnica desenvolvida e adotada por Masters e colaboradores em 1993. Para iniciar este teste, tal como o anterior, é necessário envolver o animal num pano e o membro a testar é posicionado de forma a tocar na placa aquecida (Figura 21). A duração necessária do estímulo para induzir o reflexo flexor é registada e assim determina a WRL. O valor máximo que este reflexo deve ter em membros normais dos ratos é de 4,3 segundos (Hu *et al*., 1997). Para avaliar o membro afetado este é testado três vezes consecutivas, com intervalos de dois minutos entre eles, de modo a evitar o fenómeno de sensibilização e posteriormente é realizada a média dos três registos obtidos. Devido à lesão provocada no membro este pode perder a sensibilidade, por isso foi definido um tempo máximo de 12 segundos para que o teste seja terminado caso não tenha ocorrido ainda reflexo de flexão, a fim de evitar lesões de queimadura na pele. Para o registo utiliza-se o valor de 12 segundos. Para uma correta avaliação da recuperação nociceptiva deve ser evitada a região medial da face plantar do pé do rato, de forma a limitar a influência da invasão territorial por parte do nervo safeno quando numa lesão do nervo ciático (Kingery & Vallin, 1989). Para evitar erros de leitura os animais deverão estar tranquilos, serem testados em ambiente calmo, retirando se possível todas as possíveis fontes de stress e se possível o teste deverá ser realizado pelo mesmo operador, numa sequência sempre idêntica, com o mesmo material e na mesma sala, evitando desta forma fontes de curiosidade e distração.

Estas medidas também se possível são aplicadas ao teste descrito anteriormente (Luís, 2008).

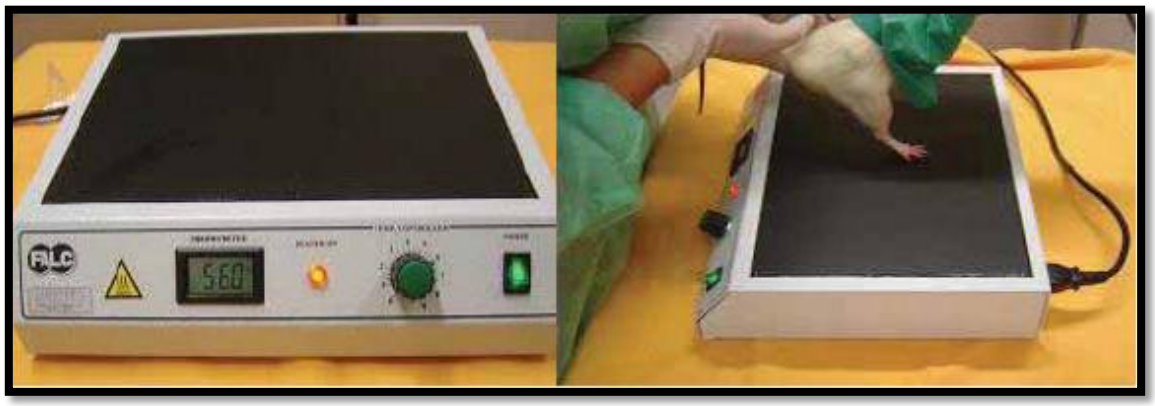

Figura 21 - Execução do teste de sensibilidade da WRL (imagem gentilmente cedida por Prof. Ana Lúcia Luís, 2008)

5.1.3. Análise do estudo das pegadas (*Walking Track Analysis*)

Em 1982, foi criado um método para quantificar a recuperação funcional do nervo ciático após lesão, utilizando como modelo experimental o rato e baseando-se no estudo das pegadas (*Walking Track Analysis*), por De Medinaceli e seus colaboradores. Estas investigações/estudos originaram o Índice de Funcionalidade do Ciático (SFI), que tem sido bastante usado como ferramenta de avaliação funcional em estudos de lesões do nervo periférico por muitos investigadores. É um método que tem várias vantagens como não precisar de equipamento especialmente dispendioso, ser de fácil execução e ter resultados precisos e fiáveis (De Medinaceli *et al*., 1982; Bain *et al*., 1989; Nichols *et al*., 2005).

5.1.3.1. Índice de Funcionalidade do Ciático

Para a realização deste teste é necessário que previamente os animais sejam treinados a andar no corredor em direção à caixa que tem como característica ter fraca luminosidade de forma a atrair o rato para o seu interior. Para obter as medidas das impressões das pegadas do rato são colocadas tiras de papel branco no corredor, com a dimensão adequada, e a face plantar dos pés dos ratos é pintada através da sua compressão numa esponja de tinta preta não tóxica (Figura 22) (Luís, 2008). Logo depois das patas posteriores do rato terem sido pintadas, este é colocado no início do corredor para que ao caminhar em direção à caixa deixe as suas pegadas nas tiras de papel (Costa *et al*., 2009).

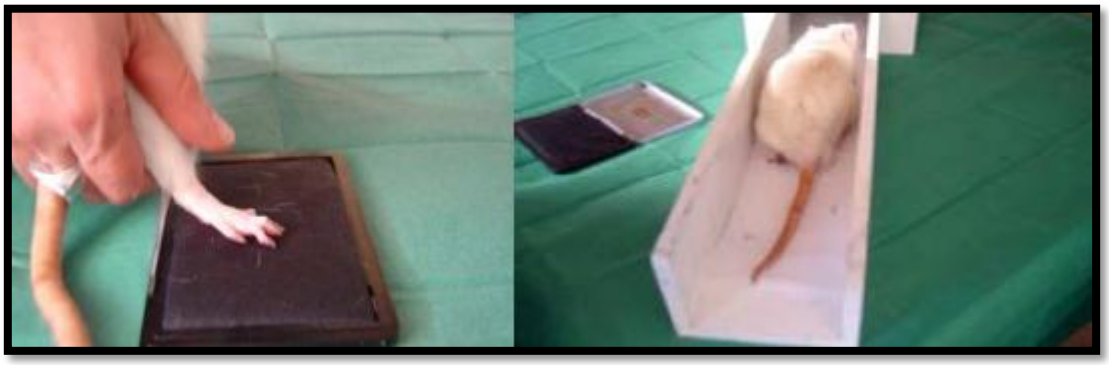

Figura 22 - Realização do teste SFI. Para a impressão das pegadas são colocadas tiras de papel branco com a dimensão adequada no corredor e a face plantar dos pés dos ratos é pintada com tinta através da impressão do pé do rato numa esponja de tinta não tóxica (imagem gentilmente cedida por Prof. Ana Lúcia Luís, 2008).

Com este índice, as pegadas são avaliadas em três parâmetros diferentes:

i) Distância do calcanhar ao terceiro dedo, comprimento da pegada (PL);

ii) Distância do primeiro ao quinto dedo (TS);

iii) Distância do segundo ao quarto dedo (ITS).

As três medidas são realizadas tanto para o membro normal (N) como para o membro experimental (E) (Figuras 23A, 23B e 24). Para obtenção do valor de SFI usa-se a fórmula complexa seguinte (Equação 2) derivada de Bain *et al*., (1989) ou de uma forma mais simplificada a Equação 3.

$$SFI = -38.3[(EPL–NPL)/NPL]+109.5[(ETS–NTS)/NTS]+13.3[(EITS–NITS)/NITS]-8.8$$

(Equação 2)

$$SFI = -38.3 \times PLF + 109.5 \times TSF + 13.3 \times ITSF – 8.8$$

(Equação 3)

O valor de SFI varia entre 0 e -100, sendo o primeiro valor referente a uma função normal e o segundo para uma disfunção absoluta, como aquela verificada imediatamente após recessão do nervo ciático (Dijkstra *et al*., 2000).

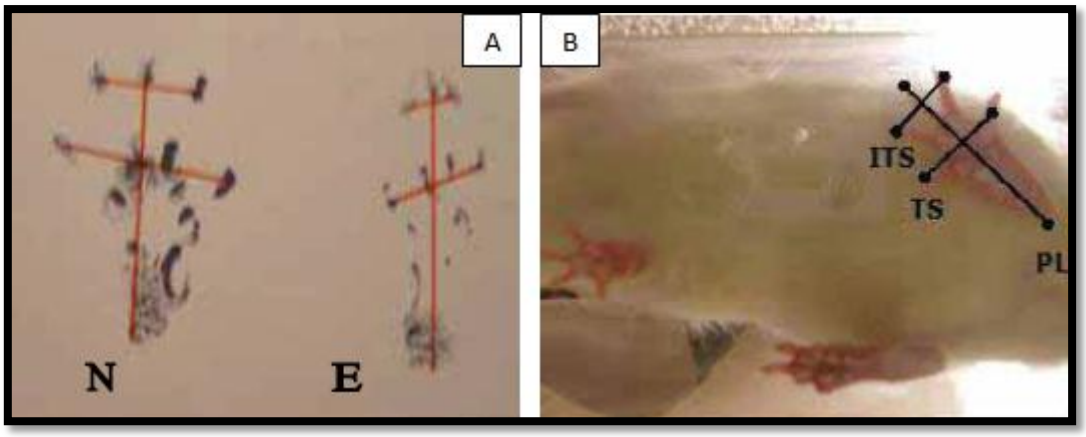

Figura 23 - Fotografia da fase de suporte do andamento do rato, tirada num corredor com impressão das pegadas em tiras de papel branco **(A)** (à esquerda, membro normal; à direita, membro experimental) ou num corredor de acrílico **(B)**. Foram assinalados os seguintes segmentos: **PL** -, comprimento da pegada; **TS** - largura da pegada; **ITS** - largura intermédia da pegada (imagem gentilmente cedida por Prof. Ana Lúcia Luís, 2008).

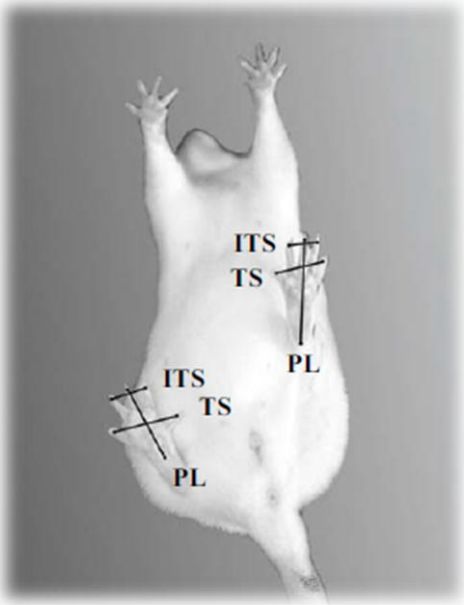

Figura 24 - Superfície plantar dos membros posteriores do rato. PL, comprimento da pegada; TS, largura da pegada; ITS, largura intermédia da pegada. A pata posterior esquerda mostra a redução dos parâmetros TS e ITS, enquanto o PL está aumentado (adaptado de Costa et al., 2009).

5.1.3.2. Índice de Funcionalidade do Ciático em condições estáticas

Devido a alguns problemas que podem surgir durante a recuperação, como são o caso das contracturas e o fenómeno de autotomia, por vezes não é possível obter pegadas que apresentem uma boa leitura para o cálculo do SFI (Meek & Coert, 2002). Para ultrapassar estas dificuldades Bervar, em 2000 propôs uma fórmula que tem

vindo a ser utilizado por alguns investigadores e é designada por Índice de Funcionalidade do Ciático em Condições Estáticas (SSI), em que não é utilizada a medida de PL (Bervar, 2000), e é representada pela Equação 4:

$$SSI = [(108.44 (TSF) + (31.85 (ITSF)] - 5.49$$

(Equação 4)

O valor de SSI também varia entre 0 e -100, sendo o primeiro valor referente a uma função normal e o segundo para uma disfunção absoluta (Dijkstra *et al*., 2000).

5.1.3.3. Limitações na análise do SFI e SSI

Esta metodologia tem sido atualmente muito utilizada no estudo do nervo mas existem alguns problemas que têm limitado o seu uso experimental. Um deles é a necessidade de fazer repetidas passagens para obter pegadas visíveis de ambos os pés. Para evitar este caso é importante treinar os animais, de forma a familiarizar o animal com o local da prova (Bain *et al*., 1989).

Devido a fenómenos de autotomia (Figura 25A), desenvolvimento de contracturas (Figura 25B), arrastamento da pegada, arrastamento da cauda sobre a pegada ou contaminação de pegadas, algumas pegadas não são possíveis de serem analisadas, isto é, de serem feitas medições (Strasberg *et al*., 1996; Varejão *et al*., 2004).

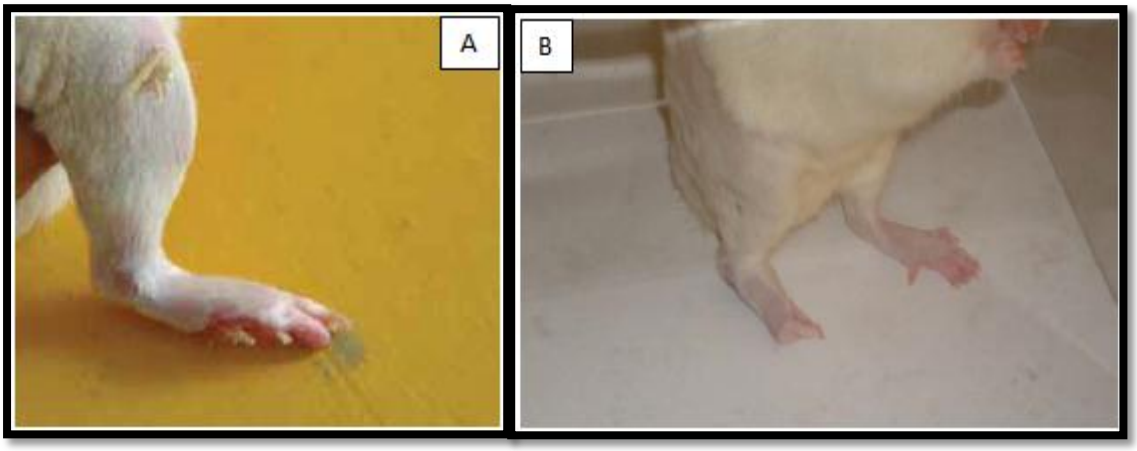

Figura 25 - Fotografias do membro posterior experimental: **(A)** fenómeno da autotomia; **(B)** contractura do membro. (Vieira, 2008)

Como resultado de uma reinervação mais rápida ou completa dos músculos flexores em detrimento dos músculos extensores surgem as contracturas articulares

em lesões do nervo ciático e que fazem com que os animais caminhem sobre o dorso do pé (Chamberlain *et al*., 2000). Para evitar o aparecimento das contracturas, muitos investigadores têm recorrido a técnicas de fisioterapia, quer através da manipulação manual do membro lesado quer ainda através da utilização de uma rede com cerca de 45º de inclinação que é colocada no interior da gaiola dos animais (Kobayashi *et al*., 1997; Varejão *et al*., 2003a). Tem-se conseguido bons resultados com a utilização de um protocolo de fisioterapia na fase inicial da regeneração, evitando a inativação muscular durante períodos prolongados (Watson *et al*., 2001; Sagiv *et al*., 2002; Varejão *et al*., 2003a).

O fenómeno de autotomia surge especialmente quando são realizadas lesões de secção do nervo ciático em ratos (Luís, 2008). É provável que este tenha origem numa disestesia dolorosa projetada nos dedos que leva à sua automutilação, em especial dos dois dedos laterais (Kauppila, 1998). Este fenómeno faz com que seja impossível obter valores para o cálculo de SFI e SSI, podendo ser evitado através da utilização de uma substância com um sabor dissuasor no local (Hadlock *et al*., 1999; Varejão, 2003). Outras estratégias podem ser utilizadas como por exemplo o uso de amitriptilina (Navarro *et al*., 1994; Rodriguez *et al*., 1999) e o alojamento na mesma gaiola de machos e fêmeas (Zellem *et al*., 1989).

Além das dificuldades descritas anteriormente podem surgir outras com menos importância mas mesmo assim alvo de muitas críticas. A subjetividade inevitável na leitura dos valores para o cálculo de SFI e SSI, torna muitas vezes necessário a repetição das passagens do rato pelo corredor para minimizar o erro de leitura/cálculo (Luís, 2008). A velocidade do andamento durante a fase de suporte influencia os valores de PL e por isso o cálculo de SFI (Walker *et al*., 1994b). A importância da análise das pegadas ser efetuada em pegadas não hesitantes e de velocidade constante, uma vez que os valores de PL variam com a velocidade do andamento (Dijkstra *et al*., 2000). O aumento de peso corporal que os animais podem apresentar ao longo do trabalho experimental pode também influenciar a medição das pegadas (Dellon & Dellon, 1991). Dellon & Dellon (1991) descreve em estirpes de ratos Sprague Dawley um fenómeno de compensação do membro normal que suporta mais peso, compensando desta forma o membro lesado. Posteriormente, este fenómeno foi rebatido por Hare *et al*. (1992), num ensaio de avaliação a longo prazo da regeneração nervosa em ratos de estirpe Lewis. É sugerido, que uma ligeira evolução favorável no membro lesado, mesmo antes da conexão funcional entre os topos distal e proximal do ciático, se deva a um fenómeno de adaptação por parte do animal (Chamberlain *et al.*, 2000). Sendo o rato utilizado como modelo experimental, é possível verificar que a

duração da fase de suporte do membro experimental é inferior à do membro normal (Dijkstra *et al*., 2000).

Em animais com o membro intacto, os valores de SFI nunca são 0, oscilando antes à volta de -10, demonstrando assim que este método não é inteiramente preciso. Daí o interesse em definir limites para a aplicação deste teste e ainda estabelecer correlações entre os resultados por meio destes testes e a histomorfometria (Luís, 2008).

5.2. Testes de avaliação morfológica

Para analisar a reparação do nervo periférico utiliza-se como principal instrumento a avaliação morfológica das fibras regeneradas através da microscopia de luz (Lundborg, 1988). Em virtude da utilização desta avaliação é possível verificar no nervo periférico que tenha sido reparado, a presença ou ausência de fibras nervosas e a sua distribuição espacial, a existência e a qualidade de mielinização, a presença e a qualificação dos tecidos vizinhos e se ocorreu a reabsorção total ou parcial dos biomateriais utilizados. Além desta avaliação permitir uma análise qualitativa, possibilita ainda uma análise quantitativa das fibras nervosas que está provado ser fundamental nos estudos de reparação do nervo periférico (Geuna *et al*., 2000; Geuna *et al*., 2001).

As fibras nervosas apresentam uma distribuição anisotrópica ao longo do nervo periférico e muitas vezes têm tendência a agrupar-se de acordo com o seu diâmetro (Torch *et al*., 1989), fazendo assim com que o processo de seleção dos campos histológicos seja um fator importante e que poderá influenciar de forma decisiva o resultado final. Esta seleção dos campos histológicos pode ser sobrestimada se escolher campos mais densos ou subestimada se escolher campos menos densos, levando assim a tirar conclusões erradas. O principal perigo para uma análise histomorfométrica é precisamente a subjetividade na escolha da amostra (Luís, 2008).

Devido ao que foi descrito anteriormente, é por isso importante, neste método de análise, estabelecer de uma forma correta a estratégia utilizada em relação à recolha da amostragem, que irá ser designada como população, na qual irá ser baseada a análise (Luís, 2008). De forma a assegurar que todas as fibras nervosas têm a mesma oportunidade de serem incluídas na amostra, uma vez que analisar todas as fibras nervosas de um nervo é um processo ineficaz, surgiu a regra da igualdade de oportunidade (Geuna, 2000). Para esta regra ser cumprida é designado o *Design-based sampling* que consiste num conjunto de regras de amostragem, com a intenção

de que todos os objetos presentes no espaço da amostragem tenham a mesma probabilidade de serem selecionados. Resultando assim numa amostragem aleatória sistemática (Geuna *et al*., 2000), que irá representar toda a fibra nervosa. Para aplicar este método é necessário escolher de forma aleatória um campo inicial e a partir dele selecionar os outros campos, através da sistematização de um processo de salto para o campo seguinte, localizado a uma distância fixa. Deverão ser selecionados preferencialmente um número relativamente elevado de campos (> 15) de menores dimensões, em vez de um número menor de campos e de maior dimensão (Schmitz, 1998).

Existe no entanto outra situação que pode também levar a que haja erros na histomorfometria do nervo periférico e que consiste na probabilidade de uma fibra de maiores dimensões poder aparecer em mais que um campo de análise, o que poderá levar a que uma fibra possa ser lida mais que uma vez (Geuna *et al*., 2001). Para evitar que esta situação aconteça, utiliza-se o método *Two-dimensional-disector* (dissecção bi-dimensional), onde é assinalado o topo de cada fibra, que representa o ponto do contorno da fibra que primeiro toca o plano de observação e somente estes são lidos e no sentido norte/sul. Como cada topo só aparece num campo e este também não representa nenhuma subjetividade para o investigador só será incluída na amostra uma vez (Geuna *et al*., 2000).

Outra técnica utilizada é a imunohistoquímica que se baseia na utilização de anticorpos que se ligam especificamente a um determinado antigénio celular, tornando-se assim visíveis ao microscópio por fluorescência ou de laser confocal, sendo necessário recorrer à utilização de determinadas sondas fluorescentes ou fluoróforos para sejam detetados os complexos antigénios-anticorpo. Através desta técnica é possível detetar os axónios em regeneração com a utilização de anticorpos anti-NF-200kd (antiproteína 200kd dos neurofilamentos) e ainda a possível migração das células de Schwann dentro dos tubos-guia ou nos biomateriais utilizando anticorpos anti-GFAP (antiproteína glial fibrilarácida), durante o período de regeneração do nervo periférico (Geuna *et al*., 2001; Varejão, 2003). Atualmente existe uma grande variedade de antigénios disponíveis, afinidades antigénio-anticorpo, tipos de anticorpo e métodos de avaliação e deteção, no entanto é necessário que o método a utilizar seja o mais apropriado para cada situação em particular. Para a microscopia óptica, é normalmente utilizada a coloração com hematoxilina-eosina (H&E) (Luís, 2008).

6. Objetivos do estudo

O principal objetivo de estudo deste trabalho consiste em estudar o valor terapêutico das MSCs derivadas da geleia de Wharton do cordão umbilical tanto *in vivo* como *in vitro*, associadas a uma membrana de poli(DL-lactide-ε-caprolactone) (PLC), em modelos experimentais de lesões de axonotmese no nervo ciático de ratos. Este trabalho foi realizado no âmbito da tese de Doutoramento da Mestre Andréa Louise Moutinho Gärtner (Instituto de Ciências biomédicas Abel Salazar – ICBAS, da Universidade do Porto – UP, sendo aqui apresentados resultados do artigo científico em fase de impressão, com a sua autorização, do qual sou co-autora e com autorização dos restantes autores.

Este objetivo geral levou a que fossem definidos objetivos mais específicos na parte do isolamento das MSCs derivadas da geleia de Wharton, uma vez que se tratava de um novo sistema celular a ser testado pelo nosso grupo experimental, tais como:

- Validar o método de transporte do cordão umbilical desde o momento do parto até ao laboratório, testando várias soluções isotónicas em que o cordão pode ser submerso para ser transportado, de maneira a que o cordão mantenha as suas características estruturais, restringindo a contaminação e multiplicação microbiana e permitindo a obtenção por isolamento mecânico e / ou enzimático de um maior número de MSCs viáveis.
- Validar o método de lavagem utilizado que antecede o processamento do cordão umbilical para criopreservação e / ou para isolamento mecânico e / ou enzimático de MSCs para a sua utilização *in vivo*.
- Validar o processamento do tecido do cordão umbilical para criopreservação, que está descrito no procedimento técnico BSK.LCV.PT.7-Edição1 do Laboratório Biosckin.
- Melhorar o protocolo de culturas celulares tanto de MSCs derivadas de uma linha celular estabelecida pela PromoCell como de MSCs isoladas a partir da geleia de Wharton do cordão umbilical (tecido do cordão umbilical fresco ou após criopreservação, segundo o procedimento técnico BSK.LCV.PT.7-Edição1 do Laboratório Biosckin).

Devido à minha integração numa equipa multidisciplinar que se dedica a temas de Medicina Regenerativa, acabei por participar em vários projetos de investigação (Projetos de Mestrado e Projetos de Doutoramento), onde a validação das técnicas acima descritas e a preparação e caracterização das MSCs isoladas da geleia de

Wharton era essencial e transversal a todos os projetos, tendo sido, por isso, definidos outros objetivos, nomeadamente:

- Testar o efeito *in vivo* do uso de MSCs isoladas da geleia de Wharton associadas a veículos biocompatíveis na regeneração do músculo tibial anterior após testes químicos, mecânicos e modelos cirúrgicos de lesão muscular. Este objetivo estabelecido no âmbito do Projeto de Doutoramento em Ciências Veterinárias do ICBAS – UP, do Licenciado Tiago de Melo Silva Ramos Pereira, Assistente Convidado do Departamento de Clínicas Veterinárias – ICBAS – UP. Propriedades morfológicas e fisiológicas do músculo-esquelético: Estudos *in vivo* com modelos de regeneração de tecido muscular.
- Realização de testes de biocompatibilidade de biomateriais desenvolvidos no âmbito do Mestrado Integrado em Bioengenharia da Faculdade de Engenharia da Universidade do Porto (FEUP) e do ICBAS-UP que poderão ser posteriormente utilizados como veículos de células MSCs tanto em lesões do nervo ciático como em lesões musculares e regeneração do tecido ósseo.

Capítulo II

Trabalho Experimental

1. Reconstrução cirúrgica do nervo periférico após lesões de axonotmese

O trabalho experimental desenvolvido em co-autoria e no âmbito dos seguintes Projetos de Doutoramento:

- Doutoramento em Ciências Veterinárias do ICBAS – UP, do Licenciado Tiago de Melo Silva Ramos Pereira, Assistente Convidado do Departamento de Clínicas Veterinárias – ICBAS – UP. Propriedades morfológicas e fisiológicas do músculo-esquelético: Estudos *in vivo* com modelos de regeneração de tecido muscular.
- Doutoramento em Ciências Veterinárias do ICBAS – UP, inserido no Programa Doutoral em Patologia e Genética Molecular, da Mestre Andréa Louise Moutinho Gärtner com a Bolsa de Doutoramento da Fundação para a Ciência e a Tecnologia (FCT) do Ministério da Ciência, da Tecnologia e do Ensino Superior (MCTES) com a referência SFRH/BD/70211/2010. Estudo funcional e morfológico da regeneração do nervo periférico recorrendo a PLGA 90:10 e células estaminais CD34+.

Os resultados foram publicados, em que o artigo e os dois capítulos de livro estão em fase de impressão, com as seguintes referências e aqui introduzidos com autorização dos restantes autores:

✓ A Gärtner, T Pereira, PAS Armada da Silva, I Amorim, R Gomes, J Ribeiro, ML França, C Lopes, B Porto, R Sousa, A Bombaci, F Fregnan, ASP Varejão, AL Luís, S Geuna, AC Maurício (2012). Use of poly(DL-lactide-e-caprolactone) PLC membranes and Mesenchymal Stem Cells for promoting nerve regeneration in an axonotmesis rat model: *in vitro* and *in vivo* analysis. Differentiation (em impressão)

✓ Andréa Gärtner, Tiago Pereira, Raquel Gomes, Ana Lúcia Luís, ML França, Stefano Geuna, Paulo Armada-da-Silva, Ana Colette Maurício (2012). Mesenchymal stem cells from extra-embryonic tissue engineering – Regeneration of Periphenal Nerve for In Advances in Biomaterials Science and Applications in Biomedicine, Editor Prof. Rosario Pignatello. InTech (ISBN 980-953-307-856-9) (em impressão)

✓ Tiago Pereira, Andréa Gärtner, Irina Amorim, Paulo Armada-da-Silva, Raquel Gomes, C Pereira, ML França, DM Morais, MA Rodrigues, MA Lopes, JD Santos, Ana Lúcia Luís, Ana Colette Maurício (2012). Biomaterials and stem cells therapies for injuries associated to skeletal muscular tissues for In Advances in

Biomaterials Science and Applications in Biomedicine, Editor Prof. Rosario Pignatello. InTech (ISBN 980-953-307-856-9) (em impressão)

2. Processamento do tecido do cordão umbilical (TCU)

O cordão umbilical aquando do nascimento pesa cerca de 40g e apresenta um comprimento entre 30-65cm e uma largura de 1,5cm. Existe, no entanto, uma grande variedade nestes dados entre os vários bebés recém-nascidos.

O armazenamento tanto do sangue como do tecido (geleia de Wharton) do cordão umbilical segue determinadas diretrizes impostas pela Netcord e pela Fundação para a Acreditação de Terapia Celular (FACT) e que são aplicadas tanto a bancos públicos como em bancos privados de criopreservação de tecidos e de SCU (como o Laboratório Criovida, Biosckin, Molecular and Cell Therapies SA, que estabeleceu um protocolo técnico e cientifico com a Universidade do Porto e onde se realizou esta parte experimental desta tese).

Apesar das regras impostas para os procedimentos é necessário que haja melhoramentos para a obtenção de um maior número de células estaminais viáveis após criopreservação e sua descongelação e limitar a contaminação microbiológica durante os procedimentos, incluindo o transporte desde o hospital / clínica onde decorreu o parto. Nesse sentido, um grupo de I&D, multidisciplinar associado a empresas públicas ou privadas de biotecnologia é essencial para o avanço de técnicas e a produção de produtos de utilização clínica.

Os próximos protocolos experimentais assim como os resultados obtidos aqui apresentados, são relativos a este tipo de estudos que foram feitos de forma a melhorar todo o procedimento do TCU, desde a recolha do cordão umbilical ao momento da sua criopreservação e posterior descongelação e isolamento de MSCs.

2.1. Validação da solução de transporte do TCU até ao laboratório de culturas celulares

Um dos tópicos importantes a ser considerado, e por isso o seu melhoramento é fundamental, é o método de transporte do cordão umbilical. É fundamental a realização de um transporte refrigerado do SCU e do TCU por um período de tempo inferior ou igual a 96 horas, através de termoacumuladores que são fornecidos com o

kit de colheita de amostras. Para evitar a perda de viabilidade do TCU testaram-se várias soluções isotónicas estéreis para que cordão umbilical colhido seja transportado imerso. Deste modo, pensa-se manter o TCU hidratado e limitar a multiplicação microbiológica durante todo o transporte até ao laboratório de criopreservação.

2.1.1. Soluções de transporte

Fragmentos de cordão umbilical com 1cm de comprimento foram coletados de doadores saudáveis após consentimento informado escrito e seguindo procedimentos validados de acordo com as diretrizes clínicas e técnicas do laboratório Biosckin (autorizado para o processamento e criopreservação de SCU e de unidades de TCU pelo Ministério da Saúde, ASST - Autoridade para os Serviços de Sangue e da Transplantação).

Estes fragmentos de cordão foram imersos por 168 horas em 4 soluções salinas estéreis diferentes à temperatura ambiente (22-24 ºC) e em refrigeração (4-6 ºC). As 4 soluções utilizadas foram (Figura 26):

- ✓ NaCl 0,9% (Labesfal,Portugal) que é o soro fisiológico com pH=4,5-7,0;
- ✓ AOSEPT®Plus (CibaVision, Portugal) contém 3% de peróxido de hidrogénio, ácido fosfórico (estabilizador), cloreto de sódio, fosfato (sistema tampão) e poloxamer (surfatante) com pH=6,8 normalmente usado para transportar e lavar as lentes de contacto;
- ✓ Dulbecco's Phosphate-Buffered Saline sem cálcio, magnésio e fenol vermelho (DPBS, Gibco, Invitrogen, Portugal) com pH=7,0-7,3;
- ✓ Hank's Balanced Salt Solution (HBSS, Gibco, Invitrogen, Portugal) com pH=5,8-6,1.

Figura 26 - Soluções salinas estéreis utlizadas. Da esquerda para a direita: NaCl, AOSept Plus, DPBS e HBSS, sendo que este último não foi utilizado a 10x como indica a embalagem mas sim a 1x.

Inicialmente, 4 fragmentos de cordão foram colocados em refrigeração (4-6 ºC) (cada um num frasco contendo uma das soluções utilizadas), outros 4 foram colocados à temperatura ambiente (22-24 ºC) (cada um num frasco contendo uma das soluções utilizadas) e 1 para controlo (fragmento sem imersão em qualquer solução e imediatamente submetido após colheita para análise histológica). Depois de conhecidos os resultados com as 4 soluções acima descritas, foi repetida a experiência apenas para as duas soluções com melhores resultados, em que foram utilizados mais 4 fragmentos de cordão, 2 para cada solução, sendo um colocado à temperatura ambiente e outro em refrigeração. No total desta experiência foi utilizado um N=13 cordões.

Passadas as 168h, os fragmentos de cordão foram recolhidos em formol 10% e processados para microscopia ótica no Laboratório de Anatomia Patológica do ICBAS-UP.

As amostras foram todas fixadas em paraformaldeído a 4%, durante 4 horas e, em seguida, lavados e conservados em DPBS até à fase seguinte. As amostras foram desidratadas e embebidas em parafina e cortadas perpendicularmente ao eixo principal do cordão umbilical com uma espessura de 10µm. Para a análise de microscopia de luz, foram coradas com hematoxilina e eosina (H&E) e observados com um microscópio vertical Leica DM400 equipado com uma câmera Leica DFC320 digital.

A integridade do TCU foi avaliada através dos seguintes parâmetros: i) retração das estruturas vasculares – vasos destacados, ii) perda de detalhe e integridade do endotélio; iii) degradação do tecido conjuntivo laxo; iv) autólise de gordura (impossível de avaliar, devido à técnica histológica utilizada), e v) perda de detalhe e integridade do mesotélio.

2.2. Validação da eficácia do método de lavagem e desinfeção do TCU

O processo de lavagem dos fragmentos de cordão umbilical é crucial para se evitar a contaminação microbiológica das culturas e para a sua correta criopreservação. Estes são obtidos após o nascimento e recolhidos em solução de transporte que não deve ultrapassar as 96 horas. Após a chegada do fragmento do cordão ao laboratório este é lavado entre 4 a 5 vezes em DPBS dependendo da sua sujidade (principalmente da presença de sangue), de seguida segue-se a desinfeção

que é realizada em etanol a 70% durante 30 segundos. Por último e antes da dissecação, o fragmento é novamente lavado em DPBS, no sentido de lavar totalmente o etanol a 70% utilizado no passo de lavagem anterior. Os vasos são dissecados do cordão umbilical enquanto este ainda está embebido em DPBS, para que se mantenha limpo caso algum vaso rompa e ocorra a saída de sangue. Para fazer a validação da lavagem do cordão umbilical utiliza-se para análise duas soluções (Figura 27):

1. Solução de lavagem 1 – solução de DPBS utilizada para o transporte do fragmento do cordão do hospital/clínica até ao laboratório. Na falta desta utiliza-se a solução da primeira lavagem das quatro em DPBS.
2. Solução de lavagem 2 – solução de DPBS que é usada para a lavagem do cordão logo após a desinfeção em etanol a 70%.

Foram utilizados 14 fragmentos de cordão (N = 14) que foram testados para contaminação microbiológica usando BacT/ALERT® (BioMérieux) (Figura 28). Cada unidade de TCU foi testada para os microrganismos aeróbios e anaeróbios e fungos, utilizando 10 ml de solução de lavagem 1 e 10 ml de solução de lavagem 2 que foram assepticamente introduzidos nos BacT/ALERT ® respetivos. Todos os procedimentos foram realizados numa câmara de fluxo laminar de cultura de tecidos em condições de esterilidade.

Figura 27 - Exemplificação do procedimento a seguir para a obtenção da solução de lavagem 1 e solução de lavagem 2.

Figura 28 – Exemplos de BacT/ALERT® . Nesta experiência apenas se utilizou os que estão assinalados com as setas. **1 -** BacT/ALERT® FA – aeróbio; **2 -** BacT/ALERT® FN – anaeróbio.

2.3. Validação do processamento e criopreservação das unidades de TCU

Os protocolos de processamento e criopreservação das unidades de TCU utilizados regem-se pelos procedimentos técnicos do laboratório Biosckin (BSK.LCV.PT.7) e foram validados para a capacidade de isolar e de expandir *in vitro* MSCs após descongelação do TCU criopreservado. Este tipo de protocolos é protegido por um acordo de confidencialidade entre o laboratório Biosckin e todos os seus investigadores envolvidos. Resumidamente, são recolhidos cordões umbilicais de doadores saudáveis (N=60), de acordo com as regras da Netcord e seguindo a lei portuguesa 12/2009 (Diário da República, lei 12/2009 de 26 de Março de 2009). A fim de assegurar a viabilidade do TCU após o parto e limitar a contaminação microbiológica das amostras, os cordões umbilicais são transportados do hospital/clínica para o laboratório a temperaturas refrigeradas e monitorizadas por um datalogger não ultrapassando as 96 horas de transporte até ao laboratório. Os TCU utilizados têm cerca de 15-20cm de comprimento e após a dissecção dos vasos sanguíneos são tratados (cortados em cubos de cerca de 0,5cm^3) e processados para criopreservação o protocolo de processamento e a composição do meio de congelação estão sujeitos a confidencialidade, conforme referido anteriormente. O TCU após processamento e manipulação e em meio de congelação é distribuído de forma equitativa por criotubos. Os criotubos são transferidos para um aparelho de congelação lento que é controlado por computador (Sylab, Consenso, Portugal). O programa utilizado tem nove etapas de congelação e é usado para definir o tempo, temperatura e taxas de otimização para o arrefecimento das MSCs. Depois de

estarem à temperatura pretendida os criotubos são mudados para o criopreservador e mantidos a -156ºC em fase gasosa de azoto líquido.

Para descongelar o TCU, os criotubos são colocados diretamente em banho de água estéril a 37 ºC e de seguida são colocados em centrifugação (150g durante 10 min). O sobrenadante obtido é cuidadosamente removido e o sedimento de células é resuspendido em meio de cultura (a composição do meio de cultura está abrangida pelo acordo de confidencialidade). Esta suspensão é transferida para frascos T75, nos quais previamente se colocou 10ml de meio de cultura. As células são deixadas a crescer durante 3 dias e é feita a sua manutenção durante a semana (2 vezes por semana) e ao fim de 12-16 dias é possível obter a confluência desejada.

3. Protocolo de culturas celulares

Foram utilizados dois tipos de células para a realização de culturas celulares:

- MSCs isoladas a partir da geleia de Wharton do cordão umbilical (culturas extemporâneas) tanto em cordões frescos como em criopreservados;
- MSCs provenientes de uma linhagem celular estabelecida (PromoCell).

É importante haver um melhoramento a nível das culturas celulares de forma a se obter um maior número de células no final e sem apresentarem qualquer tipo de contaminação.

