

DOUTORAMENTO

CIÊNCIAS VETERINÁRIAS

Cell-based therapies in
Regenerative Medicine:
The therapeutic potential of
umbilical cord and dental pulp
derived stem/stromal cells
Ana Rita Caseiro

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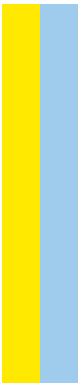
Ana Rita Caseiro. Cell-based therapies in Regenerative
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Cell-based therapies in Regenerative Medicine:
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Ana Rita Caseiro Santos



ANA RITA CASEIRO SANTOS

CELL-BASED THERAPIES IN REGENERATIVE MEDICINE:

**THE THERAPEUTIC POTENTIAL OF UMBILICAL CORD AND DENTAL PULP
DERIVED STEM / STROMAL CELLS**

Tese de Candidatura ao grau de Doutor em Ciências Veterinárias, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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DECLARATION:

The author of this Thesis declares that, in accordance with “nº2, alinea a, do Art.º 31º do Decreto-Lei nº 230/2009”, she afforded a major contribution to the design and technical execution of the work, interpretation of the results and manuscript preparation resulting in the following published, accepted and submitted articles:

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Book Chapters:

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Pinto PO, Atayde LM, Caseiro AR, Mendonça C, Santos JD, Maurício AC (2017). *Clinical application of macroporous ceramic to promote bone healing in veterinary clinical cases*. Scaffolding in Bioengineering - Materials, Technologies, Clinical Applications. Francesco Baino (Ed.), ISBN 978-953-51-5430-3, InTech.

Alvites R, AR Caseiro, ASP Varejão, AC Maurício (2017). *Olfactory Mucosa Mesenchymal Stem Cells and Biomaterials - a New Combination to Regenerative Therapies after Peripheral Nerve Injury?*. Mesenchymal Stem Cell, Phuc Van Pham (Ed.). InTech. ISBN 978-953-51-5280-4.

Pinto PO, Atayde LM, Campos JM, Caseiro AR, Pereira T, Mendonça C, Santos JD, Maurício AC (2016) *Therapeutic Strategies for Bone Regeneration: The Importance of Biomaterials Testing in Adequate Animal Models*. Advanced Materials Series - Advanced Composite Materials. Ashutosh Tiwari, Mohammad Rabia Alenezian, Seong Chan Jun (Ed.), ISBN: 978-1-119-24253-6, John Wiley and Sons

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Flores AR, Amorim I, Pedrosa SS, Caseiro AR, França M, Rêma A, Matos R, Oliveira C, Alves A, Oliveira E, Lobo-da-Cunha A, Porto B, Maurício AC, Seixas F, Gärtner F (2018), *Characterization of a short-term culture from a canine diffuse gastric carcinoma*. Journal of Translational Science 5: DOI: 10.15761/JTS.1000315

Campos JM, Sousa AC, Pinto PO, Ribeiro J, França ML, Caseiro AR, Pedrosa S, Branquinho MV, Mendonça C, Atayde LM, Santos JD, Luís AL, Maurício AC (2018). *Application of Bonelike® as synthetic bone graft in orthopaedic and oral surgery in veterinary clinical cases*. In Biomaterials Research, <https://doi.org/10.1186/s40824-018-0150-x>

Branco M, Caseiro AR, Silva DM, Amorim I, Rêma A, Pedrosa SS, Branquinho MV, Gomes PS, Fernandes MHR, Santos JD, Mauricio AC, and Sencadas V (2018), *Processing, characterization and in-vivo evaluation of PLLA fish gelatin electrospun membranes for biomedical applications*, ACS Appl. Bio Mater DOI: 10.1021/acsabm.8b00023

Alvites RA, Caseiro AR, Pedrosa SS, Branquinho MV, Varejão ASP, Geuna S, Ronchi G, Maurício AC (2018). *Peripheral nerve injury and axonotmesis: state of the art and recent advances*. Cogent Medicine, 5(1). DOI: 10.1080/2331205X.2018.1466404

Alvites RD, Caseiro AR, Pedrosa SS, Branquinho MV, Varejão ASP, Maurício AC (2018). *The Nasal Cavity of the Rat and Mouse - Source of Mesenchymal Stem Cells for treatment of Peripheral Nerve Injury*. The Anatomical Records (Hoboken). DOI: 10.1002/ar.23844.

Ribeiro J, Caseiro AR, Pereira T, Armada-da-Silva PAS, Pires I, Prada J, Amorim I, Leal Reis I, Amado S, Santos JD, Bompasso S, Raimondo S, Varejão ASP, Geuna S, Luís AL, Mauricio AC (2017). *Evaluation of PVA biodegradable electric conductive membranes for nerve regeneration in axonotmesis injuries – the rat sciatic nerve animal model*. Journal of Biomedical Materials Research Part A. doi: 10.1002/jbm.a.35998.

Alexandre N, Amorim I, Pereira T, Alvites R, Caseiro AR, Rêma A, Gonçalves A, Valadares G, Costa E, Santos-Silva A, Rodrigues M, Lopes MA, Almeida A, Santos JD, Maurício AC, Luís AL (2017) *Long term performance evaluation of small-diameter vascular grafts based on polyvinyl alcohol hydrogel and dextran and MSCs-based therapies using the ovine pre-clinical animal model*. Special issue on “Biomaterials for tissue engineering” - International Journal of Pharmaceutics. 513(1-2):332-346. pii: S0378-5173(17)30133-3. doi: 10.1016/j.ijpharm.2017.02.043.

Caseiro AR, Ribeiro J, Pereira T, Santos JD, Amorim I, Luís AL, Maurício AC (2017). *Neuro-muscular regeneration using scaffolds with mesenchymal stem cells (MSCs) isolated from human umbilical cord Wharton's jelly*. Ciência & Tecnologia dos Materiais Special Issue on Ciência e Tecnologia dos Materiais 27: 01–80.

Silva DM, Caseiro AR, Amorim I, Pereira I, Faria F, Pereira T, Santos JD, Gama F, Maurício AC (2016) *Inflammatory response to dextrin-based hydrogel associated with hMSCs, urinary bladder matrix and Bonelike® in rat subcutaneous implants*. Biomedical Materials. 11(6):065004.

Ribeiro J, Pereira T, Caseiro AR, Armada-da-Silva P, Pires I, Prada J, Amorim I, Amado S, França M, Gonçalves C, Lopes MA, Santos JD, Silva DM, Geuna S, Luís AL, Maurício AC (2015) *Evaluation of biodegradable electric conductive tubeguides and mesenchymal stem cells*. World Journal of Stem Cells, 2015. 7(6): p. 956-975, doi: 10.4252/wjsc.v7.i6.956.

Caseiro AR, Pereira T, Ribeiro J, Amorim I, Faria F, Bártolo PJ, Luís AL, Maurício AC (2015). *Neuro-muscular regeneration using scaffolds with mesenchymal stem cells (MSCs) isolated from human umbilical cord Wharton's jelly: functional and morphological analysis using rat sciatic nerve neurotmesis injury model*. Procedia Engineering - 4th International Conference on Tissue Engineering, ICTE2015, doi: 10.1016/j.proeng.2015.07.016

Pereira T, Ivanova G, Caseiro AR, Barbosa P, Bártolo PJ, et al. (2014) *MSCs Conditioned Media and Umbilical Cord Blood Plasma Metabolomics and Composition*. PLoS ONE 9(11): e113769. doi:10.1371/journal.pone.0113769

Ribeiro J, Pereira T, Amorim I, Caseiro AR, Alexandre N, Lopes M, Lima J, Gartner A, Santos JD, Bartolo P, Rodrigues JM, Mauricio AC and Luís AL (2014) *Cell Therapy with human MSCs isolated from the umbilical cord Wharton jelly associated to a PVA membrane in the treatment of chronic skin wounds*, International Journal of Medical Sciences, Volume 11, Issue 10, 11 July 2014, Pages 979-987, DOI: 10.7150/ijms.9139, EID: 2-s2.0-84905439471

Pereira T, Gartner A, Amorim I, Almeida A, Caseiro AR, Armada-da-Silva P, Amado S, Fregnan F, Varejão ASP, Santos JD, Bartolo P, Geuna S, Luís AL and Mauricio AC. (2014) *Promoting nerve regeneration in a neurotmesis rat model using poly(DL-lactide-e-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly: in vitro and in vivo analysis*. 2014. BioMed Research International, DOI: 10.1155/2014/302659, EID: 2-s2.0-84904694747

Pereira T, Armada-da Silva PAS, I Amorim I, Rêma A, Caseiro AR, Ribeiro J, Gartner A, Santos JD, Rodrigues M, Lope MA, Bártolo PJ, Luís AL, Maurício AC. (2014) *Effects of Human Mesenchymal Stem Cells isolated from the Wharton's jelly of the umbilical cord and conditioned media on skeletal muscle regeneration using a myectomy model*, Stem Cells International, doi: <http://dx.doi.org/10.1155/2014/376918>

INTELLECTUAL PROPERTY:

The work developed within the scope of the present project resulted in relevant work submitted for intellectual property protection:

Universidade do Porto internal reference: UP- 012019; INPI - Instituto Nacional da Propriedade Industrial: PAT No 20181000018334; Patent Cooperation Treaty: PCT/IB2019/052006; *Priority date*: 12.03.2019 – PT; International Publication Number: WO 2019/175773 A1, *International Publication date*: 19.09.2019), entitled:

“Compositions for use in the treatment of musculoskeletal conditions and methods for producing the same leveraging the synergistic activity of two different types of mesenchymal stromal/stem cells”

By the inventors: Jorge Miguel Pereira de Oliveira da Silva Santos, Sílvia Marlene Almeida Santos Pedrosa, **Ana Rita Caseiro**, Mariana Esteves Vieira Branquinho, Rui Damásio Alvites, Sérgio José Póvoas Camões, Joana Saraiva Rodrigues, Joana Paiva Gomes Miranda, Ana Colette Pereira de Castro Osório Maurício

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“Photopolymerized Biodegradable Copolymer Formulations for Biomedical Applications”

By the inventors: Jorge Fernando Jordão Coelho, Ana Clotilde Fonseca, Arménio Serra, José Domingos dos Santos, Ana Colette Maurício, Sílvia Santos Pedrosa, **Ana Rita Caseiro**, Mariana Vieira Branquinho, Rui Damásio Alvites.

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Abbreviations list

%: percentage	BMP-2: Bone Morphogenetic Protein 2
(v/v): volume to volume concentration	BSP: Bone Sialoprotein
°C: degrees Celsius	BVC: Bupivacaine chemical model
μM: micromolar	c: canine derived cells
¹ H-NMR: proton Nuclear Magnetic Resonance	Ca ²⁺ : Calcium ion
1-Me-His: 1-Methylhistidine	CD: Cluster of differentiation
1α,25-(OH) ₂ D ₃ : 1α,25-Dihydroxyvitamin D ₃	CDNF: Cerebral Dopamine Neurotrophic Factor
2D: two-dimensional	CFU: Colony Forming Units
3D: tri-dimensional	Cho: Choline
3-Me-His: 3-Methylhistidine	Citr: Citrulline
Ace: Acetate	CM: Conditioned Medium
ADP/ATP: Adenosine (Di/Tri Phosphate)	CNS: Central Nervous System
ADVENT: Ad Hoc Expert Group on Veterinary Novel Therapies	CNTF: Ciliary Neurotrophic Factor
AF-MSCs: Amniotic Fluid-derived Mesenchymal Stem/ Stromal Cells	CO ₂ : Carbon Dioxide
Ala: Alanine	COL1A1: Collagen Type I Alpha 1 Chain
ALP: Alkaline Phosphatase	COL3A1: Collagen Type III Alpha 1 Chain
ALPL: Alkaline Phosphatase, Liver/Bone/Kidney	Coll I: Collagen Type I
AMSC: Adipose-derived Stem cells	Coll: Collagen type
ANOVA: Analysis of variance	CPD: Citrate-Phosphate-Dextrose
APDCs/SCAPs: Stem/ Stromal Cells from human permanent teeth apical dental pulp	Cr: Creatine
ARS: Alizarin Red S	CR: Crush Injury model
AT-MSCs: Adipose Tissue-derived Mesenchymal Stem/ Stromal Cells	CSF: Colony Stimulating Factor
Avg: Average	CTX: Cardiotoxin chemical model
BA: Bile Acids	CVMP: Committee for Medicinal Products for Veterinary Use
BDNF: Brain-Derived Neurotrophic Factor	CXCR: Chemokine receptor
BHI-T: Brain Heart Infusion Broth	D ₂ O: Deuterium Oxide
BM-MSCs: Bone Marrow-derived Mesenchymal Stem/ Stromal Cells	DBBM: Deproteinized Bovine Bone Mineral
	Dex: Dexamethasone
	DF-MSCs: Dental Follicle-derived Mesenchymal Stem/ Stromal Cells
	DGS: Direção Geral de Saúde
	DMD: Duchenne' Muscular Dystrophy

DMEM: Dulbecco's Modified Eagle Medium	GDNF: Glial cell-Derived Neurotrophic Factor
DMP-1: Dentin Matrix acidic Phosphoprotein 1	GDPB: Granular Deproteinized Bovine Bone
DMSO: Dimethyl Sulfoxide	Glu/Gln: Glutamate/ Glutamine
DNA: Deoxyribonucleic Acid	GM-CSF: Granulocyte Macrophage Colony-Stimulating Factor
DPBS: Dulbecco's Phosphate-Buffered Saline	GMP: Good Manufacturing Practices
DPSCs: Dental Pulp-derived Mesenchymal Stem/ Stromal Cells	GRO(pan): Growth-regulated protein
DSPP: Dentin Sialophosphoprotein	GTN: Gastrocnemius muscle
DTSCs: Deciduous Teeth Stem/ Stromal Cells	h: human derived cells
EC: European Commission	HA/TCP/Ch: Hydroxyapatite/tricalcium-phosphate/chitosan
ECM: Extracellular Matrix	HA: Hydroxyapatite
EDTA: Ethylenediaminetetraacetic acid	HGF: Hepatocyte Growth Factor
EGF: Epidermal Growth Factor	His: Histidine
FGF-2/ bFGF: (basic) Fibroblast Growth Factor 2	HLA: Huma Leukocyte Antigen
EMA: European Medicines Agency	HT-PCL: Hyaluronic acid-Tricalcium phosphate - Polycaprolactone
EPCs: Endothelial Progenitor Cells	IFN: Interferon
EPO: Erythropoietin	IGF: Insulin-like Growth Factor
EtOH/HB: Ethanol/ Hydroxybutyrate	IGFBP-7: Insulin-like Growth Factor-Binding Protein 7
FBS: Foetal Bovine Serum	IL: Interleukin
FCS: Foetal Calf Serum	IP-10: Interferon gama-induced Protein 10
FDA: Food and Drug Administration	ISCT: International Society for Cellular Therapy
FDBA: Freeze-Dried Bone Allograft	ITS: Insulin-Transferrin-Sodium selenite
Flt-3L: fms-related tyrosine kinase3 ligand	IU: International Units
For: Formate	KH ₂ PO ₄ : Potassium dihydrogen phosphate
FOSL1: FOS Like 1	LAC: Laceration injury model
FT: Fourier Transform	Lac: Lactate
G: Birmingham wire gauge (needle size)	LIF: Leukaemia Inhibitory Factor
GAGs: Sulphated Glycosaminoglycans	Lip: Lipids
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase	LPS: Lipopolysaccharide
G-CSF: Granulocyte-Colony Stimulating Factor	LSCD: Limbal Stem Cell Deficiency
	Lys/Arg: Lysine/Arginine

M ± SD: Mean ± Standard Deviation	ON: Osteonectin
M ± SEM: Mean ± Standard Error of the Mean	OPN: Osteopontin
m: murine/ mouse derived cells	ORO: Oil Red O
Max: maximum	OSX: Osterix
MCP: Monocyte Chemotactic Protein	P: culturing passage
MDC: Macrophage-Derived Chemokine	Pax ⁷ : Muscle-specific paired box7
MDPSCs: Mobilized dental pulp stem cells	PCA: Principal Component Analysis
MDSCs: Skeletal Muscle-derived Mesenchymal Stem/ Stromal Cells	PCL: Polycaprolactone
MEPE: Matrix Extracellular Phosphoglycoprotein	PDGF: Platelet Derived Growth Factor
MHC: Major Histocompatibility Complex	PDL: Periodontal ligament
MI: Myocardial Infarction	PDL-MSCs/ PDLSCs: Periodontal Ligament-derived Mesenchymal Stem/ Stromal Cells
Min: minimum	PDT: Population Doubling Time
MIP: Macrophage Inflammatory Protein	pg/mL: picograms per millilitre
mm: millimetre	PheAla: Phenylalanine
mM: millimolar	PI: Propidium Iodine
mm ² : area in square millimetre	PICs: PW ¹⁺ / Pax ⁷⁻ interstitial cells
MPCs: Muscle Progenitor Cells	PLA: Poly Lactic acid
MRFs: myogenic regulatory factors	PLGA: Poly-lactic-glycolic acid
ms: milliseconds	pM: picomolar
MSCs: Mesenchymal Stem/ Stromal Cells	PNS: peripheral Nervous System
NGF: Nerve Growth Factor	ppm: Parts Per Million
nHA: nano Hydroxyapatite	Pro: Proline
nHAC/PLA: nano-Hidroxiapatite/ Collagen/poli-L-Lactic acid	PRP: Platelet Rich Plasma
nm: nanometres	qPCR: Quantitative Polymerase chain reaction
NMR: Nuclear Magnetic Resonance	r: rat/ rodent derived cells
NT: Neurotrophin	ra: rabbit derived cells
NURR1: Nuclear receptor related 1 protein	RA: Retinoic Acid
o: ovine derived cells	RANTES or CCL5: chemokine (C-C motif) ligand 5
O ₂ : Oxygen	RBC: Red Blood Cells
OCN: Osteocalcin	RGD: Arginylglycylaspartic acid
OD: Optical Density	rhBMP-2: recombinant human Bone Morphogenetic Protein 2
OLIG2: Oligodendrocyte transcription factor	