3.1. MSCs isoladas a partir da geleia de Wharton (culturas extemporâneas)

O TCU chegado ao laboratório é limpo na câmara de fluxo laminar segundo o protocolo de lavagem descrito anteriormente. De seguida o TCU (geleia de Wharton) é cortado em cubos de cerca de 0,5 cm^3 e os vasos são removidos por dissecção. Usando um bisturi estéril, os cubos são cortados em fragmentos de 1-2 mm sendo depois transferidos para placas de Petri previamente revestidas com poli-L-lisina (Sigma). Depois de serem distribuídos na placa acrescenta-se meio de cultura (PromoCell, C-28010) suplementado com 10% de SBF (soro bovino fetal), 0,1% de gentamicina 10mg/ml (Sigma), 0,1% de penicilina e estreptomicina (Sigma) e fungizona – anfotericina B (Gibco) (Figura 29). As placas são colocadas numa estufa (5% de CO_2, 37 ºC, NuAire) a incubar. Após 3-4 dias de incubação já se pode verificar

alguma migração de células. Uma boa confluência é obtida cerca de 15-21 dias depois.

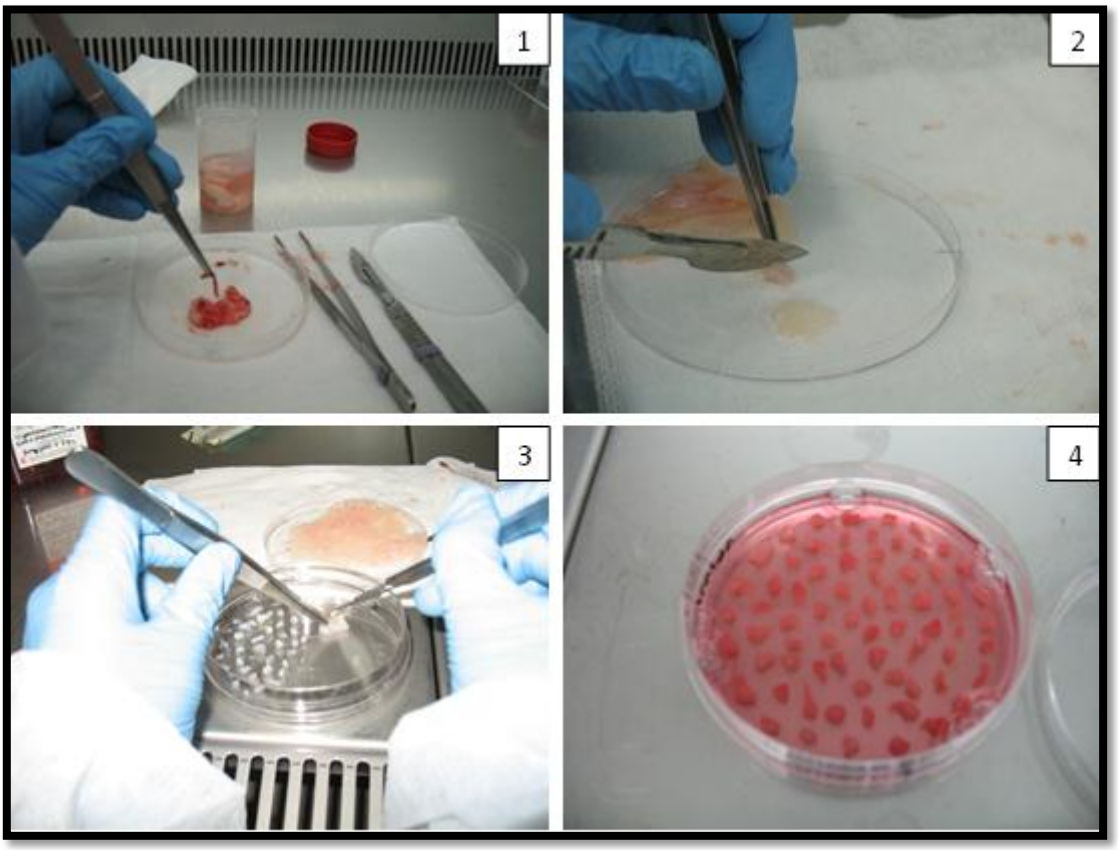

Figura 29 - Exemplificação da preparação do TCU para cultura. **1-** Remoção dos vasos do cordão umbilical. **2 –** Dissecção do TCU em unidades de 0,5 cm^3. **3 –** Colocação das unidades distribuídas pela placa de Petri. **4 –** Placa de Petri já com meio de cultura para ser colocada na estufa.

3.2. Linha celular de MSCs derivadas da geleia de Wharton (PromoCell)

As células MSCs utilizadas em estudos de regeneração de lesões de axonotmese/neurotmese, podem derivar de uma linhagem celular. Foram utilizadas células MSCs derivadas da geleia de Wharton do cordão umbilical adquiridas à PromoCell GmbH (C-12971, lote número: 8.082.606,7). Estas células estão armazenadas num criopreservador de forma a manterem a sua viabilidade. Quando são necessárias, o vial (onde as células estão contidas) é colocado em banho-maria até ao seu total descongelamento. De seguida, a suspensão de células é transferida para um frasco T75 que contém meio de cultura deste tipo de células (PromoCell, C-

28010). O frasco de cultura é mantido numa atmosfera humificada com 5% de CO_2 a 37 ºC (estufa). O meio é substituído a cada 48 horas. Quando se verifica uma confluência de 80-90% é realizada uma tripsinização. A tripsinização consiste, resumidamente, na recolha das células que são divididas no mínimo em duas partes, uma para fazer nova expansão e outra para criopreservar (para futura utilização). As células que estão destinadas a uma nova expansão são cultivadas em lamelas com poli-L-lisina (Sigma) ou em membranas de biomateriais e verifica-se que após 24 h já exibem uma confluência de 30-40%. Pretende-se depois que estas células se diferenciem em células semelhantes às do tipo neuroglial, e para isso a diferenciação é induzida com meio de cultura neurogénico (PromoCell, C-28015). O meio é normalmente substituído a cada 24 horas, durante 3 dias. A formação de células pretendidas pode ser observada após 24 horas num microscópio invertido (Zeiss, Alemanha).

Esta linha celular de células MSCs são as preferidas para serem utilizadas em ensaios *in vivo* em ratos, uma vez que o número de MSCs obtido é maior num menor tempo de cultura, não é dependente da disponibilidade de doadores e da autorização da comissão de ética do Centro Hospitalar do Porto (entidade com quem foi estabelecido um protocolo científico e fornecimento de amostras biológicas humanas - PI 0055/10 (037-DEFI/052-CES) - Projeto de Investigação ICBAS-UP) e o protocolo é realizado em menos tempo o que é vantajoso para ensaios pré-clínicos com um número elevado de animais experimentais.

A PromoCell promove uma grande quantidade de testes rígidos feitos a cada lote de MSCs, para controlo de qualidade, em que são testados para morfologia celular, taxa de aderência e viabilidade. Além disto, cada lote de células é caracterizado por análise de citometria de fluxo para um amplo painel de marcadores.

4. Participação em outros projetos:

4.1. Reconstrução cirúrgica de lesões no músculo tibial anterior

Descrição equivalente à descrita no ponto 1 deste capítulo.

4.2. Biomateriais e testes de biocompatibilidade

Como já foi referido anteriormente por vezes são usados biomateriais para a incorporação de um sistema celular na zona lesada tanto do nervo periférico como de músculo ou mesmo para reconstrução óssea. Estes biomateriais podem ser membranas ou géis e os seus componentes terão que ter todas as qualidades já referidas na introdução, para que cause o menor impacte possível no local lesado. Uma forma de avaliar o seu comportamento no local é através da reação inflamatória que poderá causar. Esta é avaliada de forma quantitativa e objetiva de acordo com o anexo E da Norma ISO 10993. Os biomateriais utilizados (Quadro 2) foram implantados no dorso dos ratos (implantes subcutâneos) sendo posteriormente suturada a pele, normalmente com pontos isolados (Figura 30). Passados 15 dias foram recolhidas as amostras em formol 10% e enviadas para o laboratório onde são tratadas e coradas (com H&E) de forma a poderem ser observadas ao microscópio em lâminas para posterior avaliação. Esta avaliação é feita com base na tabela E.1 e E.2 que se encontram no anexo E da Norma ISO 10993. Consiste essencialmente na contagem de células tipo ou resposta inflamatória sendo depois classificado conforme a contagem de 0 a 4 (Quadro 3). O resultado final obtido é depois traduzido no grau de irritabilidade que a membrana/gel apresenta para o animal.

Quadro 2 - Biomateriais testados. Nota: o G1 e G2 têm exatamente a mesma composição, o que variou foi o modo da sua produção: no G1, o PEG é adicionado após a reação da pectina com o quitosano e no G2, o PEG é adicionado à pectina antes da adição do quitosano.

Designação	Membrana/Gel	Composição
Alg-AH	Gel	alginato + ácido hialurónico
Alg	Gel	alginato
AH	Gel	ácido hialurónico
AQ	Gel	quitosano
G1	Gel	pectina + quitosano + polietilenoglicol
G2	Gel	pectina + quitosano + polietilenoglicol
PLGA	Membrana	poly(lactic-co-glycolic acid)
PLGA+1%	Membrana	poly(lactic-co-glycolic acid) + 1% flurbiprofeno
PEC	Membrana	pectina + quitosano + policaprolactona
PVA	Membrana	ácido polivinílico
PLGA-HA	Membrana	poly(lactic-co-glycolic acid) + hidroxiapatite
PLC	Membrana	policaprolactona
PLC-HA	Membrana	Policaprolactona + hidroxiapatite

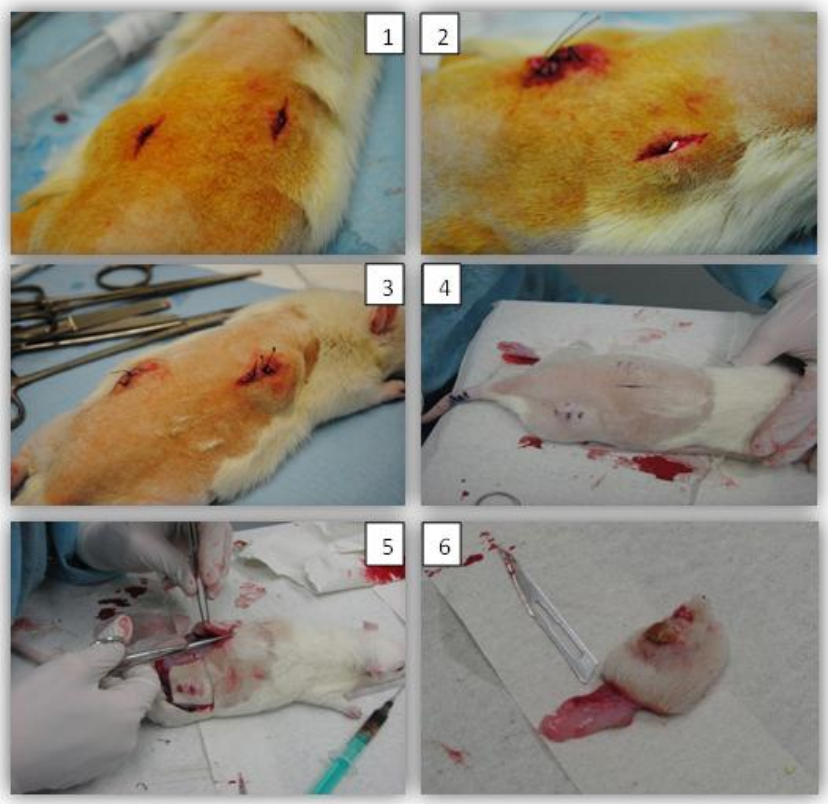

Figura 30 - Procedimento para implantação dos biomateriais e recolha da amostra para avaliação. **1 –** Abertura do dorso dos ratos para implantação dos biomateriais; **2 –** Implantação dos biomateriais; **3 –** Locais onde os biomateriais foram colocados suturados; **4 –** Depois de 15 dias, os cortes estão completamente cicatrizados; **5 –** Recolha das amostras; **6 –** Exemplo de uma amostra que é levada para o laboratório para ser tratada e corada.

Quadro 3 - Quadros retirados do anexo E da Norma ISO 10993, que são utilizados para a avaliação da reação inflamatória.

For examples of scoring systems used for biological evaluation of degradable materials, see Reference [19].

Table E.1 — Examples of a histological evaluation system — Cell type/response

Cell type/response	Score				
	0	1	2	3	4
Polymorphonuclear cells	0	Rare, 1-5/phf [a]	5-10/phf	Heavy infiltrate	Packed
Lymphocytes	0	Rare, 1-5/phf	5-10/phf	Heavy infiltrate	Packed
Plasma cells	0	Rare, 1-5/phf	5-10/phf	Heavy infiltrate	Packed
Macrophages	0	Rare, 1-5/phf	5-10/phf	Heavy infiltrate	Packed
Giant cells	0	Rare, 1-2/phf	3-5/phf	Heavy infiltrate	Sheets
Necrosis	0	Minimal	Mild	Moderate	Severe

[a] phf = per high powered (400 ×) field.

Table E.2 — Examples of a histological evaluation system — Response

Response	Score				
	0	1	2	3	4
Neovascularisation	0	Minimal capillary proliferation, focal, 1-3 buds	Groups of 4-7 capillaries with supporting fibroblastic structures	Broad band of capillaries with supporting structures	Extensive band of capillaries with supporting fibroblastic structures
Fibrosis	0	Narrow band	Moderately thick band	Thick band	Extensive band
Fatty infiltrate	0	Minimal amount of fat associated with fibrosis	Several layers of fat and fibrosis	Elongated and broad accumulation of fat cells about the implant site	Extensive fat completely surrounding the implant

Capítulo III

Resultados e Discussão

1. Reconstrução cirúrgica do nervo periférico após lesões de axonotmese

O trabalho experimental desenvolvido em co-autoria e no âmbito dos seguintes Projetos de Doutoramento:

- Doutoramento em Ciências Veterinárias do ICBAS – UP, do Licenciado Tiago de Melo Silva Ramos Pereira, Assistente Convidado do Departamento de Clínicas Veterinárias – ICBAS – UP. Propriedades morfológicas e fisiológicas do músculo-esquelético: Estudos *in vivo* com modelos de regeneração de tecido muscular.
- Doutoramento em Ciências Veterinárias do ICBAS – UP, inserido no Programa Doutoral em Patologia e Genética Molecular, da Mestre Andréa Louise Moutinho Gärtner com a Bolsa de Doutoramento da Fundação para a Ciência e a Tecnologia (FCT) do Ministério da Ciência, da Tecnologia e do Ensino Superior (MCTES) com a referência SFRH/BD/70211/2010. Estudo funcional e morfológico da regeneração do nervo periférico recorrendo a PLGA 90:10 e células estaminais CD34+.

Os resultados foram publicados, em que o artigo e os dois capítulos de livro estão em fase de impressão, com as seguintes referências e aqui introduzidos com autorização dos restantes autores:

✓ A Gärtner, T Pereira, PAS Armada da Silva, I Amorim, R Gomes, J Ribeiro, ML França, C Lopes, B Porto, R Sousa, A Bombaci, F Fregnan, ASP Varejão, AL Luís, S Geuna, AC Maurício (2012). Use of poly(DL-lactide-e-caprolactone) PLC membranes and Mesenchymal Stem Cells for promoting nerve regeneration in an axonotmesis rat model: *in vitro* and *in vivo* analysis. Differentiation (em impressão)

✓ Andréa Gärtner, Tiago Pereira, Raquel Gomes, Ana Lúcia Luís, ML França, Stefano Geuna, Paulo Armada-da-Silva, Ana Colette Maurício (2012). Mesenchymal stem cells from extra-embryonic tissue engineering – Regeneration of Periphenal Nerve for In Advances in Biomaterials Science and Applications in Biomedicine, Editor Prof. Rosario Pignatello. InTech (ISBN 980-953-307-856-9) (em impressão)

✓ Tiago Pereira, Andréa Gärtner, Irina Amorim, Paulo Armada-da-Silva, Raquel Gomes, C Pereira, ML França, DM Morais, MA Rodrigues, MA Lopes, JD Santos, Ana Lúcia Luís, Ana Colette Maurício (2012). Biomaterials and stem cells therapies for injuries associated to skeletal muscular tissues for In Advances in

Biomaterials Science and Applications in Biomedicine, Editor Prof. Rosario Pignatello. InTech (ISBN 980-953-307-856-9) (em impressão)

2. Processamento do TCU

2.1. Validação da solução de transporte do TCU até ao laboratório de culturas celulares – resultados da análise histológica

Assim que os fragmentos de cordão foram colocados nos frascos com as respetivas soluções notou-se logo uma alteração nos que continha a solução AOSEPT®Plus. Verificou-se que o fragmento começou a efervescer e a flutuar na solução, enquanto que nos outros o fragmento depositava-se no fundo do frasco sem qualquer alteração visível macroscopicamente (Figura 31). Deduziu-se assim que a solução provavelmente não seria a mais indicada, o que foi mais tarde foi comprovado com a análise histológica (Figura 32).

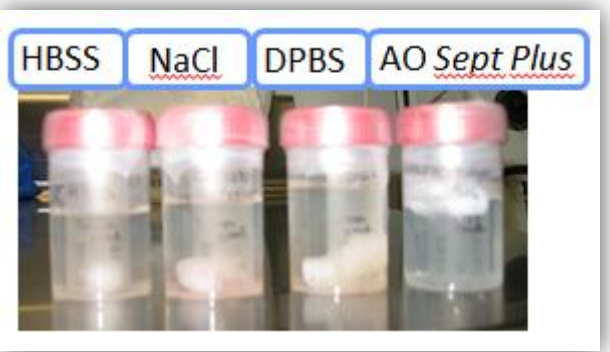

Figura 31 - Fragmentos colocados nos frascos com a sua respetiva solução, onde é de notar o 'comportamento' do fragmento do cordão na solução AOSept Plus.

As primeiras amostras foram analisadas com a ajuda da Drª Irina Amorim (Laboratório de Anatomia Patológica do ICBAS – UP) e obtiveram-se os resultados presentes no quadro 4.

Quadro 4 - Resultados da primeira fase da experiência. Legenda: TA – temperatura ambiente (22-24 ^{o}C); REFR – refrigerado (4-6 ^{o}C); + presente; +/- moderado; - ausente; IA impossível de avaliar.

	Vasos destacados (retração das estruturas vasculares	Perda de detalhe e integridade do endotélio	Degradação do tecido conjuntivo laxo	Autólise da gordura	Perda de detalhe e integridade do mesotélio
Soro fisiológico (TA)	+/-	+	+/-	IA	+/-
Soro fisiológico (REFR)	+/-	+	+/-	IA	+/-
HBSS (TA)	+/-	+/-	+/-	IA	+/-
HBSS (REFR)	+/-	+/-	-	IA	-
DPBS (TA)	+/-	+/-	-	IA	-
DPBS (REFR)	-	+/-	-	IA	-
AO Sept Plus (TA)	++	+/-	++	IA	++
AO Sept Plus (REFR)	+/-	++	++	IA	++
Controlo	+	++	+	IA	++

Como os parâmetros analisados são fatores negativos é de esperar que as soluções com melhores resultados apresentem na sua maioria o símbolo – (ausente). Assim podemos concluir, através da análise do quadro 1, que as soluções DPBS e HBSS (em temperatura refrigerada) foram as que obtiveram melhores resultados. De forma a confirmar e a verificar qual das duas era decididamente a melhor, foi repetida a experiência apenas para estas duas soluções, obtendo-se os resultados apresentados no quadro 5.

Quadro 5 - Resultados da confirmação de resultados da experiência. Legenda: TA – temperatura ambiente (22-24 ^{o}C); REFR – refrigerado (4-6 ^{o}C); + presente; +/- moderado; - ausente; IA impossível de avaliar.

	Vasos destacados (retração das estruturas vasculares	Perda de detalhe e integridade do endotélio	Degradação do tecido conjuntivo laxo	Autólise da gordura	Perda de detalhe e integridade do mesotélio
HBSS (TA)	-	+	+/-	IA	+
HBSS (REFR)	+/-	-	-	IA	-
DPBS (TA)	+/-	-	+/-	IA	+
DPBS (REFR)	-	-	-	IA	-

Analisando estes últimos resultados vê-se que ambas as soluções continuam a ter avaliações muito idênticas em termos da manutenção da estrutura histológica, sendo por isso mais vantajoso a utilização de uma delas em comparação com o controlo, que não utiliza qualquer tipo de solução no transporte do cordão. O facto de ambas as soluções apresentarem destacamento de vasos pode ser um ponto positivo, pois assim ajuda na dissecção dos vasos durante o procedimento.

A avaliação apresentada nas tabelas anteriores foi feita através da observação dos cortes histológicos dos fragmentos de cordão umbilical em microscópio ótico avaliando-se os parâmetros pré-definidos (Figura 32, 33, 34, 35 e 36). A avaliação é feita através da média entre os vários cortes (N=13 cordões) e não apenas de um.

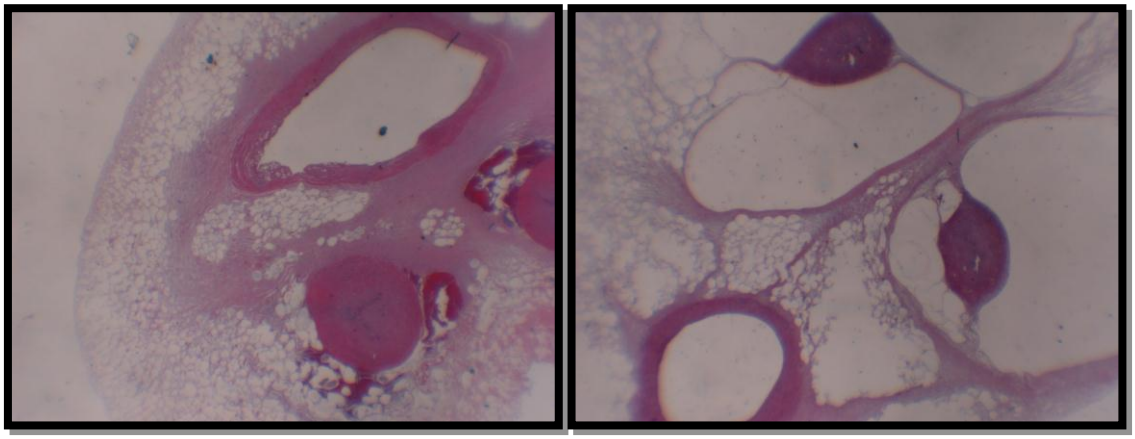

Figura 32 - Exemplos de cortes histológicos do fragmento do cordão umbilical em AOSept Plus REFR (esquerda) ou AOSept Plus TA (direita). É possível verificar a total destruição do tecido. (Ambas tiradas à lupa)

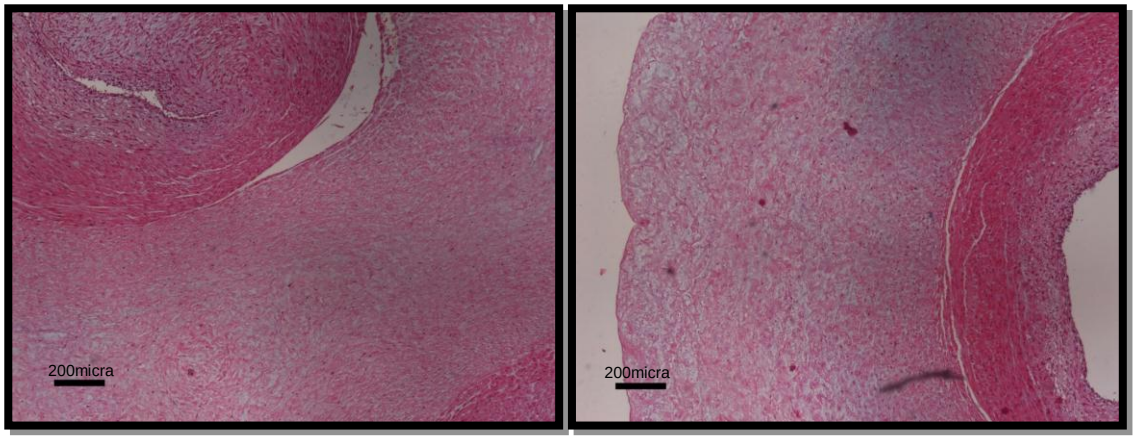

Figura 33 - Exemplos de cortes histológicos do fragmento do cordão umbilical em DPBS REFR (esquerda) ou DPBS TA (direita). Imagens relativas à 1ª experiência, no entanto as da 2ª experiência são idênticas. (Ambas tiradas com uma ampliação 40x)

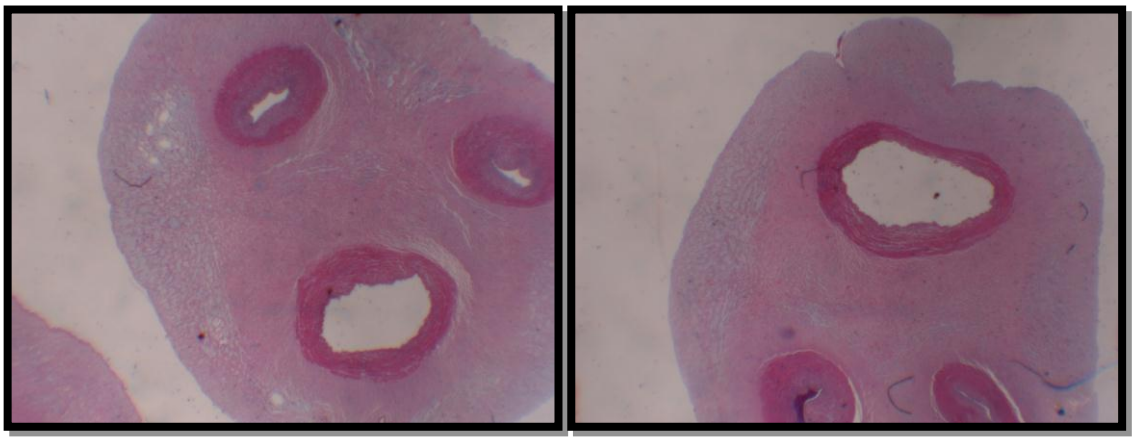

Figura 34 - Exemplos de cortes histológicos do fragmento do cordão umbilical em NaCl REFR (esquerda) ou NaCl TA (direita). (Ambas tiradas à lupa)

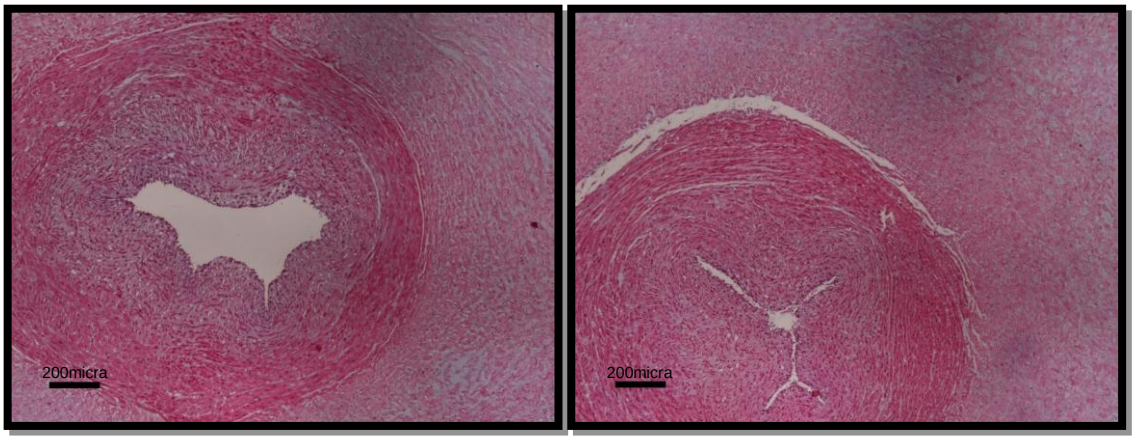

Figura 35 - Exemplos de cortes histológicos do fragmento do cordão umbilical em HBSS REFR (esquerda) ou HBSS TA (direita). Imagens relativas à 1ª experiência, no entanto as da 2ª experiência são idênticas. (Ambas tiradas com uma ampliação 40x)

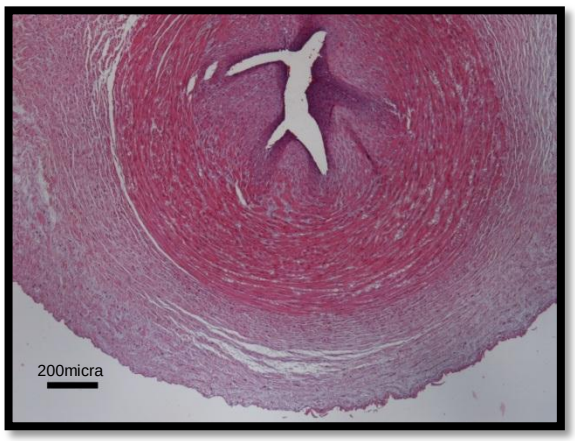

Figura 36 - Exemplo de corte histológico do fragmento do cordão umbilical usado como controlo, em que não foi usada nenhuma solução de transporte. (Ampliação 40x)

2.2. Validação da eficácia do método de lavagem e desinfeção do TCU – resultados da análise microbiológica por BacT/ALERTS®

Os BacT/ALERTS® com as amostras das soluções 1 e 2 conforme foi descrito anteriormente, foram enviados para um laboratório de análises clínicas (Laboratório Prof. Ernesto Morais, Porto, Portugal), obtendo-se os resultados descritos no quadro 6. A cada amostra foi atribuída uma letra sendo que esta pode ser, por exemplo, A1 ou A2 se for da solução de lavagem 1 ou 2, respetivamente.

Quadro 6 – Resultados microbiológicos.

	Hemocultura		
	Em aerobiose		Em anaerobiose
Solução	Estirpe bacteriana	Fungos	Estirpe bacteriana
A1	positivo - *Staphylococcus lugdunensis*	negativo	positivo - *Staphylococcus lugdunensis*
A2	negativo	negativo	negativo
B1	positivo - *Escherichia coli*	negativo	positivo - *Escherichia coli*
B2	negativo	negativo	negativa
C1	positivo - *Staphylococcus lugdunensis*	negativo	positivo - *Staphylococcus lugdunensis*
C2	negativo	negativo	negativo
D1	negativo	negativo	negativo
D2	negativo	negativo	negativo
E1	positivo - *Enterococcus faecalis*	negativo	positivo - *Enterococcus faecalis*
E2	negativo	negativo	negativo
F1	positivo - *Staphylococcus epidermidis*	negativo	positivo - *Staphylococcus epidermidis*
F2	negativo	negativo	negativo
G1	negativo	negativo	negativo
G2	negativo	negativo	negativo
H1	negativo	negativo	positivo - *Bacteroides vulgatus*
H2	negativo	negativo	negativo
I1	negativo	negativo	positivo – *Staphylococcus epidermidis*
I2	positivo – *Staphylococcus coagúlase*	negativo	negativo
J1	positivo – *Staphylococcus coagúlase*	negativo	positivo – *Staphylococcus coagúlase*
J2	negativo	negativo	negativo
K1	positivo – *Escherichia coli*	negativo	positivo – *Escherichia coli*
K2	negativo	negativo	negativo
L1	positivo – *Escherichia coli*	negativo	positivo – *Escherichia coli*
L2	negativo	negativo	negativo
M1	positivo – *Escherichia coli*	negativo	positivo – *Escherichia coli*
M2	negativo	negativo	negativo
N1	positivo – *Streptococcus sanguinis*	negativo	positivo – *Streptococcus sanguinis*
N2	negativo	negativo	negativo

Analisando os resultados obtidos, verificamos que todas as amostras que apresentavam contaminação microbiana na solução de lavagem 1, passaram a não apresentar nenhuma contaminação depois da lavagem (solução de lavagem 2). Na amostra H e I isto não é verificado, mas segundo o resultado obtido pode-se concluir que não foi erro do método de lavagem mas sim erro do operador no momento da preparação dos BacT/ALERTS® ou troca de resultados no laboratório, não sendo por isso contadas na eficiência do método. Fora estas duas exceções, pode-se concluir então que o método de lavagem é 100% eficaz na eliminação microbiológica (incluindo bactérias aeróbias e anaeróbias, leveduras e fungos).

2.3. Validação do processamento do TCU

Devido ao aumento da lise de eritrócitos ou contaminação microbiológica durante a cultura de células em algumas amostras de TCU criopreservadas não foi possível isolar as MSCs nessas mesmas amostras. No entanto foram observadas MSCs num microscópio invertido (Zeiss, Alemanha) em diferentes pontos de expansão em inúmeras amostras de TCU processadas e criopreservadas (resultados abrangidos por acordo de confidencialidade e ainda em análise). Estes resultados permitiram concluir que os protocolos de tratamento e arrefecimento para criopreservação de unidades de TCU são adequados para preservar a sua viabilidade, uma vez que foi possível isolar e expandir as MSCs após descongelação apropriada e na presença das condições de cultura adequadas.

2.4. Culturas celulares

Com os protocolos definidos anteriormente foi possível isolar MSCs da geleia de Wharton do cordão umbilical (Figura 37) assim como fazer cultura da linha celular de células MSCs da PromoCell (Figura 38).

Depois de obtermos a confluência pretendida na cultura de MSCs da linhagem da PromoCell foi possível que estas fossem diferenciadas em células do tipo neuroglial (Figura 39), na presença de meio neurogénico (PromoCell, C-28015) ao fim de 96 horas.

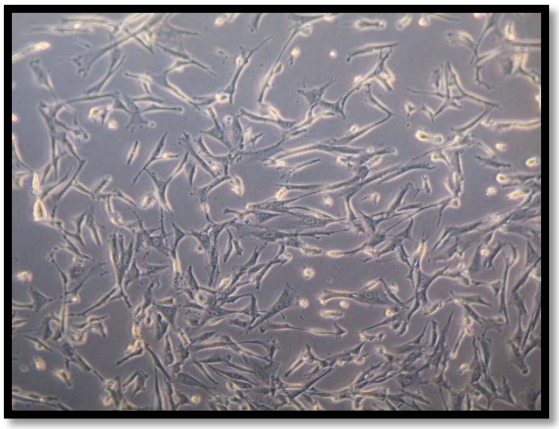

Figura 37 - MSCs isoladas a partir de geleia de Wharton apresentando uma forma semelhante às células mesenquimatosas com uma morfologia plana poligonal (100x).

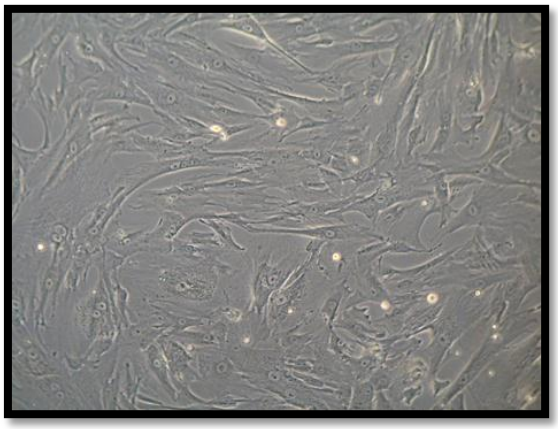

Figura 38 - Linha celular de MSCs a partir de geleia de Wharton (PromoCell) apresentando uma forma semelhante às células mesenquimatosas com uma morfologia plana poligonal (100x).

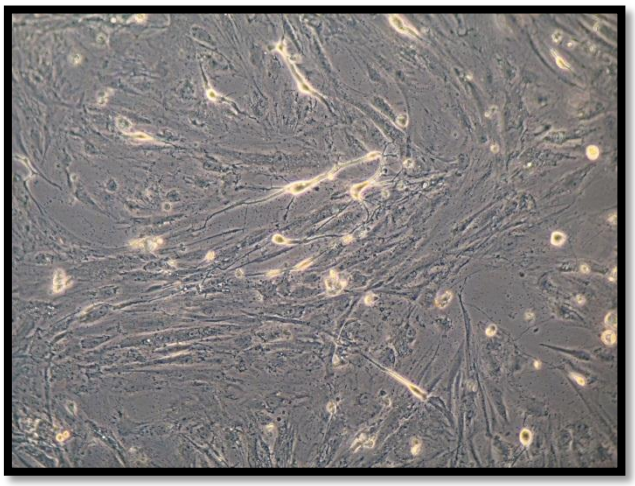

Figura 39 - Linha celular de MSCs a partir de geleia de Wharton (PromoCell) após 96 horas de incubação em meio de cultura neurogénico. As células tornaram-se muito longas e há formação de células do tipo neuroglial (100x).

2.4.1. Análise do cariótipo

Foi realizada uma análise de cariótipo de forma a verificar a existência de alterações a nível cromossómico nas MSCs indiferenciadas da geleia de Wharton (de ambos os protocolos). Feita essa análise verificou-se que não houve alterações estruturais, demonstrando que esta linha celular não tem características neoplásicas e é estável durante os processos de cultura celular, em termos de número e da estrutura dos cromossomas somáticos e sexuais (46, XY) (Figura 40).

Quanto às MSCs diferenciadas em células tipo neuroglial, como não foram encontradas células em metáfase, não foi possível analisar o cariótipo.

Figura 40 - Metafases selecionadas de células MSCs indiferenciadas isoladas da geleia de Wharton, que mostra o número normal de cromossomos (46, XY) (1000x).

2.4.2. Imunocitoquímica

Para a confirmar a obtenção de MSCs indiferenciadas e a diferenciação em meio neurogénico em células do tipo neuroglial (células obtidas a partir do TCU criopreservado ou MSCs da Promocell), recorreu-se à técnica de imunocitoquímica utilizando marcadores específicos, nomeadamente o GFAP (marcador das células de glia), o GAP-43 (relacionado com o crescimento neuronal) e o NeuN (marcador para núcleos de neurónios) (Figura 41).

Podemos verificar através da realização de imunocitoquímica que as MSCs indiferenciadas, como era de esperar não apresentaram marcação por parte dos marcadores GFAP, GAP-43 e NeuN. Após 96h em meio de cultura neurogénico foi realizada nova marcação, o qual se verificou que as células do tipo neuronal obtidas eram marcadas positivamente pelos três marcadores.

Podemos assim afirmar que a diferenciação em células do tipo neuroglial foi conseguida a partir das MSCs do TCU (fresco e criopreservado) e da PromoCell (N=57).