RICE: rest, ice/ cold, compression and elevation	TSP: Sodium Trimethylsilyl-[2,2,3,3-d4]-Propionate
RNA: Ribonucleic Acid	Tyr: Tyrosine
RQ: Relative Quantification	U/mL: units per millilitre
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction	UCB: Umbilical Cord Blood
RUNX: Runt Related Transcription Factor2	UCB-MSCs: Umbilical Cord Blood-derived Mesenchymal Stem/ Stromal Cells
SC: Schwann Cells (Chapter II)	UCBP: Umbilical Cord Blood Plasma
sCD40L: soluble CD40 ligand	UCB-PRP: Umbilical Cord Blood-derived Platelet Rich Plasma
SCs: Satellite Cells	UCBS: Umbilical Cord Blood Serum
SD: Standard Deviation	UC-MSCs: Umbilical Cord Stroma/ Wharton's Jelly-derived Mesenchymal Stem/ Stromal Cells
SDF-1: Stromal Derived Growth Factor	UCT: Umbilical Cord Tissue
SEM: Standard Error of the Mean	Val/Leu/Ile: Valine, Leucine, Isoleucine
SHEDs: Stem cells from Human Exfoliated Deciduous teeth	VCAM-1: Vascular Cell Adhesion Protein 1
SkM-MSCs: Skeletal Muscle-derived Mesenchymal Stem/ Stromal Cells	VEGF: Vascular Endothelial Growth Factor
SL: Soleus muscle	VGFR: VEGF receptor
SMF: Static magnetic field	VML: Volumetric Muscle Loss/ Lesion model
SM-MSCs: Synovial Membrane-derived Mesenchymal Stem/ Stromal Cells	WBC: White Blood Cells
SPP1: Secreted Phosphoprotein 1	xg: number of times the gravitational force
STRO-1: Stromal Cell Precursor Surface Antigen	α -Glu: α -Glucose
SVDs: Singular value Decomposition	α MEM: Minimum Essential Medium Eagle - Alpha Modification
TA: Tibialis anterior muscle	α -SMA: alfa Smooth Muscle Actin
TCP: Tricalcium phosphate	β -Glu: β -Glucose
TGF: Transforming Growth Factor	μ g/mL: micrograms per millilitre
Thr: Threonine	μ L: microliter
TNC: Total Nucleated Cells	μ M: micromolar
TNF: Tumoral Necrosis Factor	
Try: Tryptophan	

Resumo geral

Os sistemas biológicos possuem uma capacidade intrínseca de reparação e regeneração tecidual, mas a eficácia dessa resposta pode ser limitada por múltiplos fatores, resultando na regeneração incompleta ou inadequada dos tecidos. Apesar de importantes desenvolvimentos técnicos e científicos alcançados nesta área nas últimas décadas, não foram ainda identificadas estratégias definitivas para superar as limitações dos mecanismos orgânicos nativos ou das técnicas médicas e cirúrgicas atualmente disseminadas. Mais recentemente, o reconhecimento do papel das populações de Células Estaminais/ Estromais Mesenquimatosas nas estratégias regenerativas impulsionou uma nova linha de investigação para a otimização dos processos de reparação e regeneração tecidual.

O presente projeto teve como objetivo contribuir para o avanço do conhecimento científico na área de investigação da Medicina Regenerativa, focando o desenvolvimento de sistemas celulares inovadores para aplicação terapêutica, visando a regeneração de tecidos moles problemáticos, como os tecidos neuromusculares.

Primeiramente, os mecanismos que presidem aos processos de regeneração tecidual foram investigados e o estado da arte das abordagens regenerativas foi definido para cada tecido-alvo (**Chapter I – Section 1.1 - Mesenchymal Stem/ Stromal Cells application for skeletal muscle repair and regeneration** e **Section 1.2 - Mesenchymal Stem/ Stromal Cells application for peripheral nerve repair and regeneration**). Globalmente, a associação de populações de natureza estaminal/ estromal mesenquimatosa resultou em vantagem regenerativa por meio de ações específicas em cada um dos estágios de reparação/ regeneração do tecido muscular esquelético e do sistema nervoso periférico.

Considerando a multiplicidade de fontes celulares reportadas, a investigação concentrou-se na seleção das fontes celulares mais adequadas, considerando os requisitos técnicos e biológicos impostos para a aplicação terapêutica efetiva dos sistemas desenvolvidos. As Células Estaminais/ Estromais Mesenquimatosas derivadas do tecido da polpa dentária foram, portanto, propostas como candidatas, e métodos adequados para o seu isolamento e cultura foram propostos e validados (**Chapter II - Section 2.1 - Dental Pulp Stem/ Stromal Cells as an innovative source for therapies development**). Determinou-se que o método de explante seria o mais adequado para o isolamento celular, principalmente devido à limitação na quantidade de tecido pulpar disponível para processamento em cada dente. Foram isoladas populações de proliferação rápida, cromossómicamente estáveis, que demonstraram possuir as

características fenotípicas e funcionais desejadas das populações estaminais/estromais mesenquimatosas (expressão de marcadores de superfície, incluindo das principais moléculas de histocompatibilidade, e capacidade de diferenciação em tecidos específicos).

Após a confirmação de que as populações isoladas apresentavam as características necessárias, a investigação prosseguiu no sentido do refinamento do sistema celular, para atender aos requisitos regulatórios aplicáveis para sua utilização clínica, e para compreender os mecanismos que presidem aos seus efeitos regenerativos. Em ambas as perspectivas, o sistema derivado de células dentárias foi avaliado em comparação com a população derivada do estroma do cordão umbilical, previamente estabelecida e explorada pelo grupo de investigação.

Visando a implementação de métodos de cultura isentos de xeno-elementos, o plasma do sangue do cordão umbilical foi proposto como um substituto para o suplemento convencionalmente utilizado em cultura celular, o soro fetal bovino. Para o efeito, foram utilizadas técnicas de ressonância magnética nuclear, a fim de avaliar a composição metabolómica dos suplementos (**Chapter III – Section 3.1. - Metabolomic profile of human Umbilical Cord Blood Plasma and Foetal Bovine Serum, and their effects on human Mesenchymal Stem/ Stromal Cells populations, Part I**). Ambos os suplementos apresentaram perfis de metabolitos comparáveis, com exceção para o teor de glucose (isoformas α e β), o qual foi determinado como o principal componente divergente entre os dois suplementos, tendo sido detetado em maiores quantidades nas amostras de plasma do sangue do cordão umbilical. Outras diferenças subtis também foram destacadas, como os níveis de lactato, treonina, valina/ leucina/ isoleucina (aminoácidos essenciais), alanina, glutamato/ glutamina e prolina (aminoácidos não essenciais), que foram predominantemente identificados no soro fetal bovino. Quanto ao conteúdo em fatores bioativos, os suplementos foram analisados através da análise por multiplexação (**Chapter III – Section 3.2 - Bioactive factors content of human Umbilical Cord Blood Plasma and Foetal Bovine Serum: a preliminary comparative characterization**). Foram apenas extraídas conclusões preliminares deste ensaio, devido às diferentes espécies de origem dos suplementos analisados e à disponibilidade limitada de métodos reativos entre espécies. Os resultados sugeriram que o plasma do sangue do cordão umbilical forneceria níveis superiores de diversos fatores bioativos, incluindo o TGF- β 1, o GRO α , a MCP-1, o MDC, o PDGF-AA, o PDGF-BB e RANTES. Adicionalmente, o desempenho comparativo dos suplementos foi investigado para a cultura das populações de Células Estaminais/ Estromais Mesenquimatosas derivadas do estroma do cordão umbilical e da polpa dentária (**Chapter III – Section 3.1 -**

Metabolomic profile of human Umbilical Cord Blood Plasma and Foetal Bovine Serum, and their effects on human Mesenchymal Stem/ Stromal Cells populations, Part II). O plasma do sangue do cordão umbilical proporcionou taxas de proliferação superiores às obtidas com soro fetal bovino, sem induzir atividade senescente nas células em cultura. No entanto, o plasma do sangue do cordão umbilical foi associado ao aumento dos eventos de apoptose precoce e morte celular, acompanhando o crescente conteúdo de suplemento no meio. Estes eventos foram particularmente observados nas Células Estaminais/ Estromais Mesenquimatosas derivadas do estroma do cordão umbilical. Ambas as populações demonstraram manter o padrão de expressão de marcadores fenotípicos, embora tenham sido observadas modificações internas na expressão génica, em resposta às diferentes formulações de meio. Também se determinou que a capacidade de diferenciação multilinhagem das populações celulares foi afetada pelo plasma do sangue do cordão umbilical, que bloqueou a diferenciação osteogénica enquanto promoveu a diferenciação adipogénica. Observou-se ainda que a cultura de micromassa mantida em meio suplementado com plasma do sangue do cordão umbilical (controlos indiferenciados) apresentou maior deposição de matriz condrogénica em relação às células suplementadas com soro fetal bovino. As diferenças observadas foram atribuídas ao teor diferencial de glucose observado nas duas opções de suplemento, que se relacionaram fortemente com os métodos utilizados para a colheita do sangue do cordão umbilical utilizado para a produção do plasma, bem como devido ao teor distinto de fatores bioativos presente.