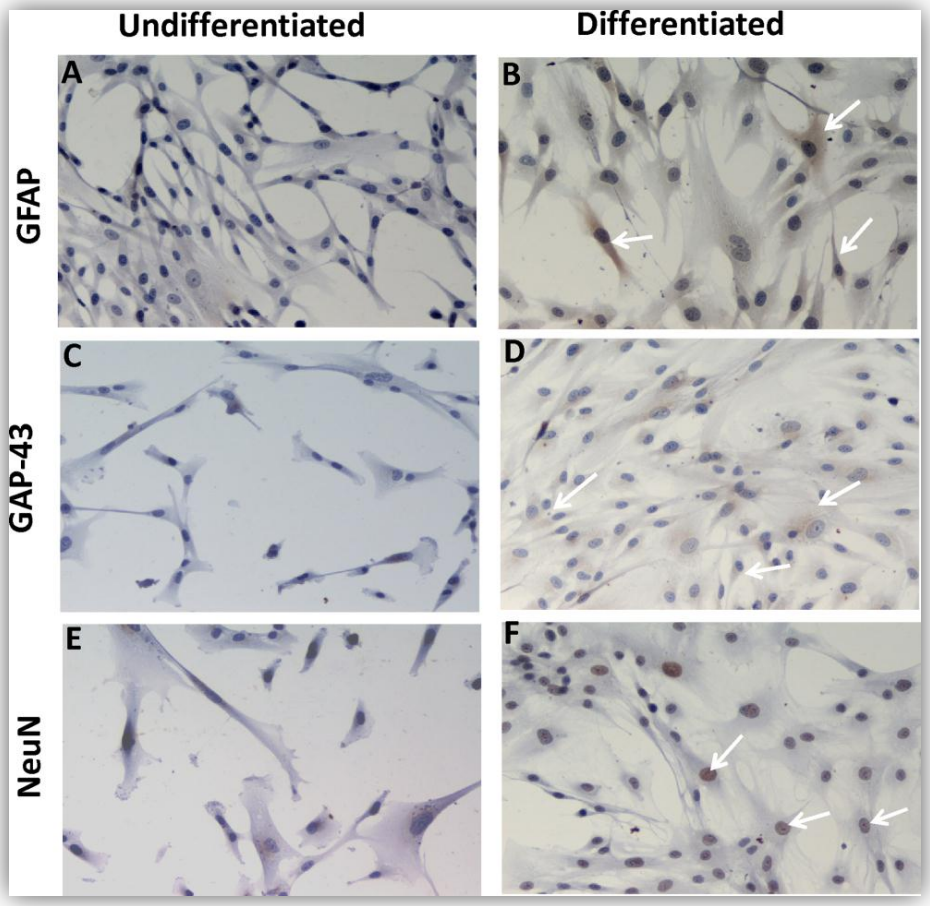

Figura 41 - Teste de imunocitoquímica utilizando os marcadores GFAP, GAP-43 e NeuN. (200x)

3. Participação em outros projectos:

3.1. Reconstrução cirúrgica de lesões no músculo tibial anterior

Descrição equivalente à descrita no ponto 1 deste capítulo.

3.2. Biomateriais – Implantes subcutâneos para avaliação de citocompatibilidade

Para ser feita a avaliação são analisados diferentes cortes histológicos, normalmente três, mas pode variar conforme o estado da amostra. Isto é, a patologista responsável (Drª Irina Amorim, Laboratório de Anatomia Patológica – ICBAS-UP) verificar que todas as lâminas estão muito homogéneas apenas pode ser avaliado um

corte. Em cada corte são escolhidos normalmente três campos onde se faz a avaliação, este número também pode variar conforme a amostra. Às vezes é necessário avaliar mais um campo para confirmação de resultados.

A amostra onde foi colocado o gel Alg-AH (Quadro 2) ainda apresenta vestígios do gel demonstrando que mesmo depois de 15 dias este ainda não foi totalmente degradado, podendo ser considerado um ponto negativo (Quadro 7 e Figura 42).

Quadro 7 - Avaliação ao gel Alg-AH segundo anexo E da Norma ISO 10993.

	Corte 1			Corte 2			Corte 3			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	0	0	1	0	0	0	1	1	0	0,33
Linfócitos	3	3	3	3	3	2	3	3	3	2,89
Plasmócitos	2	2	3	3	3	2	2	2	2	2,33
Macrófagos	4	4	4	4	4	4	3	4	4	3,89
Células gigantes	0	0	0	0	0	0	0	1	0	0,11
Necrose	0	0	0	0	0	0	0	0	0	0,00
SUB-TOTAL (x2)										**19,11**
Neovascularização	3	2	2	3	2	3	3	4	3	2,78
Fibrose	1	1	0	2	2	2	1	2	1	1,33
Infiltração lipidica	0	0	0	0	0	0	0	0	0	0,00
SUB-TOTAL										**4,11**
									TOTAL:	**23,22**

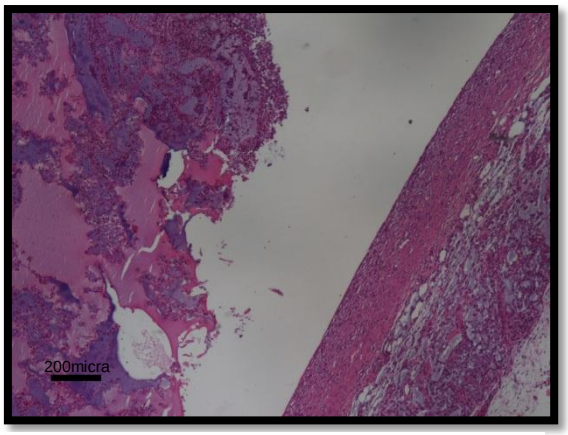

Figura 42 - Corte histológico da amostra com Alg-AH. (40x)

Quanto ao gel Alg (Quadro 2) foi necessário avaliar mais um campo para confirmar os resultados. Foi possível verificar que este gel causou uma reação inflamatória sem a formação de cápsula a envolver, não se apresenta cicatrizado e é possível ver vestígios de material no local (Quadro 8 e Figura 43).

Quadro 8 - Avaliação ao gel Alg segundo anexo E da Norma ISO 10993.

	Corte 1				
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	Campo 4	**Média**
Neutrófilos (PMN)	0	0	1	0	0,25
Linfócitos	3	3	4	3	3,25
Plasmócitos	3	3	1	1	2,00
Macrófagos	3	3	4	4	3,50
Células gigantes	1	0	0	0	0,25
Necrose	0	0	0	0	0,00
SUB-TOTAL (x2)					**18,50**
Neovascularização	2	3	2	3	2,50
Fibrose	0	0	0	0	0,00
Infiltração lipidica	0	0	0	0	0,00
SUB-TOTAL					**2,50**
				TOTAL:	**21,00**

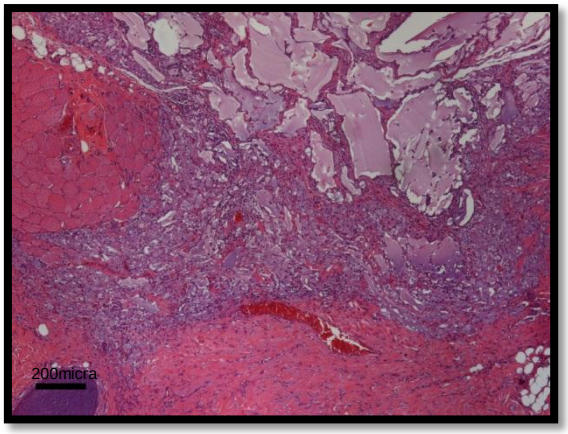

Figura 43 - Corte histológico da amostra com Alg. (40x)

A implantação do gel AH (Quadro 2) fez com que se formasse pus na zona da sua aplicação, isto é, criou um abcesso (Quadro 9 e Figura 44).

Quadro 9 - Avaliação ao gel AH segundo anexo E da Norma ISO 10993.

	Corte 1			Corte 2			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	4	4	3	3	3	3	3,33
Linfócitos	3	3	3	3	2	3	2,83
Plasmócitos	2	2	1	2	2	2	1,83
Macrófagos	3	3	2	3	2	3	2,67
Células gigantes	0	1	0	3	0	1	0,83
Necrose	0	0	0	0	0	0	0,00
SUB-TOTAL (x2)							**23,00**
Neovascularização	1	2	2	2	2	3	2,00
Fibrose	1	2	3	1	1	2	1,67
Infiltração lipidica	0	0	0	0	0	0	0,00
SUB-TOTAL							**3,67**
						TOTAL:	**26,67**

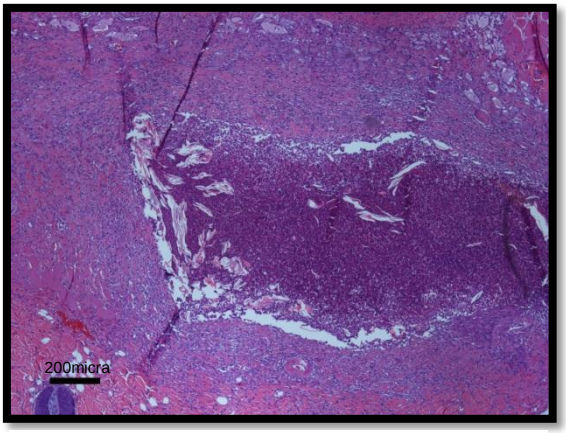

Figura 44 - Corte histológico da amostra com AH. (40x)

O implante de gel AQ (Quadro 2) causou uma reação inflamatória aguda, tendo comprometido o músculo (Quadro 10 e Figura 45).

Quadro 10 - Avaliação ao gel AQ segundo anexo E da Norma ISO 10993.

	Corte 1			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	1	1	1	1,00
Linfócitos	3	3	4	3,33
Plasmócitos	3	2	2	2,33
Macrófagos	3	4	3	3,33
Células gigantes	2	3	2	2,33
Necrose	0	0	0	0,00
SUB-TOTAL (x2)				**24,67**
Neovascularização	2	1	2	1,67
Fibrose	1	0	1	0,67
Infiltração lipidica	0	0	0	0,00
SUB-TOTAL				**2,33**
			TOTAL:	**27,00**

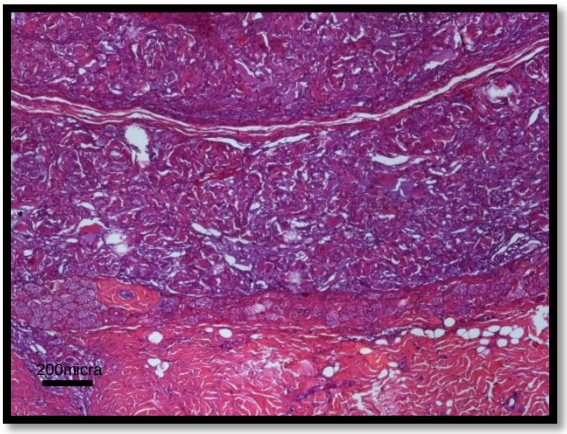

Figura 45 - Corte histológico da amostra com AQ. (40x)

O G1 e G2 (Quadro 2) são constituídos pelos mesmos materiais, no entanto, o método de fabrico foi diferente. O G1 (Quadro 11 e Figura 46) ao contrário do outro apresentou a formação de quistos foliculares que podem ser causados por uma agulha, no entanto o G2 (Quadro 12 e Figura 47) por apresentar necrose é o pior gel que foi utilizado.

Quadro 11 - Avaliação ao gel G1 segundo anexo E da Norma ISO 10993.

	Corte 1			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	2	1	1	1,33
Linfócitos	3	3	4	3,33
Plasmócitos	2	2	3	2,33
Macrófagos	3	4	3	3,33
Células gigantes	3	1	1	1,67
Necrose	0	0	0	0,00
SUB-TOTAL (x2)				**24,00**
Neovascularização	1	2	1	1,33
Fibrose	2	0	2	1,33
Infiltração lipidica	0	0	0	0,00
SUB-TOTAL				**2,67**
			TOTAL:	**26,67**

Quadro 12 - Avaliação ao gel G2 segundo anexo E da Norma ISO 10993.

	Corte 1			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	3	3	1	2,33
Linfócitos	3	3	3	3,00
Plasmócitos	3	3	3	3,00
Macrófagos	3	4	3	3,33
Células gigantes	0	1	2	1,00
Necrose	1	1	0	0,67
SUB-TOTAL (x2)				**26,67**
Neovascularização	3	3	3	3,00
Fibrose	1	1	2	1,33
Infiltração lipidica	0	0	0	0,00
SUB-TOTAL				**4,33**
			TOTAL:	**31,00**

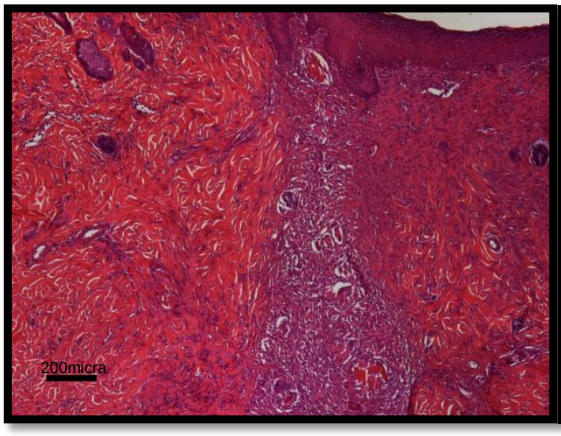

Figura 46 - Corte histológico da amostra com G1. (40x)

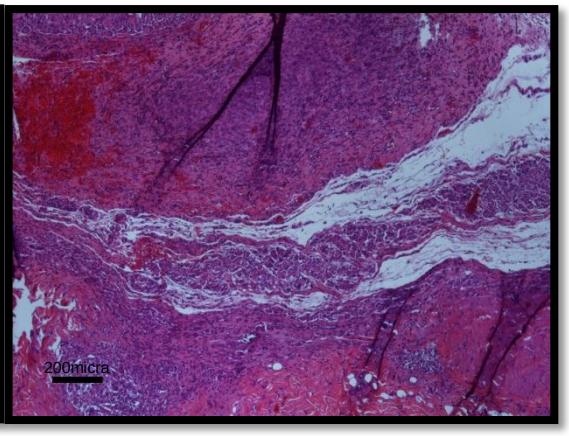

Figura 47 - Corte histológico da amostra com G2. (40x)

A implantação da membrana de PLGA (Quadro 2) causou uma resposta inflamatória bem localizada circundada por uma cápsula fibrosa. Foi notado que esta reação não se encontra em plano subcutâneo mas sim a nível do músculo. Os valores obtidos para células gigantes nos campos 1 e 2 do corte 2, foram verificados à volta de pelos (local normal destas células na presença de material estranho) (Quadro 13 e Figura 48).

Quadro 13 - Avaliação à membrana PLGA segundo anexo E da Norma ISO 10993.

	Corte 1			Corte 2			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	0	1	1	1	1	1	0,83
Linfócitos	2	3	3	3	3	3	2,83
Plasmócitos	2	3	3	2	0	1	1,83
Macrófagos	3	3	3	3	3	3	3,00
Células gigantes	1	0	1	2	2	2	1,33
Necrose	0	0	1	0	0	0	0,17
SUB-TOTAL (x2)							**20,00**
Neovascularização	3	3	3	2	3	3	2,83
Fibrose	1	1	2	2	2	2	1,67
Infiltração lipidica	0	0	0	0	0	0	0,00
SUB-TOTAL							**4,50**
						TOTAL:	**24,50**

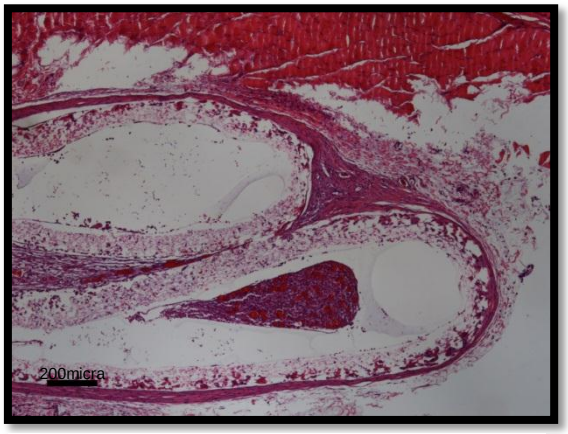

Figura 48 - Corte histológico da amostra com PLGA. (40x)

A implantação da membrana PLGA+1% (Quadro 2) causou uma pequena hemorragia no local, não se verificando mais nenhuma reação além da inflamatória (Quadro 14 e Figura 49).

Quadro 14 - Avaliação à membrana PLGA+1% segundo anexo E da Norma ISO 10993.

	Corte 1			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	1	0	0	0,33
Linfócitos	3	3	2	2,67
Plasmócitos	3	3	3	3,00
Macrófagos	3	3	3	3,00
Células gigantes	3	3	3	3,00
Necrose	0	0	0	0,00
SUB-TOTAL (x2)				**24,00**
Neovascularização	3	3	3	3,00
Fibrose	1	2	1	1,33
Infiltração lipidica	0	0	0	0,00
SUB-TOTAL				**4,33**
			TOTAL:	**28,33**

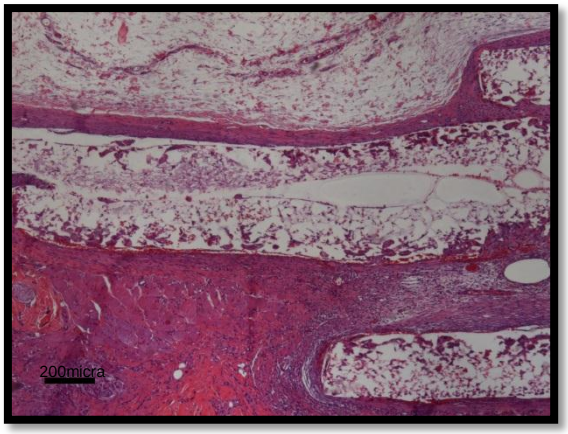

Figura 49 - Corte histológico da amostra com PLGA+1%. (40x)

Na amostra onde foi implantada a membrana de PEC (Quadro 2) verifica-se uma cápsula fibrosa muito espessa contendo muitos neutrófilos, o que equivale a ter pus e apresenta ainda algum material dentro da cápsula (Quadro 15 e Figura 50).

Quadro 15 - Avaliação à membrana PEC segundo anexo E da Norma ISO 10993.

	Corte 1			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	3	3	3	3,00
Linfócitos	3	3	3	3,00
Plasmócitos	2	3	2	2,33
Macrófagos	3	3	3	3,00
Células gigantes	0	0	2	0,67
Necrose	0	0	0	0,00
SUB-TOTAL (x2)				**24,00**
Neovascularização	3	3	3	3,00
Fibrose	2	2	1	1,67
Infiltração lipidica	0	0	0	0,00
SUB-TOTAL				**4,67**
			TOTAL	**28,67**

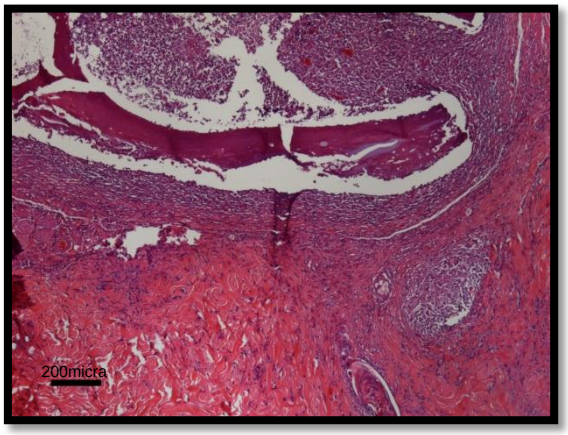

Figura 50 - Corte histológico da amostra com PEC. (40x)

No local da colocação do implante da membrana de PVA (Quadro 2) encontra-se um quisto folicular que pode ter sido causado por uma picada de agulha e verifica-se a existência de uma crosta purulenta (Quadro 16 e Figura 51).

Quadro 16 - Avaliação à membrana PVA segundo anexo E da Norma ISO 10993.

	Corte 1			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	3	3	3	3,00
Linfócitos	3	3	3	3,00
Plasmócitos	1	0	3	1,33
Macrófagos	1	2	2	1,67
Células gigantes	0	0	0	0,00
Necrose	0	0	0	0,00
SUB-TOTAL (x2)				**18,00**
Neovascularização	3	3	3	3,00
Fibrose	2	2	2	2,00
Infiltração lipidica	0	0	0	0,00
SUB-TOTAL				**5,00**
			TOTAL:	**23,00**

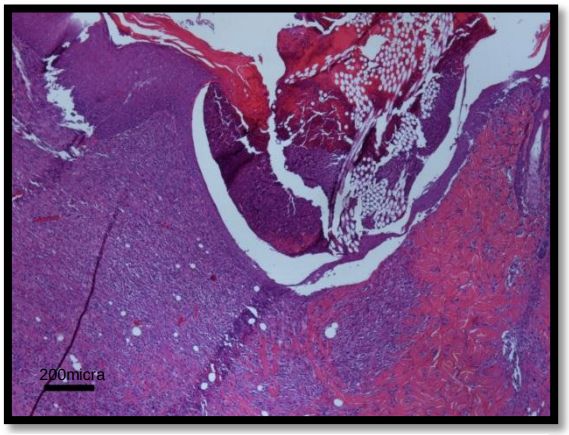

Figura 51 - Corte histológico da amostra com PVA. (40x)

Para a membrana de PLGA-HA (Quadro 2) foram analisados 2 cortes, no entanto o segundo não se considera pois este já se encontra em fase de reparação, e não é isso que se pretende avaliar (Quadro 17 e Figura 52).

Quadro 17 - Avaliação à membrana PLGA-HA segundo anexo E da Norma ISO 10993.

	Corte 1			Corte 2			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	3	3	3	0	0	1	3,00
Linfócitos	3	3	3	3	3	3	3,00
Plasmócitos	2	2	3	2	2	1	2,33
Macrófagos	3	3	3	2	2	2	3,00
Células gigantes	3	3	3	0	1	0	3,00
Necrose	0	0	0	0	0	0	0,00
SUB-TOTAL (x2)							**28,67**
Neovascularização	3	3	3	4	3	4	3,00
Fibrose	1	1	1	2	2	2	1,00
Infiltração lipidica	0	0	0	0	0	0	0,00
SUB-TOTAL							**4,00**
						TOTAL:	**32,67**

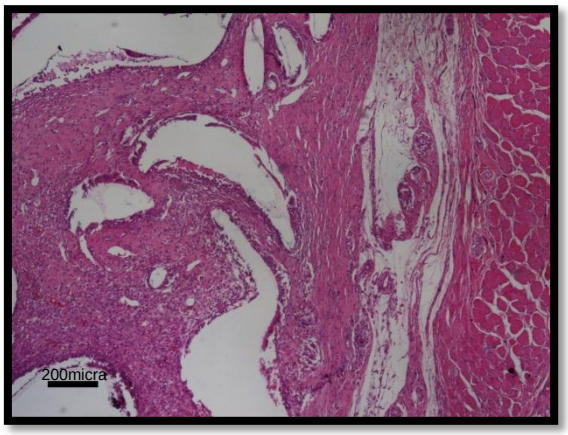

Figura 52 - Corte histológico da amostra com PLGA-HA. (40x)

No caso da membrana de PLC (Quadro 2) é encontrada uma cápsula fina à volta do local onde foi colocado o implante (Quadro 18 e Figura 53).

Quadro 18 - Avaliação à membrana PLC segundo anexo E da Norma ISO 10993.

	Corte 1			Corte 2			Corte 3			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	1	2	0	0	0	0	1	1	1	0,67
Linfócitos	3	3	3	1	2	1	3	3	3	2,44
Plasmócitos	1	2	1	1	1	1	2	1	2	1,33
Macrófagos	1	2	2	2	1	1	3	3	2	1,89
Células gigantes	0	0	0	0	0	0	0	0	0	0,00
Necrose	0	0	0	0	0	0	0	0	0	0,00
SUB-TOTAL (x2)										**12,67**
Neovascularização	3	3	3	4	4	3	4	3	3	3,33
Fibrose	1	1	1	3	3	2	3	2	2	2,00
Infiltração lipidica	0	0	0	0	0	0	0	0	0	0,00
SUB-TOTAL										**5,33**
									TOTAL:	**18,00**

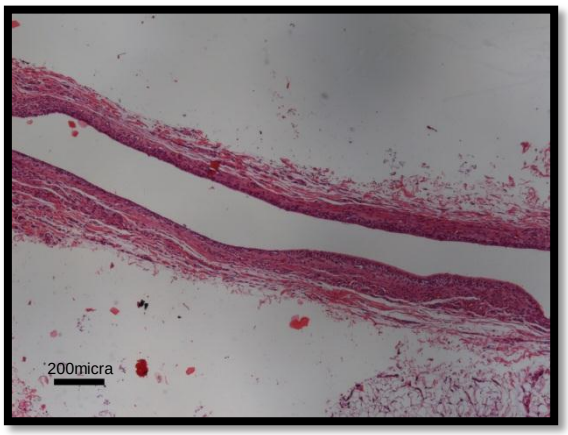

Figura 53 - Corte histológico da amostra com PLC. (40x)

A quantidade de células gigantes que se verifica no local de implantação da membrana de PLC-HA (Quadro 2) é devido na sua maioria à presença de corpos estranhos como é o caso de pêlos (Quadro 19 e Figura 54).

Quadro 19 - Avaliação à membrana PLC-HA segundo anexo E da Norma ISO 10993.

	Corte 1			Corte 2			Corte 3			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	1	0	2	1	1	0	1	0	1	0,78
Linfócitos	3	3	3	3	3	3	3	3	3	3,00
Plasmócitos	2	2	3	2	2	2	2	2	1	2,00
Macrófagos	3	4	4	4	3	3	4	3	3	3,44
Células gigantes	0	0	3	2	0	0	0	1	0	0,67
Necrose	0	0	0	0	0	0	0	0	0	0,00
SUB-TOTAL (x2)										**19,78**
Neovascularização	4	3	3	3	3	3	3	2	2	2,89
Fibrose	2	2	2	3	3	3	2	1	1	2,11
Infiltração lipidica	0	0	0	0	0	0	0	0	0	0,00
SUB-TOTAL										**5,00**
									TOTAL:	**24,78**

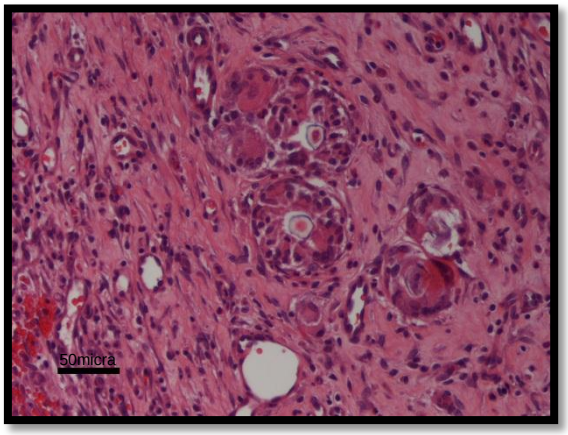

Figura 54 - Corte histológico da amostra com PLC-HA. (200x)

No tecido subcutâneo dos ratos existem células típicas de uma resposta inflamatória mas que nada têm a ver com isso, daí ser necessário também a avaliação de pele normal como controlo para subtrair aos valores conseguidos por parte dos biomateriais. Foram feitos dois controlos:

- Controlo normal: foi retirada uma amostra da pele do rato e enviada para avaliação, sem qualquer tipo de tratamento (Quadro 20 e Figura 55).
- Controlo *sham*: o dorso do rato foi aberto e sem ser lá colocado nada foi suturado. Assim que cicatrizou (15 dias depois) foi recolhida uma amostra desse local para avaliação e também foi recolhida uma amostra do local suturado de onde se retirou o controlo normal (Quadro 21 e Figura 56).

O valor controlo usado vai ser a média entre o valor do controlo normal e controlo *sham.*

Quadro 20 - Avaliação ao controlo normal segundo anexo E da Norma ISO 10993.

	Corte 1			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	0	0	0	0,00
Linfócitos	1	1	1	1,00
Plasmócitos	1	0	0	0,33
Macrófagos	0	1	1	0,67
Células gigantes	0	0	0	0,00
Necrose	0	0	0	0,00
SUB-TOTAL (x2)				**4,00**
Neovascularização	1	1	1	1,00
Fibrose	0	0	0	0,00
Infiltração lipidica	0	0	0	0,00
SUB-TOTAL				**1,00**
			TOTAL:	**5,00**

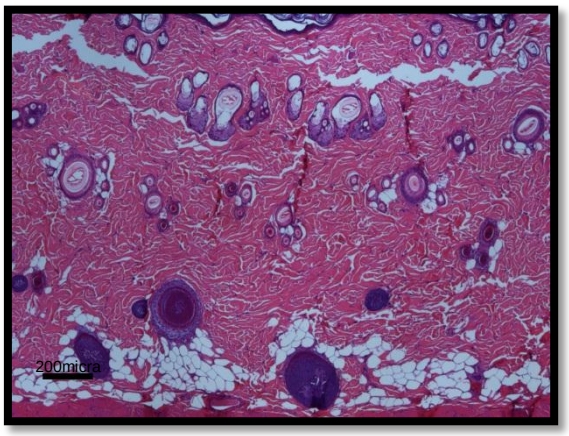

Figura 55 - Corte histológico da amostra controlo normal. (40x)

Quadro 21 - Avaliação ao controlo *sham* segundo anexo E da Norma ISO 10993.

	Corte 1				Corte 2			
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	Campo 4	Campo 1	Campo 2	Campo 3	**Média**
Neutrófilos (PMN)	1	1	1	0	0	1	1	0,71
Linfócitos	3	3	3	3	3	3	3	3,00
Plasmócitos	3	3	2	3	3	3	3	2,86
Macrófagos	3	3	3	3	3	3	3	3,00
Células gigantes	2	2	1	2	2	2	1	1,71
Necrose	0	0	0	0	2	0	1	0,43
SUB-TOTAL (x2)								**23,43**
Neovascularização	3	2	3	3	2	2	3	2,57
Fibrose	1	2	2	1	2	1	1	1,43
Infiltração lipidica	0	0	0	0	0	0	0	0,00
SUB-TOTAL								**4,00**
							TOTAL:	**27,43**

	Corte 1			Corte 2			Corte 3	
Células Tipo/Resposta	Campo 1	Campo 2	Campo 3	Campo 1	Campo 2	Campo 3	Campo 1	**Média**
Neutrófilos (PMN)	3	3	3	1	1	1	1	1,86
Linfócitos	3	3	3	3	3	3	3	3,00
Plasmócitos	3	3	3	3	3	3	3	3,00
Macrófagos	3	3	3	2	3	3	3	2,86
Células gigantes	0	0	0	0	1	2	1	0,57
Necrose	0	0	0	0	0	0	0	0,00
SUB-TOTAL (x2)								**22,57**
Neovascularização	3	3	3	3	3	3	3	3,00
Fibrose	2	2	2	3	3	2	1	2,14
Infiltração lipidica	0	0	0	0	0	0	0	0,00
SUB-TOTAL								**5,14**
							TOTAL:	**27,71**

Total:	27,57

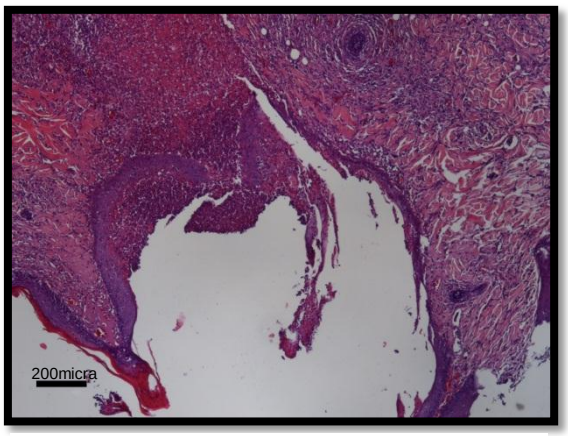

Figura 56 - Corte histológico da amostra controlo *sham*. (40x)

Depois de todas as avaliações terem sido feitas, foi descontado o valor médio entre os controlos que é 16,29 ((27,57+5,00)/2). Com o valor obtido já se consegue classificar o implante utilizando a Escala de avaliação (Figura 57).

Escala:
0,0 - 2,9 = Não irritante
3,0 - 8,9 = Ligeiramente irritante
9,0 - 15,0 = Moderadamente irritante
>15 = Severamente irritante

Figura 57 - Escala de avaliação para classificar o grau de irritabilidade do implante subcutâneo.

Com os resultados obtidos (Quadro 22) podemos afirmar que a membrana de PLC é a melhor para ser utilizada como implante subcutâneo uma vez que não é considerada irritante (causa pouca reação inflamatória). O mesmo já não se pode dizer da membrana PLGA-HA pois é severamente irritante. O fabrico dos implantes com reagentes de grau médico (muito mais dispendiosos) e a sua esterilidade são alguns dos aspetos que podem ser mudados para os implantes não serem tão irritantes, podendo mesmo fazer com que os implantes classificados de ligeiramente irritante possam passar a não irritantes. Nunca deve ser esquecido o fator de que cada animal pode ter uma reação inflamatória própria, variando de uns para outros.

Quadro 22 - Resultados e conclusões da avaliação dos implantes subcutâneos.

Implantes:	Resultado parcial	Valor controlo	Resultado final	Conclusão
PLC	18	16,29	1,71	Não irritante
Alg	21	16,29	4,71	Ligeiramente irritante
PVA	23	16,29	6,71	Ligeiramente irritante
Alg-AH	23,22	16,29	6,93	Ligeiramente irritante
PLGA	24,5	16,29	8,21	Ligeiramente irritante
PLC-HA	24,78	16,29	8,49	Ligeiramente irritante
PLGA 600	26,33	16,29	10,04	Moderadamente irritante
G1	26,67	16,29	10,38	Moderadamente irritante
AH	26,67	16,29	10,38	Moderadamente irritante
AQ	27	16,29	10,71	Moderadamente irritante
PLGA+1%	28,33	16,29	12,04	Moderadamente irritante
PEC	28,67	16,29	12,38	Moderadamente irritante
G2	31	16,29	14,71	Moderadamente irritante
PLGA-HA	32,67	16,29	16,38	Severamente irritante

4.

Use of poly(DL-lactide-ε-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly of the umbilical cord for promoting nerve regeneration in axonotmesis: *in vitro* and *in vivo* analysis

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Abbreviations:

NMR-nuclear magnetic resonance; SFI-sciatic functional index; EPT-extensor postural thrust; WRL-withdrawal reflex latency; PNS-peripheral nervous system; PLC-poly(DL-lactide-ε-caprolactone); PLGA-poly(lactic-coglycolic acid)

ABSTRACT

Cellular systems implanted into an injured nerve may produce growth factors or extracellular matrix molecules, modulate the inflammatory process and eventually improve nerve regeneration. In the present study, we evaluated the therapeutic value of human umbilical cord matrix MSCs (HMSCs) on rat sciatic nerve after axonotmesis injury associated to Vivosorb® membrane. During HMSCs expansion and differentiation in neuroglial-like cells, the culture medium was collected at 48, 72 and 96h for nuclear magnetic resonance (NMR) analysis in order to evaluate the metabolic profile. To correlate the HMSCs ability to differentiate and survival capacity in the presence of the Vivosorb® membrane, the [Ca2+]i of undifferentiated HMSCs or neuroglial-differentiated HMSCs was determined by the epifluorescence technique using the Fura-2AM probe. The Vivosorb® membrane proved to be adequate to be used as scaffold associated with undifferentiated HMSCs or neuroglial-differentiated HMSCs. In vivo testing was carried out in adult rats where a sciatic nerve axonotmesis injury was treated with undifferentiated HMSCs or neuroglial differentiated HMSCs with or without the Vivosorb® membrane. Motor and sensory functional recovery was evaluated throughout a healing period of 12 weeks using sciatic functional index (SFI), extensor postural thrust (EPT), and withdrawal reflex latency (WRL).

Stereological analysis was carried out on regenerated nerve fibers. In vitro investigation showed the formation of typical neuroglial cells after differentiation, which were positively stained for the typical specific neuroglial markers such as the GFAP, the GAP-43 and NeuN.

NMR showed clear evidence that HMSCs expansion is glycolysis-dependent but their differentiation requires the switch of the metabolic profile to oxidative metabolism. In vivo studies showed enhanced recovery of motor and sensory function in animals treated with transplanted undifferentiated and differentiated HMSCs that was accompanied by an increase in myelin sheath. Taken together, HMSC from the umbilical cord Wharton jelly might be useful for improving the clinical outcome after peripheral nerve lesion.

KEY WORDS: axonotmesis, stem cells, mesenchymal stem cells, neuroglial-like cells, nerve regeneration, Wharton jelly.

1. INTRODUCTION

A full understanding of nerve regeneration, especially complete functional achievement and organ reinnervation after nerve injury, still remain the principle goal of regenerative medicine.

The reliability of animal models is crucial for peripheral nerve research. Because of its peripheral nerve size, the rat sciatic nerve has been the most commonly experimental model used in studies concerning the peripheral nerve regeneration and possible therapeutic approaches (Ronchi et al., 2009). Although sciatic nerve injuries themselves are rare in humans, this experimental model provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets (Amado et al., 2008; Mackinnon et al., 1985). Focal crush causes axonal interruption but preserves the connective sheaths (axonotmesis). After this kind of injury, regeneration is usually successful, in a short latency (1-2 day), axons regenerate at a steady rate along the distal nerve supported by the reactive Schwann cells (SCs) and the preserved endoneural tubules which enhance axonal elongation and facilitate adequate reinnervation (Luis et al., 2007).

Tissue engineering of peripheral nerves associates biomaterials, like chitosan, PLC, and other biomaterials, some of them, previously studied by our group (Amado et al., 2008; Luis et al., 2008a; Maurício et al., 2011) to cellular systems, able to differentiate into neuroglial –like cells or even by modulating the inflammatory process, which might improve nerve regeneration, in terms of motor and sensory recovery, and also, by shortening the healing period avoiding regional muscular atrophy. Cell transplantation has been proposed as a method of improving peripheral nerve regeneration (Chen et al., 2007). SCs, MSCs, embryonic stem cells, marrow stromal cells are the most studied support cells candidates. Schwann cell transplantation enhance axon outgrowth both in vitro (Schlosshauer et al., 2003) and in vivo (Keilhoff et al., 2006).Transplantation of viable SCs offers better results than the sheer release of growth factors, because SCs have the above mentioned regeneration promoting effect (Bhatheja and Field, 2006; Fansa et al., 1999; Hall, 1978; Ide, 1996; Madduri and Gander, 2010; Muir, 2010; Schmitte et al., 2010). The generation of sufficient amounts of SCs for auto-transplantation, however, requires a significant time for cell culture.

Current concepts include mitogenic substances to enhance cell yield. Due to their side effects they should be avoided in clinical therapy. Moreover, functional nerves have to be sacrificed as SCs donor resulting in loss of sensation, scarring and, possibly, neuroma formation. The limitations of harvesting autologous SCs, such as

donor nerve sacrifice and donor site morbidity, have led to investigation of other cell types that provide similar trophic support to axon regeneration (Ladak et al., 2011).

On the other hand, MSCs have become one of the most interesting targets for Tissue Regeneration including the peripheral nerves. Like bone marrow stromal cells and other mesenchymal cells, they are plastic adherent, stain positively for markers of the mesenchymal (CD10, CD13, CD29, CD44, CD90, and CD105) and negatively for markers of the hematopoietic lineage (Wang et al., 2004).These cells are capable of self-renewal with sustained proliferation in vitro and can differentiate into multiple mesodermal cells, including neuron-like cells (Park et al., 2010). The high plasticity and low immunogenicity of these cells, turn them into a desirable form of cell therapy for the injured nervous system without requiring the use of immunosuppressive drugs during the treatments (Thuret et al., 2006).

MSCs have been isolated from various other origins, including skin, hair follicle, periosteum, amniotic fluid, umbilical cord blood and adipose tissue. Harvest and expansion of MSCs are straight forward techniques (Colter, 2000; Gnecchi and Melo, 2009), which are not only feasible regarding the laboratory approach but also regarding their application with the patient. With appropriate stimuli and environmental conditions MSCs exhibit a respectable plasticity (Joshi and Enver, 2002). They may even differentiate into non-mesenchymal lineages, including myelinating cells of the peripheral nervous system (PNS) (Dezawa et al., 2001; Tohill et al., 2004). Beside their easy expansion in culture their plasticity makes MSCs an ideal source for tissue repair and tissue engineering. Bone marrow represents the most commonly used tissue source of adult MSCs. Bone marrow MSCs (BMSCs) have been applied for cell based therapies; however, due to the limited number of BMSCs available for autologous use and the possibility of donor site morbidity and the decreased number of BMSCs along the adult life, there is a need to identify alternative MSCs sources.

A recently reported potential alternative tissue source of MSCs is the connective tissue (Wharton's Jelly) of human umbilical cord (UC). Different harvesting procedures have led to UC-derived cells that exhibit a neuronal phenotype (Fu et al., 2006; Mitchell et al., 2003; Sarugaser et al., 2005; Wang et al., 2004) and have potential utility in treatment of neurodegenerative diseases (Weiss et al., 2006; Weiss et al., 2003), indicating the versatility of this cell source. Interestingly, these cells, which are major histocompatibility complex (MHC) class II negative, not only express both an immunoprivileged and immunomodulatory phenotype, but their MHC class I expression levels can also be manipulated (Sarugaser et al., 2005), making them a potential cell source for MSC-based therapies. In addition, these cells represent a non controversial source of primitive mesenchymal progenitor cells that can be harvested after birth,

cryogenically stored, thawed, and expanded for therapeutic uses (Maurício et al., 2011).

The aim of this study was to explore the therapeutic value of human UC matrix (Wharton's jelly) derived MSCs (HMSC) both in vitro and in vivo, associated to a Poly(DL-lactide-ε- caprolactone) PLC (Vivosorb®) membrane, on a rat sciatic nerve axonotmesis experimental model.

2. MATERIALS AND METHODS

2.1. Poly(DL-lactide-ε-caprolactone) (PLC) membranes

Poly(DL-lactide-ε-caprolactone) (PLC) membranes (Vivosorb®) were purchased from Polyganics BV, Groningen, Netherlands (FS01-006/20 Lot: FSA2009092311).

2.2. Cell Culture and in vitro differentiation of HMSC from Wharton's jelly umbilical cord

Human MSC from Wharton's jelly UC (HMSCs) were purchased from PromoCell GmbH (C-12971, lot-number:8082606.7). Cryopreservated cells were cultured and maintained in a humidified atmosphere with 5% CO2 at 37ºC. Mesenchymal Stem Cell Medium (PromoCell, C-28010) was replaced every 48 hours. At 90% confluence, cells were harvested with 0.25% trypsin with EDTA (Gibco) and passed into a new flask for further expansion. HMSCs at a concentration of 2500 cell/ml were cultured and after 24 hours cells exhibited 30-40% confluence. Differentiation was induced with MSC neurogenic medium (Promocell, C-28015). Medium was replaced every 24 hours during 3 days. The formation of neuroglial-like cells was observed after 24 hours in an inverted microscope (Zeiss, Germany).

Intracellular free Ca2+ concentration ([Ca2+]i) was measured in Fura-2-loaded cells by using dual wavelength spectrofluorometry as previously described (Amado et al, 2008). The measurements were performed on undifferentiated HMSCs after confluence was obtained and on neuroglial-differentiated HMSCs, cultured on Vivosorb® discs in order to correlate the HMSCs ability to differentiate and survival capacity in the presence of the Vivosorb® membrane.

2.3 Cytogenetic analysis of Human MSC from Wharton's jelly

HMSC cell line (differentiated) from Wharton's jelly was studied for cytogenetic analysis at passage 5. When confluence was reached, culture medium was changed and supplemented with 4µg/ml colcemid solution (stock solution, Cat. no. 15212-012, Gibco). After 4 hours, HMSCs were collected and suspended in 8ml of 0.075M KCl solution supplemented with bovine fetal serum (BFS). Then the suspension was incubated in 37◦C for 35 minutes. After centrifugation (1500 rpm), 8ml of the fixative methanol:glacial acetic acid at 6:1 was added and mixed together, and the cells were again centrifuged. After 2 rounds of fixation, 2 new rounds were performed with the fixative methanol:glacial acetic acid at 3:1. After the last centrifugation, the HMSC suspension was spread onto very well glass cleaned slides.

Analysis was performed by one scorer on Giemsa-stained cells.

2.4. Immunocytochemistry

At passage 3, HMSCs were trypsinized, washed and re-suspended in Mesenchymal Stem Cell Medium (PromoCell, C-28010) at a concentration of 1x105cell/ml. HMSCs were fixed with paraformaldehyde at 4ºC for 15 min and washed with distilled water before permeabilization in 0.5% Triton-X100. Non-specific binding was blocked using blocking solution (PBS containing 1% bovine serum albumin (BSA)) for 1 hour at room temperature. HMSCs were then incubated 2 hours at room temperature with primary antibodies of rabbit anti-growth associated protein-43 (GAP-43, 1:200) (Chemicon, AB5220), rabbit anti-glial fibrillary acidic protein (GFAP, 1:500) (Chemicon, AB5804) and mouse anti-neuronal nuclei (NeuN, 1:100) (Chemicon, MAB377). After washing, HMSCs were incubated 15 minutes with secondary antibodies goat anti-rat IgG (Millipore, AP136P) and goat anti-rabbit IgG (Millipore, 12-348MN). After several washes in PBS, HMSCs were incubated with horseradish peroxidase (HRP)-coupled streptavidin for 10 min. DAB (diaminobenzidine) served as chromogen.

2.5. NMR spectroscopy

During HMSCs expansion and differentiation to neuroglial-like cells, 160 µL of the culture medium was collected at 48, 72 and 96 hours (N =5) for nuclear magnetic resonance (NMR) analysis. 1H-NMR spectra of the collected samples were acquired at 14.1 T, 25ºC, using a Varian 600 MHz spectrometer equipped with a 3 mm indirect detection probe with z-gradient (Varian, Palo Alto, CA) by standard methods. Solvent-

suppressed 1H-NMR spectra were acquired with 6 kHz sweep width, using 14 s delay for allowing total proton relaxation, 3s water pre-saturation, 45° pulse angle, 3.5 seconds acquisition time, and at least 64 scans. 1HNMR spectroscopy was performed and the following metabolites were determined: lactate, doublet located at 1.33 ppm; alanine, doublet at 1.45 ppm; and H1-α glucose, doublet at 5.22 ppm. Sodium fumarate (final concentration of 2 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution. The relative areas of 1H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSproTM NMR spectral analysis program (Acorn, NMR Inc, Fremont, CA).

2.6. Surgical procedure

For the in vivo testing, Sasco Sprague adult rats (Charles River Laboratories, Barcelona, Spain) were divided in groups of 6 animals each: A group of 6 animals was used as control without any sciatic nerve injury (Group 1 – Control).In Group 2 the crushed sciatic nerve did not have any other intervention (Group 2 - Crush). In Group 3, the axonotmesis lesion of 3 mm was enwrapped with a PLC (Vivosorb®) membrane (Group 3 – CrushPLC). In Group 4, the crushed sciatic nerve was infiltrated in the lesion area with a suspension of 1500 HMSCs (in a total volume of 50 µl) (Group 4 – CrushCell), in Group 5, the crushed sciatic nerve was encircled by a PLC (Vivosorb®) membrane covered with a monolayer of non (Group 5 – CrushCellNonDifPLC) and in Group 6 the axonotmesis lesion of 3 mm was enwrapped with a PLC (Vivosorb®) membrane covered with a monolayer of differentiated HMSCs (neurogliallike cells) (Group 6 – CrushCellDifPLC). The standardized crush injury was carried out with the animals placed prone under sterile conditions and the skin from the clipped lateral right thigh scrubbed in a routine fashion with antiseptic solution. The surgery procedure was the one previously described (Luís et al., 2007; Luis et al., 2007). The standard crush injury was performed by a non-serrated clamp (Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria), exerting a constant force of 54 N for a period of 30 s, 10 mm above the bifurcation into tibial and common peroneal nerves inducing a 3mm axonotmesis lesion. To prevent autotomy, a deterrent substance was applied to rat right foot.

No local or systemic signs of rejection or foreign body were observed in the experimental animals transplanted with PLC membranes and HMSCs. There was no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period of 12 weeks after the surgical procedure.

2.7. Functional assessment

All animals were tested preoperatively (week 0), and every week until the end of the 12-week follow-up time.

2.7.1. Motor performance and nociceptive function

Motor performance and nociceptive function were evaluated by measuring extensor postural thrust (EPT) and withdrawal reflex latency (WRL), respectively (Luis et al., 2007; Luis et al., 2008a; Luis et al., 2008b). For EPT test, the affected and normal limbs were tested 3 times, with an interval of 2 minutes between consecutive tests, and the 3 values were averaged to obtain a final result. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values were incorporated into an equation (Equation (1)) to derive the percentage of functional deficit, as described in the literature(Koka and Hadlock, 2001):

$$\% \text{ Motor deficit} = [(NEPT - EEPT) / NEPT] \times 100 \quad (1)$$

The nociceptive withdrawal reflex (WRL) was adapted from the hotplate test developed by Masters et al. (1993) (Masters et al., 1993) and described elsewhere (Luis et al., 2007; Luis et al., 2008a; Luis et al., 2008b). Normal rats withdraw their paws from the hotplate within 4s or less(Hu et al., 1997). The cutoff time for heat stimulation was set at 12 seconds to avoid skin damage to the foot (Varejao et al., 2003).

2.7.2. Sciatic functional index (SFI)

For SFI, animals were tested in a confined walkway measuring 42 cm long and 8.2 cm wide, with a dark shelter at the end, as previously described (Luis et al., 2007; Luis et al., 2008a; Luis et al., 2008b). Several measurements were taken from the footprints: (i) distance from the heel to the third toe, the print length (PL); (ii) distance from the first to the fifth toe, the toe spread (TS); and (iii) distance from the second to the fourth toe, the intermediary toe spread (ITS). For SFI, all measurements were taken from the experimental (E) and normal (N) sides. The mean distances of three measurements were used to calculate the following factors:

$$\text{Toe spread factor (TSF)} = (ETS - NTS) / NTS$$

Intermediate toe spread factor (ITSF) = (EITS – NITS) / NITS

Print length factor (PLF) = (EPL - NPL) / NPL

SFI was calculated as described by Bain et al. (Bain et al., 1989) according to the following equation:

SFI = -38.3(EPL – NPL) / NPL + 109.5(ETS – NTS) / NTS + 13.3(EIT – NIT) / NIT – 8.8 = (-38.3 × PLF) + (109.5 × TSF) + (13.3 × ITSF) – 8.8 (2)

For SFI, an index score of 0 is considered normal and an index of -100 indicates total impairment. When no footprints were measurable, the index score of -100 was given (Dijkstra et al., 2000).

2.8. Sciatic nerve stereology

Nerve samples (10-mm-long sciatic nerve segments distal to the crush site and from unoperated controls) were processed for quantitative morphometry of myelinated nerve fibers (Raimondo et al., 2009). Fixation was carried out using 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1M Sorensen phosphate buffer for 6-8 hours and resin embedding was obtained following Glauerts' procedure (Scipio et al., 2008). Series of 2-µm thick semi-thin transverse sections were cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained by Toluidine blue. Stereology was carried out on a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). Systematic random sampling and D-disector were adopted using a protocol previously described (Geuna et al., 2004; Geuna et al., 2000). Fiber density and total number of myelinated fibers were estimated together with fiber and axon diameter and myelin thickness.

2.9. Statistical analysis

Data of functional tests are reports as means and standard deviations (SD) at each time point, including pre-operatively, and each experimental group. Differences between time points and between groups were tested by two-way analysis of variance (ANOVA) using a mixed model of within- (time of recovery) and between-subjects

(experimental groups) factors. In cases of significant main effect of experimental group (between-subjects factor), pairwise comparisons were further conducted using the post hoc Tukey's HSD test using IBM SPSS statistics19 software package (SPSS, Inc.). For stereology, statistical comparisons of quantitative data were subjected to one-way ANOVA test using the software —Statistica per discipline biomedichell (McGraw-Hill, Milan, Italy). In RMN studies, comparison of data between different experimental groups was performed by One-way ANOVA followed by Newman-Keuls posthoc test using GraphPad Prism Software, (San Diego California USA). In all cases differences at $p<0.05$ were considered significant.

3. RESULTS

3.1. Cytocompatibility of Vivosorb® membranes and confirmation of HMSCs differentiation into neuroglial-like cells and karyotype analysis.

Results obtained from epifluorescence technique are referred to measurements from undifferentiated HMSCs and neuroglial-differentiated HMSCs which correspond to [Ca2+]I from cells that did not begin the apoptosis process (data not shown). The undifferentiated HMSCs cultured on Vivosorb® membranes reached confluence and exhibited a normal starlike shape with a flat morphology in culture. In the presence of neurogenic medium, the formation of neuroglial-like cells was observed after 24 hours. According to these results, it is reasonable to conclude that Vivosorb® membranes are a viable substrate for undifferentiated HMSCs culture or neuroglial-differentiated HMSCs adhesion, multiplication and differentiation.

The phenotype of HMSCs was assessed by PromoCell. Rigid quality control tests are performed for each lot of PromoCell HMSCs isolated from Wharton's jelly of UC. HMSCs were tested for cell morphology, adherence rate and viability. Furthermore, each cell lot was characterized by flow cytometry analysis for a comprehensive panel of markers, such as PECAM (CD31), HCAM (CD44), CD45, and Endoglin (CD105). The HMSCs exhibited a mesenchymal-like shape with a flat and polygonal morphology. During expansion the cells became long spindle-shaped and colonized the whole culturing surface (Figure 1A). After 96 hours of culture in neurogenic medium, we observed a morphological change. The cells became exceedingly long and there was a formation of typical neuroglial-like cells with multi-branches and secondary branches (Figure 1B). The differentiation was tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN in neurogliallike differentiated MSCs.

Undifferentiated HMSCs were negatively labeled to GFAP, GAP43 and NeuN (Figure 2A, 2C and 2E, respectively). After 96 hours of differentiation the attained cells were positively stained for glial protein GFAP (Figure 2B) and for the growthassociated protein GAP-43 (Figure 2D). All nuclei of neuroglial-like cells were also labeled with the neuron specific nuclear protein NeuN (Figure 2E) demonstrating successful differentiation of HMSCs in neuroglial-like cells.

Undifferentiated HMSCs exhibited a normal star-like shape with a flat morphology. After in vitro differentiation, HMSCs morphology changed into typical neuroglial-like pattern with multi-branches and secondary branches (Figure 1B). Giemsa-stained cells of differentiated HMSC cell line at passage 5 were analyzed for cytogenetic characterization. However, no metaphases were found, therefore the karyotype could not be established. The karyotype of undifferentiated HMSCs was determined previously and no structural alterations were found demonstrating absence of neoplastic characteristics in these cells, as well as chromosomal stability to the cell culture procedures.

3.2 Glycolysis is stimulated during HMSCs expansion but not during differentiation

During the 96 hours expansion of undifferentiated HMSCs, the glucose consumption was 4.4 ± 0.05 pmol/cell, while during differentiation the cells consumed 5.0 ± 1.1 pmol/cell. Interestingly, although the glucose consumption was very similar in both conditions after the 96 hours cell culture, the glucose consumption rate was only similar between the 48 and 72 hours. Undifferentiated HMSCs mostly consumed glucose in the first 48 hours at a rate of 0.5 ± 0.004 pmol/h/cell per hour, while during differentiation, the glucose consumption rate lowered to just 0.08 ± 0.01 pmol/h/cell per hour. Between the 72 and 96 hours of culture, the glucose consumption was also increased in undifferentiated cells (0.21 ± 0.04 pmol/h/cell) when compared with cells undergoing differentiation (0.08 ± 0.04 pmol/h/cell).As expected, the lactate production increased during HMSCs expansion. After 96 hours, the lactate production was 26 ± 1.1 pmol/cell during expansion, while during cell differentiation the total lactate production was 6.0 ± 0.6 pmol/cell. The lactate production rate during HMSCs expansion was increased during the first and the last hours of expansion. In the first 48 hours, the rate was 0.2 ± 0.03 pmol/h/cell and between the 72 and 96 hours the rate was 0.5 ± 0.04 pmol/h/cell. Interestingly, during the 96 hours of differentiation the

lactate production rate was almost stable although between the 48 and 96 hours of differentiation there was not a production but a slight consumption of lactate.

3.3 Lactate and alanine metabolism is altered during differentiation of HMSCs

Alanine production was significantly decreased during HMSCs differentiation. During the 96 hours expansion, the cells produced 6.9 ± 0.7 pmol/cell while after differentiation the alanine production was 2.2 ± 0.4 pmol/cell. During expansion and differentiation, the alanine was mostly produced during the first hours as seen by the alanine production rate. During the first 48 hours, undifferentiated HMSCs produced alanine at a rate of 0.10 ± 0.003 pmol/h/cell while during differentiation, in that period, the alanine production rate was 0.02 ± 0.008 pmol/h/cell. The lactate/alanine ratio was significantly decreased after 48 and 72 hours in undifferentiated HMSCs. The ratio decreases from 3.5 ± 0.3 at time zero of expansion to 2.1 ± 0.1 and 2.5 ± 0.3 in the following 48 and 72 hours, respectively. After 96 hours, HMSCs differentiated into neuroglial-like cells presented a significantly lower lactate/alanine ratio (2.6 ± 0.5) than undifferentiated cells (3.9 ± 0.2).

3.4. Functional analysis

3.4.1. Nociceptive function evaluated by withdrawal reflex latency (WRL)

The withdrawal reflex requires intact thermal and nociceptive innervations of the hindpaw. Early after sciatic axonotmesis, the withdrawal response could not be recorded and WRL values rose to the cutoff time of 12 seconds (Table 1). WRL progressively reduced approaching the normal values at week 12. There was a significant effect of time in WRL scores during the healing period [$F(10,330) = 168.32$, $p = 0.000$]. An overall main effect of kind of treatment was also noticed in WRL results during the healing time [$F(5,33) = 22.786$, $p = 0.000$]. WRL values were significantly different in groups treated with the PLC membrane, alone or in combination with undifferentiated and differentiated HMSCs (Group 5 – CrushCellNonDifPLC; Group 6 – CrushCellDifPLC; Group 3 – CrushPLC) with the groups not receiving the PLC membrane ($p < 0.05$). In this case, however, the WRL values seem to indicate better functional recovery in the groups with the cellular system (Group 5 –

CrushCellNonDifPLC; Group 6 – CrushCellDifPLC), demonstrating the positive results concerning the cellular system treatment (Table 1).

3.4.2. Motor performance by measuring extensor postural thrust (EPT)

The EPT measures the antigravity response when animals are lowered and the hindlimb is loaded. In control animals and before injury, EPT scores center around zero, showing similar forces in both sides. In the week following sciatic axonotmesis, EPT scores increased to near maximal values, indicating almost complete loss of contractile force in the affected hindlimb. EPT scores then recovered steadily in the following weeks to normal values. Therefore, a significant effect of time in EPT scores during the healing period was found [$F(10,330) = 136.09$, $p = 0.000$]. Also, differences in EPT scores between the experimental groups were significant [$F(5,33) = 48.678$, $p = 0.000$]. Further pairwise tests showed that the EPT values in the untreated axonotomized group (Group 2) were similar to the other axonotomized groups treated with different combinations of PLC membranes and cellular systems (Group 5; Group 6). The groups treated with cells and PLC membrane (Group 5; Group 6), however, presented significant differences in EPT values compared to the group treated with PLC membrane only (Group 3) ($p < 0.05$), with positive results associated with the cellular system treatment. The group treated with infiltrated undifferentiated HMSCs (Group 4) presented worse recovery of EPT values, when compared to the group where these cells (Group 5; Group 6) were applied locally, by means of a PLC membrane ($p< 0.05$). There were no significant differences between the groups with PLC membranes plus undifferentiated and differentiated HMSCs (Group 5; Group 6) (Table 2).

3.4.3. Sciatic functional index (SFI)

The SFI score demonstrated severe deficit in the 2 weeks following sciatic nerve crush in all experimental groups. In the following weeks, SFI values improved progressively ending up with values indistinguishable from control animals by week 12 of recovery. Therefore, a significant main effect of time was demonstrated by ANOVA [$F(10,330) = 268.937$, $p = 0.000$]. ANOVA also demonstrated a significant effect of group [$F(5,33) = 52.632$, $p = 0.000$]. Further pairwise comparisons located the group

effect in the Group 4, with this group displaying delayed recovery of SFI compared to the Group 2 (p=0.000) and Group 2 (p=0.004) (Table 3).

3.5. Sciatic nerve stereology

Figure 3 shows the histological appearance of the nerve fibers in the different experimental groups. As expected after a crush injury that does not interrupt the nerve continuity, axon regeneration occurred in all experimental groups. Results of the stereological analysis of regenerated nerve fibers are reported in Table 4. Statistical analysis showed that all axonotmesis groups have a significantly ($p < 0.05$) higher number and density of regenerated nerve fibers compared to control. By contrast, in all axonotmesis groups, nerve fibers showed a significantly ($p < 0.05$) smaller diameter, of both axon and fiber, and myelin thickness. As far as numerical differences among axonotmesis groups are concerned, statistical analysis did not reveal any significant ($p > 0.05$) difference for any of the morphological predictors of nerve recovery except for myelin thickness that was significantly ($p < 0.05$) higher in Group 5 and Group 6.

4. DISCUSSION

Peripheral nerve tissue engineering associates biomaterials to cellular systems, able to differentiate into neuroglial-like cells, which might improve motor and sensory recovery. SCs, MSCs, embryonic stem cells, marrow stromal cells are the most studied support cells candidates. The cellular systems implanted into the injured nerve may produce growth factors or extracellular matrix (ECM) molecules, or may modulate the inflammatory process, to improve nerve regeneration (Amado et al., 2010; Gu et al., 2011; Luis et al., 2007; Luis et al., 2008a; Simoes et al., 2010). To implant cultured cells into defective nerves (with axonotmesis and neurotmesis injuries), there are two main techniques. The cellular system may be directly injected into the neural scaffold which has been interposed between the proximal and distal nerve stumps or around the crush injury (in neurotmesis and axonotmesis injuries, respectively). It can also be performed by pre-adding the cells to the neural scaffold via injection or co-culture (in most of the cellular systems, it is allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured nerve (Maurício et al., 2011).

The majority of natural biomaterials used in clinical applications (for instance, peripheral nerve injuries) such as hyaluronic acid, collagen, and gelatin are derived from animal sources. In spite of thorough purification methods, these materials bear the

inherent risk of transfer of viral diseases and may cause immunological body reactions while synthetic biomaterials are not associated with these risks (Maurício et al., 2011). Results obtained from epifluorescence technique that measure the [Ca2+]i and the cell culture morphology of undifferentiated HMSCs and neuroglial-differentiated HMSCs cultured on Vivosorb® membranes evidence that this biomaterial is a viable substrate for undifferentiated HMSCs culture or neuroglial differentiated HMSCs adhesion, multiplication and differentiation.

Our data showed that PLC does not deleteriously interfere with the nerve regeneration process, as a matter of fact, the information on the effectiveness of PLC membranes and tubeguides for allowing nerve regeneration was already provided experimentally and with patients (Maurício et al., 2011). PLC becomes hydrophilic by water uptake, which increases the permeability of the polymer. This is important in the control of nutrient and other metabolite transport to the surrounding healing tissue. A few weeks after implantation, the mechanical strength gradually decreases and loss of molecular weight occurs as a result of the hydrolysis process. In approximately 24 months, PLC degrades into lactic acid and hydroxycaproic acid which are both safely metabolized into water and carbon dioxide and/or excreted through the urinary tract. In contrast to other biodegradable polymers, PCL has the advantage of not creating an acidic and potentially disturbing micro-environment, which is favorable to the surrounding tissue (Luis et al., 2007).

Extra-embryonic tissues, as stem cell reservoirs, offer many advantages over both embryonic and adult stem cell sources. Extra-embryonic tissues, collectively known as the afterbirth, are routinely discarded at parturition, so little ethical controversy attends the harvest of the resident stem cells populations. Most significantly, the comparatively large volume of extra-embryonic tissues and easy manipulation hypothetically increases the number of stem cells that can be isolated (Marcus and Woodbury, 2008). The UC contains two arteries and one vein protected by a proteoglycan rich connective tissue called Wharton's jelly. Within the abundant extracellular matrix of Wharton's jelly resides a recently described stem cell population. In average, 400 000 cells can be isolated per UC, which is significantly greater than the number of MSCs that can be routinely isolated from adult bone marrow. In vitro, Wharton's jelly MSCs cells are capable of differentiation to multiple mesoderm cell types including skeletal muscle and neurons (Fu et al., 2006; Wang et al., 2004). Generation of clinically important dopaminergic neurons has also been reported (Fu et al., 2006). HMSCs isolated from Wharton's jelly can be easily and ethically obtained and processed compared with embryonic or bone marrow stem cells. These stem cells may be a valuable source in the repair of the peripheral nervous system with capacity

to differentiate into neuroglial-like cells (Fu et al., 2006; Yang et al., 2008). The transplanted HMSCs were also able to promote local blood vessel formation and release the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Fu et al., 2006; Wang et al., 2004). Previous results obtained by our research group using N1E-115 cells in vitro differentiated into neuroglial-like cells to promote regeneration of axonotmesis and neurotmesis lesions in the rat model showed that there was no significant effect in promoting axon regeneration and, when N1E-115 cells were cultured inside a PLGA scaffold used to bridge a nerve defect, they can even exert negative effects on nerve fiber regeneration (Luis et al., 2008a; Maurício et al., 2011). The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by spaceoccupying effect could have hindered the positive effect of local neurotrophic factor release leading a negative outcome on nerve regeneration (Amado et al., 2010; Amado et al., 2008; Luis et al., 2008a; Maurício et al., 2011). Thus, N1E-115 cells did not prove to be a suitable candidate cellular system for treatment of nerve injury after axonotmesis and neurotmesis(Amado et al., 2010; Amado et al., 2008; Luis et al., 2008a; Maurício et al., 2011) and their application is limited only to research purposes as a basic scientific step for the development of other cell delivery systems, due to its neoplastic origin. In this study, we used a PLC membrane to deliver undifferentiated and in vitro differentiated HMSCs from the UC Wharton jelly and we compared this delivery approach with direct infiltration of these undifferentiated HMSCs in suspension, in the rat sciatic nerve axonotmesis model. The cellular systems implanted into the injured nerve may produce growth factors or ECM molecules, or may modulate the inflammatory process, to improve nerve regeneration or even replaced the injured neural and SCs (Amado et al., 2010; Amado et al., 2008; Luis et al., 2008a; Maurício et al., 2011). The differentiated HMSCs karyotype could not be established, once no dividing cells were obtained at passage 5, which can be in agreement with the degree of differentiation. The karyotype analysis of undifferentiated HMSCs previously determined and published elsewhere, excluded the presence of neoplastic signs, thus supporting the suitability of our cell culture and differentiation procedures. This concern also resulted from our previous experience with N1E-115 neoplastic cell line and the negative results we obtained in the treatment of axonotmesis and neurotmesis injuries (Amado et al., 2010; Amado et al., 2008; Luis et al., 2008b; Maurício et al., 2011). Nevertheless, our cultured undifferentiated HMSCs showed normal morphology when inspected with an inverted microscope. These cells presented a star-like shape with a flat morphology, characteristic of the MSCs.

A consequence of cell metabolism during in vitro expansion and differentiation is that culture conditions are constantly changing. The comprehension and optimization of the expansion and differentiation process of HMSCs will contribute to maximization of cell yield, reduction of cell culture and a decrease of total process costs (Gong et al., 2009; Kirouac and Zandstra, 2008). In culture conditions, cells consume mostly glucose as substrate for the generation of cellular energy (ATP). A high rate of glucose consumption is necessary for cell growth and maintenance. In fact, it has been proposed that cells undergoing high proliferation rates rely essentially on glycolysis to generate ATP, producing considerable amounts of lactate (Vander Heiden et al., 2009). This process, known as Warburg effect, has been described to occur in HMSCs during expansion (Dos Santos et al., 2010; Funes et al., 2007). Our results obtained from the in vitro testing of this cellular system show that during 96 hours expansion, the undifferentiated HMSCs consumed glucose and produce, as expected, high concentration of lactate as a metabolic sub-product which is consistent with the Warburg effect and glycolysis stimulation. It was also described that MSCs do not require oxidative phosphorylation to survive (Dos Santos et al., 2010; Funes et al., 2007) instead, hypoxia prolongs the lifespan of these cells, increases their proliferative capacity and reduces differentiation(Fehrer et al., 2007). Several studies in recent years investigated the biological activity of HMSCs and their in vitro differentiation in neuroglial-like cells (Fu et al., 2004; Mitchell et al., 2003). In this study, HMSCs were in vitro differentiated with neurogenic medium. After 96 hours the regular mesenchymal-like shape of the cells changed and the cells became exceedingly long.

Morphologically it was observed the formation of neuroglial-like cells after differentiation which were positively stained for the typical specific neuronal markers (Choong et al., 2007; Fu et al., 2004; Mitchell et al., 2003) such as the GFAP, the GAP-43 and the NeuN attesting a clear successful differentiation. The morphologic and biochemical characteristics of neuroglial-like cells are already described but the mechanism by which stem cells differentiate into neuroglial-like cells is still unknown. In our experimental conditions, HMSCs that undergo differentiation in neuroglial-like cells, consumed significantly less glucose and produced significantly less lactate than HMSCs that undergo expansion. These major differences allow us to conclude that during HMSCs differentiation in neuroglial-like cells the glycolytic process, which proved to be the crucial metabolic mechanism during HMSCs expansion, is switched to oxidative metabolism. Several factors, such as inhibition of oxidative phosphorylation due to defects in mitochondrial DNA, dysfunctional Krebs cycle, or inactivation of p53 have been suggested to contribute to the glycolytic switch occurring during cellular expansion and differentiation (Funes et al., 2007). Recently, it has been shown that

umbilical-cord derived mesenchymal stem cells adapt their oxygen consumption and energy metabolism to the available oxygen concentrations (Lavrentieva et al., 2010). In our conditions, HMSCs consumed glucose at higher rates during the first 48 hours of expansion.

After that period the glucose consumption rate decreases although there was not a reduction in lactate production rate. Interestingly, during HMSCs differentiation in neuroglial-like cells, lactate production rate is significantly lower and between the 72 and 96 hours, there was not a lactate production rate but rather consumption. The conversion of pyruvate in lactate is an NADH-dependent reduction but pyruvate may also be converted into alanine via transaminase reaction, through the enzyme alanine aminotransaminase (Yang et al., 2002). Surprisingly, during HMSCs expansion, the alanine production significantly increased but the same was not noted during differentiation in neuroglial-like cells. However, the lactate/alanine ratio was lower at 48 and 72 hours during HMSCs expansion than at the same periods of differentiation. After 96 hours this ratio was decreased in differentiated cells. The appearance of more alanine than lactate in the media of neuroglial-like cells can be associated with a reduced redox cytosolic state (low ratio NADH/NAD+) which may point to a role of oxidative stress during the differentiation of HMSCs in neuroglial-like cells, however further investigation is needed to prove this suggestion. Our results show clear evidences that HMSCs expansion is dependent of glycolysis while their differentiation in neuroglial-like cells requires the switch of the metabolic profile to oxidative metabolism. Also important may be the role of oxidative stress during this process.

Here we also tested the efficacy of our combined biomaterial and cellular system approach in the in vivo treatment of sciatic nerve crush injury. Following transection, axons show staggered regeneration and may take substantial time to actually cross the injury site and enter the distal nerve stump (Brushart et al., 2002) Although delayed axonal elongation might be caused by growth inhibition originating from the distal nerve itself, growth-stimulating influences may overcome axons stagger. More robust and fast nerve regeneration is expected to result in better reinnervation and functional recovery. As a potential source of growth promoting signals, HMSCs transplantation is expected to have a positive outcome. Our results showed that the use of either undifferentiated or differentiated HMSCs enhanced the recovery of sensory and motor function. The observation that in both cell-enriched experimental groups myelin sheath was thicker, suggest that HMSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration (Geuna et al., 2009).

5. CONCLUSION

We conclude that HMSCs isolated from the Wharton's jelly of the UC delivered through PLC membranes might thus be regarded a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, such as digital nerves. In addition, these cells represent a non controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses, including nerve injuries like axonotmesis and neurotmesis.

6. Tables and figures captions

Table 1 - Values in seconds (s) were obtained performing Withdrawal Reflex Latency (WRL) test to evaluate the nociceptive function. This test has been performed pre-operatively (week- 0), and every week after the surgical procedure until week-12. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group (N = 6).

Table 2 - Values of Motor Deficit were obtained performing Extensor Postural Thrust (EPT) test. This test has been performed preoperatively (week-0), and every week after the surgical procedure until week-12. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group (N = 6).

Table 3 - Sciatic Function Index (SFI) measured pre-operatively (week-0), and every week after the surgical procedure until week-12. An index score of 0 is considered normal and an index of 100 indicates total impairment. The measurements of the print length (PL), the toe spread (TS), and the intermediary toe spread (ITS), were taken from the experimental (E) and normal (N) sides. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group (N = 6).

Table 4 - Histomorphometrical assessment of normal (Group 1 - Control) and regenerated sciatic nerves submitted to a standardized sciatic nerve crush injury with non-serrated clamp (week-12 post-traumatic). Results are presented as mean and standard deviation (SD). N corresponds to the number of rats within the experimental group (N = 6).

Figure 1 – HMSCs from Wharton's jelly exhibiting a mesenchymal-like shape with a flat polygonal morphology (A). After72h of culture in neurogenic medium the cells became exceedingly long and there is a formation of typical neuroglial-like cells with multibranches. (B) (Magnification: 100x).

Figure 2 – Undifferentiated HMSC cells from the Wharton's jelly presenting a negative staining for: (A) GFAP which is a glial cell marker; (C) GAP-43 which is related with axonal outgrowth and (E) NeuN which is a marker for nucleus of neurons. Neuroglial-like cells obtained from HMSCs in vitro differentiated with neurogenic medium exhibiting a positive staining for: (B) GFAP; (D) GAP-43 and (F) NeuN (Magnification: 200x).

Figure 3 – Histological appearance of the regenerated nerve fibers in the different experimental groups: Control (A), Crush (B), CrushPLC (C), CrushCell (D), CrushCellNonDifPLC (E), CrushCellDifPLC (F) (Magnification: 1,000x).

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ETHICAL APPROVAL: This work is original in that it has not been published before or submitted for publication elsewhere, and will not be submitted elsewhere before a decision has been taken as to its acceptability in this Journal where you are Editor. Each author meets the criteria for authorship and assumes the corresponding responsibility. In this study, laboratory animals were used. All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance

with the European Communities Council Directive of November 1986 (86/609/EEC), and the NIH guidelines for the care and use of laboratory animals have been observed.