Considerando que a secreção de fatores bioativos é sugerida como a principal via para o efeito regenerativo das Células Estaminais/ Estromais Mesenquimatosas no músculo esquelético e no tecido nervoso periférico, a capacidade secretora das Células Estaminais/ Estromais Mesenquimatosas do estroma do cordão umbilical e da polpa dentária foi investigada (***Chapter IV – Section 4.1: Metabolomic and Bioactive Factors Secretory profile of human Mesenchymal Stem/ Stromal Cells populations***). A espectroscopia por ressonância magnética nuclear foi aplicada ao meio condicionado produzido pelas Células Estaminais/ Estromais Mesenquimatosas, observando-se a dinâmica dos metabolitos energéticos durante o período de condicionamento. Uma avaliação adicional do conteúdo de fatores bioativos do secretoma foi realizada visando um amplo painel de fatores de crescimento, quimiocinas e citocinas, que incluiu moléculas pró-angiogénicas, anti-apoptóticas, pró-mitogénicas, antifibróticas e quimiotáticas. As diferenças mais significativas na secreção das duas populações celulares foram encontradas nos níveis de FST, HGF, G-CSF, IL-8 e MCP-1 (secretadas de forma mais expressiva pelas células derivadas do estroma do cordão

umbilical), enquanto as células derivadas da polpa dentária proporcionaram elevados níveis de VEGF-A (e FST). O potencial regenerativo do secretoma foi investigado em relação aos efeitos pró-angiogénico e quimiotático de seus conteúdos. Concluiu-se que o perfil secretório distinto não resultou em efeitos significativamente diferentes no comportamento de populações de células endoteliais (proliferação *in vitro*, migração e capacidade de formação de tubos) e que ambos os perfis de secreção preveniram a senescência e a apoptose, e promoveram a quimiotaxia das próprias populações celulares.

Os resultados individuais de cada tarefa do projeto foram finalmente integrados e discutidos, e foram propostas direções futuras para a continuação de desenvolvimento de conhecimentos (**Chapter V – General Discussion, Conclusions and Future directions**). Em resumo, foi demonstrado que populações apresentando as características de Células Estaminais/ Estromais Mesenquimatosas podem ser facilmente isoladas de dentes adultos, apresentando características comparáveis às originadas de outros tecidos, nomeadamente do estroma do cordão umbilical. Estas populações celulares demonstraram ainda serem suscetíveis de se integrar em métodos de cultura isentos de xeno-elementos, particularmente utilizando produtos derivados do sangue do cordão umbilical (plasma) em substituição de componentes derivados de animais, classicamente utilizados na cultura de células de mamífero. Os métodos isentos de xeno-elementos demonstraram sustentar a proliferação celular, mas também demonstraram afetar as populações em estudo, nomeadamente quanto à sua expressão génica e capacidade de diferenciação, vinculando o desenvolvimento posterior destes sistemas a uma investigação profunda do impacto de tais modificações na segurança e desempenho regenerativo dos sistemas celulares. Em relação aos mesmos sistemas celulares utilizando métodos clássicos de cultura (suplementação soro fetal bovino), uma extensa análise do secretoma foi apresentada, sendo pioneira na abordagem comparativa da metabolómica e da secreção de fatores bioativos das duas fontes celulares. Apesar das diferenças significativas identificadas no perfil de secreção de fatores bioativos, os ensaios *in vitro* e *in vivo* indicaram que ambas as populações celulares projetam ter um desempenho semelhante na promoção dos eventos angiogénicos em múltiplos cenários regenerativos, incluindo o músculo esquelético e nervo periférico. Esses sistemas inovadores podem ser adicionalmente desenvolvidos visando não apenas a aplicação em medicina humana, mas também considerando outras espécies veterinárias clinicamente relevantes, como equinos e canídeos, que apresentam patologias e desafios terapêuticos semelhantes.

General abstract

Biological systems hold an inherent capacity for tissue repair and regeneration, but the effectiveness of this response may be limited by multiple factors, resulting in suboptimal regenerative outcomes. Despite relevant technical and scientific developments on the topic over the last decades, no definitive strategies have yet been proposed to overcome the limitations of the native organic mechanisms or currently employed medical and surgical techniques. More recently, the reconnaissance of the role of Mesenchymal Stem/ Stromal Cells populations in the regenerative strategies propelled a new line of research towards optimised tissue repair and regeneration.

The current project aimed to contribute to scientific advancements in the Regenerative Medicine field of research, towards the development of innovative cellular systems for therapeutic application, targeting the regeneration of challenging soft tissues, such as the neuromuscular tissue.

In the first instance, the mechanisms presiding to the tissue regeneration processes were investigated, and the 'state of the art' on regenerative approaches was defined for each tissue target (**Chapter I – Section 1.1 - Mesenchymal Stem/ Stromal Cells application for skeletal muscle repair and regeneration** and **Section 1.2 - Mesenchymal Stem/ Stromal Cells application for peripheral nerve repair and regeneration**). General observations indicated that the association of populations of mesenchymal stem/ stromal nature resulted in improved regenerative outcomes by specific effects impacting on each of the repair/ regeneration stages in skeletal muscle and peripheral nervous systems.

Considering the multitude of cellular sources reported, research focused on the selection of suitable cellular sources considering the technical and biological requisites imposed for the effective therapeutic application of the developed systems. The dental pulp tissue derived Mesenchymal Stem/ Stromal Cells were therefore selected as candidates, and isolation and culturing methods were proposed and validated (**Chapter II - Section 2.1 - Dental Pulp Stem/ Stromal Cells as an innovative source for therapies development**). It was determined that the explant/ outgrowth method would be the most adequate for cellular isolation, mostly due to the limited dental pulp tissue available for processing in each tooth. Fast expanding populations were obtained, while maintaining their chromosomal stability, and determined to present the phenotypical and functional characteristics desired of such populations (surface markers expression, including that of major histocompatibility molecules, and tissue-specific differentiation capacities).

Once determined that the populations isolated presented the necessary characteristics, research followed towards the refinement of the cellular system, to comply with the

regulatory requisites applicable for clinical application, and to understand the mechanisms presiding to its regenerative effects. In either perspective, the dental-derived system was assessed in comparison to the classical umbilical cord stroma-derived population, previously established and explored by the research group.

Aiming at the implementation of xeno-free culturing methods, the umbilical cord blood plasma was proposed as a substituent for the conventional xenogeneic culture supplement, foetal bovine serum. For the purpose, nuclear magnetic resonance techniques were employed to assess on the metabolomic composition of the supplements (**Chapter III – Section 3.1. - Metabolomic profile of human Umbilical Cord Blood Plasma and Foetal Bovine Serum, and their effects on human Mesenchymal Stem/ Stromal Cells populations, Part I**). Both supplements presented comparable metabolite profiles, except for the glucose (α and β isoforms) content, which was determined as the principal component to distinguish the two supplements. This metabolite was detected in higher amounts in the umbilical cord blood plasma samples. Other subtle differences were also highlighted, such as in lactate, threonine, valine/leucine/isoleucine (essential aminoacids), alanine, glutamate/ glutamine and proline (non-essential aminoacids), which were dominantly identified in the foetal bovine serum. Regarding the bioactive factors content, the supplements were assayed through multiplexing analysis (**Chapter III – Section 3.2 - Bioactive factors content of human Umbilical Cord Blood Plasma and Foetal Bovine Serum: a preliminary comparative characterization**). Only preliminary conclusions were drawn from this assay, due to the distinct species origin of the sampled supplements and limited availability of cross-species reactive methods. The umbilical cord blood plasma was suggested to provide superior levels of diverse bioactive factors, including TGF- β 1, GRO α , MCP-1, MDC, PDGF-AA, PDGF-BB and RANTES. Further, the comparative performance of the supplements was investigated for the culturing of the umbilical cord stroma- and dental pulp-derived Mesenchymal Stem/ Stromal Cells populations (**Chapter III – Section 3.1 - Metabolomic profile of human Umbilical Cord Blood Plasma and Foetal Bovine Serum, and their effects on human Mesenchymal Stem/ Stromal Cells populations, Part II**). The umbilical cord blood plasma provided superior proliferations rates than foetal bovine serum, while not inducing senescent activity in the cultured cells. However, the umbilical cord blood plasma was associated with increased early apoptosis and cellular death, accompanying increasing media content of the supplement. These events were particularly observed in the umbilical cord stroma-derived Mesenchymal Stem/ Stromal Cells. Both populations demonstrated to maintain their phenotypical markers expression pattern, although internal modifications in gene expression in response to the

different media formulations were observed. The multilineage differentiation capacity was also determined to be impacted by the umbilical cord blood plasma, which hindered osteogenic differentiation and promoted adipogenic differentiation. It was further observed that the micromass cultured maintained in umbilical cord blood plasma supplemented media (undifferentiated controls) presented increased chondrogenic matrix deposition as compared to foetal bovine serum supplemented cells. The observed differences were attributed to the differential glucose content observed in the two supplement options, that significantly related to the collection methods of the umbilical cord blood utilised for the plasma production, as well as to the bioactive factor differential content.