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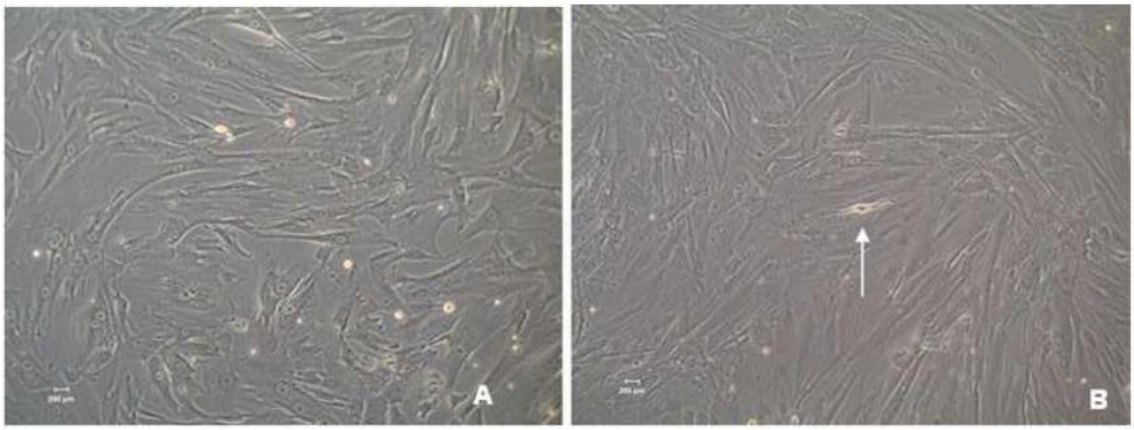

Figure 1

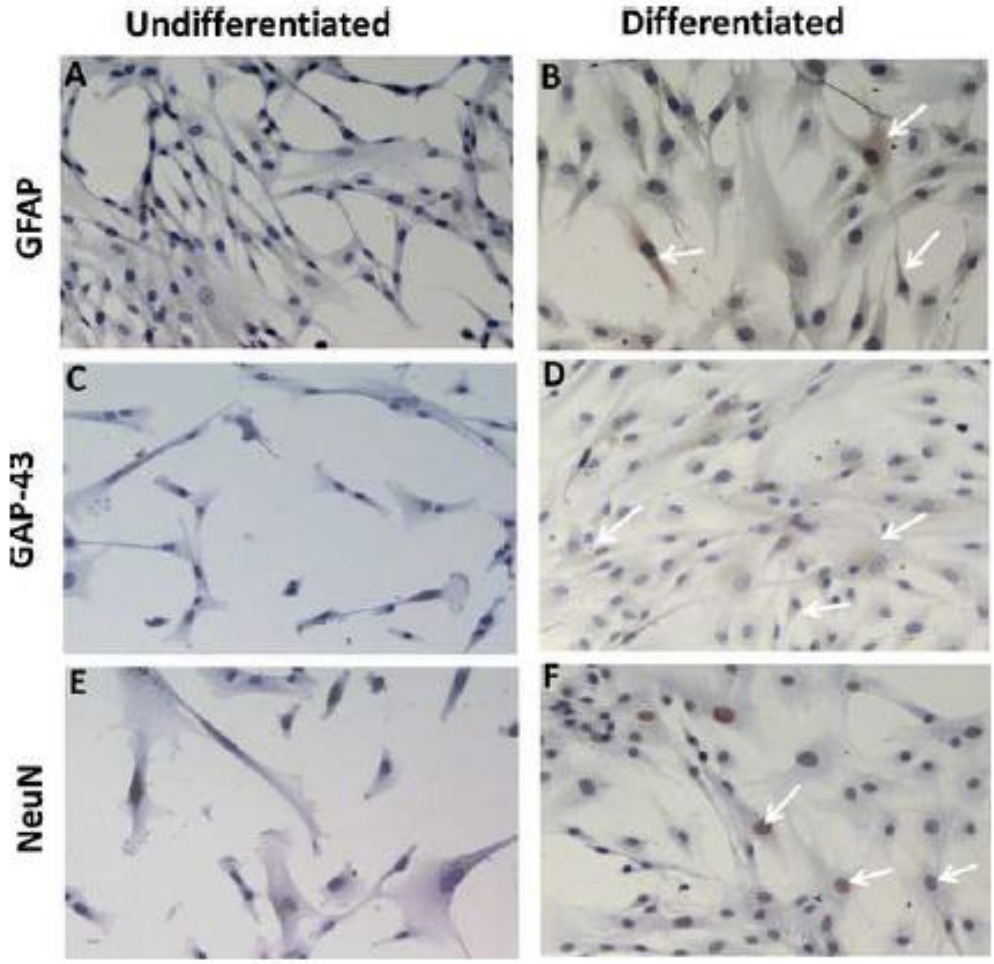

Figure 2

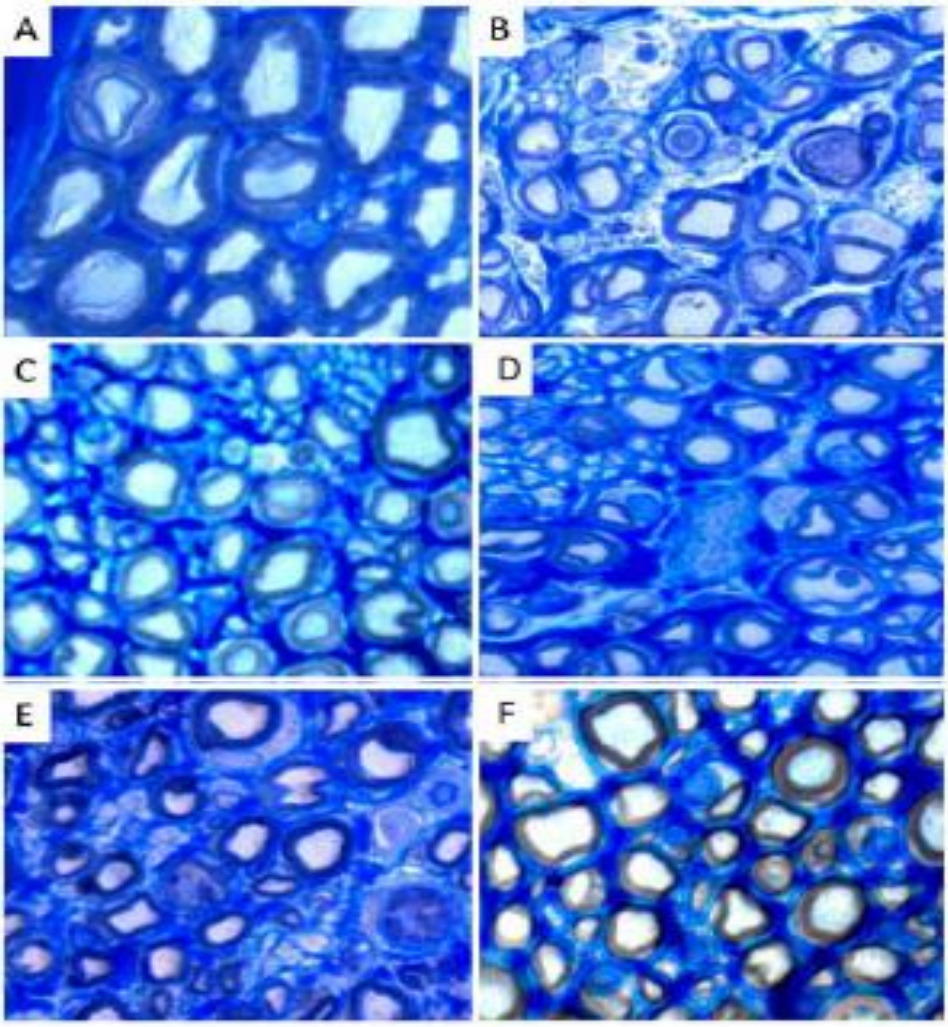

Figure 3

	Week 0 Mean ±SEM	Week 1 Mean ±SEM	Week 2 Mean ±SEM	Week 3 Mean ±SEM	Week 4 Mean ±SEM	Week 5 Mean ±SEM	Week 6 Mean ±SEM	Week 7 Mean ±SEM	Week 8 Mean ±SEM	Week 10 Mean ±SEM	Week 12 Mean ±SEM
Controlo	3.00 ±0.63	3.00 ±0.63	2.83 ±0.41	2.67 ±0.52	3.33 ±0.52	3.33 ±0.52	3.00 ±0.00	3.17 ±0.98	3.17 ±0.41	2.50 ±0.55	2.83 ±0.41
Crush	1.67 ±0.52	8.83 ±4.22	6.83 ±4.31	4.83 ±3.25	4.50 ±3.02	2.67 ±0.82	2.33 ±0.52	2.33 ±0.52	2.33 ±0.82	2.17 ±0.41	2.00 ±0.00
CrushPLC	3.22 ±0.87	11.16 ±0.90	9.46 ±1.18	7.83 ±1.41	6.83 ±1.09	5.54 ±0.51	4.80 ±0.32	4.45 ±0.26	4.20 ±0.50	3.55 ±0.29	3.04 ±0.48
CrushCell	1.43 ±0.53	10.86 ±1.68	9.29 ±2.29	7.43 ±1.81	5.43 ±2.15	3.43 ±0.79	2.57 ±0.53	2.14 ±0.38	1.86 ±0.38	1.57 ±0.53	1.29 ±0.49
CrushCell NonDiffPLC	3.70 ±1,28	12.00 ±0.00	10.83 ±2.09	9.45 ±2.63	7.15 ±2.99	6.41 ±2.91	5.56 ±1.57	4.90 ±0.85	4.42 ±1.30	3.48 ±0.82	3.01 ±0.59
CrushCell DiffPLC	3.68 ±1.04	12.00 ±0.00	11.80 ±0.52	9.93 ±2.06	8.60 ±1.37	7.34 ±0.50	5.66 ±0.98	5.09 ±0.75	4.69 ±0.62	3.90 ±0.63	3.01 ±0.59

Table 1

Group 1 – Control (without any sciatic nerve injury), n=6

Group 2 – Crush (axonotmesis injury without any other intervention), n=6

Group 3 – CrushPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane), n=7

Group 4 – CrushCell (axonotmesis lesion infiltrated with a suspension of 1250-1500 HMSCs, n=7

Group 5 – CrushCellNonDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with a monolayer of nondifferentiated HMSCs), n=6

Group 6 – CrushCellDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with differentiated HMSCs), n=7

	Week 0 Mean ±SEM	Week 1 Mean ±SEM	Week 2 Mean ±SEM	Week 3 Mean ±SEM	Week 4 Mean ±SEM	Week 5 Mean ±SEM	Week 6 Mean ±SEM	Week 7 Mean ±SEM	Week 8 Mean ±SEM	Week 10 Mean ±SEM	Week 12 Mean ±SEM
Controlo	0.01 ±0.04	0.01 ±0.06	0.00 ±0.03	-0.01 ±0.04	-0.01 ±0.03	0.02 ±0.06	0.03 ±0.03	0.02 ±0.03	0.00 ±0.03	-0.02 ±0.07	0.00 ±0.02
Crush	0.05 ±0.06	0.84 ±0.02	0.82 ±0.05	0.71 ±0.05	0.54 ±0.06	0.42 ±0.10	0.36 ±0.07	0.15 ±0.08	0.10 ±0.13	0.02 ±0.11	0.00 ±0.06
CrushPLC	0.00 ±0.17	0.84 ±0.13	0.84 ±0.14	0.75 ±0.16	0.66 ±0.15	0.52 ±0.17	0.38 ±0.11	0.32 ±0.06	0.25 ±0.05	0.16 ±0.04	0.07 ±0.09
CrushCell	0.01 ±0.01	0.91 ±0.01	0.84 ±0.02	0.72 ±0.02	0.54 ±0.04	0.47 ±0.03	0.35 ±0.03	0.26 ±0.02	0.15 ±0.03	0.07 ±0.01	0.03 ±0.01
CrushCell NonDiffPLC	0.05 ±0.12	1.00 ±0.00	0.94 ±0.02	0.86 ±0.05	0.73 ±0.19	0.45 ±0.27	0.16 ±0.12	0.18 ±0.13	0.10 ±0.08	0.04 ±0.09	0.01 ±0.12
CrushCell DiffPLC	0.05 ±0.22	1.00 ±0.00	0.94 ±0.05	0.84 ±0.07	0.65 ±0.16	0.40 ±0.23	0.20 ±0.12	0.11 ±0.09	0.05 ±0.05	0.06 ±0.07	0.02 ±0.06

Table 2

Group 1 – Control (without any sciatic nerve injury), n=6

Group 2 – Crush (axonotmesis injury without any other intervention), n=6

Group 3 – CrushPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane), n=7

Group 4 – CrushCell (axonotmesis lesion infiltrated with a suspension of 1250-1500 HMSCs, n=7

Group 5 – CrushCellNonDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with a monolayer of nondifferentiated HMSCs), n=6

Group 6 – CrushCellDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with differentiated HMSCs), n=7

	Week 0 Mean ±SEM	Week 1 Mean ±SEM	Week 2 Mean ±SEM	Week 3 Mean ±SEM	Week 4 Mean ±SEM	Week 5 Mean ±SEM	Week 6 Mean ±SEM	Week 7 Mean ±SEM	Week 8 Mean ±SEM	Week 10 Mean ±SEM	Week 12 Mean ±SEM
Controlo	-12.43 ±2.13	-9.17 ±2.04	-8.59 ±2.85	-8.54 ±2.01	-8.06 ±2.52	-9.92 ±4.18	-7.59 ±3.74	-7.16 ±3.11	-7.84 ±2.35	-10.03 ±1.17	-8.80 ±0.97
Crush	-13.15 ±7.72	-69.80 ±14.27	-71.00 ±10.78	-39.85 ±13.09	-28.88 ±10.91	-28.95 ±15.55	-26.13 ±6.31	-13.67 ±6.48	-10.62 ±7.96	-6.72 ±5.48	-5.33 ±4.30
CrushPLC	-2.50 ±18.55	-85.29 ±9.53	-69.25 ±10.17	-59.62 ±14.80	-44.89 ±6.62	-28.16 ±6.44	-22.83 ±6.79	-19.39 ±6.98	-14.72 ±7.36	-10.27 ±4.26	-7.93 ±2.78
CrushCell	-5.48 ±3.40	-92.88 ±9.55	-85.20 ±10.56	-53.92 ±11.22	-42.92 ±6.99	-38.88 ±6.65	-32.34 ±8.41	-28.29 ±7.64	-22.19 ±4.69	-16.33 ±2.47	-9.14 ±3.67
CrushCell NonDiffPLC	-1.69 ±20.41	-81.08 ±5.40	-83.79 ±5.45	-62.74 ±17.39	-26.63 ±6.74	-23.25 ±6.31	-13.58 ±17.49	-11.30 ±16.25	-19.62 ±14.35	-10.02 ±5.02	-5.47 ±2.94
CrushCell DiffPLC	-12.48 ±6.79	-76.94 ±6.68	-74.16 ±7.46	-56.42 ±16.18	-34.44 ±5.59	-27.08 ±3.05	-26.61 ±4.50	-24.09 ±8.12	-18.19 ±8.35	-10.85 ±3.30	-8.56 ±4.87

Table 3

Group 1 – Control (without any sciatic nerve injury), n=6

Group 2 – Crush (axonotmesis injury without any other intervention), n=6

Group 3 – CrushPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane), n=7

Group 4 – CrushCell (axonotmesis lesion infiltrated with a suspension of 1250-1500 HMSCs, n=7

Group 5 – CrushCellNonDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with a monolayer of nondifferentiated HMSCs), n=6

Group 6 – CrushCellDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with differentiated HMSCs), n=7

Group	Fiber Density (N /mm2)	Fiber Number (N)	Fiber diameter (µm)	Axon diameter (µm)	Myelin thickness (µm)
Control	15,905±287	7,666±190	6.66±0.12	4.26±0.07	1.19±0.03
Crush	20,109±1,232	10,644±423	4.99±0.19	3.48±0.10	0.76±0.05
CrushPLC	23,349±4,278	11,532±1,086	4.55±0.36	3.36±0.17	0.60±0.10
CrushCell	20,200±4,971	9,806±2,695	5.31±0.69	3.74±0.49	0.78±0.10
CrushCellNondiffPLC	20,945±1,162	10,909±1,584	5.11±0.42	3.39±0.25	0.86±0.11
CrushCellDiffPLC	23,593±5,611	9,640±1,784	4.93±0.43	3.25±0.37	0.84±0.05

Table 4

Group 1 – Control (without any sciatic nerve injury), n=6

Group 2 – Crush (axonotmesis injury without any other intervention), n=6

Group 3 – CrushPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane), n=7

Group 4 – CrushCell (axonotmesis lesion infiltrated with a suspension of 1250-1500 HMSCs, n=7

Group 5 – CrushCellNonDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with a monolayer of nondifferentiated HMSCs), n=6

Group 6 – CrushCellDifPLC (axonotmesis lesion of 3 mm enwrapped with a PLC membrane covered with differentiated HMSCs), n=7

5.

Chapter Number

Mesenchymal stem cells from extra-embryonic tissues for tissue engineering – Regeneration of the Peripheral Nerve

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1. INTRODUCTION

Recent advances in Regenerative Biology and Regenerative Medicine are impressive and in the last years the scientific community has witnessed the emergence of many new concepts and discoveries. Until a few years ago, biological tissues were regarded as unable of extensive regeneration, but nowadays organs and tissues like the brain, spinal cord or cardiac muscles appear as capable to be reconstructed, based on “stem cells” [1].

Stem cell research has sparked an international effort due to the variety of possible uses in clinical procedures to treat diseases and improve health and life expectancy. Stem cell research has crossed a century journey and has evolved greatly even in its own definition. In 1967, Lajtha defined that in the adult stem cells could only be found in regenerative organs, such as blood, intestine, cartilage, bone and skin. Nowadays, these cells are considered to exist even in tissues with no commitment to regeneration such as the central nervous system [1, 2].

2. STEM CELLS

Stem cells are undifferentiated cells, with endless self-renewal sustained proliferation *in vitro* and multilineage differentiation capacity [3]. This *in vitro* multilineage differentiation

capacity has targeted these cells with extreme importance for use in tissue and cell-based therapies.

The first stem cell appearance is in the early zygotic cells, which are totipotent and give rise to the blastocyst. They are capable to differentiate into all cell and tissue types. With differentiation, cells become less capable of self-renewal and differentiation in other cell type becomes more limited [1]. Stem cells can be loosely classified into 3 broad categories based on their growth behavior and isolation time during ontogenesis: embryonic, fetal and adult.

Embryonic stem cells (ESCs) were first observed in a pre-implantation embryo by Bongso and colleagues in 1994 [4]. Since then, many cell lines and a multiplicity of tissues have been successfully derived from ESCs and tested in several animal disease models [5-7]. Nevertheless, post-transplantation immune-rejection has been a major problem. Many studies are being conducted to avoid this major issue. This could be resolved by personalizing tissues through somatic nuclear transfer (NT) or induced pluripotent stem cells (iPSC) techniques [8], but the teratoma development in animals is still a concern and a serious problem [9]. In order to overcome the limitations placed by ESCs and iPSCs, a variety of adult stem cell populations has been recently isolated and characterized for their potential clinical use. While still multipotent, adult stem cells have long been considered restricted, giving rise only to progeny of their resident tissues [9]. *In vivo,* adult stem cells exist in a quiescent state, located in almost all tissues, until mediators activate them to restore and repair injured tissues. These cells are surrounded by mature cells that have reached the end line in terms of differentiation and proliferation [10]. Stem cell research focuses on the development of cell and tissue differentiation, so as characterization techniques, for tissue and cell identification with marker patterns. Such protocols are essential for regenerative therapies [11].

2.1. MESENCHYMAL STEM CELLS

The development of cell-based therapies for cartilage [12] and skin [13] reconstruction marks the beginning of a new age in tissue regeneration. Mesenchymal stem cells (MSCs) have become one of the most interesting targets for tissue regeneration due to their high plasticity, proliferative and differentiation capacity together with their attractive immunosuppressive properties. MSCs present low immunogenicity and high immunosuppressive properties due to a decreased or even absence of Human Leucocyte Antigen (HLA) class II expression [14]. Research in this field has brought exciting promises in many disorders and therefore in tissue regeneration. Currently the

differentiation potential of MSCs in multilineage end-stage cells is already proven, and their potential for treatment of cardiovascular [15], neurological [16], musculoskeletal [17, 18], and cutaneous [19] diseases is now well established. Fibroblast colony-forming units or marrow stromal cells, currently named MSCs, were first isolated in 1968 from rat bone marrow [20]. These cells were clonogenic, formed colonies when cultured, and were able to differentiate *in vitro* into bone, cartilage, adipose tissue, tendon, muscle and fibrous tissue. Since then many other tissues have been used to isolate these cells. MSCs can be obtained from many different tissues, including bone marrow, adipose tissue, skeletal muscle, umbilical cord matrix and blood, placental tissue, amniotic fluid, synovial membranes, dental pulp, fetal blood, liver, and lung [21]. The concept of MSCs is based on their ability to differentiate into a variety of mesodermal tissues and was first proposed by Caplan in 1991 [22] and further validated by additional research in 1999 [23]. Due to the many different methods and approaches used for MSCs culture, the Mesenchymal and Tissue Stem Cell Committee, of the International Society for Cellular Therapy (ISCT), recommended several standards do define MSCs [24]. Therefore, MSCs are defined as presenting: i) plastic adherent ability; ii) absence of definitive hematopoietic lineage markers, such as CD45, CD34, CD14, CD11b, CD79α, CD19 and class-II Major Histocompatibility Complex (MHC) molecules, specially HLA-DR; and expression of nonspecific markers CD105, CD90 and CD73 iii) ability to differentiate into mesodermal lineage cells, osteocytes, chondrocytes and adipocytes. Along with mesodermal differentiation, it has been demonstrated the capacity of MSCs to differentiate into ectodermal cell lines, as neurons [25, 26], keratocytes [27] and keratinocytes [28], so as endodermal cell line, like hepatocytes [29, 30] and pancreatic β-cells [31]. Moreover, they also possess anti-inflammatory and immunomodulation properties and trophic effects [32, 33]. Increasing evidence now demonstrates that the therapeutic effects of MSCs do not lay only on the ability to repair damage tissue, but also on the capacity of modulating surrounding environment, by secretion of multiple factors and activation of endogenous progenitor cells [34, 35]. Compared with ESCs and other tissue specific stem cells, MSCs are more advantageous. Moreover some studies have demonstrated that MSCs have a higher chromosomal stability and lower tendency to form tumors and teratomas, compared to other stem cells [36, 37].

Although they present similar biological characteristics, it cannot be ignored the existing of some disparities, as differences in, expansion potential under same culture conditions and age-related functional properties [38]. Compared to ESCs, MSCs isolated from the umbilical cord matrix (Wharton's jelly) have many advantages, such as shorter population doubling time, easy culture in plastic flasks, good tolerance by

the immune system, so that transplantation into non-immunesuppressed animals does not induce acute rejection, anticancer properties, [9] and most important lack of tumorigenic activity. As well as ESCs, these cells are originated from the inner cell mass of the blastocyst but with a major difference: they do not raise ethical controversies, since they are collected from tissues usually discarded at birth [39].

2.1.1. MSCs SOURCES AND VALIDATION OF TRANSPORT AND PROCESSING PROTOCOLS

Bone marrow, adipose tissue, umbilical cord blood and umbilical cord matrix have been considered the main sources of MSCs for tissue engineering purposes. Among these sources, bone marrow represents the main source of MSCs for cell therapy. However, the proliferative capacity [40-43], differentiation potential and clonal expandability [44] of MSCs derived from bone marrow decrease significantly with age, gender and seeding density, and the number of cells per marrow aspirate is usually quite low [3, 45]. It is still a mystery if MSCs ageing is due to factors intrinsic or extrinsic to the cells. Many possible reasons have been described in an attempt to explain MSCs ageing. Possible extrinsic factors include: reduced synthesis of proteoglycans and glycosaminoglycans reducing proliferation and viability [46], and production of glycosylated end products, inducing apoptosis and reactive oxygen species [47]. Intrinsic factors causing MSCs ageing might include: cell senescence-associated *β*-galactosidase and higher expression of p53 and pathway genes p21 and BAX, resulting in blunted proliferation potential [43]. Regarding seeding density, many authors suggest that lower seeding densities induce faster proliferation rates [48, 49]. This has been explained by contact inhibition in higher seeding densities [49], and higher nutrient availability per cell in lower seeding densities [49]. Use of bone marrow MSCs has disadvantages; donors are submitted to invasive harvest of bone marrow. This raises the need to find alternative sources of MSCs for autologous and allogenic use. Candidate tissue sources should provide MSCs displaying high proliferative and differentiation potency [50].

Extra-embryonic tissues are a good alternative to adult donor. This tissues, such as, amnion, microvillus, Wharton's jelly and umbilical cord perivascular cells, are routinely discarded at child-birth, so little ethical and religious controversy attends the harvesting of the resident stem cell populations. The comparatively large volume of extra-embryonic tissues increases the chance of isolating suitable amounts of stem cells, despite the complex and expensive procedures needed for their isolation. Some protocols use enzymatic digestion while others use enzyme-free tissue explant

methods that require longer culture time [51]. There are also MSCs in cord blood (CB), but many studies report low frequency of these cells and unsuccessful isolation. However, Zhang and colleagues were able to isolate MSCs from CB with a 90% successful rate when CB volume was ≥ 90ml and a transport time until storage was ≤ 2hours [51].

In recent years, MSCs derived from umbilical cord matrix, Wharton's jelly has attracted much interest. Wharton's jelly is a mature mucous tissue and the main component of the umbilical cord, connecting the umbilical vessels to the amniotic epithelium. Umbilical cord derives from extra-embryonic or embryonic mesoderm; at birth it weights about 40g and measures approximately 30-65 cm in length and 1.5cm in width [52]. Anyway, individual differences are observed within newborn babies. Fong and colleagues characterized Wharton's Jelly stem cells and found the presence of both embryonic and MSCs, targeting this source as unique and of valuable use for clinical applications. MSCs from the Wharton's jelly can be cultured with little or even no major loss trough at least 50 passages [53].

CB and more recently, umbilical cord tissue (UCT) have been stored cryopreserved in private and public cord blood and tissue banks worldwide in order to obtain hematopoietic and MSCs and, although guidelines exist (Netcord – Foundation for the Accreditation of Cellular Therapy), standardized procedures for CB and UCT transport from the hospital / clinical to the laboratory, storage, processing, cryopreservation and thawing are still awaited. These may be critical in order to obtain higher viable stem cells number after thawing and limit microbiological contamination.

Our research group focused in determining whether UCT storage and transport from the hospital / clinical to the laboratory at room temperature (RT) or refrigerated (4-6ºC) and immersed in several sterile saline solutions affects the UCT integrity in order to be cryopreserved. The umbilical cord contains two arteries and one vein, which are surrounded by mucoid connective tissue, and this is called the Wharton's jelly. The cord is covered by an epithelium derived from the enveloping amnion. The interlaced collagen fibers and small, woven bundles are arranged to form a continuous soft skeleton that encases the umbilical vessels. In the Wharton's jelly, the most abundant glycosaminoglycan is hyaluronic acid, which forms a hydrated gel around the fibroblasts and collagen fibrils and maintains the tissue architecture of the umbilical cord by protecting it from pressure [54].

One centimeter-long fragments of umbilical cords (N = 12) were collected from healthy donors after written informed consent and following validated procedures according to the clinical and technical guidelines of the Private Bank Biosckin, Molecular and Cell Therapies, SA (authorized for processing and cryopreserving CB and UCT units by the

Portuguese Minister of Health, ASST – Autoridade para os Serviços de Sangue e de Transplantação). The 1 cm fragments were immersed for 168 hours in 4 different sterile saline solutions at RT (22-24ºC) and refrigerated (4-6ºC): NaCl 0.9% (Labesfal, Portugal), AOSEPT®-PLUS (Ciba Vision, Portugal), Dulbecco's Phosphate-Buffered Saline without calcium, magnesium and phenol red (DPBS, Gibco, Invitrogen, Portugal) and Hank's Balanced Salt Solution (HBSS, Gibco, Invitrogen, Portugal). The preservative-free, aqueous AOSEPT® PLUS solution contains hydrogen peroxide 3%, phosphonic acid (stabiliser), sodium chloride, phosphate (buffer system), and poloxamer (surfactant), and is usually used to transport and wash contact lenses. After 168 hours, the fragments were collected in 4% of paraformaldehyde and processed for light microscopy. The samples were fixed in 4% paraformaldehyde for 4 hours and then washed and conserved in phosphate buffer saline (PBS) until embedding. The specimens were dehydrated and embedded in paraffin and cut at 10 μm perpendicular to the main umbilical cord axis. For light microscope analysis, sections were stained with haematoxylin and eosin (HE) and observed with a Leica DM400 microscope equipped with a Leica DFC320 digital camera. The UCT integrity was evaluated through the following parameters: i) detachment of vessels and retraction of vascular structures; ii) loss of detail and integrity of the endothelium; iii) connective tissue degradation; iv) autolysis of fat (impossible to assess, due to histological technique); and v) loss of detail and integrity of the mesothelium. It was concluded that the best transport solutions were HBSS or DPBS at a temperature of 4-6ºC since those maintained the histological structure of UC evaluated through those 5 parameters previously referred (Figure 1 and Figure 2).

[Figure 1]

[Figure 2]

As a matter of fact, the UC immersed for 168 hours in DPBS and HBSS at refrigerated temperature presented integrity of the histological structure comparable to a UC collected and processed for histological analysis immediately after birth (Figure 3). With DPBS, a slight retraction of the vessels was noted, which is advantageous since the vessels are stripped and discarded before cryopreservation of the UCT. It was concluded that the transport of the UC from the hospital / clinic to the cryopreservation laboratory should be performed with the UC immersed in DPBS or HBSS at refrigerated temperatures.

[Figure 3]

The isolation and culture of MSCs from the Wharton's jelly has been performed by our research group in order to obtain undifferentiated MSCs and *in vitro* differentiated into neural-like cells to be tested in axonotmesis and neurotmesis lesions of the rat sciatic nerve. The isolation has been performed by enzyme-free tissue explant and enzymatic isolation. Despite our standard approaches, we are aware that there are still significant variations that exist between laboratories' protocols, which must be taken into account when comparing results using other methodologies. There is a wide range of individual differences among donor tissues also and our protocols usually use 15 - 20 cm of UC. While most UC samples will provide a reasonable number of MSCs using the provided protocols, some samples may result in sub-optimal cell isolation and expansion. The reasons behind this phenomenon still remain to be clarified, but as we have previously mentioned, the temperature and the time of transport from the hospital / clinic to the cryopreservation laboratory is crucial.

Irrespective of the specific protocol, the washing procedure of the umbilical cord fragments is crucial in order to avoid microbiological contamination of the cultures. After obtaining the written informed consent from the parents, fresh human umbilical cords are obtained after birth and collected in HBSS or DPBS at 4-6ºC, as it was previously described. After washing the umbilical cord unit 4 times in rising DPBS, disinfection is performed in 75% ethanol for 30 seconds. Finally, and before the dissection step, umbilical cord unit is washed in DPBS. The vessels are usually stripped with UC unit still immersed in DPBS. Once washing step in MSCs isolation and culture is essential to achieve good UCT units for cryopreservation and future clinical use, washing protocol was validated. DPBS from the first washing step (used immediately after collection for transportation of the unit to the laboratory – *washing step 1 solution*) and DPBS used in washing step after disinfection in 75% ethanol (*washing step 6 solution*) from 14 umbilical cord units (N = 14) collected from healthy donors and transported from the hospital/clinical at 4-6ºC in less than 72hours were tested for microbiological contamination using BacT/ALERT® (bioMérieux). Each unit was tested for aerobic and anaerobic microorganisms and fungi using 10 ml of the *washing step 1 solution* and *washing step 6 solution* which were aseptically introduced into the BacT/ALERT® testing flasks. All procedures were performed in a laminar flow tissue culture hood under sterile conditions. All the units that presented microbial contamination in DPBS obtained from the first washing step (*washing step 1 solution*) presented no contamination in the analysis performed to DPBS from the last washing step immediately performed before MSCs isolation or UCT cryopreservation (*washing*

step 6 solution). The following microorganisms were identified in the DPBS solution from the first washing step: *Staphylococcus lugdunensis* (N = 2); *Staphylococcus epidermidis* (N = 1); *Staphylococcus coagulase* (N = 2); *Escherichia coli* (N = 4); *Enterococcus faecalis* (N = 1); and *Streptococcus sanguinis* (N = 1). The DPBS solution from the first washing step (*washing step 1 solution*) from 3 units was negative for microbial contamination (N = 3). These results permitted us to conclude that the washing protocol was 100% efficient in what concerns microbiological elimination (including aerobic and anaerobic bacteria, yeast and fungi).

Once the transport and washing protocols were validated, it was important to isolate and expand *in vitro* the MSCs from the UCT units for pre-clinical trials.

In the "enzymatic procedure" we use collagenase type I (Sigma-Aldrich). With the written informed consent from the parents, fresh human umbilical cords were obtained after birth and stored in HBSS (Gibco, Invitrogen, Portugal) for 1–48 hours before tissue processing to obtain MSCs. After removal of blood vessels, the mesenchymal tissue is scraped off from the Wharton's jelly with a scalpel and centrifuged at 250 g for 5 minutes at room temperature and the pellet is washed with serum-free Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Portugal). Next, the cells are centrifuged at 250 g for 5 minutes at room temperature and then treated with collagenase (2 mg/ml) for 16 hours at 37ºC,washed, and treated with 2.5% trypsin-EDTA solution (Sigma-Aldrich) for 30 minutes at 37ºC with agitation. Finally, the cells are washed and cultured in DMEM (Gibco, Invitrogen, Portugal) supplemented with 10% fetal bovine serum (FBS), glucose (4.5 g/l), 1% (w/v) penicillin and streptomycin (Sigma), and 2.5 mg/ml amphotericin B (Sigma) in 5% CO_2 in a 37ºC incubator (Nuaire). Around 2×10^5 cells are plated into each T75 flask in 10 ml culture medium. Cells are allowed to attach and grow for 3 days. To remove the non-adherent cells or fragments, the flasks are gently washed using pre-warmed DPBS after which 10 ml of pre-warmed culture medium is added. The culture medium is changed every third day (or twice per week). Confluence (80-90%) is normally reached at day 12–16, and the cells are removed with pre-warmed trypsin-EDTA solution (4 ml per flask), for 10 min at 37°C. The cells are plated onto poly-l-lysine coated glass coverslips (in 6- or 24-well tissue culture plates) or on biomaterials used in the nerve reconstruction. Normally, 5000 cells/cm^2 are plated on the coverslips or on the membranes (Figure 4).

[Figure 4]

In our "enzyme-free tissue explant protocol" for isolation of MSCs, enzymatic digestion is not employed. The mesenchymal tissue (Wharton's jelly) is diced into cubes of about

0.5 cm^3 and the remaining vessels are removed by dissection. Using a sterile scalp, the cubes are diced in 1-2 mm fragments and transferred to a Petri dish pre-coated with poly-l-lysine (Sigma) with Mesenchymal Stem Cell Medium (PromoCell, C-28010) supplemented with 1% (w/v) penicillin and streptomycin (Sigma), and 2.5 mg/ml amphotericin B (Sigma) and cultured in 5% CO_2 in a 37ºC incubator (Nuaire). Some tissue fragments will allow cell migration from the explants in 3 - 4 days incubation. Confluence is normally obtained 15-21 days after.

The laboratory's processing and cryopreservation protocols of the UCT units following the technical procedures of Biosckin, Molecular and Cell Therapies S.A. (BSK.LCV.PT.7) were validated for the ability of isolating and expanding *in vitro* MSCs after cryopreserved UCT thawing. The protocols of processing and cryopreservation of the UCT are protected by a Confidentiality Agreement between Biosckin, Molecular and Cell Therapies S.A. and all the involved researchers. Briefly, the UCT collected from healthy donors (N = 60), and according to Netcord guidelines and following the Portuguese law 12/2009 (*Diário da República, lei 12/2009 de 26 de Março de 2009*) is diced into cubes of about 0.5 cm^3 and the remaining vessels are removed by dissection. In order to ensure the viability of the UCT after parturition and limit the microbiological contamination of the samples, the umbilical cords were transported from the hospital / clinic to the laboratory at refrigerated temperatures monitored by a datalloger in less than 72 hours. The UCT units from 15-20 centimeters-long umbilical cords and after the blood vessels dissection are treated and processed for cryopreservation using a cryoprotective solution (freezing medium). The UCT units are transferred to a computer-controlled slow rate freezer (Sylab, Consensus, Portugal) and a nine-step freezing program is used to set up the time, temperature, and rates specifically optimized for the human umbilical cord-MSCs cooling. To thaw frozen cells, the cryovials are transferred directly to a 37°C water bath. Upon thawing in less than a minute, the cell suspension is centrifuged at 150 × g for 10 min, and the supernatant is gently removed and the cell pellet is resuspended in culture medium. In some UCT cryopreserved units it was not possible to isolate MSCs due to increase number of erythrocytes' lysis or microbiological contamination during cell culture. The MSCs morphology was observed in an inverted microscope (Zeiss, Germany) at different points of expansion. These results permitted to conclude that the processing and cooling protocols used for UCT units' cryopreservation were adequate to preserve the UCT viability since it was possible to isolate and expand MSCs after appropriate thaw and in presence of adequate cell culture conditions.

An established and ready-to-use Human MSC cell line was also employed for promoting axonotmesis and neurotmesis lesions regeneration. Human MSCs from

Wharton's jelly umbilical cord were purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). Cryopreservated cells are cultured and maintained in a humidified atmosphere with 5% CO_2 at 37ºC. Mesenchymal Stem Cell Medium (PromoCell, C-28010) is replaced every 48 hours. At 80-90% confluence, cells are harvested with 0.25% trypsin with EDTA (Gibco) and passed into a new flask for further expansion. MSCs at a concentration of 2500 cells/ml are cultured on poli-D-lysine coverslips (Sigma) or on biomaterials membranes and after 24 hours cells exhibit 30-40% confluence. Differentiation into neuroglial-like cells is induced with MSC neurogenic medium (Promocell, C-28015). Medium is normally replaced every 24 hours during 3 days. The formation of neuroglial-like cells can be observed after 24 hours in an inverted microscope (Zeiss, Germany) (Figure 5 and Figure 6).

[Figure 5]

[Figure 6]

This established human MSC cell line is preferred for *in vivo* testing in rats, since the number of MSCs obtained is higher in a shorter culture time, it is not dependent on donors availability and ethic committee authorization, and the protocol is much less time consuming which is advantageous for pre-clinical trials with a large number of experimental animals. As a matter of fact, there is no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period after the surgical procedure. The phenotype of MSCs was assessed by PromoCell. Rigid quality control tests are performed for each lot of PromoCell MSCs isolated from Wharton's jelly of umbilical cord. MSCs are tested for cell morphology, adherence rate and viability. Furthermore, each cell lot is characterized by flow cytometry analysis for a comprehensive panel of markers.

The MSCs isolated with the two protocols described (and from fresh and the cryopreserved UCT units) and from the established Promocell cell line exhibited a mesenchymal-like shape with a flat and polygonal morphology. During expansion the cells became long spindle-shaped and colonized the whole culturing surface. After 96 hours of culture in neurogenic medium, cells changed in morphology. The cells became exceedingly long and there was a formation of typical neuroglial-like cells with multi-branches and secondary branches. Giemsa-stained cells of differentiated MSC cell line at passage 5 were analyzed for cytogenetic characterization. However, no metaphases were found, therefore the karyotype could not be established. The karyotype of undifferentiated HMSCs was determined previously and no structural alterations were

found demonstrating absence of neoplasic characteristics in these cells, as well as chromosomal stability to the cell culture procedures [55, 56]. The differentiated MSCs karyotype could not be established, since no dividing cells were obtained at passage 5, which can be in agreement with the degree of differentiation. The karyotype analysis of undifferentiated MSCs previously determined, excluded the presence of neoplasic cells, thus supporting the suitability of our cell culture and differentiation procedures. This concern also resulted from our previous experience with N1E-115 neoplasic cell line and the negative results we obtained in the treatment of axonotmesis and neurotmesis injuries [57-59]. Nevertheless, undifferentiated MSCs from the Wharton's jelly culture (obtained from either protocol or from the Promocell cell line) showed normal morphology when inspected with an inverted microscope (Figure 7).

The differentiation was tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN by neural-like cells attained from HwMSCs. Undifferentiated MSCs were negatively labeled to GFAP, GAP-43 and NeuN. After 96 hours of differentiation the attained cells were positively stained for glial protein GFAP and for the growth-associated protein GAP-43. All nucleus of neural-like cells were also labeled with the neuron specific nuclear protein called NeuN showing that differentiation of MSCs in neural-like cells was successfully achieved for MSCs obtained from UCT (fresh and cryopreserved) and for the Promocell MSC cell line (Figure 8) [55].

2.1.2. DIFFERENTIATION INTO NEUROGLIAL-LIKE CELLS

MSCs express nestin, a maker for neural and other stem cells [60, 61] and can be differentiated in adipose tissue, bone, cartilage, skeletal muscle cells, cardiomyocyte-like cells, and neuroglial-like cells [54, 55, 60, 62], presenting great potential to biomedical engineering applications. These cells fit into the category of primitive stromal cells and because they are abundant and inexpensive, they might be very useful for regenerative medicine and biotechnology applications.

By employing neuron-conditioned media, sonic hedgehog and fibroblast growth factor 8, MSCs isolated from the Wharton's jelly can be induced toward dopaminergic neurons. These cells have been transplanted into hemiparkinsonian rats where they prevented the progressive degeneration/behavioral deterioration seen in these rats [63]. Rat MSCs isolated from the Wharton's jelly when transplanted into brains of rats with global cerebral ischemia significantly reduced neuronal loss, apparently due to a rescue phenomenon [64]. Neuronal differentiation of human MSCs could also provide cells to replace neurons lost due to neurodegenerative diseases. Recent studies

showed that transplanted MSCs-derived neurons become electrophysiologically integrated within the host neural tissue [65]. However, all these therapeutic applications need uniform and reproducible regulation.

A consequence of cell metabolism during *in vitro* expansion is that culture conditions are constantly changing. The comprehension and optimization of the expansion and differentiation process will contribute to maximization of cell yield, reduced need of cell culture, and a decrease in total processing costs [66, 67] Elucidation of regulatory mechanisms of MSCs differentiation will allow optimization of *in vitro* culture and their clinical use in the treatment of neural-related diseases. Research is being performed to optimize expansion process parameters in order to grow MSCs in a controlled, reproducible, and cost-effective way [68]. Metabolism is certainly one of these parameters.

3. REGENERATION AND IN VIVO TESTING

With the world wide global increase in life expectancy, a variety of disabling diseases with large impact on human population are arising. This includes cardiovascular, neurological, musculoskeletal, and malignancies. Therefore, it is imperative that new and more effective treatment methods are developed to correct for these changes. Further research with experimental animal systems is required to translate to in vivo cell-based therapy that has been extensively investigated in vitro [1]. Stem cell biology is probably the golden key for cell therapies and regenerative medicine. Regeneration is the physical process where remaining tissues organize themselves to replace missing or injured tissues *in vivo* [39].

It has been speculated that once MSCs have the potential to differentiate into several tissues, they might be responsible for turnover and maintenance of adult tissues, just like hematopoietic stem cells have this role in blood cells [69]. First, it was believed that after injection of MSCs to injured place cells would migrate to the damaged site and differentiate into ones with the appropriate function for repairing, so MSCs could mediate tissue repair through there multilineage capacity replacing damaged cells. Subsequent studies have suggested that the mechanism used by MSCs for tissue repairing is not really this way. This new idea was reinforced by the confirmation that this cells homed to damaged site, particularly to spots of hypoxia, inflammation and apoptosis [70, 71].

Recent studies demonstrated that transplanted MSCs modified the surrounding tissue microenvironment, promoting repair with functional improvement by secretion factors (known as paracrine effect), stimulation of preexisting stem cells in the original tissue

and decreasing of inflammation and immune response [72]. Other studies have demonstrated that MSC-conditioned media by itself could have therapeutic effects. All this data suggest that MSC apply a reparative effect on injured side through its paracrine effects [73].

It is necessary to overcome some barriers before a cell-based therapy becomes routine in clinics, including the cell number and the administration way of treatment. MSCs are difficult to be maintained stable in culture for long time, but due to their short doubling time, if at the outset many cells are harvest they may be properly scaled up in primary culture, never forgetting the ideal seeding number [39].

MSCs are an attractive candidate for cell-based regenerative therapy; the evidence is that currently there are 139 trial registries for MSC therapy 27 of which are based on umbilical cord MSCs [74].

3.1. NERVE REGENERATION

After Central Nervous System (CNS) lesions, Peripheral Nervous System (PNS) injuries are the ones with minor successes in terms of functional recovery. These kinds of injuries are frequent in clinical practice. About two centuries ago it was assumed that these nerves would never regenerate. Indeed, scientific and clinical knowledge greatly increased in this area. Nevertheless, a full understanding of axonal recovery and treatment of nerve defects, especially complete functional achievement and organ reinnervation after nerve injury, still remains the principle challenge of regenerative biology and medicine [75, 76].

3.1.1. Nerve repair

Many peripheral nerve injuries can only be dealt through reconstructive surgical procedures. Despite continuous refinement of microsurgery techniques, peripheral nerve repair still stands as one of the most challenging tasks in neurosurgery, as functional recovery is rarely satisfactory in these patients [76]. Direct repair should be the procedure of choice whenever tension-free suturing is possible; however, patients with loss of nerve tissue, resulting in a nerve gap, are considered for a nerve graft procedure. In these cases, the donor nerves used for grafting are commonly expendable sensory nerves. This technique, however, has some disadvantages, with the most prominent being donor site morbidity, that may lead to a secondary sensory deficit and occasionally neuroma and pain. In addition, no donor and recipient nerve diameters often occurs which might be the basis for poor functional recovery. Alternatives to peripheral nerve grafts include cadaver nerve segments allografts, end-to-side neurorrhaphy, and entubulation by means of autologous non-nervous tissues,

such as vein and muscles [76]. One advantage of these allografts compared with the autografts is the absence of donor site morbidity and theoretically the unlimited length of tissue available [77]. Experimental work from a number of laboratories has emphasized the importance of entubulation for peripheral nerve repair to manage nerve defects that cannot be bridged without tension (neurotmesis with loss of nerve tissue). Nerves will regenerate from the proximal nerve stump towards the distal one, whereas neuroma formation and ingrowth of fibrous tissue into the nerve gap are prevented. [78]. The reliability of animal models is crucial for PN research, including therapeutic strategies using biomaterials and cellular systems. As a matter of fact, rodents, particularly the rat and the mouse, have become the most frequently used animal models for the study of peripheral nerve regeneration because of the widespread availability of these animals as well as the distribution of their nerve trunks which is similar to humans [79]. Because of its PN size, the rat sciatic nerve has been the most commonly experimental model used in studies concerning the PN regeneration and possible therapeutic approaches [80]. Functional recovery after PN injury is frequently incomplete, even with adequate microsurgery, so, many research and clinical studies have been performed including biomaterials for tube-guides. Since the 80's, Food and Drug Administration (FDA) has approved a variety of these biomaterials both natural and synthetic. The ideal biomaterial nerve graft should increase number, length and speed of axon regeneration [77]. It should be: i) biocompatible, not toxic neither present undesired immunologic response; ii) permeable enough to permit nutrient and oxygen diffusion and allows cell support systems; iii) flexible and soft to avoid compression; iv) biodegradable, the ideal rate is to remain intact during axon regeneration across nerve gap and after degrade softly and v) technically reproducible, transparent, easy to manipulate, and sterilize [81]. Currently 3 types of materials are available for nerve reconstruction: non-resorbable, natural resorbable and synthetic resorbable. Polyvinil alcohol hydrogel (PVA) is an example of a non-absorbable biomaterial. It combines water in similar proportions to human tissue, with PVA providing a stable structure easy to sterilize, which is a main advantage of this materials, but has some limitations such as: nerve compression and suture tension after regeneration due to its non-resorbable nature [77]. Collagen type I from humans or animals, is an example of a natural resorbable device, which has some advantages such as: i) easy to isolate and purify, ii) good adhesiveness for supporting cell survival an proliferation, iii) has been proven to be highly biocompatible and support nerve regeneration *in vivo*. On the other hand, offers some immune response requiring the use of immunosuppressive drugs or pre-treatment of the material before clinical use [77]. Poly (DL-lactide-ε-caprolactone) (PLC) a synthetic resorbable material

is the only transparent device approved by FDA, important characteristic for the surgeon that facilitates the insertion of the nerve stumps across the nerve gap, but, on the other hand it is not flexible [77]. Chitosan, PLC, collagen, poly(L-lactide) and poly(glycolide) copolymers (PLGA) and others, some of them, previously studied by our group [57, 58, 82] were associated to cellular systems, which are able to differentiate into neuroglial-like cells or capable of modulating the inflammatory process, improved nerve regeneration, in terms of motor and sensory recovery, and also shortening the healing period after axonotmesis and neurotmesis, avoiding regional muscular atrophy [57, 58, 82].

Researches with acellular nerve allografts, as alternative for repairing peripheral nerve defects have been reported. These nerve allografts remove the immunoreactive SCs and myelin however preserve the internal structure of original nerve, containing vital components such as collagen I, laminin and growth factors essential for repairmen of the lesions [83]. Acellular grafts remain insufficient, due to the increasing extent of nerve damages. Also, viable cells are necessary for debris removal and environmental regeneration reestablishment [83].

Cell transplantation, such as Schwann cells (SCs) transplantation has been proposed as a method of improving peripheral nerve regeneration [84]. SCs are peripheral glial cells that enwrap axons to form myelin with a central role in neuronal function. When there is damage in PNS, SCs are induced to mislay myelin, proliferate and segregate numerous factors, including cytokines responsible for reproducing a microenvironment suitable for supporting axon regeneration [85, 86]. They also have a vital participation in endogenous repair, reconstructing myelin, which are essential for functional recovery [85, 86]. SCs, MSCs, ESCs, marrow stromal cells are the most studied support cells candidates. SCs transplantation enhance axon outgrowth both *in vitro* [87] and *in vivo* [88]. Although to achieve an adequate amount of autologous SC, a donor nerve is necessary and a minimum of 4-8 weeks for *in vitro* expansion. Umbilical cord MSCs may be the perfect cell model as supplement for nerve grafts, once they are easily obtained, with no ethical controversy and can differentiate into neuglial-like cells [83]. Matuse and collaborators induced MSCs from the umbilical cord into SCs capable of supporting peripheral nerve regeneration and myelin reconstruction *in vivo*. They transplanted these SCs into injured sciatic nerve, and proved that these cells maintained their differentiated phenotype *in vivo*, and contributed for axonal regeneration and functional recovery [89].

In our studies we aimed to explore the therapeutic value of human umbilical cord matrix (Wharton's jelly) derived MSCs, undifferentiated and differentiated in neuroglial-like cells, both *in vitro* and *in vivo*, associated to a variety of biomaterials such as, Poly (DL-

lactide-ε-caprolactone) PLC (Vivosorb®) membrane, and Chitosan type III on rat sciatic nerve axonotmesis and neurotmesis experimental model. For cell transplantation into injured nerves (with axonotmesis and neurotmesis injuries), there are two main techniques. The cellular system may be directly inoculated into the neural scaffold which has been interposed between the proximal and distal nerve stumps or around the crush injury (in neurotmesis and axonotmesis injuries, respectively); or the cells can be pre-added to the neural scaffold via inoculation or co-culture (in most of the cellular systems, it is allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured nerve [82].

Our PLC studies [55] demonstrated that this biomaterial does not interfere negatively with the nerve regeneration process, in fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already provided experimentally and with patients [82]. PLC becomes hydrophilic by water uptake, which increases the permeability of the polymer. This is essential for the control of nutrient and other metabolite transportation to the surrounding healing tissue. A few weeks after implantation, the mechanical power gradually decreases and there is a loss of molecular weight as a result of the hydrolysis process. Nearly in 24 months, PLC degrades into lactic acid and hydroxycaproic acid which are both safely metabolized into water and carbon dioxide and/or excreted through the urinary tract. In contrast to other biodegradable polymers, PCL has the advantage of not creating an acidic and potentially disturbing micro-environment, which is favorable to the surrounding tissue [90]. Chitosan has attracted particular attention in medical areas due to its biocompatibility, biodegradability, and low toxicity, low cost, improvement of wound-healing and antibacterial properties. Moreover, the potential use of chitosan in nerve regeneration has been demonstrated both *in vitro* and *in vivo* [57, 91]. Chitosan is a partially deacetylated polymer of acetyl glucosamine obtained after the alkaline deacetylation of chitin [57, 82]. While chitosan matrices have low mechanical strength under physiological conditions and are unable to maintain a predefined shape after transplantation, their mechanical properties can be improved by modification with a silane agent, namely γ-glycidoxypropyltrimethoxysilane (GPTMS), one of the silane-coupling agents which has epoxy and methoxysilane groups. The epoxy group reacts with the amino groups of chitosan molecules, while the methoxysilane groups are hydrolyzed and form silanol groups. Finally, the silanol groups are subjected to the construction of a siloxane network due to the condensation. Thus, the mechanical strength of chitosan can be improved by the cross-linking between chitosan, GPTMS and siloxane network. By adding GPTMS and employing a freeze-drying technique, we have previously obtained chitosan type III membranes (hybrid chitosan membranes)

with pores of about 110 µm diameter and about 90% of porosity, and which were successful in improving sciatic nerve regeneration after axonotmesis and neurotmesis [56, 57, 82].

The induction of a crush injury in rat sciatic nerve provides a very realistic and useful model of damage for the study of the role of numerous factors in regenerative processes [57]. Focal crush causes axonal interruption but preserves the connective sheaths (axonotmesis). After axonotmesis injury regeneration is usually successful, after a short (1-2 day) latency, axons regenerate at a steady rate towards the distal nerve stump, supported by the reactive SCs and the preserved endoneural tubules enhance axonal elongation and facilitate adequate reinnervation [92]. Our research group has been testing the efficacy of combining biomaterials and cellular systems in the treatment of sciatic nerve crush injury [57-59, 82, 90, 91, 93-95]. Following transection, axons show staggered regeneration and may take substantial time to actually cross the injury site and enter the distal nerve stump [60]. Although delayed axonal elongation might be caused by growth inhibition originating from the distal nerve itself, growth-stimulating influences may overcome axons stagger. More robust and fast nerve regeneration is expected to result in better reinnervation and functional recovery. As a potential source of growth promoting signals, MSCs transplantation is expected to have a positive outcome. Our results showed that the use of either undifferentiated or differentiated HMSCs enhanced the recovery of sensory and motor function in axonotmesis lesion of the rat sciatic nerve [56]. Neurotmesis must be surgically treated by direct end-to-end suture of the two nerve stumps or by a nerve graft harvested from elsewhere in the body in case of tissue loss. To avoid secondary damage due to harvesting of the nerve graft, a tube-guide can be used to bridge the nerve gap. Acutely after sciatic nerve transection there is a complete loss of both motor and thermal sensory function. Sensory and motor deficit then progressively decrease along the post-operative. From a morphological point of view, nerve regeneration occurs if Wallerian degeneration is efficient and is substituted by re-growing axons and the accompanying viable SCs [96, 97]. The axon regeneration pattern is improved by using appropriate biomaterials for the tube-guide design, like chitosan type III and PLC and cellular systems like MSCs from the Wharton jelly [57, 90, 91, 95]. The surgical technique and the time for the reconstructive surgery is also crucial for the nerve regeneration after neurotmesis [57, 90, 91, 95].

3.2. Assessment of nerve regeneration in the sciatic nerve rat model

Although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor [94, 98-100]. Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling [79] do not necessarily predict the reestablishment of motor and sensory functions [100-103]. Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery [100]. In this sense, research on peripheral nerve injury needs to combine both functional and morphological assessment. The use of biomechanical techniques and rat's gait kinematic evaluation is a progress in documenting functional recovery [104]. Indeed, the use of biomechanical parameters has given valuable insight into the effects of the sciatic denervation/reinnervation, and thus represents an integration of the neural control acting on the ankle and foot muscles, which is very useful and accurate to evaluate different therapeutic approaches [103-105].

3.2.1. Functional Assessment

After injury and treatment of animals, follow-up results are very important for analysis of functional recovery. Animals are tested preoperatively (week 0), and every week during 12 and 20 weeks, for axonotmesis and neurotmesis of the rat sciatic nerve, respectively. Motor performance and nociceptive function are evaluated by measuring extensor postural thrust (EPT) and withdrawal reflex latency (WRL), respectively [55, 58, 94]. For EPT test, the affected and normal limbs are tested 3 times, with an interval of 2 minutes between consecutive tests, and the 3 values are averaged to obtain a final result. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values are incorporated into an equation (Equation (1)) to derive the percentage of functional deficit, as described in the literature [106]:

$$\% \text{ Motor deficit} = [(NEPT - EEPT) / NEPT] \times 100 \qquad (1)$$

The nociceptive withdrawal reflex (WRL) was adapted from the hotplate test developed by Masters et al. (1993) [107]. Normal rats withdraw their paws from the hotplate within 4s or less. The cutoff time for heat stimulation is set at 12 seconds to avoid skin damage to the foot.

For Sciatic Functional Index (SFI), animals are tested in a confined walkway that they cross, measuring 42 cm long and 8.2 cm wide, with a dark shelter at the end. Several

measurements are taken from the footprints: i) distance from the heel to the third toe, the print length (PL); ii) distance from the first to the fifth toe, the toe spread (TS); and iii) distance from the second to the fourth toe, the intermediary toe spread (ITS). In the static evaluation (SSI) only the parameters TS and ITS, are measured. For SFI and SSI, all measurements are taken from the experimental (E) and normal (N) sides. Prints for measurements are chosen at the time of walking based on precise, clear and completeness of footprints. The mean distances of three measurements are used to calculate the following factors (dynamic and static):

$$\text{Toe spread factor (TSF)} = (\text{ETS} - \text{NTS}) / \text{NTS} \quad (2)$$

$$\text{Intermediate toe spread factor (ITSF)} = (\text{EITS} - \text{NITS}) / \text{NITS} \quad (3)$$

$$\text{Print length factor (PLF)} = (\text{EPL} - \text{NPL}) / \text{NPL} \quad (4)$$

SFI is calculated as described by Bain et al. [108] according to the following equation:

$$\text{SFI} = -38.3(\text{EPL} - \text{NPL}) / \text{NPL} + 109.5(\text{ETS} - \text{NTS}) / \text{NTS} + 13.3(\text{EIT} - \text{NIT}) / \text{NIT} - 8.8 = (-38.3 \times \text{PLF}) + (109.5 \times \text{TSF}) + (13.3 \times \text{ITSF}) - 8.8 \quad (5)$$

For SFI and SSI, an index score of 0 is considered normal and an index of -100 indicates total impairment. When no footprints are measurable, the index score of -100 is given [109]. In each walking track 3 footprints are analyzed by a single observer, and the average of the measurements is used in SFI calculations.

Ankle kinematics analysis is carried out prior nerve injury, at week-2 and every 4 weeks during the 12 or the 20-week follow-up time, for axonotmesis and neurotmesis lesions, respectively. The motion capture is performed with 2 digital high speed cameras (Oqus, Qualysis®) at a rate of 200 images per second, and Qualisys Track Manager software (QTM, Qualysis®). The cameras operate on a infra-red light frequency ensuring a high level of accuracy on the determination of reflective marker position and a position residual of less than 2.7 mm was obtained. Cameras are usually positioned to not recorder significant signal deflection during the test and four reflective markers were placed at the skin of the rat right hindlimb at the proximal edge of the tibia, the lateral malleolus and the fifth metatarsal head. Advanced analysis of the 2-D movement (sagittal plan) data is performed with Visual3D software (C-Motion®, Inc). The rats' ankle angle is determined using the scalar product between a vector representing the

foot and a vector representing the lower leg. With this model, positive and negative values of position of the ankle joint (Θº) indicate dorsiflexion and plantarflexion, respectively. For each step cycle the following time points are identified: midswing, midstance, initial contact (IC) and toe-off (TO) [104, 109-113] and are time normalized for 100% of step cycle. The normalized temporal parameters are averaged over all recorded trials. Angular velocity of the ankle joint (Ω º/s) is also determined where negative values correspond to dorsiflexion. A total of 6 walking trials for each animal with stance phases lasting between 150 and 400 ms are considered for analysis, since this corresponds to the normal walking velocity of the rat (20–60 cm/s) [104]. Animals walk on a Perspex track with length, width and height of respectively 120, 12, and 15 cm. In order to ensure locomotion in a straight direction, the width of the apparatus is adjusted to the size of the rats during the experiments.

3.2.2. Morphologic Assessment

Nerve samples are processed for quantitative morphometry of myelinated nerve fibers [114]. Fixation is usually carried out using 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1M Sorensen phosphate buffer for 6-8 hours and resin embedding is obtained following Glauerts' procedure (Scipio et al., 2008). Series of 2-µm thick semi-thin transverse sections are cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained by Toluidine blue. Stereology is carried out on a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). Systematic random sampling and D-disector is always adopted using a protocol previously described [115, 116]. Fiber density and total number of myelinated fibers is estimated together with fiber and axon diameter and myelin thickness.

3.3. RESULTS

3.3.1. Differentiation and metabolism of MSCs from Wharton’s jelly

In our experimental studies we expanded undifferentiated MSCs from human umbilical cord Wharton’s jelly that exhibited a normal star-like shape with a flat morphology in culture (Figures 4 and 5). To prevent the possibility of eventual mutations due to expansion artifacts, a total of 20 Giemsa-stained metaphases of these cells, were analyzed for numerical aberrations. Sporadic, non-clonal aneuploidy was found in 3 cells (41-45 chromosomes). The other 17 metaphases had 46 chromosomes (Figure 7). The karyotype was determined in a completely analyzed G-banding metaphase. No structural alterations were found. The karyotype analysis to the MSCs cell line derived

from Human Wharton jelly demonstrated that this cell line has not neoplasic characteristics and is stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes [55].

[Figure 7]

We differentiated MSC from Wharton's Jelly into neuroglial-like cells. After 96 hours of incubation in neurogenic medium, we observed a morphological change. The cells became exceedingly long and there was a formation of typical neural-like cells with multi-branches and secondary branches (Figure 6). The differentiation was tested based on the expression of typical neuronal markers such as GFAP, GAP-43 and NeuN by neural-like cells attained from HwMSCs. Undifferentiated MSCs were negatively labeled to GFAP, GAP-43 and NeuN (Figure 8A,C,E). After 96 hours of differentiation the attained cells were positively stained for glial protein GFAP (Figure 8B) and for the growth-associated protein GAP-43 (Figure 8D). All nucleus of neural-like cells were also labeled with the neuron specific nuclear protein called NeuN (Figure 8F) showing that differentiation of MSCs in neural-like cells was successfully achieved [55].

[Figure 8]

The *in vitro* expansion and differentiation of MSCs for clinical cell-based therapy is a very expensive and long process that needs standardization. Although pre-clinical and clinical data demonstrated the safety and effectiveness of MSCs therapy in some pathologies such as neurological, there are still questions surrounding the mechanism of action. In our research work we aimed to disclose the possible role of metabolism not only in the MSCs maintenance and expansion but also during the differentiation in neural-like cells [55]. MSCs maintenance and differentiation, to neural-like cells, depends on metabolic modulation. *In vitro*, glucose is the most widely used substrate for the generation ATP which is essential for cell growth and maintenance. It has been proposed that cells undergoing high proliferation rates depend on glycolysis to generate ATP, known as Warburg effect, although this pathway is less effective than the oxidative phosphorylation in terms of ATP production [117]. Our results showed that during expansion, the undifferentiated MSCs consume glucose and produce high concentration of lactate as a metabolic sub product which is consistent with the Warburg effect and glycolysis stimulation. MSCs do not require oxidative phosphorylation to survive as alternative, hypoxia extends the lifespan, increases their

proliferative ability and reduces differentiation [118]. The morphologic and biochemical characteristics of neural-like cells are already described but the mechanism by which stem cells differentiate into neural-like cells is still unknown. In our research work, MSCs that undergone differentiation into neural-like cells, consumed significantly less glucose and produced significantly less lactate than MSCs that undergone only expansion. These major differences allow us to conclude that during MSCs differentiation in neural-like cells the glycolytic process, which proved to be the crucial metabolic mechanism during MSCs expansion, is switched to oxidative metabolism [55].

Our results show clear evidences that MSCs expansion is dependent of glycolysis while their differentiation in neural-like cells requires the switch of the metabolic profile to oxidative metabolism. Also important may be the role of oxidative stress during this process. This work is a first step to identify key metabolic-related mechanisms responsible for human MSCs from the Wharton's jelly expansion and differentiation [55].

The lack of standardization of MSCs isolated from the Wharton's jelly culture conditions has limited some progress in scientific and clinical research. Understanding these MSCs metabolism during expansion, as well as determining molecular and biochemical mechanisms for differentiation is of great significance to develop new effective stem cell-based therapies.

4. Biomaterial and cellular system association – discussion and final remarks

We recently *in vivo* tested using the rat model, the efficacy of biomaterials and cellular system association in treatment of sciatic nerve axonotmesis and neurotmesis injury. Following transection, axons show staggered regeneration and may take substantial time to cross the injured site and enter the distal nerve stump [119]. However delayed axonal elongation might be caused by growth inhibition originated from the distal nerve itself, growth-stimulating influences may overcome axons stagger. As a potential source of growth promoting signals, MSCs transplantation is expected to give a positive outcome. Our results showed that the use of either undifferentiated or differentiated MSCs in axonotmesis lesion boosted the recovery of sensory and motor function. In both cell-enriched experimental groups we observed that the myelin sheath was thicker, this suggests that MSCs might apply their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration [120]. Also results from *in vivo* testing previously performed by our research group showed that infiltration of MSCs from the Wharton's jelly, or the combination of chitosan type III

membrane enwrapment and MSCs enrichment after nerve crush injury provide an advantage to post-traumatic nerve regeneration [56, 57]. Chitosan type III was developed as a hybrid of chitosan by adding GPTMS. A synergistic effect of an extra permeability and physicochemical properties of chitosan type III and the presence of silica ions may be responsible for the good results in post-traumatic nerve regeneration promotion observed in the sciatic nerve after axonotmesis and neurotmesis [57, 91]. The substantial improvement of axonal regeneration found in sciatic nerve crush enwrapped by chitosan type III membranes and for bridging nerve gaps after neurotmesis [57, 91], suggests that this biomaterial may not just work as a simple mechanical device but instead may induce nerve regeneration. The neuroregenerative properties of chitosan type III may be explained by the effect on SCs proliferation, axon elongation and myelinization [55, 91]. Our data also showed that PLC does not deleteriously interfere with the nerve regeneration process, as a matter of fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already provided experimentally and with patients [82]. The MSCs from the Wharton's jelly may be a valuable source in the repair of the peripheral nervous system with capacity to differentiate into neuroglial-like cells. The transplanted MSCs are also able to promote local blood vessel formation and release the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) [55]. Previous results obtained by our research group using N1E-115 cells *in vitro* differentiated into neuroglial-like cells to promote regeneration of axonotmesis and neurotmesis lesions in the rat model showed that there was no significant effect in promoting axon regeneration and, when N1E-115 cells were cultured inside a PLGA scaffold used to bridge a nerve defect, they can even exert negative effects on nerve fiber regeneration. The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading a negative outcome on nerve regeneration. Thus, N1E-115 cells did not prove to be a suitable candidate cellular system for treatment of nerve injury after axonotmesis and neurotmesis and their application is limited only to research purposes as a basic scientific step for the development of other cell delivery systems, due to its neoplasic origin [57-59, 91, 93]. The MSCs isolated from the Wharton´s jelly through PLC and chitosan type III membranes might be a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, such as digital nerves. The results from our experimental work [55, 56] showed that the use of either undifferentiated or neuroglial-like differentiated MSCs enhanced the recovery of sensory and motor function of the rat sciatic nerve. The observation that in

both cell-enriched experimental groups myelin sheath was thicker, suggest that MSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration [120]. In addition, these cells represent a non-controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses, including nerve injuries like axonotmesis and neurotmesis. The time and temperature of the transport (and the saline solution used) of the UC units from the hospital / clinic to the laboratory is crucial for a successful outcome considering MSCs isolation and proliferation from fresh and cryopreserved UCT. It is highly recommend that the transport from the clinic to the hospital should be refrigerated, and the UC units should be immediately immersed in a sterile saline solution like HBSS or DPBS.

5. FIGURE LEGENDS

Figure 1 – Cross section of an umbilical cord transported immersed in DPBS at the refrigerated temperature of 4-6ºC. Samples were stained with haematoxylin and eosin (HE). Magnification: 10X.

Figure 2 – Cross section of an umbilical cord transported immersed in DPBS at the refrigerated temperature of 4-6ºC. Samples were stained with haematoxylin and eosin (HE). The UCT integrity was quality evaluated through the following parameters: i) detachment of vessels and retraction of vascular structures; ii) loss of detail and integrity of the endothelium; iii) connective tissue degradation; iv) autolysis of fat (impossible to assess, due to histological technique); and v) loss of detail and integrity of the mesothelium. Magnification: 40X.

Figure 3 - Cross section of an umbilical cord collected and processed for histological analysis immediately after birth under optimal conditions according to Netcord guidelines. Stained with haematoxylin and eosin (HE). Magnification: 40X.

Figure 4 - MSCs isolated from Wharton's jelly using the "enzymatic protocol" exhibiting a mesenchymal-like shape with a flat polygonal morphology. Magnification: 100x.

Figure 5 – MSC cell line from Wharton's jelly (PromoCell) exhibiting a mesenchymal-like shape with a flat polygonal morphology. Magnification: 100x.

Figure 6 - MSC cell line from Wharton's jelly (PromoCell) after 72h of incubation in neurogenic medium. The cells became exceedingly long and there is a formation of typical neuroglial-like cells with multibranches. Magnification: 100x.

Figure 7 – Selected metaphases from undifferentiated MSC cells isolated from Wharton's jelly, showing the normal number of chromosomes (46, XY). Magnification: 1000X.

Figure 8 – Undifferentiated MSC cells from the Wharton's jelly presenting a negative staining for: (A) GFAP which is a glial cell marker; (C) GAP-43 which is related with axonal outgrowth and (E) NeuN which is a marker for nucleus of neurons. Neuroglial-like cells obtained from HMSCs in vitro differentiated with neurogenic medium exhibiting a positive staining for: (B) GFAP; (D) GAP-43 and (F) NeuN. Magnification: 200x [55].

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Figure 1

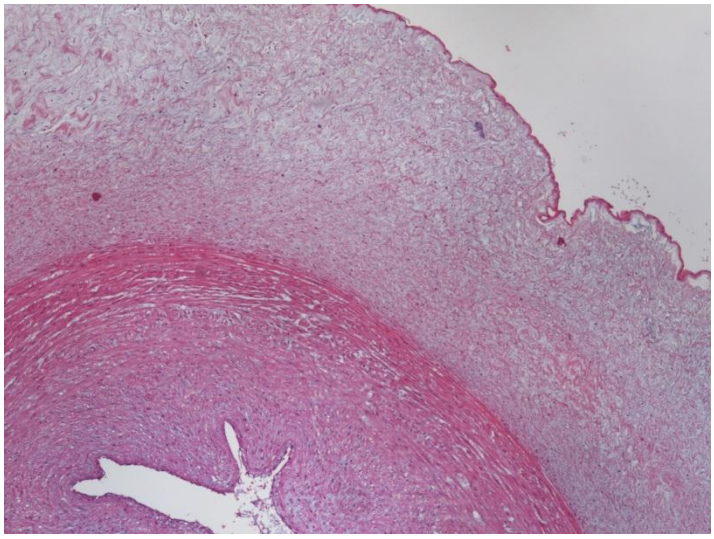
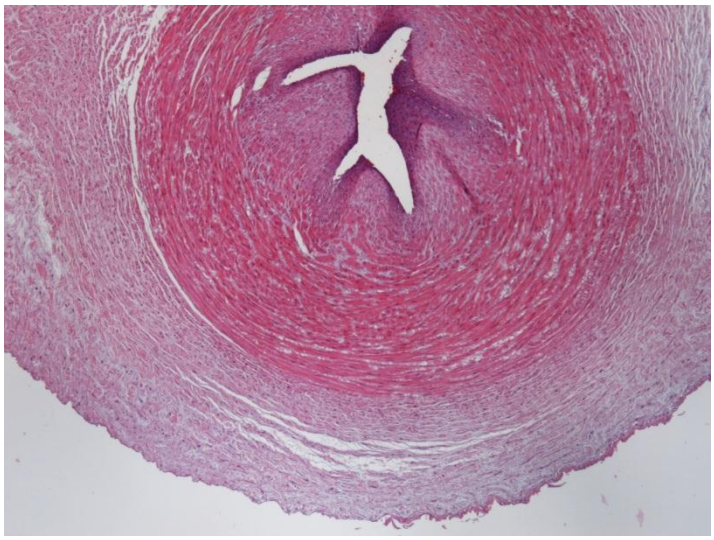


Figure 2



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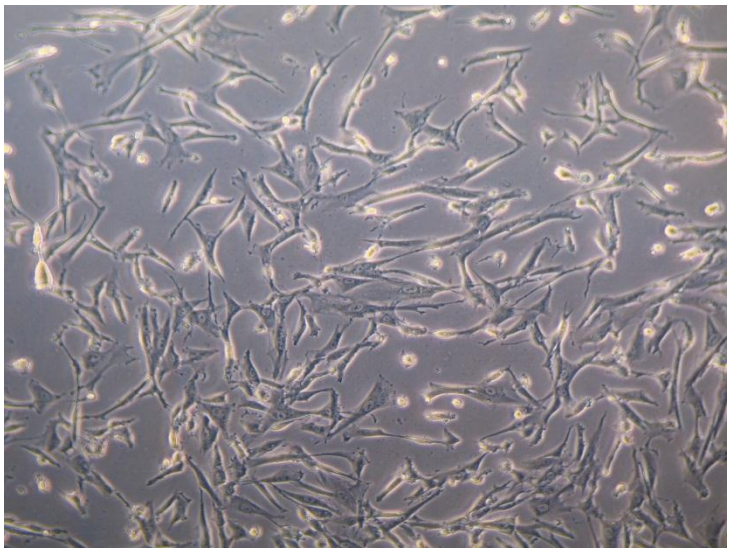

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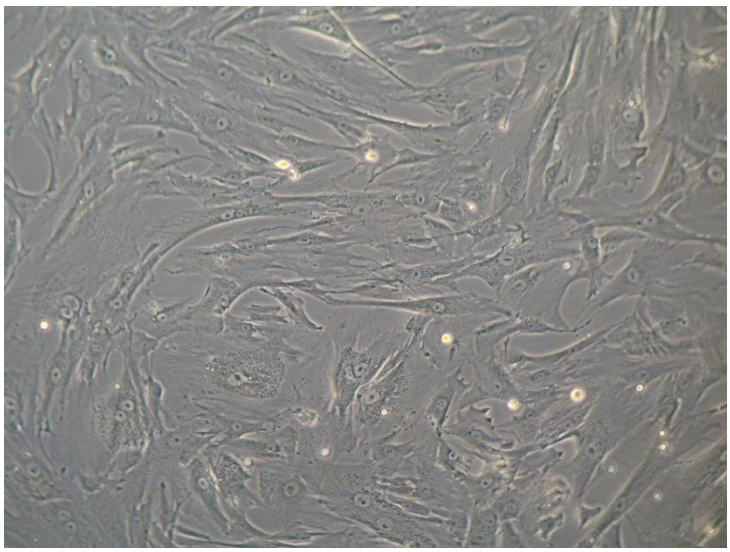
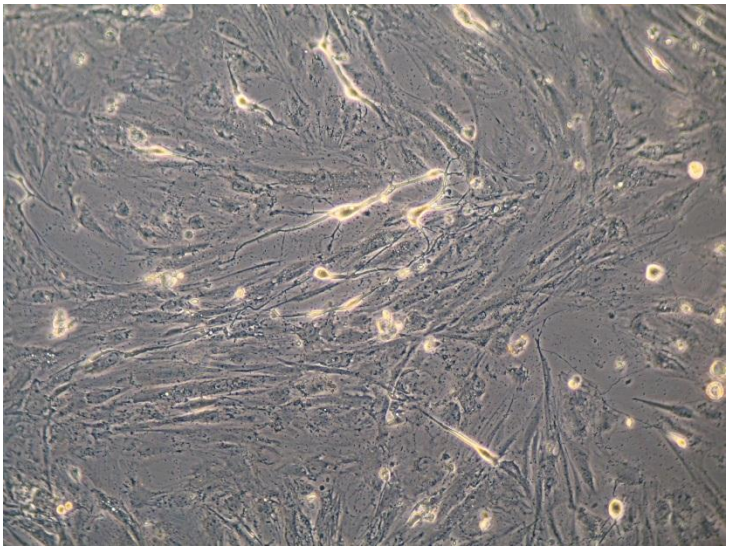


Figure 5



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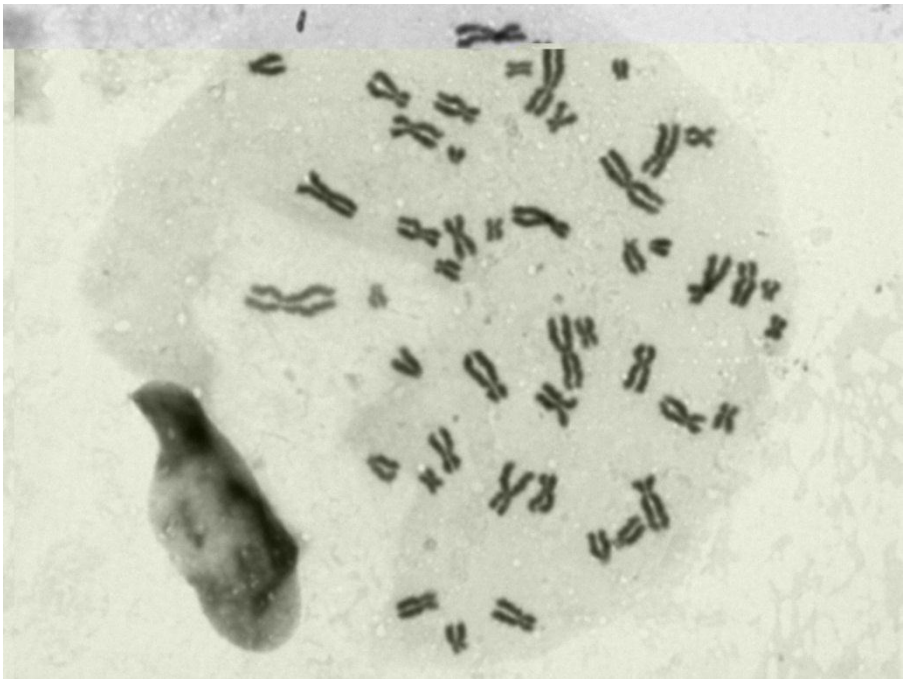

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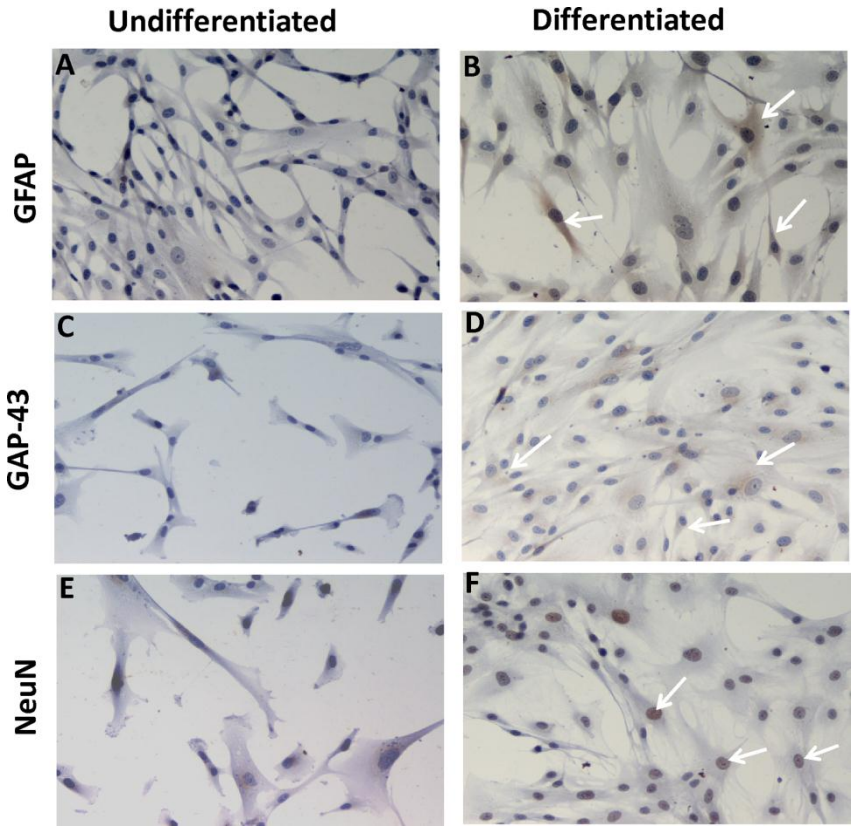

Figure 8

6.