Considering that the secretion of bioactive cues is suggested as the main pathway for the regenerative effect of Mesenchymal Stem/ Stromal Cells on skeletal muscle and peripheral nervous tissue, the secretory capacity of the umbilical cord stroma- and dental pulp-derived Mesenchymal Stem/ Stromal Cells was investigated (**Chapter IV – Section 4.1: Metabolomic and Bioactive Factors Secretory profile of human Mesenchymal Stem/ Stromal Cells populations**). Nuclear magnetic resonance spectroscopy was applied to the conditioned media produced by the Mesenchymal Stem/ Stromal Cells. The dynamics of energetic metabolites was observed and discussed through the conditioning period. Additional evaluation of bioactive factors content of the secretome was endured (through multiplexing techniques) targeting an extensive panel of growth factors, chemokines and cytokines, which included pro-angiogenic, anti-apoptotic, pro-mitogenic, antifibrotic, and chemoattractant molecules. The most significant differences in the secretion of both cellular populations were found on FST, HGF, G-CSF, IL-8 and MCP-1 (most expressively secreted by umbilical cord stroma-derived cells), while dental pulp-derived cells provided high levels of VEGF-A (and FST). The regenerative potential of the secretome was further investigated towards the pro-angiogenic and chemotactic effects of its contents. It was concluded that the distinct secretory cocktail did not result in significantly different effects on endothelial cell populations behaviour (*in vitro* proliferation, migration and tube formation capacity) and that both secretome profiles forestalled senescence and apoptosis, and presented chemoattractant properties for the cellular populations themselves.

Individual results of each task of the project are finally integrated and discussed, and future directions for continued knowledge development are proposed accordingly (**Chapter V – General Discussion, Conclusions and Future directions**). In brief, it was demonstrated that populations presenting Mesenchymal Stem/ Stromal Cells

characteristics could be easily isolated from adult teeth, presenting comparable features to those originating from other organic tissues, namely from the umbilical cord stroma. These cellular populations were further demonstrated to be amenable of integration in xeno-free culturing methods, particularly utilising umbilical cord blood products (plasma) in substitution of animal-derived components classically employed in mammalian cell culture. The xeno-free methods demonstrated to sustain cellular proliferation, but were also demonstrated to affect Mesenchymal Stem/ Stromal Cells populations, namely regarding their gene expression and multilineage differentiation capacity, binding further development to a deep investigation of such modifications and on their impact on the safety and regenerative performance of the cellular systems. Regarding the same cellular systems using classical culturing methods (foetal bovine serum supplementation), an extensive analysis on the metabolomic and bioactive factor secretion profile was presented, pioneering on the comparative approach on the metabolomic and bioactive factor secretion of the two cellular sources. Despite the significant differences identified in the bioactive secretion profile, *in vitro* and *in vivo* assays indicated that both cellular populations are expected to perform similarly in the promotion of the angiogenic events in regenerative scenarios in multiple tissues, including the skeletal muscle and peripheral nerve. These innovative systems can be further developed targeting not only human application but also considering other clinically relevant veterinary species, such as equine and canine, that present similar pathologies and therapeutic challenges.

Chapter I

The therapeutic potential of Mesenchymal Stem/ Stromal Cells in Regenerative Medicine

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Introductory note

In the event of tissue damage, intrinsic healing mechanisms are activated, and direct cellular actions are driven towards the restoration of the native tissue structure and function. However ubiquitous in all body tissues, these mechanisms may be inherently limited (as is the case of central and peripheral nervous systems) or be precluded by the extent or duration of the damage events (as occurs in the skeletal muscle or bone tissue)(Pereira Lopes et al., 2006, Walsh and Midha, 2009, Merritt et al., 2010). In either scenario, tissue healing capacity may be further inhibited by host-related factors, such as age, gender and general health status (Jarvinen et al., 1983, Pola et al., 2004, Gopinath and Rando, 2008, Giordani and Puri, 2013). Current therapeutic approaches (of medical/ conservative or surgical nature) fail to achieve the desired recovery in most cases (Järvinen et al., 2005, Hoke, 2006, Järvinen et al., 2007) and, as such, new strategies are being explored.

One of the fast-expanding fields aiming at new therapeutic developments is the Regenerative Medicine through which innovative biomaterials, cellular systems and bioactive cues are being explored as independent or combined strategies to optimise tissue repair and regeneration.

In the cellular systems universe, the most obvious cellular sources to explore would be derived from native tissue populations, of either differentiated or undifferentiated nature (Koning et al., 2009, Tedesco et al., 2010, Armada-da-Silva et al., 2014). However, significant limitations preclude the widespread utilisation of this approach, due to the very limited availability and complex isolation methods. There is also reported loss of regenerative potential of these local populations when *ex vivo* cultured (Yaffe, 1968, Pereira Lopes et al., 2006, Page et al., 2011). These and other factors exacerbated the need to pursue alternative sources (Tamaki et al., 2005, Pereira Lopes et al., 2006, Page et al., 2011).

Mesenchymal Stem/ Stromal Cells (MSCs) present themselves as promising cellular systems to address this need. They can be obtained from a variety of tissue sources and have been investigated and demonstrated to bare noteworthy benefits to tissue regeneration *in vivo* (Maurício et al., 2011, Pereira et al., 2014). A dominant part of these beneficial effects is assigned to the secretory capacity of these cells, through which they produce and release an array of bioactive molecules that impact the course of tissue healing (Caplan and Dennis, 2006, da Silva Meirelles et al., 2009).

Regarding the nomenclature of these populations as Stem or Stromal cells, a debate field is open within the scientific community. The classical and widely accepted definition is the one proposed by the International Society for Cellular Therapy (ISCT), in which Mesenchymal Stem Cells present plastic adherence when *ex vivo* cultured, expression of cluster of differentiation (CD) 105, CD73 and CD90 surface molecules and absence of hematopoietic markers (CD45, CD14, CD19, CD34 and Human Leukocyte Antigen (HLA)-DR), and the capacity for trilineage differentiation (into adipocytic, osteocytic and chondrocytic lineages)(Dominici et al., 2006). In parallel, an alternative scientific current regards these as not representing 'true' stem cells, since indisputable evidence of some critical features has not yet been presented, such as the asymmetric cell division and complete lineage renewal. Therefore, the term Mesenchymal Stromal Cell is proposed to better describe the conventionally isolated populations (Prockop and Oh, 2012). This dispute is well represented in the presented work, as earlier publications did reflect the classical trend of Mesenchymal Stem Cells definition, while most recent works acknowledge the conflicting visions on the correct term to define the cellular populations herein explored.

In this Chapter, a detailed review of literature on the potential application of MSCs-based therapy in neuromuscular lesions is addressed, individually covering the particularities of the skeletal muscle and peripheral nerve tissues, as potential target tissues to newly developed systems application.

The particular repair and regenerative mechanisms of each tissue are explored, substantiating on the significant bioactive factors for each healing stage, since this is the process in which MSCs are proposed to interact and modulate, thus resulting in boosted regenerative outcomes.

1.1. Mesenchymal Stem/ Stromal Cells application for skeletal muscle repair and regeneration

Trends in Mesenchymal Stem Cells' Applications for Skeletal Muscle Repair and Regeneration

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Trends in Mesenchymal Stem Cells' Applications for Skeletal Muscle Repair and Regeneration

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Additional information is available at the end of the chapter

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Abstract

Skeletal muscle injuries are quite frequent in traumatic scenarios, such as war injuries or road- or work-related accidents. The skeletal muscle has good regenerative ability, but the extent or recurrence of muscle injury might impair complete structural and functional recovery. Severe tissue loss overwhelms skeletal muscle's intrinsic regenerative capabilities and culminates in the development of noncontractile fibrous tissue scar. Conservative RICE -based and surgical treatments show limited efficacy in terms of improving these severe cases outcomes, pressing the need for new approaches on skeletal muscle's therapy. Since the first suggestions of the potential of mesenchymal stem cells for regenerative medicine and tissue engineering, many applications have been explored for a variety of tissues and diseases, including the skeletal muscle, which is the focus of this literature review.