Chapter Number

Biomaterials and stem cell therapies for injuries associated to skeletal muscular tissues

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1. INTRODUCTION

Skeletal muscle injuries are common in humans, particularly in athletes and it is important to develop new methods to improve muscle regeneration. Skeletal muscle has good regenerative ability, but the extent of muscle injury might prevent complete regeneration, especially in terms of functional recovery. Severe lesions, like those originated by trauma associated with loss of healthy muscular tissue and development of fibrous tissue scar and irreversible muscular atrophy after long-term peripheral nervous injuries are examples of those situations where regeneration is limited. An alternative approach for the restoration of the damaged skeletal muscular tissue, considered to be an ultimate treatment of some traumatic or degenerative diseases, is the transplantation of stem cells that limit the fibrosis and the atrophy of the involved muscle masses, and even imply the myocytes regeneration and local revascularization [1]. Stem cells and regenerative medicine is a fast emerging field with rapid strides of progress and focus on human health. Successful clinical use of stem cells in regenerative medicine depends on 3 important features: i) stem cells can grow and

divide indefinitely, ii) stem cells can differentiate into specialized cell types, like skeletal muscle; and iii) the stem cells can be delivered to the site of lesion associated to biomaterials, nowadays available with excellent characteristics concerning biocompatibility.

The purpose of this review is to describe the current research lines in the skeletal muscle regeneration field, with special emphasis to the work performed by our research group in testing different biomaterials and cellular therapies, emphasizing the use of mesenchymal stem cells (MSCs) isolated from the Wharton's jelly of the umbilical cord. We also focused our research in developing skeletal muscular lesion models which could be reproducible. It is important to state, that a multidisciplinary team has a crucial role in the development of these biomaterials associated to cellular systems, and in pre-clinical tests. MSCs comprise a rare population of multipotent progenitor cells with a great therapeutic potential since they are capable of self-renewal and multi-lineage differentiation. Due to this ability, MSCs appear to be an attractive tool in the context of Tissue Engineering and cell-based therapy concerning skeletal muscle regeneration. Several biomaterials associated to MSCs from the Wharton's jelly of the umbilical cord have been tested in standard lesions of the rat muscle and the results of these tests will be discussed here. The umbilical cord matrix is an important and safe source of MSCs with positive effects in nerve and skeletal muscle regeneration, with no ethical or technical issues.

2. SKELETAL MUSCLE TISSUE

2.1. Basic structure and terminology

The muscle fibers are the basic contractile units of skeletal muscles. They are individually surrounded by a connective tissue layer and grouped into bundles to form a skeletal muscle [1] Each muscle is surrounded by a layer of dense connective tissue - the epimysium - which is continuous with the tendon. The muscle is composed of numerous bundles of muscle fibers – fascicles - which are separated from each other by another connective tissue layer named perimysium. The endomysium is the connective tissue that separates individual muscle fibers from each other. Mature muscle cells are termed muscle fibers or myofibers. Each myofiber is a multinucleate syncytium formed by fusion of immature muscle cells termed myoblasts. In the cytoplasm of each myofiber – the sarcoplasm - lays the contractile apparatus of the cell which is composed of sarcomeres arranged in series to form myofibrils, which give myofibers their striated appearance. The sarcomeres contain a number of proteins, including alpha-actinin — which is the major constituent of the Z band — and actin and

myosin, which are the major components of the thin and thick filaments, respectively. The sarcoplasm, located between the myofibrils, is called the intermyofibrillar network and contains the mitochondria, lipid, glycogen, T-tubules, and sarcoplasmic reticulum [1-3]. Skeletal muscles are highly vascularized to provide essential nutrients for muscle function. As the myofiber matures, it is contacted by a single motoneuron and expresses characteristic molecules for contractile function, principally different myosin heavy chain isoforms and metabolic enzymes. Both the motoneuron and the myoblast origin have been implicated to play a role in specifying the myofiber contractile properties, although the precise mechanisms remain to be defined [1].

2.1.1. Fiber type

Individual adult skeletal muscles are composed of a mixture of myofibers with different physiological properties, ranging from a slow-contracting/fatigue-resistant type to a fast-contracting/non-fatigue-resistant type. The proportion of each fiber type within a muscle determines its overall contractile properties [1]. The slow contracting *soleus* muscle is rich in myofibers expressing the slow type I myosin heavy chain isoform, whereas the fast contracting plantaris muscle is devoid of slow type I myofibers [1-3]. The most informative methods to delineate muscle fiber types are based on specific myosin profiles, specially the myosin heavy chain (MHC) isoform complement. According to the major MHC isoforms found in adult mammalian skeletal muscles, the following pure fiber types exist: slow type I with MHCIb, and three fast types, namely type IIA with MHCIIa, type IID with MHCIId and type IIB with MHCIIb [4]. Despite having different physiological properties, the basic mechanism of muscle contraction is similar in all myofiber types and is the result of a “sliding mechanism” of the myosin-rich thick filament over the actin-rich thin filament after neuronal activation [5]. The connective tissue framework in skeletal muscle combines the contractile myofibers into a functional unit, in which the contraction of myofibers is transformed into movement via myotendinous junctions at their ends, where myofibers attach to the skeleton by tendons. Thus the functional properties of skeletal muscle depend on the maintenance of a complex framework of myofibers, motor neurons, blood vessels, and extracellular connective tissue matrix [1].

2.2. Regeneration of the skeletal muscle

Regeneration is a unique adaptation of skeletal muscle that occurs in response to injury. Following direct trauma or disease, the regeneration of skeletal muscle results in restoration, to some degree, of the original structure and function of the muscle tissue [6]. Skeletal muscle regeneration is a physiological response of the tissue to traumatic

or pathological injuries and its progress depends on the type of damaged muscle and the extent of the injury. Under normal conditions, the regenerated muscle is morphologically and functionally indistinguishable from undamaged muscle [1]. Regeneration resembles the process of formation of skeletal muscle during embryogenesis. Skeletal myogenesis begins in the somites where multipotencial mesodermal cells commit to the myogenic lineage. These mononucleated myoblasts then fuse and form multinucleated cells (myotubes) that ultimately develop into mature myofibers [1, 7]. During the course of muscle development, a distinct subpopulation of myoblasts fails to differentiate and remains associated with the surface of the developing myofiber as quiescent muscle satellite cells (SCs) in fully developed mature skeletal tissue [1, 7].

2.2.1. Satellite Cells and other cells involved in regeneration of skeletal muscle tissue

During regeneration and muscle repair, SCs fuse together or to the existing fibers to form new muscle fibers [8]. Although the number of SCs is greatly reduced in aged muscle, those remaining maintain an intrinsic capacity to regenerate the muscle tissue as efficiently as in younger muscles. A vital condition for successful regeneration is the presence of SCs in the uninjured portions of the basal membrane of the myofiber, along with its ability for reinnervation and revascularization. After a skeletal muscle injury, myofibers become completely desintegrated via myolysis and the SCs are realeased from the basal membrane. From this point SCs start to divide and are capable of differentiating into muscle fibers, reestablishing myofiber's architecture and restoring the muscle function [9, 10]. In post-natal skeletal muscle, PW1 expression is detected in SCs and a subset of interstitial cells and is markedly up-regulated during muscle regeneration [11]. These interstitial multipotent stem cells are extralaminal and exhibit fibroblastic morphology but do not express the same myogenic markers such as Pax7 [10]. $PW1^{+}/Pax7^{-}$ interstitial cells (PICs) are myogenic *in vitro* and efficiently contribute to skeletal muscle regeneration *in vivo* as well as generating satellite cells and PICs. PICs show bipotential behavior *in vitro,* generating both smooth and skeletal muscle. Isolated PICs do not express Pax7 or MyoD, but they convert to a $Pax7^{+}/MyoD^{+}$ state before forming skeletal muscle *in vitro.* PICs are not derived from a Pax3-expressing parental cell and thus do not share a satellite cell lineage; however, PICs do express Pax3 upon conversion to skeletal muscle. PICs are a key cell population that cannot be recruited into the skeletal muscle lineage in the absence of Pax7 function and is likely to contribute to the Pax7 muscle phenotype during postnatal

growth. PICs are as abundant as SCs in muscle tissue and correspond to the only population of PW1+/Pax7– cells *in vivo*, requiring Pax7 for their myogenic capacity [11]. PDGFRα+ mesenchymal progenitor cells located in the muscle *interstitium* were also identified as being distinct from SCs. Of the muscle-derived cell populations, only PDGFRα+ cells show efficient adipogenic differentiation both *in vitro* and *in vivo*, being strongly inhibited by the presence of satellite cell-derived myofibres. These results suggest that PDGFRα+ mesenchymal progenitors are the major contributor to ectopic fat cell formation in skeletal muscle that is more conspicuous in perimysium and particularly in perivascular space. The balance between satellite cell-dependent myogenesis and PDGFRα+ cell-dependent adipogenesis, rather than multipotency of satellite cells, has a considerable impact on muscle homeostasis [12]. Hematopoietic and dendritic cells are also present in the perimysium of the skeletal tissue, as well as some lymphocytes and macrophages [10].

2.2.2. Myogenic differentiation

Cells derived from Pax3-expressing cells are myofibres and SCs [11]. Once activated, SCs express factors involved in the specification of the myogenic program, such as Pax-7, desmin, MNFα, Myf5, MRF4 and MyoD. Activated SCs enter the cell cycle and proliferate as indicated by the expression of factors involved in cell cycle progression, such as PCNA and by the incorporation of BrDU. Recently, miRNAs have also been reported to regulate gene expression in skeletal muscle. Upon activation, SCs generate fusion-competent myoblasts and can self-renew at least to a limited extent. Any interruption in the proliferation or fusion of myoblasts, or any alterations in the extracellular matrix leads to the development of fibrosis, compromising the establishment of the correct muscular function [8, 10]. Proliferative MyoD and/or Myf5 positive myogenic cells are termed myoblasts. Both SCs and myoblasts increase their cytoplasmic-nuclear ratio and can migrate along myofibers. Proliferating myoblasts withdraw from the cell cycle to become terminally differentiated myocytes that express the “late” myogenic regulatory factors (MRFs), Myogenin and MRF4, and subsequently muscle-specific genes such as MHC and muscle creatine kinase (CK_M), and stopping Pax7 expression. Myogenic subpopulations have also been identified by their enriched M-cadherin and CD34 expression. M-cadherin can be considered to be a reliable marker for both quiescent and activated SCs. Once fusion of myogenic cells is completed, newly formed myofibers increase in size and myonuclei move to the periphery of the muscle fiber [1, 10, 13].

2.2.3. Degeneration

This scenario changes dramatically when the muscle is damaged, in which muscle degeneration after acute injury is characterized by myofiber necrosis and is followed by inflammation, tissue reconstruction and remodeling [10]. The necrosis is triggered by disruption of the myofiber sarcolemma resulting in increased myofiber permeability. The disruption of myofiber integrity is reflected by increased serum levels of muscle proteins, such as CK (usually restricted to the myofiber cytosol) [1]. It has been hypothesized that increased Ca^{2+} influx after sarcolemmal or sarcoplasmic reticulum damage results in a loss of Ca^{2+} homeostasis and increased Ca^{2+}-dependent proteolysis that drives tissue degeneration resulting in focal or total autolysis depending on the extent of the injury [1].

2.2.4. Inflammation

The early regenerative response in skeletal muscle is similar to that in other tissues and requires the coordinated regulation of inflammation, extracellular matrix remodeling, and myofiber growth [14]. The early phase of muscle injury is usually accompanied by the activation of mononucleated cells, mainly inflammatory cells and myogenic cells. Factors released by the injured muscle activate inflammatory cells residing within the muscle, which in turn provide the chemotactic signals to circulating immune cells. Neutrophils are the first immune cells to invade the injured muscle, with a significant increase in their number being observed as early as 1–6h after myotoxin or exercise-induced muscle damage. After neutrophil infiltration and 48h post-injury, macrophages become the predominant inflammatory cell type within the site of injury. Macrophages infiltrate the injured site and through phagocytosis remove cellular debris and may affect other aspects of muscle regeneration by activating myogenic cells [1]. Testosterone has a documented ability to modulate the activity of immune, fibroblast, and myogenic precursor cells, which are all components of regeneration [14].

3. SKELETAL MUSCLE INJURY MODELS

In order to study the process of muscle regeneration in a controlled and reproducible way, it was necessary to develop experimental models of muscle injury [1]. In this sense, a variety of experimental models that compromise skeletal muscle function or destroy this tissue is available. Each of the injury models can potentially have a different effect on the fate of resident cells and circulating cells within the muscle bed after the trauma [10]. A large number of studies, involving a variety of experimental injuries, such as injection of myotoxic agents, crush, ischemia, denervation and muscular dystrophies, demonstrate the unique ability of skeletal muscle for

regeneration, irrespectively the precise method used to induce the initial injury [15]. In this review we will focus on chemical and mechanical models of skeletal muscle injury, adding a new model of muscle injury based on surgical myectomy, developed in order to mimic severe losses of skeletal muscle mass. Other models, like exercise and denervation, will also be outlined. The latter is not a model of injury but else of skeletal muscle disuse but that also can be used to investigate skeletal muscle remodeling. There is a variety of other genetic models that are essential in studying diseases like Duchenne muscle dystrophy (Mdx mouse is currently the most widely used in this case) but will not be discussed in this review.

3.1. Chemical methods of skeletal muscle injury

The use of myotoxins, such as bupivacaine (Marcaine), cardiotoxin (CTX), and notexin (NTX) is perhaps the easiest and most reproducible way to induce muscle injury and regeneration. Myotoxins are also widely used to induce skeletal muscle injury because their inoculation by intramuscular injection does not require complex surgery. Several chemical agents are known to produce skeletal muscle damage. Severe muscle fiber damage, like breakdown of sarcolemma and myofibrils, has been described after intramuscular injections of 0.75% bupivacaine, 2% mepivacaine, or 2% lidocaine associated to epinephrine [18]. While lidocaine can cause rapid destruction of skeletal muscle fibers, long-acting anesthetics, like bupivacaine, are more often used to cause skeletal muscle injury in rodents [19].

3.1.1. Bupivacaine

The bupivacaine injection procedure is simple and quick, does not involve extensive surgery, and induces a regeneration process which is qualitatively similar to that observed in other model systems. Doses of 1.5 and 1% wt/vol produce significant levels of muscle injury and subsequent regeneration, but these doses also produce large regions of ischemic muscle tissue. Doses of 0.75 and 0.5% bupivacaine are also effective in inducing regeneration and produce little or no ischemia [16]. Muscle fiber necrosis is extremely rapid after induced bupivacaine injury [14]. Injection of the drug into small skeletal muscles of rat or mouse leads to immediate and massive myonecrosis followed by phagocytosis of necrotic debris and a rapid and apparently complete regeneration of muscle fibers 3-4 wk after injection. The peak isometric twitch and tetanic tensions produced by rat fast-twitch *extensor digitorum longus* muscle injected with bupivacaine returns to normal values by 21 d after injection [17]. Morphological analysis has shown that many indexes of successful regeneration in healthy muscle can be completed within 2–3 weeks of recovery from injury [14]. The

sequence of fiber breakdown induced by bupivacaine is similar to that of progressive muscular dystrophy [18] and it is also striking that the same types of muscle fibers are spared by both Duchenne's muscular dystrophy and bupivacaine toxicity. It has been suggested that bupivacaine may disrupt Ca^{2+} homeostasis *in vivo*, triggering Ca^{2+}-activated cellular death pathways that include proteolysis. This suggestion is supported by the findings that (i) bupivacaine affects sarcoplasmic reticulum function *in vitro*, (ii) extracellular Ca^{2+} omission delays the morphological changes and decreases the protein degradation rate that are observed in isolated rat soleus muscle exposed to bupivacaine, and (iii) bupivacaine uncouples isolated rat liver and heart mitochondria and decreases mitochondrial membrane potential and oxygen consumption both in cultured fibroblasts and Ehrlich tumor cells [19]. Extracellular Ca^{2+} plays a part in mediating the muscle damage caused by bupivacaine but other factors must also be involved [20]. For example macrophage invasion is necessary for complete degeneration of myofibrillar components [21]. Saito and Nonaka [22] injected 0,5ml of 0,5% bupivacaine after *soleus* muscle exposure of Wistar rats, and observed that SCs proliferation began at almost the same time as following muscle crush injuries. Bupivacaine is still commonly used for the purpose of studying the mechanisms of skeletal muscle regeneration following injury [7, 14, 15, 22].

3.1.2. Cardiotoxin and Notexin

Snake venom is known for a long time to directly affect the skeletal muscle, producing fibrillation, contractures and depolarization of the sarcolemma. Although initially ascribed to the phospholipase A content of this venom, muscle contracture and depolarization seems to be related to the cardiotoxic action of cobra venom [23]. Notexin (NTX) is a phospholipase A2 neurotoxin peptide extracted from snake venoms that block neuromuscular transmission by inhibition of acetylcholine release. Cardiotoxin (CTX) is also a peptide isolated from snake venoms, but it is a protein kinase C-specific inhibitor that appears to induce the depolarization and contraction of muscle cells, disruption of membrane structure, and lysis of various cell types [1]. CTX is postulated to be neurotoxic as its injection destroys neuromuscular junctions [24]. However, CTX might cause direct destruction of muscle tissues [25]. Snake CTX polypeptide is now known to be a potent inducer of muscle contracture with phospholipase A likely acting in accelerating the action of CTX rather than in augmenting it [23, 26]. Dantrolene antagonizes CTX-induced contractures, suggesting a role for Ca^{2+} derived from the sarcoplasmic reticulum in CTX action. CTX rapidly lowers the threshold for Ca^{2+}-induced Ca^{2+} release in heavy sarcoplasmic reticulum fractions. The mechanism of action involved in contractures of skeletal muscle appears

to be related to the immediate and specific effect of CTX (Ca^{2+} release by the sarcoplasmic reticulum) [27, 28].

A more recent study by Gutiérrez and Ownby [25] focused on the role of PLA_2 as important myotoxic components in these venoms suggesting that myotoxic PLA_2s binds to acceptors in the plasma membrane leading to its disruption and pronounced Ca^{2+} influx which, in turn, initiates a complex series of degenerative events associated with contracture, activation of calpains and cytosolic Ca^{2+}-dependent PLA_2s, and mitochondrial Ca^{2+} overload. Fourie, Meltzer [30] already had suggested that the biological effects of CTX could be a consequence of inhibition of plasma membrane (Ca^{2+} + Mg^{2+})-ATPases. The local myonecrosis is often associated with other effects, such as hemorrhage, blistering and edema, in a complex pattern of local tissue damage. Apart from membrane-active CTXs, snake venom hemorrhagic metalloproteinases also cause myonecrosis, but the mechanism involved is likely to be an indirect one, probably related to ischemia [25]. CTX is a useful model for muscle regeneration that does not influence muscle architecture like basal lamina or microvasculature, making the regeneration process less complicated than other models like crush, where for example, inadequate blood supply might result in an increase of fibrosis. CTX injection also results in faster and more extensive muscle degeneration, and an earlier start of the reconstruction phase, than muscle crushing [24].

3.2. Mechanical methods of skeletal muscle injury

Crush injuries of the skeletal muscle can occur in considerable numbers following natural disasters or acts of war and terrorism. They can also occur sporadically after industrial accidents or following periods of unconsciousness from drug intoxication, anesthesia, trauma or cerebral events [31]. Crushing as a method of inducing muscle injury and regeneration was first described by Bassaglia and Gautron [32], and has since been used in several published research studies [24]. Muscle damage occurs at three distinct stages: at the time of the initial mechanical crushing force, during the period of ischemia and during the period of reperfusion [31]. It has been hypothesized that ischemia is the primary instigator of local muscle damage following crush injuries [33]. However, studies have shown that although skeletal muscle tissue can survive circulatory ischemia for 4h, the mechanical force sustained in crushing, along with ischemia, causes skeletal muscle death in only 1 h. Studies of enzyme release suggest that most damage to myocytes occurs during the reperfusion stage rather than the ischemic stage [31]. Animal models of muscle injury should closely mimic the clinical situation. Among these models open crush lesion have been used frequently, allowing

standardized evaluation of regeneration in a selected muscle. For application of the trauma, either forceps or custom-made devices have been used. There are two types of muscle-crush models described in the literature: the segmental crush and the complete crush, where only 4-6% of the fibers remain intact [34]. There are different forms to accomplish the segmental crush model but most of them include the use of a surgical instrument (hemostatic clamp e.g.) to produce a standardized closing force in a specific area of a muscle causing a compression contusion injury [35]. One of the important steps of this procedure is denervation, which makes the initial steps of regeneration less painful for the animal. Skeletal muscle contusion can also be performed without skin incision by dropping a mass over a selected muscle. This technique was used by Iwata, Fuchioka [36] employing a 640g mass dropped from a 25 cm height onto an impactor (diameter 10 mm) placed on the belly of the rat medial *gastrocnemius*. This procedure damaged around 47% of the entire cross-sectional area of both medial and lateral *gastrocnemius*. At day 2 post-injury, an intense inflammatory response and necrotized myofibers with infiltrated mononuclear cells were observed. No myotubes were found at this stage. However, a number of regenerative myotubes were detected at days 7, 14, and 21 days post-injury. This study also showed that normal locomotion recovers prior to isometric force and complete regeneration of the injured muscle [36]. The main disadvantage of the complete muscle crush is the potential damaging of myoneural junctions which triggers not only regeneration of muscle substance but also initial innervation deficits. These deficits always lead to impaired healing [34]. Histological analysis of muscle regeneration after crush injury shows an initial phase of inflammation followed by SCs activation, myotube regeneration and fibrosis of the muscle. It has been shown that development of fibrotic tissue is one of the main factors affecting the recovery of muscle function after traumatic muscle injury [34]. In a qualitative assessment performed by our group we tested the open crush lesion in the *tibialis anterior* (TA) muscle of adult Sasco Sprague rats. Different standardized force intensities, durations of muscle compression (30 seconds and 1 minute) and time points (3, 8, 15 and 21 days post-surgery) were considered for the histological evaluation of skeletal muscle injury. Hematoxilin-eosin (HE) and Masson's trichrome staining were employed in this preliminary study. At day 3 post-surgery, myofiber damage was evident and the lymph nodes were reactive due to the active inflammatory process. The presence of fibrosis was evident only following 15 days from the initial injury. This evaluation revealed that the crush model was not the most appropriate for *in vivo* evaluation of cellular therapies for skeletal muscle regeneration aid, since the extent of this injury type did not present the magnitude required to accurately appreciate the biological effects of MSCs utilization [38].

3.3. Myectomy and myotomy

The loss of a portion of a skeletal muscle poses a unique challenge for regeneration of muscle tissue and restoration of its normal structure and function [39]. In the event of large-scale soft tissue traumas, extensive loss of full-thickness native tissue architecture renders the wound site unable to support normal regeneration process. In severe tissue injuries the acute inflammatory response is followed by formation of a provisional fibrin matrix derived from trauma-associated blood clotting and this matrix is then infiltrated by type I collagen-producing fibroblasts [40]. In order to mimic those situations, new experimental models have been developed in which a defined portion of the muscle tissue is removed, creating a myectomy defect within the muscle. For example, Merrit et al. [39] removed a 0.5 x 1.0 cm or a 1.0 x 1.0 cm fraction of the *gastrocnemius* muscle of rats, creating a small and large defect respectively. This was accomplished lacerating the lateral side of the muscle with a #9 scalpel blade. We have recently developed a novel experimental muscle injury model in the TA muscle of adult Sasco Sprague rat, by using a biopsy punch to create a standardized myectomy defect. Sasco Sprague male rats with 250-300g were used and after a standardized 5 mm diameter myectomy lesion in the mid-belly of the *tibialis anterior* muscle, the defect was completely filled with different vehicles and/or biomaterials, cellular suspensions containing $1x10^6$ human MSCs isolated from Wharton's jelly and conditioned media (Figure 1). This concentrated media contains trophic factors secreted by MSCs during cell culture. In our research work, the myectomy model proved to be the most appropriate for a comprehensive and standard evaluation of the rat skeletal muscular regeneration ability. The regeneration process in other models of lesion, like simple muscle crush, did not present the magnitude required to accurately appreciate the biological effects of MSCs [38].

[Figure 1]. Biopsy punch for myectomy lesion creating a 5 mm Ø defect in the rat *tibialis anterior* (TA) muscle.

Another less invasive model of muscle injury has been used in a number of studies by producing a laceration injury (myotomy) [42-44]. In some cases this was obtained by a partial thickness (50%) cut of gastrocnemius muscles in mice at 60% of their length from their distal insertion, through 75% of their width and then sutured with a modified Kessler stitch and simple sutures using a PDS 7.0 wire (Ethicon, Somerville, New Jersey) [43]. Other studies used a full-thickness (100%) cut though 50% of the *gastrocnemius* muscle width [44]. The advantages of this model are its reproducibility and the ability to apply consistently precise injections into the laceration site [43].

3.4. Denervation (indirect model)

Innervation regulates skeletal muscle mass and muscle phenotype and peripheral nerve injury in the rat is a widely used model to investigate nerve regeneration and can also be employed as a model of muscle inactivity and muscle atrophy. Changes in the muscles may contribute to functional deficit after nerve injury [47]. Denervation induces muscle atrophy and 25 months post denervation muscle fibers cross sectional area of the *extensor digitorium longus* (EDL) muscle diminish to only 2.5% of control animals although their fascicular organization is maintained [47]. The effect of denervation on muscle atrophy is both activity-dependent and activity-independent since the degree of hindlimb muscle atrophy after spinal isolation (activity-independent nerve influence) is less when compared to the atrophy caused by removal of all nerve influences by transecting the sciatic nerve [9]. Two basic mechanisms are responsible for denervation-induced muscle atrophy. First, there is augmented activity of the ubiquitin-proteasome pathway and proteolysis [48]. Second, there is cell death and myonuclei apoptosis conjugated with decreased capacity of satellite cell-dependent reparative myogenesis [49]. Together with atrophy, denervated/reinnervated muscles undergo phenotypical changes and conversion between muscle fiber types [50]. The relative increase in type I or type II muscle fibers following denervation seems to depend on the type of muscle fibers predominant in the muscle, with type II muscle fibers (fast fibers) increasing in proportion in *soleus* (considered a slow muscle) and type I muscle fiber number increasing in *gastrocnemius* and TA muscles [51]. Likewise, the degree of muscle fiber atrophy in short-term denervation (4 weeks) has been noticed to be greater in the muscle fiber type that is more abundant in the affected muscles [52]. Earlier studies suggested that that it was possible that denervated muscles could have increased muscle plasticity due to acceleration in the early myoblastic stages of muscle regeneration. Nevertheless McGeachie and Grounds [53] data proved that very few precursors were proliferating in denervated muscle within 30 h after injury, and the onset of myogenesis at 30 h was essentially the same in denervated and innervated muscle. They compared the onset of DNA synthesis in muscle precursors in denervated and innervated muscle of adult BALBc mice regenerating after a simple cut injury. This study concluded that although denervation of skeletal muscles causes an increase in SCs and connective tissue cell turnover, it does not “prime” the general population of muscle precursors to start synthesizing DNA more rapidly after injury than in innervated muscle [53]. After sciatic nerve transection at an adult age, electromyography (EMG) patterns in hindlimb muscles during locomotion remained highly abnormal even after recovery periods lasting 15 or 21 weeks [54]. This may be a limitation when using denervated muscles as a model of muscle injury since

regeneration might be affected for a very prolonged period. Like already mentioned, other models of skeletal muscle injury, like complete crush or myectomy, can be accompanied by denervation since these traumatic models may possibly damage peripheral nerves or myoneural junctions. This might also be an undesirable occurrence in the standardization of these models of muscle injury. In fact, our preliminary work using TA myectomy showed that few animals developed severe muscle force deficit after 4 weeks recovery, suggesting that damaged of the supplying nerve occurred in these animals subset.

In our research group, standard peripheral nerve injuries in the rat sciatic nerve model have been performed [57-63] in order to evaluate different therapeutic approaches including several biomaterials and cellular systems to promote sensitive and functional recovery of the nerve. A standard crush injury is performed by a non-serrated clamp (Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria), exerting a constant force of 54 N for a period of 30s, 10mm above the bifurcation into tibial and common peroneal nerves, inducing a 3mm axonotmesis lesion [57-63]. In order to induce a standard neurotmesis lesion in the rat sciatic nerve model, considered a more serious lesion, under deep anaesthesia, the right sciatic nerve is exposed through a skin incision extending from the greater trochanter to the distal mid-half followed by a muscle splitting incision. After nerve mobilisation, a transection injury is performed (neurotmesis) using straight microsurgical scissors. The nerve is injured at a level as low as possible, in general, immediately above the terminal nerve ramification. To prevent autotomy, a deterrent substance should be daily applied to rat right foot [57-63]. Both experimental injuries induce severe motor deficit and loss of sensory function, evaluated by measuring extensor postural thrust (EPT) and withdrawal reflex latency (WRL), respectively [57-63]. Sensory and motor deficit then progressively decreased along the post-operative, depending on the therapeutic approach used. Very promising results were obtained with chitosan type III membranes and MSCs isolated from the umbilical cord matrix. In addition, we also perform kinematic analysis of the rat walk which is a more sensitive behavioral test. This analysis is increasingly being used to assess functional recovery in peripheral nerve research because of its higher accuracy and better relationship with histological outcome [57-63]. We should bare in mind that locomotion is also of higher functional relevance since it involves integrated function of both the motor and sensory systems and their respective components, such as skeletal muscles, sensory endings, efferent and afferent nerve fibers and integrative centers within the central nervous system. Muscles innervated by sciatic nerve branches include both dorsiflexors and plantarflexors and, although in our published studies we focused our kinematic analysis

only in the stance phase, we now prefer to include analysis of the ankle joint motion also during the swing phase in order to provide additional information [59]. Denervation can be a very useful model of skeletal muscle injury for some experimental studies but some limitations might be pointed out in studies that attempt to focus exclusively on the muscular regeneration process. Nevertheless and as demonstrated by several studies, this muscular regeneration process is highly dependent of the neural supply and the nerve regeneration itself can be influenced by the damaged muscle tissue.

4. TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Every day thousands of clinical procedures are performed to replace or repair tissues in the human body that have been damaged through disease or trauma that use tissue engineering technology. The use of constructs for tissue engineering (TE) and regenerative medicine are promising innovative therapies that can address several clinical situations. These constructs are often combination of cells, scaffolds and biological factors. Although there are only a few commercial products currently in the market for cell/drug delivery, probably because each type of cell requires its own specific encapsulating microenvironment with cell-specific material properties and spatially controlled bioactive features, a vast amount of research is being performed worldwide on all aspects of tissue engineering/regenerative medicine exploring polymer materials. To implant cells into defective skeletal muscles, there are two main techniques. The cellular system may be directly injected into the scaffold which is localized in the injury site. It can also be performed by pre-adding the cells to the scaffold via injection or co-culture (in most of the cellular systems, cells are allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured muscle. In case of multiple sites of injury, the systemic administration of cells capable of reaching damaged tissues would be an interesting alternative [64].

4.1. Scaffolds and Biomaterials

Scaffolds, which are used to deliver cells, drugs, and genes into the body, can take on various forms from porous solid devices to injectable networks, such as a typical three-dimensional porous matrix, a nanofibrous matrix, a hydrogel, and microspheres. Although solid scaffolds provide a mechanically strong matrix for seeded cells, hydrogel scaffolds and microspheres are becoming increasingly popular in TE. The spherical nature maximizes the surface area, and the small volume of beads facilitating biomolecular transport. Regarding hydrogels, they have a similar microstructure to the extracellular matrix (ECM) and allow good physical integration into the defect by the use of minimally invasive approaches for material and cell/drug delivery. The biological,

chemical, topography features and mechanical properties, as well as the degradation kinetics of hydrogels, can be tailored depending on the application [65-68]. Aligned nanoscale and microscale topographic features in scaffolds have been also reported to influence the alignment of cells. For example, this alignment is an important requirement of functional skeletal muscle since it leads to alignment of myoblasts and cytoskeletal proteins and promote myotube assembly along the nanofibres and microgrooves to mimic the myotube organization in muscle fibres [65, 68-70]. Scaffold are used successfully in various fields of tissue engineering such as bone formation, periodontal regeneration, cartilage development, as artificial corneas, in tendon repair and in ligament replacement. In addition, the incorporation of drugs (i.e., inflammatory inhibitors and/or antibiotics) into scaffolds or specific molecules to provide adequate signals to the cells is also possible [71] Depending on the medical applications, scaffolds requirements will depend on its function. Hydrogels can be used as a physical barrier to protect the cells from hostile extrinsic factors before delivery, or be used as a matrix to drug controlled release or cell adhesion, growth and differentiation to further improve the secretion of therapeutic proteins from cells. In fact cells are capable of delivering drugs in response to an external stimulus, which is highly advantageous to maintain homeostasis for patients suffering from chronic diseases. For the first application, the scaffold needs: i) to be biocompatible, by minimizing the patients' immune response, which is detrimental to cell viability, hydrogel stability, and mass transport. Ideally, the scaffold should evoke no or only minimal fibrous tissue reaction, macrophage activation, and cytokine and cytotoxic agent release ii) to have controllable degradability, being the degradation products not toxic and eliminated easily from the implantation site by the body, and iii) to have mechanical properties that are sufficient to shield cells from tensile forces without inhibiting biomechanical cues to cells through mechanotransduction pathways that mediate tissue homeostasis, morphogenesis, cell growth, contractility, differentiation, and pathophysiology [70-72]. For the second applications further requirements are needed, mainly: i) a microstructure that allows for the influx of nutrients and oxygen toward the encapsulated cells and prevents the efflux of therapeutic molecules and cellular wastes away from the scaffold; this is assured through adequate pore size distribution and its interconnectivity. A high surface:volume ratio should be suitable for cell/drug attachment; ii) adequate drug binding affinity to allow a controllable drug released to be stable when incorporated in the scaffold at a physiological conditions; iii) bioadhesion, to allows cells and tissues to adhere to scaffolds. Some hydrogels such as fibrin or collagen inherently exhibit bioadhesive properties, but others do not and therefore linker molecules that enable covalent or non-covalent molecular interactions between

the scaffold and its surroundings are incorporated; iv) the mechanical properties of the scaffold, commonly controlled with the polymer concentration and molar ratio between polymers and cross-linking molecules, should match those of the tissue at the implantation site as well as the degradation rate that should match the rate of tissue regeneration [73-77].

4.1.1. Hydrogel scaffolds

Hydrogels, three-dimensional (3D) networks of hydrophilic polymers, are appealing for biological applications because of their high water content, high permeability, biocompatibility, and the ability of be placed into critical defects in a minimally invasive manner [78]. They are being used in a wide range of tissues, including cartilage, bone, muscle, fat, liver, and neurons. For use in drug/cell delivery, hydrogels should be low-viscosity solutions prior to gelling, which is crucial to maintain cell viability during the encapsulation process, and should rapidly gel in the human body. These properties can be fine-tuned through variations in the chemical structure and cross-linking density in hydrogels. Injectable hydrogels can be formed *in situ* by either chemical or physical cross-linking methods [79, 80]. Physical cross-linked hydrogels are capable of phase transition in response to external stimuli such as temperature, pH or both [81]. Chemically cross-linked hydrogels are prepared through photopolymerization, disulfide bond formation, or reaction between thiols and acrylate or sulfone. The latter hydrogels undergo significant volume changes compared to the first ones [81-83]. The pH/temperature-sensitive hydrogels show several advantages over thermo-sensitive ones, such as the absence of clogging during injection and avoidance of pH decreased caused by degradation. The pH/temperature-sensitive copolymer hydrogels can be prepared by combining a pH-sensitive moiety with a temperature-sensitive block. For example, if acidic sulfamethazine oligomers (OSMs) are coupled with thermosensitive poly(e-CL-co-LA)-PEG-poly-(e-CL-co-LA) triblock copolymers a pH/temperature-sensitive hydrogels (OSM-PCLA-PEG-PCLA-OSM) is produced. Photopolymerized hydrogel systems have been reported to provide better temporal and spatial control over the gelation process [79, 81, 84].

4.1.2. Biomaterials for scaffold fabrication

A wide variety of natural and synthetic materials have been used to prepare injectable hydrogels. Natural polymers, which are either components of or have macromolecular properties similar to the natural ECMs, are known to often undergo rapid degradation upon contact with body fluids or medium and show batch-to-batch variation. Synthetic hydrogels offer improved control of the matrix architecture and chemical composition,

no immunogenicity, consistent supply of large quantities, but tend to have lower biological activity. Therefore, modification of natural and synthetic derived hydrogels is usually required [79]. A natural biodegradable 3D scaffold can be made of acellular muscle ECM but it's fragile and difficult to handle [85]. Another natural biodegradable scaffold can be created by using fibrin, which leads to a process much similar to wound healing, in which fibrin forms a temporary scaffold to serve tissue regeneration and then is replaced by the physiological ECM. Fibrin has the additional advantage that it binds growth factors [70].

Fibrin hydrogels are made from commercially purified allogeneic fibrinogen and purified thrombin, and have been used in a variety of tissue engineering applications. Its main disadvantages reported to be shrinkage of the gel, low mechanical stiffness and its rapid degradation can be overcome by incorporating other polymers such as gelatin, hyaluronic acid, and chondroitin-6-sulfate. Fibrin glue is clearly distinguished from fibrin hydrogels that are prepared from purified fibrinogen and thrombin. Despite the commercial fibrin glue is available in standardized quality; autologous fibrin glue is cheaper and has no viral transmission and prion infection [86]. Tisseel® VH, is a fibrin glue commercialized by Baxter, and consists of a two-component fibrin biomatrix with highly concentrated human fibrinogen to produce fibrin gel from a blood sample and is safeto be used in TE. FloSeal® is another commercial hemostatic matrix with potential in TE, and consists of a cross-linked bovine-derived Gelatin Matrix component and a human-derived Thrombin component. Literature reports that myoblasts seeded on fibrin gels have been shown to differentiate into contracting muscle fibres and to demonstrate a normal length–tension and force–frequency relationship [87, 88].