Current research has focused on the influence of nonmuscular MSCs on promoting tissue healing and limiting fibrotic scar formation, as well as on the modulation of the inflammatory response to injury. The most popular source of MSCs is, without a doubt, the bone marrow. However, MSC populations are present in virtually all body tissues, and alternative sources have been proposed, such as the adipose tissue, synovial membrane, dental pulp, and even umbilical cord tissue.

MSCs from various sources have been demonstrated as capable of *in vitro* differentiation into myogenic lineages, through adequate stimuli, displaying phenotypical markers of native skeletal muscle cells. In addition, *in vivo* applications suggest they are capable of integrating host muscular tissues, even when delivered systemically.

MSCs are capable of secreting a wide range of active molecules to their surrounding media, including growth factors, chemokines, and cytokines. Most of these growth factors have been associated to the skeletal muscle's regenerative process, and their efficiency has been demonstrated to increase when applied in spatial and temporal coordination. Hence, the combination of molecules secreted by MSCs gained interest as modulator of inflammatory, fibrotic, and regenerating events. This has been proposed as possibly the primary mode of action of undifferentiated MSCs into a lesion site, providing controlled release of such components. Concurrently to the implantation of undifferentiated cells, it has also been hypothesized that the application of secretion products alone (termed as Conditioned Medium) display similar if not improved effects on skeletal tissue regeneration, as it does in other damaged tissues.

Keywords: Skeletal muscle, skeletal muscle regeneration, cellular therapies, mesenchymal stem cells, biomaterials, secretome

1. Introduction

Skeletal muscle accounts for nearly half of the human body mass [1], and inherited and acquired pathologies are often observed in clinical practice.

Given their impact on quality and life expectancy of patients, severe forms of degenerative muscular diseases, such as Duchenne muscular dystrophy (DMD), have been one of the hot topics of skeletal muscle regeneration research, and encouraging results have been obtained through the application of mesenchymal stem cells (MSCs), giving hope for the development of new therapies that can effectively improve the quality of life of affected patients [2–7].

Acquired muscle affections are seemingly more common in active humans, greatly associated to sports practice, but also quite frequent in other traumatic sceneries, such as road or work-related accidents or war injuries [8–11]. Muscle damage can result from ischemia and denervation, to contusion, sprain damage, laceration, avulsion, and other severe tissue losses.

Skeletal muscle has a good regenerative ability, but the extent or recurrence of these insults might impair complete myofibers regeneration, limiting structural and functional recovery of the affected muscle groups. Severe tissue loss usually supplants skeletal muscle's intrinsic regenerative capabilities [8] and culminates in the development of noncontractile fibrous tissue scar [12]. Other well-known factor to impact the intrinsic capacity of skeletal muscle to respond to injury events is the age of the patient [13], affecting both intrinsic cellular mechanisms and their involving niche, hindering their effectiveness upon activation [14]. The regeneration potential of skeletal muscle depends on a multitude of cell types that, upon exposure to specific cues, cooperate to regenerate the damaged tissue, generating a coordinated tissue response [15]. Under particular conditions, such as chronic diseases and aging, the ability of these cells to support the regenerative response declines, leading to maladaptive responses, e.g., the formation of fibrotic scars and fatty infiltration [15].

Current recommendations for skeletal muscle lesions management rely on empirical application of conservative RICE-based (rest, ice/cold, compression and elevation) and surgical treatments [10, 16] but show limited efficacy in terms of improving severe cases outcomes, pressing the need for new approaches on skeletal muscle's therapy.

Presently, biomedical research is working in various fronts toward complete restoration of structure and function of damaged muscles, converging efforts in the areas of biomaterial development, cell systems applications, and bioactive molecules aiming at filling the defect and recovering the esthetics of the body part, as well as its function.

One of the strategies being intensely explored involves the application of muscle resident and nonmuscular stem cells in search for faster and more effective recovery from severe injuries, restoring both tissue structure and function [17, 18].

2. Skeletal muscle structure and intrinsic healing mechanisms

The basis of skeletal muscle structure and regeneration have been extensively revised in literature, and only a brief description and emphasis to strategic "key points" will be given herein [9, 16].

Skeletal muscle is composed of a mixture of muscle-specific cells, nerves, blood vessels, and connective tissue support matrix. Skeletal muscle tissue-specific cells are multinucleated structures holding complex and highly organized contraction machinery enclosed within the plasma membrane (sarcolemma), and a single cell is termed as myofiber. According to their contractile properties, myofibers can be classified into three types. Type 1 myofibers are slow contracting and fatigue resistant, type 2A myofibers are fast contracting and have intermediate resistance to fatigue, and type 2B myofibers are fast contracting and have poor fatigue resistance. The function and training of a specific muscle or muscle group determine their composition in terms of fiber type content.

The extracellular matrix (ECM) supporting the myofibers (basal lamina or basement membrane) is composed of a vast set of proteins, such collagen, fibronectin, laminin, and other glycoproteins [9]. Myofibers are bound together by connective tissue sheaths (the endomysium, perimysium, and epimysium) associating them at three upscaling levels from involving a single myofiber to the whole muscle belly [16].

The healing process following skeletal muscle injury is classically divided into three inter-related and time-dependent phases, conveying the destruction, repair, and remodeling of the affected tissue. The first phase (destruction phase) is defined by the rupture and necrosis and degeneration of the myofibers (mainly mediated by alterations of the sarcolemma and loss of calcium homeostasis) and associated neurovascular structures and ECM, by the formation of a hematoma (between the damaged/ruptured and retracted muscle cells) and the initiation of the inflammatory cells response [9, 16]. Other authors distinguish a primary hemostatic stage, preceding the inflammatory reaction [19]. The inflammatory phase becomes evident within 24 hours after injury and comes up until approximately 3 days after the event. It is defined by a

sequential influx of neutrophils and macrophages to the site, engaging in the phagocytosis of the debris on site and the release of a cascade of mediatory cytokines [9, 20].

The following phase (repair phase) includes the phagocytosis of the debris resulting from the damaged/necrotized tissue, the kickoff of the regeneration of the myofibers, and the production of a connective-tissue scar by migrating fibroblasts and neurovascular regrowth [16].

At this stage, satellite cells (SCs) assume a preponderant role. These cells, firstly identified in the early 1960s [21], constitute a population of myogenically committed but undifferentiated cells, residing between the basal lamina and the muscle fiber, assuming functions of maintenance of tissue homeostasis and regeneration. Muscle-specific paired box 7 (Pax7) is a hallmark of postnatal myogenesis capacity and commonly characterizes SCs populations [15]. Upon injury, SCs are activated and undergo one of two fates: differentiation into myogenic cells or “stem-like” division, maintaining the pool of available cells for intervention in future events of injury. These two courses of the SCs population relate to their Myf5 transcription factor expression: the dominant population Pax7⁺/Myf5⁺ undergoes myogenic differentiation while the minor Pax7⁺/Myf5⁻ population remains undifferentiated replenishing the SCs niche [22]. Other populations with stem cells’ characteristics have been identified within the muscle tissue, such as mesangioblasts and PICs (PW1⁺/Pax7⁻ interstitial cells). Further details on intrinsic regenerative populations associated to skeletal muscle and SCs origins and dynamics can be found elsewhere [23–27].

In the final remodeling phase, the regenerated tissue matures and the formed fibrous connective tissue reorganizes and contracts [16]. This stage is highly significant for the outcome of the whole process and fine regulation of the late fibrotic events turns essential [28–30]. Especially after severe tissue loss, the fibrin matrix derived from the clotting process and inflammatory response requires remodeling into collagen type 1 network, produced by fibroblastic cells [31]. The development of definitive fibrosis at a lesion site begins at approximately 2 weeks after injury and progresses over time. Exacerbated fibrosis prejudices the repair and remodeling phases hindering muscle regeneration and full functional recovery [32].

Although fibrosis is mostly referred to as a negative aspect of the healing process, evidence suggests that a certain level of fibrosis acts as support matrix to new tissue ingrowth, promoting proper realignment of the myofibers and the myofibrils, and maintaining a degree of mechanical properties on the regenerating tissue [32]. Also, reports of “functional fibrosis” support its importance to the process, by contributing to a certain stance to the force distribution along the muscle or muscle group, preventing continued overload of the remaining skeletal muscle tissue, and contributing to functional recovery unrelated to effective skeletal muscle tissue regeneration [33].

3. MSCs’ sources for skeletal muscle regeneration

In the ever-growing field of regenerative medicine and tissue engineering, stem cells are posing as one of the main characters in the most recent therapeutic strategies [17, 18].

Given the presence of resident stem and SCs within the skeletal muscle tissue, native tissue skeletal muscle-derived MSCs (MDSCs) would appear as the favorite source for regeneration therapies [34]. In response to muscle damage, the SCs population is activated by the released biomolecules and begins to proliferate and originates large numbers of muscle progenitor cells (MPCs), which will in turn contribute to skeletal muscle structure reconstruction. At the same time, not all SCs derive into MPCs but rather self-renew, contributing to the replenishment of the quiescent cells within the muscle tissue [35, 36].

Many authors have explored skeletal muscle tissue-specific cells for repair and regenerative purposes, as summarized by Koning et al. [37]. Despite of their demonstrated benefits in several settings [38], the practical use of postnatal skeletal muscle progenitors or SCs is limited due to decreased cell availability (requiring the harvest of large volumes of healthy tissue for adequate numbers) [31, 39]. The expansion of MDSCs is possible, but as it has long been known, it leads to dedifferentiation of early committed myogenic cells [40] and loss of potential. As little as 1 day in culture following isolation and sorting hinders its engraftment potential and contribution to regeneration events, hence turning it difficult to attain relevant cell numbers for implantation [34]. Confirming this loss of potential, the implantation of freshly isolated SCs in numbers as low as 250 cells outperforms the use of as many as 1.5×10^5 MPCs of first passage derived from SCs expansion [35]. This initial boost provided by seldom expanded MPCs, however, does not seem to sustain for long term effects [41].

Consequently, current research has focused on the influence of nonmuscular MSCs on promoting tissue healing and limiting fibrotic scar formation, as well as on the modulation of the inflammatory response to injury.

From the first suggestion of MSCs' potential for regenerative medicine and tissue engineering, many applications have been explored for a variety of tissues and diseases [42], including the skeletal muscle, which is the focus of this literature review. Our research group has dedicated to the development of MSC-based cellular therapies for application on several body tissues, from peripheral nerve to blood vessels and skin wounds [43–53], including for skeletal muscle volumetric loss lesions [54].

Since their first descriptions as a specific cell population in the late 1960s [55–57], knowledge on MSCs' features and potential has grown exponentially [58], as have the effective medical applications in which these are beneficial. The MSC population from the bone marrow (BM-MSCs) was the first to be characterized [55], and at the time, stem cells were thought to be exclusive to organs with fair regenerative capacity, such as the blood, intestine, bone, and skin. Nowadays, we are aware that they are present in virtually all the body tissues, in variable numbers, mostly remaining in a quiescent state until activated by significant events, ensuring a certain degree of defense against damage and disease [56, 59, 60].

The most significant features of MSCs are their clonogenic and proliferative capacities, while remaining genetically stable and in undifferentiated state, and their differentiation abilities [58], into various mesodermal, ectodermal, and endodermal cell types [61].

Through the years, significant progress has been made toward MSC characterization, and in an effort to standardize and unite the scientific community, the Mesenchymal and Tissue Stem

Cell Committee of the International Society for Cellular Therapy (ISCT) gathered a series of recommendations regarding the acceptable criteria for the definition of “mesenchymal stem cell” populations. Specifically, MSCs are determined to be characterized by (i) plastic adherent ability; (ii) absence of definitive hematopoietic lineage markers, such as CD45, CD34, CD14, CD11b, CD79- α , CD19, and class II major histocompatibility complex (MHC) molecules, specially human leukocyte antigen (HLA)-DR, and expression of nonspecific markers CD105, CD90, and CD73; and (iii) ability to differentiate into mesodermal lineage cells, osteocytes, chondrocytes, and adipocytes [62].

Another appealing point on MSC research is their immune features. Unlike terminally differentiated cells, MSCs are somehow immunologically privileged, avoiding the use of additional immunosuppressive supplements during the treatments, which are mostly (although not exclusively) deleterious for intrinsic regeneration mechanisms [42, 63]. One of the main mediators of immune responses is the HLA-II, of which MSCs present only neglectable levels, deeming them immunologically privileged [64]. This is a key point, considering the difficulty of finding matching donors among the human population and the challenges of harvesting sufficient numbers of cells from one patient upon necessity [65]. Hence, the lack of HLA-II opens the possibility of using directly obtained or banked cells from consenting healthy donors from the same species, designated as allografts [66]. Given their peculiar immune features, the xenogenic implantation of human-derived cells in appropriate nonimmunosuppressed animal models is feasible [52, 54, 67] and provides valuable information on their behavior and effect on experimental stages that more closely mimic clinical practice reality [63].

In addition, immunomodulatory actions have also been attributed to MSCs, by controlling and modifying host immune response, either locally (by blunting the tissue response at the implantation site [54]) or systemically (ameliorating signals of severe immunological disturbances, such as chronic inflammatory, autoimmune diseases or graft-versus-host-disease) [63, 68].

The bone marrow is without a doubt the most widely explored source for MSCs for therapeutic purposes. The bone marrow is harvested from the patient or consenting donor, and the adherent MSCs are isolated and expanded until desired numbers are attained for the intended application. The harvesting procedure is however highly invasive and potentially painful, motivating the search for more easily accessible sources. Furthermore, the “quality” of the isolate cells strongly depends on the age, gender, and health status of the patient or donor [65]. Adipose tissue and synovial membrane are also valid sources, and harvesting tissue for cell isolation is mostly associated to primary interventions for esthetical and/or medical reasons. [2, 39]. Other sources of MSCs are gaining ground for the minimally invasive nature of their harvest, as well as for the lesser ethical concerns surrounding their tissues of origin, namely, the stromal tissue of the umbilical cord [66] and the dental pulp [69] (Figure 1). The collection of the tissue sources for these implicates lesser ethical and technical issues since they were mostly discarded as medical waste following birth or dental procedures [66, 67, 69, 70]. Another alternative method could be the collection of MSCs from postmortem tissues. MSCs have been successfully isolated from the bone marrow, skeletal muscle, neural tissue, and dental pulp of deceased donors [71, 72]. These options, however, comply with similar if not aggravated ethical, legal, and even social and religious concerns to conventional MSC sources [71].

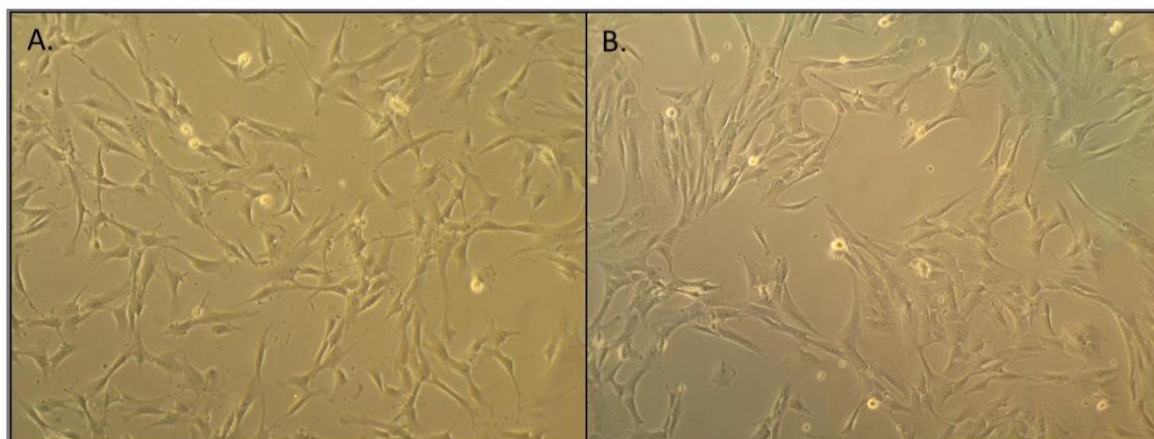


Figure 1. Morphological similarities between (A) DPSCs and (B) UC-MSCs (magnification: $\times 100$).

Focusing on the skeletal muscle repair and regeneration, and apart from muscle-derived cells, the most popular source of MSCs is still the bone marrow. However, MSC populations are present in virtually all body tissues, and alternative sources have been proposed, such as adipose tissue, synovial membrane [2, 73, 74], dental pulp [4, 69, 75], and even umbilical cord tissue [76, 77]. Cells from these sources display comparable phenotypical features regarding their “stemness” potential (they are plastic adherent, positive for markers of the mesenchymal and negative for the hematopoietic lineage markers). As previously referred, they are similarly amenable of differentiation into mesodermal and endodermal cell lineages, including adipocytes, chondrocytes, osteoblasts, endothelial cells, and hepatocytes [61]. Nonetheless, MSCs from distinct sources are not completely identical, whether regarding phenotypical markers expression, proliferation rates, or even multilineage differentiation aptitudes [74].