Alginate is the designation given to a natural family of biodegradable, biocompatible, hydrophilic and non-toxic polysaccharides extracted from some marine algae and some microorganisms. Alginates are linear block co-polymers composed of two different monomers, β-D-mannuronic acid (M) and α-L-glucuronic acid (G), which are linked by (1-4) glycosidic bonds. The main property of alginate that potentiates its use in different areas, it is its ability to bind some divalent cations such as Ca^{2+} in the carboxylic groups which provides the gelation of the alginate solution. The properties of the gel are dependent of the ratio between M and G monomers (M:G ratio); if the proportion of the G monomer is predominant, a strong brittle gel it obtained, whereas if the proportion of the M monomer is predominant, the formed gel will be weaker, but more flexible, because there are less junction zones between the polymer chains. As alginate is a polyelectrolyte, more specifically a polyanion, it can be ionically associated with a polycation existent in the same solution through hydrogen bonding or electrostatic interactions, forming a polyelectrolyte complex [89, 90]. Cell-encapsulating calcium

cross-linked alginate hydrogels have been extensively studied because alginate molecules are anionic polysaccharides and do not associate with many proteins. Since alginate itself is inert for cell attachment and spreading, the cell adhesion properties can be tailored by linking molecules such as RGD peptides to its backbone [91]

Chitosan is a natural and hydrophilic copolymer, and it is composed by two monomeric units, D-glucosamine and N-acetyl-D-glucosamine linked by β(1–4)-glycosidic bond. This linear polysaccharide has been widely studied in medical applications due to its biocompatibility, biodegradability, non-toxicity, fungistaticity, antimicrobial activity, non-carcinogenicity, notable affinity to proteins, promotion of cell adhesion as well as proliferation and differentiation [67]. Chitosan results from the alkaline deacetylation of the chitin and its solubility is mainly influenced by its molecular weight and degree of deacetylation. Some methods have been developed to lower the molecular weight of chitosan by hydrolysis of the polymeric chains, in order to produce chitosan salts which are soluble in water. Chitosan-based hydrogels have been gelled via glutaraldehyde cross-linking, UV irradiation, and thermal variations [81].

Hyaluronic Acid (HA) is a natural, hydrophilic and non-sulfated glycosaminoglycan. This polymer is a linear polysaccharide, in which the repeating unity is a disaccharide composed by two monomers, D-glucuronic acid and N-acetyl-D-glucosamine, linked through alternating β1,3 and β1,4 glycosidic bonds. HA has been used as a biomaterial in various medical applications, due to its biocompatibility, biodegradability, and non-immunogenicity. HA is the main component existent in the extracellular matrix (ECM) of living tissues, namely in the connective, epithelial and neural. This polymer, due to its structural and biological properties, has the ability of mediate the cell signalling and behaviour, and the matrix organization. HA is able of interact with some cell surface receptors, being involved in the tissue hydrodynamics, cell migration and proliferation. Several strategies have been reported to prepare HA-based hydrogels [92, 93].

Among the most widely used synthetic polymers for scaffolds, either alone or copolymerized with synthetic or natural polymers, as biodegradable polymers are polyglycolide, polylactide and its copolymer poly(lactide-co-glycolide), polyphosphazene, polyanhydride, poly(propylene fumarate), polycyanoacrylate, polycaprolactone, polydioxanone, and polyurethanes, and as non-biodegradeable polymers are included polyvinyl alcohol (PVA), polyhydroxyethymethacrylate, and poly(N-isopropylacrylamide) [71, 94].

The majority of natural biomaterials used in clinical applications are derived from animal or human cadavers' sources. In spite of thorough purification methods, these materials bear the inherent risk of transfer of viral diseases and may cause immunological body reactions while synthetic biomaterials are not associated with

these risks. So, a critical issue in this type of cellular transplants is the search for an optimal vehicle to provide the ideal environment for cell hosting and for the release and conduction of molecules to the site of injury for cell-host interaction. Taking this into account we evaluated different biomaterials as vehicles for the cellular system intended to be tested for skeletal muscle regeneration using our myectomy model injury model in the rat. Plasma derived substances, hemostatic matrix solutions and hydrogels (Figure 2 and Figure 3) were tested and the *in vivo* response was compared histologically according to the International Standard ISO 10993-6 (*see* 5.1.3). The following procedure was done under sterile conditions. For preparation of the spherical hydrogel, the polymer solution is prepared by adding in a ratio of 1:1 (V/V), a sodium alginate aqueous solution 7% (m/V) to a sodium hyaluronate aqueous solution 0.5% (m/V), under magnetic stirring. Afterwards the polymer solution is inserted into an insulin syringe and a droplet is released into an excess of cerium nitrate solution 135mM, in order to obtain a cross-linked polymer sphere of approximately 60 µl of volume. Cerium nitrate and sodium hyaluronate solution were sterilized by microfiltration (0.22 µm membrane) and sodium alginate powder is sterilized in an autoclave (120ºC for 15 minutes) previous to the solution preparation (Figure 2 and Figure 3). These tested biomaterials including the spherical hydrogel were not only used as vehicles but their properties were also evaluated and optimized to find a suitable matrix for the cellular implants.

[Figure 2]. Hydrogel preparation (alginate, hyaluronic acid and cerium).
[Figure 3]. Application of a spherical hydrogel containing $1x10^6$ MSCs from the Wharton's jelly, in the 5 mm Ø TA myectomy defect.

4.2. Cells

There is evidence both from animal studies and clinical investigations that cell therapy involving different types of stem cells application is promising as means to promote regeneration of skeletal muscles following severe injuries. Technical or/and ethical difficulties in obtaining sufficient and appropriate stem cells from the bone marrow or from embryos (obtained from assisted reproduction techniques or somatic nuclear transfer - cloning) have limited the application of this type of therapy. Stem cells are known as an undifferentiated population, with endless self-renewal and sustained proliferation *in vitro* and multilineage differentiation ability [95]. The *in vitro* multilineage differentiation is the concept that gives these cells an extreme priority for use in tissue and cell-based therapies. Stem cells can be loosely classified into 3 categories based

on their functional role: hematopoietic stem cells, mesenchymal stem cells (MSCs) and embryonic stem cells [95].

MSCs have become one of the most exciting targets for tissue regeneration due to their high plasticity, proliferative and multilineage differentiation capacity. These cells are capable of differentiating into adipose, bone cartilage and muscle. Among all this notable characteristics, MSCs reveal other properties of great importance, they present low immunogenicity and high immunosuppressive properties due to a decreased or even absence HLA Class II expression [96]. Differentiation potential of MSCs in multilineage end-stage cells has been proven, so as the treatment potential in musculoskeletal disorders [97, 98]. Since their first isolation in 1968, from rat bone marrow [99], MSCs have been isolated with success from almost all tissue sources: skeletal muscle, adipose tissues, synovial membranes, umbilical cord matrix and blood, placental tissue, amniotic fluid among others. Along with differentiation capacity, an increasing amount of data has demonstrated that the MSCs have the capacity of modulating the surrounding environment, by secretion of multiple factors and activation of endogenous progenitor cells [100, 101].

4.2.1. Umbilical cord

From our data and from previously published experimental work, the development of cell therapies associated to biomaterials is a promising tool for increase skeletal muscle regeneration, avoiding the irreversible loss of function and limit the fibrous scar tissue presence [57-59]. Recent years have witnessed an explosion in the number of adult stem cells populations isolated and characterized. While still multipotent, adult stem cells have long been considered restricted, giving rise only to progeny of their resident tissues. Recently, and currently controversial studies have challenged this dogma, suggesting that adult stem cells may be far more plastic than previously appreciated [102, 103]. Extra-embryonic tissues as stem cell reservoirs offer many advantages over both embryonic and adult stem cell sources. The umbilical cord matrix is an important and safe source of MSCs with positive effects in nerve and skeletal muscle regeneration, with no ethical or technical issues. MSC isolated from umbilical cord matrix (Wharton's jelly), as well as embryonic stem cells (ESCs) are originated from inner cell mass of blastocyst [104]. Comparing with ESCs, MSCs have shorter population doubling time; can be easily cultured in plastic flasks, are well tolerated by immune system; therefore transplantation of these cells into non-immune-suppressed animals does not induce acute rejection. Most important, these cells do not originate teratomas [104]. Like bone marrow stromal cells and other MSCs, the MSCs from the

Wharton's jelly are plastic adherent, stain positively for markers of the mesenchymal lineage (CD10, CD13, CD29, CD44, CD90, and CD105) and negatively for markers of the hematopoietic lineage. These MSCs are capable of self-renewal with sustained proliferation *in vitro* and can differentiate into multiple mesodermal cells. The high plasticity and low immunogenicity of these cells turn them into a desirable form of cell therapy for the injured musculoskeletal tissue without requiring the use of immunosuppressive drugs during the treatments. Interestingly, these cells, which are HLA class II negative, not only express both an immuno-privileged and immuno-modulatory phenotype, but their HLA complex class I expression levels can also be manipulated, making them a potential cell source for MSC-based therapies. In addition and as previously referred, these cells represent a non-controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses. MSCs from the Wharton's jelly display a high proliferative rate and plasticity, being able to differentiate into adipocytes, osteoblasts, chondrocytes, cardiomyocites, neurons, and glia. More recently, Conconi et al. [105] demonstrated thar CD105(+)/CD31(-)/KDR(-) cells are able not only to differentiate *in vivo* towards the myogenic lineage as demonstrated by the co-localization of HLA 1 and sarcomeric tropomyosine antigens, but also to contribute to the muscle regenerative process. These cells were found to differentiate *in vitro* into myoblast-like cells, expressing Myf5 and MyoD after 7 and 11 days of myogenic induction, respectively. The timing of expression of Myf5 and MyoD in CD105(+)/CD31(-)/KDR(-) cells is similar to that described during embryonic development and in myoblast cultures [105].

Using the myectomy model we tested the use of Human MSCs isolated from the Wharton's jelly in order to improve skeletal muscle regeneration. The cells were directly infiltrated into the lesion or delivered by different vehicles including Floseal®, Tisseel®, carboximetilcellulose (Sigma) and spherical hydrogel (own fabrication). MSC from Wharton's jelly were purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). The MSCs are cultured and maintained in a humidified atmosphere with 5% CO_2 at 37ºC. Mesenchymal Stem Cell Medium, PromoCell (C-28010) is replaced every 48 hours. At 90% confluence, cells are harvested with 0.25% trypsin with EDTA (GIBCO) and passed into a new flask for further expansion. MSCs at a concentration of 2 x 10^5cells are cultured exhibiting a 90% confluence after 3-4 days. The application of human MSCs in rats is possible without inducing any immunossupression in the experimental animals. The MSCs exhibited a normal star-like shape with a flat morphology in culture (Figure 4). A total of 20 Giemsa-stained metaphases of these cells, were analyzed for numerical aberrations. Sporadic, non-clonal aneuploidy was

found in 3 cells (41-45 chromosomes) the other 17 metaphases had 46 chromosomes. The karyotype was determined in a completely analyzed G-banding metaphase and no structural alterations were found [57]. The karyotype analysis to the MSCs cell line derived from Human Wharton jelly demonstrated that this cell line hasn't neoplasic characteristics and is stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes. Also, the morphologic characteristics of these cells in culture, observed in an inverted microscope, are normal. These cells presented a star-like shape with a flat morphology, characteristic of the MSCs been adequate to be used in *in vivo* rat experimental model [57]. The MSCs karyotype was studied in order to be sure that these cells did not present any number or structure chromosome abnormalities due to isolation and cell culture procedures before *in vivo* application. These concerned was due to the negative effects that some cellular systems, like the ESCs present, inducing the production of teratomas. The cellular systems implanted into the injured skeletal muscle improved the skeletal muscle regeneration since these cells produce growth factors, ECM molecules, and even modulate the inflammatory process.

[Figure 4]. Undifferentiated MSCs from Wharton's jelly, exhibiting a star-like shape with a flat morphology (100x magnification).

5. EVALUATION OF MUSCLE REGENERATION

Muscle biopsies should be considered in order to obtain careful clinical assessment or for investigation purposes. After the collection of the muscle samples, they should be immediately equally divided in three. One sample should be placed into formalin (for hematoxilin and eosin - HE), another sample should be fixed into 2.5% purified glutaraldehyde in 0.1M Sorensen phosphate buffer (for electron microscopy - EM) and the other sample should remain unfixed and refrigerated (for histochemistry, biochemistry/genetics analysis).

5.1. Routine histological evaluation

Routine evaluation of the muscle biopsy sample involves the examination of formalin-fixed, paraffin processed sections and unfixed frozen sections with standard histological and enzyme histochemical stains at the light microscopic level. HE is the routine histological stain used for evaluation of basic tissue organization and cellular structure. For HE, the whole piece of tissue should be fixed in a clamp and after the tissue is infiltrated with wax, both longitudinal and cross sections must be cut before

embedding. Five levels should be obtained, especially in cases suspected of vasculitis. The parameters that can be evaluated are: the type of inflammatory infiltrate present; examination of the structure of vessels walls (vasculitis and/or fibrinoid necrosis); presence of endomysial and perimysial fibrosis/fatty infiltration; the range of fiber caliber; presence of angulated fibers; increase in number of centrally located nuclei; central capillary migration; split fiber; group atrophy; necrotic/myopathic (degenerating) fibers; atrophic fibers; regenerating fibers; target fibers; whorl fibers and ring fibers. The rounding of fiber contour and the variation of fiber diameter should also be analyzed, however they are better evaluated with frozen sections (Figure 5).

[Figure 5]**.** HE staining of TA muscles 15 days post myectomy (A - control) and application of fibrin (B), hydrogel (C), Floseal® (D).

5.1.1. Morphological analysis

Long-standing histological characteristics are still used to identify the mammalian skeletal muscle regeneration process. On muscle cross-sections, these fundamental morphological characteristics are newly formed myofibers of small caliber and with centrally located myonuclei. Newly formed myofibers are often basophilic (reflecting high protein synthesis) and express embryonic/developmental forms of MHC (reflecting de novo fiber formation). On muscle longitudinal sections and in isolated single muscle fibers, central myonuclei are observed in discrete portions of regenerating fibers or along the entire new fiber, suggesting that cell fusion is not diffuse during regeneration but rather focal to the site of injury [1]. Cross-sectional area (CSA) analysis is one of the features that can be assessed. This can be achieved with imaging software processing (Scio Image, ImageJ) of HE-stained muscle sections. A predefined number of fibers is traced per sample and should be determined as appropriate by the examination of no additional changes in standard deviation. The classification of small and large fibers can be determined for example by setting three standard deviations from the mean CSA for the uninjured group at different time points [14]. The CSA and number of myotubes can be used to estimate the development degree of muscle regeneration following injury.[36]

5.1.2. Collagen quantification

Collagen content in the wound bed can be calculated by image analysis of Masson's Trichrome-stained histological images taken at a predefined image magnification. Color separations must be performed and an analysis threshold must be established for each image series collected using the same brightness and white balance settings. Output

images showing only computed blue coverage must be compared to the color images to ensure the representation of truly blue color due to collagen staining. As a control the analysis must be performed on uninjured (control) skeletal muscle tissue sections stained with Masson's Trichrome and collected using the same camera and threshold settings to confirm a collagen content of zero for control tissue. The ratio of blue pixels above the threshold to total pixels in the image is used to calculate the collagen content for each image [40].

5.1.3. International Standard (ISO 10993-6)

The International Standard (ISO 10993-6) specifies test methods for the assessment of the local effects after implantation of biomaterials intended for use in medical devices. These implantation tests are not intended to evaluate or determine the performance of the test specimen in terms of mechanical or functional loading. The local effects are evaluated by a comparison of the tissue response caused by the tested implant to that caused by the control. The objective of the test methods is to characterize the history and evolution of the tissue response after implantation of a medical device/biomaterial including final integration or resorption/degradation of the material. The test sample shall be implanted into the tissues most relevant to the intended clinical use of the material. For short-term testing, animals such as rodents or rabbits are commonly used. During the first two weeks after implantation the reaction due to the surgical procedure itself may be difficult to distinguish from the tissue reaction evoked by the implant and for that reason in our study we collected the muscle samples 15 days after implantation. For degradable/resorbable materials the test period shall be related to the estimated degradation time of the test product. In our case the majority of the vehicles/matrices tested the degradation time is less than 4 days. In the absence of complete degradation, absorption, or restoration to normal tissue structure and function, the overall data collected may be sufficient to allow characterization of the local effects after implantation. A sufficient number of implants shall be inserted to ensure that the final number of specimens to be evaluated will give valid results. The evaluation of the biological response must be accomplished by documenting the macroscopic and histopathological responses as a function of time. The responses to the test sample must be compared to the responses obtained at the control sample or sham operated sites. The scoring system used for the histological evaluation shall take into account the extent of the area affected, either quantitatively (e.g. in micrometres) or semi-quantitatively (Annex E of this Sandard) [44, 106]. The biological response parameters, which shall be assessed and recorded, include: i) the extent of fibrosis/fibrous capsule (layer in μm) and inflammation; ii) the degeneration as

determined by changes in tissue morphology; iii) the number and distribution as a function of distance from the material/tissue interface of the inflammatory cell types, namely polymorph nuclear neutrophilic leucocytes, lymphocytes, plasma cells, eosinophils, macrophages and multinucleated cells; iv) the presence, extent and type of necrosis; v) other tissue alterations such as vascularization, fatty infiltration, granuloma formation and bone formation; vi) the material parameters such as fragmentation and/or debris presence, form and location of remnants of degraded material; vii) the quality and quantity of tissue ingrowth, for porous and degradable implant materials [106]. Under the conditions of the study and following the results for the mentioned parameters in the semi-quantitative scoring system (Annex E of this Sandard), the test sample is considered as non-irritant (0,0 up to 2,9), slight irritant (3,0 up to 8,9), moderate irritant (9,0 up to 15,0), severe irritant (> 15) to the tissue as compared to the negative control sample [106]. This test method is used for assessing the biological response of muscle tissue to an implanted material (Annex C of this Standard). As already mentioned, the method compares the biological response to implants of test specimens with the biological response to implants of control specimens. The control materials are those used in medical devices of which the clinical acceptability and biocompatibility characteristics have been established [106]. In our study we developed an adaptation of this Standard by considering the control as the group where the surgical procedure (myectomy) was performed without any biomaterial or cell implantation (Figure 6). Although the surgical technique may profoundly influence the result of any implantation procedure, we assumed that our standardized myectomy lesion could be considered as the *Control* group since we were able to determine the local effects of the different implants by their comparison to the minor effects of the surgical procedure. The surgeries were executed under general anesthesia with a xylazine (1.25 mg/100 g BW im) and ketamine (9 mg/100 g BW im) combination [38].

[Figure 6]**.** ISO 10993-6 scoring for the groups tested. The *Control* group obtained a score of 14.7 (in blue). Scorings above the *Control* group were considered as non-irritant (in yellow), slight irritant (in orange) and moderate irritant (in red).

5.2. Histochemistry

For histochemistry a basic panel should be performed, preferentially in frozen sections. Depending on the objectives, an extended panel can be done, concerning the study of some molecules or enzyme processes such as the already mentioned Masson's

Trichrome-stain, ATPase; NADH-TR or Esterase (Bancroft&Stevens). When necessary, other special stains can be performed on paraffin sections.

5.3. Immunohistochemistry

In general, the immunohistochemical stains are utilized for the diagnosis of various muscular dystrophies. They also may help to determine the subtypes of inflammatory cells within an infiltrate or for other investigation purposes. Specific skeletal muscle markers such as myosin heavy chain and desmin can be applied in order to clearly identify this tissue. The distinction of SCs, considered as the reservoir of myogenic precursor cells, from other cells must be made (like plasma cells, which may be occasionally seen under the basal lamina in pathologic conditions). SCs can be can be easily demonstrated by immunostaining for N-CAM; they also express vimentin. Activated SCs generally express Myo-D and myogenin [107]. The regenerating fibers express N-CAM, MyoD and myogenin, and also embryonic and neonatal isoforms of myosin heavy chain. In contrast to mature fibers, MCH class I histocompatibility complex is expressed in regenerating fibers.

5.4. Immunofluorescence

For the preparation of TA muscles for immunofluorescence they should be embedded in Tissue-Tek OCT compound. Sections are cut at 10 µm using a Leica CM1850 cryostat and placed onto Surgipath microscope slides. Laminin-α2 chain is detected with a 1:500 dilution of rabbit anti-laminin-α2 (2G) polyclonal antibody. The laminin-α1 chain is detected with a rat anti-laminin-α1 monoclonal antibody. Primary rabbit antibodies are detected with a 1:500 dilution of fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody and the rat monoclonal antibody is detected with 1:500 dilution of fluorescein isothiocyanate-conjugated anti-rat secondary antibody. In all immunofluorescence experiments, secondary only antibody controls are included to test for specificity. For mouse monoclonal antibodies, endogenous mouse immunoglobulin is blocked with a mouse-on-mouse (MOM) kit. A 1-µg/ml concentration of tetramethylrhodamine-conjugated wheat germ agglutinin (WGA) is used to define muscle fibers. To examine immune response, cytotoxic T cells are detected with fluorescein isothiocyanate-labeled rat anti-mouse CD8a and macrophages are detected with fluorescein isothiocyanateconjugated anti-mouse F4/80 at 1:1000 (Figure 7 and Figure 8) [50].

[Figure 7]. Double imunofluorescence staining for laminin and CD31.
[Figure 8]. $Pax7^{+}$ SC counterstained with DAPI.

5.5. Electron microscopy

Electron microscopic (EM) examination of the glutaraldehyde-fixed portion of the biopsy is performed when the light microscopic studies are inconclusive. Thus, it is reserved for selected circumstances in which the pathologist determines that EM has the potential of contributing significantly to determining a specific diagnosis. A specimen placed in glutaraldehyde must be small, approximately 1-2 mm in width and depth, allowing the complete tissue penetration by this fixative. Glutaraldehyde makes tissue brittle and interferes with immunohistochemical studies, so it is not appropriate for the paraffin specimen. With EM, other muscle cell parameters can be analyzed in detail: the myofibril architecture; the plasma and sacolemmal membrane; the mitochondria (size, density and shape); T-tubule; amount of lipid; nucleus; phagocytic granules and amount of glycogen. EM is extremely useful in some cases: to identify inclusions primarily found by light microscopy; to help in the characterization of stored material found on light microscopy and define its intracellular localization; to analyze structural abnormalities found by light microscopy; can assist in the diagnosis of mitochondrial myopathy or seeking evidence to support a diagnosis of dermatomyositis (EM can be used to look for tubuloreticular inclusion in endothelial cells when light microscopic fails to reveal it).

5.6. Contraction force measurement

To obtain an estimate of total TA muscle strength reduced by the injury and possibly recovered by the cell/vehicle implants, contractile force due to electrical stimulation can be measured before injury, after injury and at the time of sacrifice (at different time points) for non-implanted and implanted animals. This can be accomplished with the animals under general anesthesia and by anchoring the knee joint using a custom clamping system anchored to the floor of the surgical stereomicroscope stand and attaching a silk ligature to the cleft between digits 1 and 2 that must be anchored to a transducer at the other end. This can also be executed by cutting the TA tendon just before the insertion at the ankle and tying it with a 4.0 nylon suture attached to the isometric transducer. The exposed muscle is stimulated using 2 custom needle electrodes placed at the proximal muscle surface. Electrical stimulation of the TA muscle is applied at 5 volts, 4 ms pulse duration, at 500 ms intervals and the resultant tetanic force recorded (200 points(s) using a BioPac MP-100 (Harvard Apparatus) and accompanying software (Acknowledge^{TM}). The muscle must be kept hydrated during the procedure using sterile saline. Maximum tetanic force is measured by reducing the stimulation interval to 20 ms, generating continuous stimulation simulating tetanus

condition. Another method of applying the electrical stimulation can be obtained by exposing the sciatic nerve with an incision in the hamstring region The tibial nerve is cut just after the sciatic nerve splits into the tibial and peroneal nerves to eliminate any contraction from the *gastrocnemius* muscle causing background in the force data. The exposed sciatic nerve is then laid over two electrodes with a small piece of parafilm and should also be kept moist with periodic treatment of mineral oil. Stimulation is made using a supra-maximal square-wave pulse of 0.1 ms duration. Measurements are performed at the length at which maximal extension is obtained during the twitch and the data should be recorded for sub-maximal and maximal isometric force. Specific maximal force should be quantified by correcting for muscle mass [40, 98].

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Figure 1

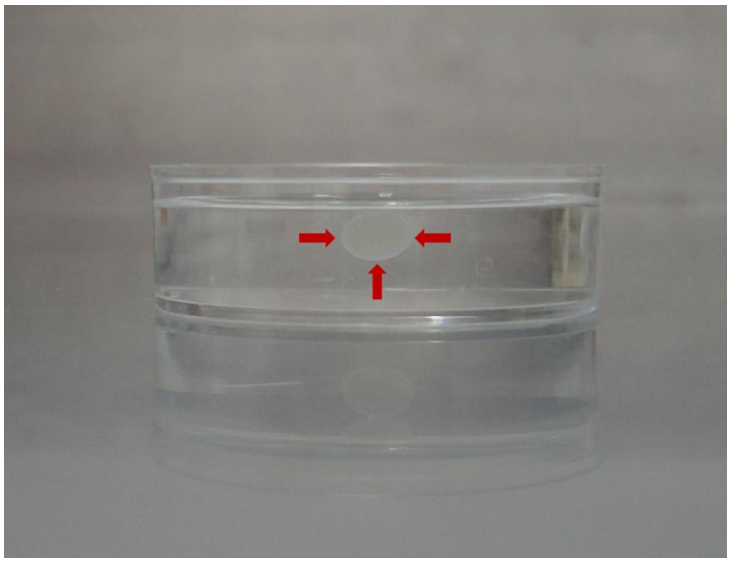


Figure 2

Figure 3

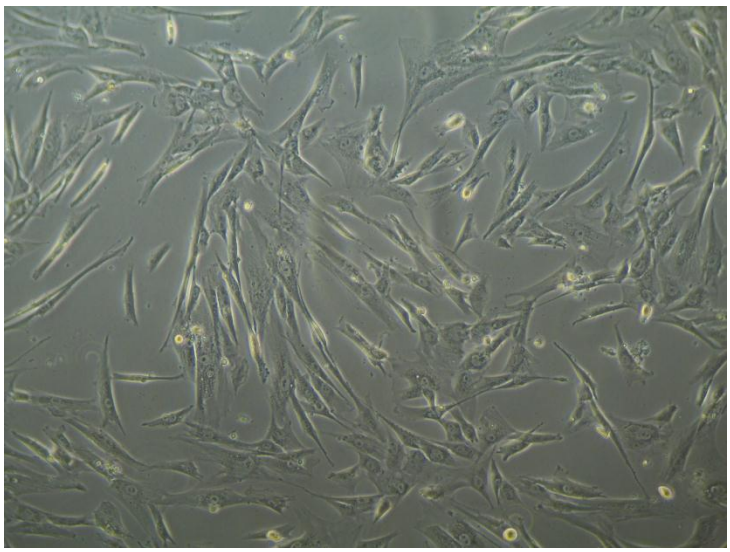
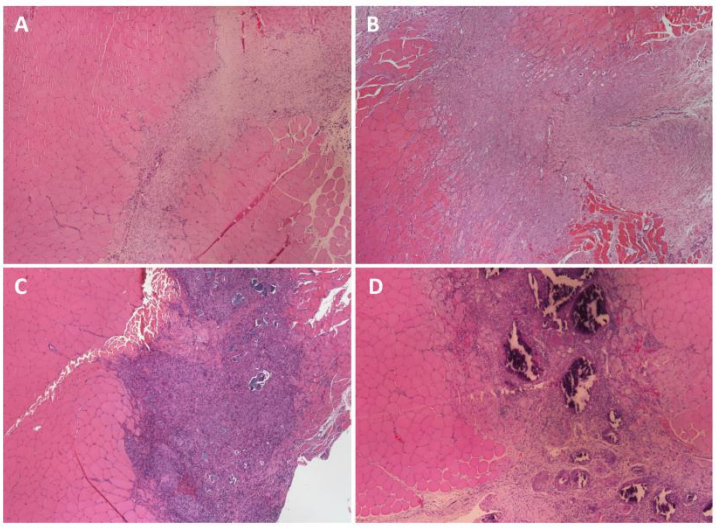


Figure 4



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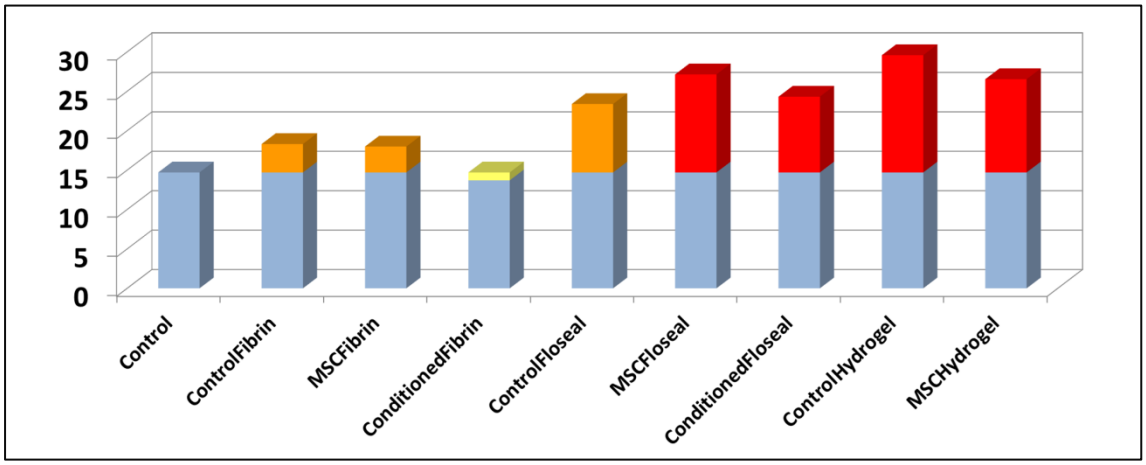

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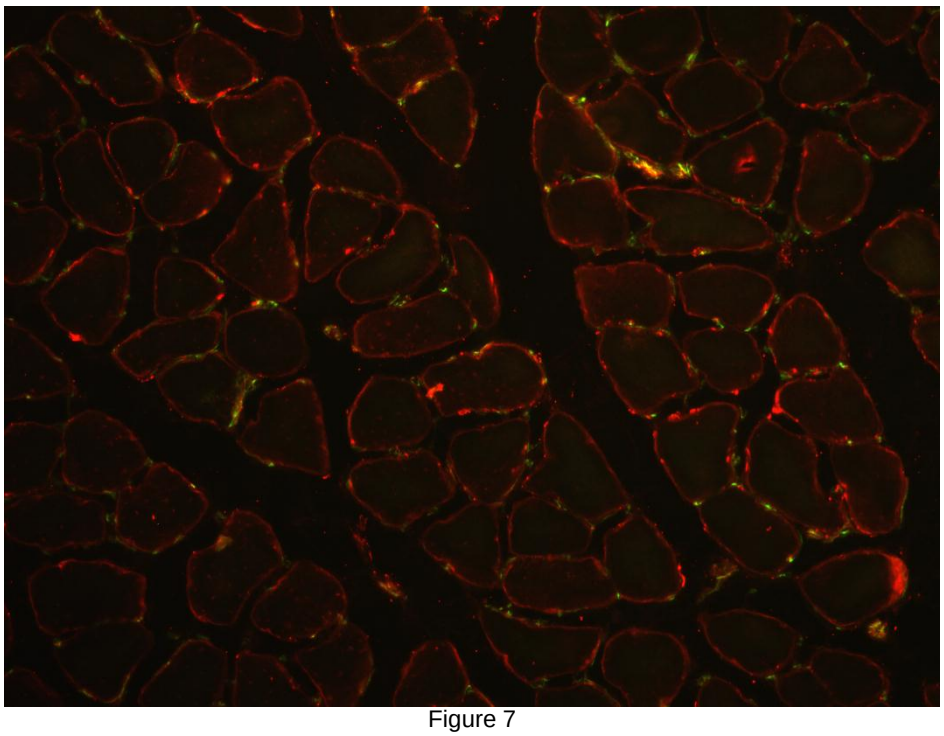

Figure 7

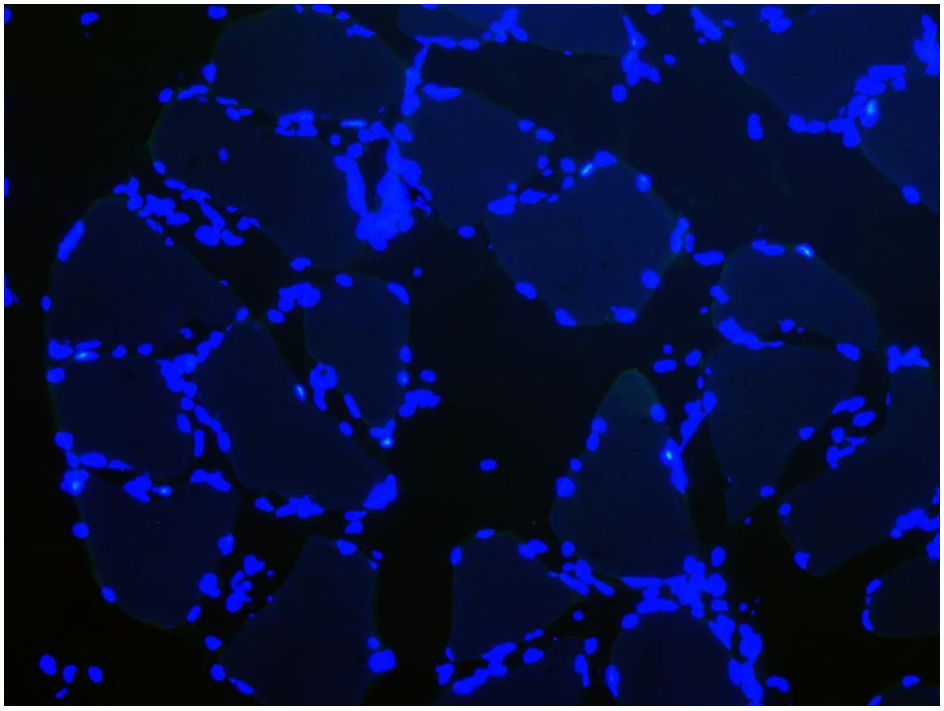

Figure 8

Capítulo IV

Conclusões e Perspetivas futuras

Para a realização deste trabalho experimental que engloba uma equipa multidisciplinar composta por um grande número de investigadores, primeiramente foram definidos os objetivos de estudo, seguido da seleção da metodologia a ser utilizada, o que levou à obtenção de resultados, com base nos quais podemos resumir as seguintes conclusões:

- As MSCs isoladas a partir do TCU associadas a membranas de PLC, podem ser consideradas um instrumento potencialmente valioso para melhorar o resultado clínico após lesões de axonotmese. Além disso, estas células representam uma fonte não controversa de células progenitoras mesenquimatosas primitivas, que podem ser colhidas após o nascimento, criopreservadas e caso sejam descongeladas podem ser expandidas para fins terapêuticos.
- O transporte do fragmento do cordão umbilical desde o hospital/clínica até ao laboratório deve ter em atenção pelo menos dois aspetos: refrigeração e imersão numa solução. O fragmento deve vir totalmente imerso numa solução de DPSB ou HBSS a temperaturas refrigeradas de forma a manter a sua integridade histológica e o período de transporte não deve ultrapassar as 96 horas. Como os kits para recolha de SCU e TCU já vêm providos de termoacumuladores, o problema da refrigeração é ultrapassado sendo por isso apenas necessário o fornecimento de uma embalagem individual de DPBS ou HBSS.
- A lavagem do fragmento do cordão umbilical aquando da sua chegada ao laboratório e antes do seu processamento e criopreservação é de extrema importância para evitar contaminações microbiológicas das amostras. Este método consiste na lavagem do fragmento 4 a 5 vezes em solução de DPBS (dependendo do grau de contaminação, especialmente com sangue), uma passagem por álcool 70% (para desinfeção) e por fim mais uma passagem por em DPBS (para retirar o álcool).
- Os protocolos de processamento e de criopreservação de unidades de TCU, adaptados pelo laboratório Biosckin, são adequados para preservar a sua viabilidade, uma vez que foi possível isolar e expandir as MSCs após descongelação apropriada e na presença de condições de cultura adequadas às células.
- Os protocolos definidos para culturas celulares de MSCs são eficientes tanto para isolar e expandir células a partir da geleia de Wharton do cordão umbilical como para fazer cultura de uma linhagem de MSCs como aquela da Promocell.
- Esta linha celular de células MSCs da Promocell são adequadas para serem utilizadas em ensaios in vivo em lesões de axonotmese e lesões traumáticas musculares no modelo experimental de rato, uma vez que o número de MSCs obtido é maior num menor tempo de cultura, não é dependente da disponibilidade de doadores

e da autorização da comissão de ética dos Hospitais / Clínicas. Além disso, o protocolo de expansão é realizado em menos tempo o que é vantajoso para ensaios pré-clínicos com um número elevado de animais experimentais, quando comparado to o protocolo de isolamento a partir de TCU fresco ou criopreservado.

- A utilização de MSCs da geleia de Wharton em lesões de miectomia do músculo tibial anterior no modelo experimental do rato apresentam resultados promissores, sendo prioridade a identificação de um veículo adequado para a aplicação destas células neste tipo de lesão muscular traumática.
- Com a avaliação do grau de inflamação que um biomaterial pode causar na zona do animal onde é implantado, neste caso no dorso dos ratos, foi possível classificar qual o mais irritante para o animal e permite avaliar a biocompatibilidade de um material antes da realização da sua utilização em ensaios pré-clínicos (ensaios animais) e ensaios clínicos. A membrana de PLC demonstrou não ser irritante e por isso pode ser considerada uma boa escolha para ser implantada numa lesão em associação com um sistema celular como as MSCs. Por outro lado a membrana de PLGA-HA é severamente irritante, o que seria prejudicial a sua implantação no local da lesão.

Os resultados obtidos ao longo do nosso trabalho vêm comprovar a importância de haver um estudo longitudinal que incluí:

- Desenvolvimento de sistemas celulares, capazes de serem diferenciados *in vitro* em células neuronais (modelo experimental de células estaminais – células estaminais de origem extra-fetal – células estaminais hematopoiéticas do sangue do cordão umbilical e células estaminais mesenquimatosas da geleia de Wharton);
- Desenvolvimento de biomateriais reabsorvíveis (PLGA, PLC, PVA, quitosano, colagénio) para utilização em lesões do nervo periférico, em lesões traumáticas do músculo esquelético e em preenchimentos de defeitos ósseos;
- Experimentação *in vivo* dos *scaffolds* desenvolvidos, em lesões de axonotmese e de neurotmese (com e sem perda de substância) no modelo rato;
- Desenvolvimento e adequação da análise funcional (incluindo análise cinemática) e morfológica;
- Transferência de conhecimento e de procedimentos para a aplicação clínica em Medicina e em Medicina Veterinária.

Todas as investigações que têm sido realizadas têm como objetivo obter melhores resultados, de modo a que os procedimentos utilizados possam ser extrapolados para a Medicina Humana.

Este estudo em conjunto com os inúmeros estudos que existem nesta área esperam contribuir para um avanço na Engenharia de Tecidos. A Engenharia de Tecidos é um campo interdisciplinar de investigação que aplica os princípios da engenharia e de ciências da vida para o desenvolvimento de substitutos biológicos que restauram, mantém ou melhoram a função dos tecidos (Langer & Vacanti, 1993).

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Capítulo V

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