3.1. Evidence of the *in vitro* myogenic differentiation potential of MSCs

MSCs from various nonmuscular sources have been demonstrated as capable of *in vitro* differentiation into myogenic lineages, through adequate stimuli, displaying phenotypical markers of native skeletal muscle cells [61]. However, nonmuscular MSCs depict hardly any spontaneous tendency toward myodifferentiation (0.001%) [6], unlike the “MSC-like” populations residing in the muscle tissue that show a degree of lineage commitment to myogenic formation, undergoing spontaneous differentiation in standard culture [31]. Not all MSC sources seem to show the same tendency or predisposition toward this differentiation pathway upon stimuli. As an example, the myogenic potential of MSCs from the synovial membrane (SM-MSCs) seems more limited than for chondrogenesis, osteogenesis, or adipogenesis since only limited number of individually expanded clones presented myotube formation capacity, suggesting subpopulations with specific tendencies for lineage commitment [73]. MSCs isolated from the umbilical cord stromal tissue display limited intrinsic tendency toward myogenic lineage differentiation, expressing diminished levels of pluripotency and specific myogenic markers involved in such process (such as Oct-4, Nanog, Pax-7, MyoD and myogenin, and M-cadherin), and they do not seem to spontaneously differentiate toward this lineage

[76]. Conversely, when cocultured with differentiating myoblasts, differentiation can be observed [76].

Cells undergoing myodifferentiation paths sequentially express characteristic transcription factors belonging to the myogenic regulatory factors (MRFs) family, such as MyoD and myosin (early a terminal differentiation markers) that are expressed with a well-defined time course depending on how far long the process has progressed [39, 78], replicating the embryogenesis of skeletal muscle tissue [15].

MSCs from the bone marrow, adipose tissue (AT-MSCs), synovial membrane, and dental pulp (DPSCs) are also capable of fusing to myoblasts in coculture systems. Although multinucleated hybrid myotubes generally appear only at low frequencies in the total population [6, 38, 74, 75] it results in the detection of muscle-specific gene expression in stromal cells, which is turned on through myogenic fusion [79].

In alternative to direct contact coculture, trans-well settings can also efficiently induce differentiation into skeletal muscle of AT-MSC subpopulations, via a fusion-independent mechanism, leading to the expression of aforementioned differentiating myotubes markers. This suggests that the differentiating myoblast can promote MSC myogenesis through secreted biomolecules that can effectively cross the trans-well filter and exert action on the MSC receptors. Nonetheless, the differentiation efficiency did not match the direct contact settings, deeming cell-to-cell direct interaction a key factor and suggesting that these two mechanisms act in complementary ways [6].

Further away from the coculture system, the supplementation of MSCs with conditioned medium (CM) from both mature muscle cells and primary precursors induced differentiation toward myogenic phenotypes [80]. CM from injured skeletal muscle also influences MSC proliferation, in a dose-dependent manner, and promotes myogenic lineage differentiation into the characteristic morphologies and transcription factors sequential expression. The medium from undamaged muscle did not elicit such responses, demonstrating that the injury event triggers the secretion of essential signaling biomolecules that modulate intervenient cells' fate *in situ* and are capable of modulating exogenous cells, such as BM-MSCs [78].

In 5-azacytidine (5-Aza)-induced differentiation, enriched umbilical cord stroma (UC-) MSCs, adherent fraction of umbilical cord blood (UCB-MSCs), periodontal ligament-derived MSCs (PDL-MSCs), SM-MSCs, AT-MSCs, BM-MSCs, and skeletal muscle-derived MSCs (SkM-MSCs) also begin displaying suggestive myoblast-like shape and fusing into multinucleated immature myofibers, expressing early muscular markers, such as Myf5 and then MyoD [2, 39, 67, 70, 77, 81]. The spontaneous twitching of multinucleated fused differentiating myotubes has also been described after 9–10 days culture [78]. Although classical myodifferentiation protocols rely on the pathway triggering by 5-Aza-induced DNA methylation, this is known to cause epigenetic changes, possibly precluding further advancements into clinical applications. In an alternative approach, differentiation can be successfully induced using a more “physiological” induction medium, composed of defined growth factors, such as bFGF, VEGF, and IGF-1, and it can successfully induce BM-MSCs into multinucleated myotube-like structures, with striated cytoplasm and replicating specific expression patterns of the myogenic pathway,

similarly to other induction techniques [61]. Similar behavior is also observed in MSCs cultured in promyogenic medium containing dexamethasone and hydrocortisone [82].

3.2. Evidence on the application of MSCs for *in vivo* skeletal muscle regeneration

Further, *in vivo* applications suggest that, to variable extent, MSCs are capable of integrating host muscular tissues, being identifiable at the lesion site shortly after implantation (Figure 2) [54, 67, 75–77, 83–85]. The long time observation of administered MSCs in host muscle has also been reported, even when delivered systemically [2, 4]. Intra-arterial delivery appears more adequate for systemic MSC delivery, in detriment of intravenous routes. Keeping in mind the circulatory anatomy, this was an expected observation since venous routes implicate increased systemic dilution effects, as well as significant entrapment of cells within the pulmonary capillary bed [86].

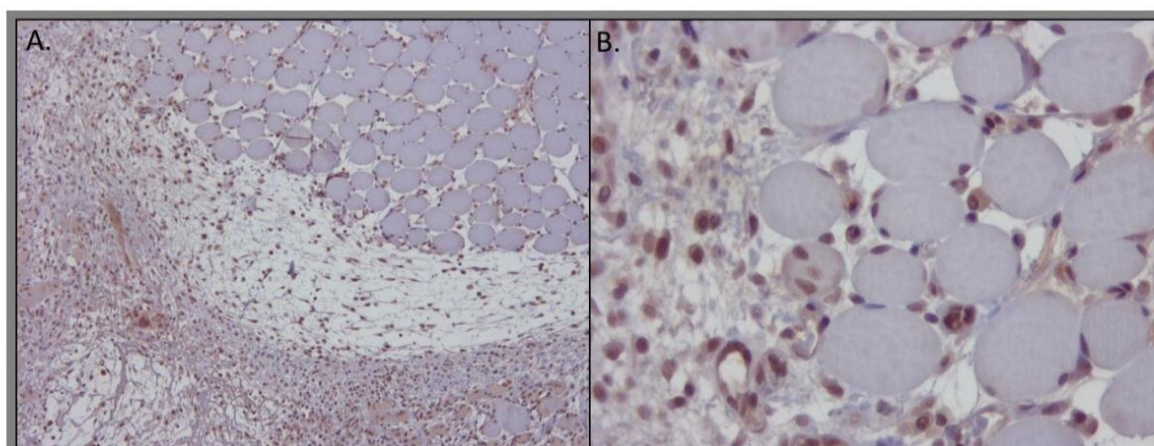


Figure 2. Human-derived UC-MSCs engrafted within injured skeletal muscle tissue 4 days after implantation. Immunohistochemistry staining for human nuclei (hNu) antigen (blue stained cells). Magnification: (A) $\times 100$, (B) $\times 400$.

It is set that the engraftment potential of MSCs into a damaged tissue is not absolute. The numbers or percent of cells identified at different time points following injection invariably decreases [76, 85, 87, 88], down to nearly as little as 10% of the initially delivered numbers in a couple of months [88].

MSCs positively influence recovery of chemically induced muscle damage [76, 77], nonvolumetric laceration [89] as well as in crush injuries [90, 91]. Although on occasions the delivered cells could not be identified on site as differentiated entities or fused to host cells or, when so, only to low degrees, the observed benefits further suggest that their contribution to the regeneration of skeletal muscle might rely on mechanisms other than fusion to myofibers after differentiation [89, 90].

UC-MSCs also engraft in the resident skeletal muscle tissue and are identifiable up to 14 days after administration and in some extent differentiate into cells expressing sarcomeric troponin and myosin antigens [67]. When administered in undifferentiated state MSCs seem to replicate

embryonic myogenesis events, as they do following *in vitro* induction [67], triggering gene expression characteristic of myogenic differentiation pathways [2].

SM-MSCs are detectable integrated within host tissue for up to 6 months after either local or systemic delivery, demonstrating their preferential homing ability to the injured tissues since greater numbers were harbored by the injured muscle, although they could seldom be identified in other body systems [2]. At longer terms after BM-MSC [88] or UC-MSC [76] administration, about 5% of myofibers were of hybrid nature and could be identified along the whole muscle length [88]. The hybrids' formations seem to be a progressive process since hybrid myofibers represent a much smaller fraction (under 1%) in the regenerating muscle at shorter time points [74]. The administration of these cells granted increased muscle mass and mature fiber formation when compared to untreated muscles [76]. AT-MSCs also contribute to enhanced regeneration, reducing fibrosis and improving histological and functional features after only 4 weeks. However MSCs alone did improve the process, their association to a biomaterial vehicle and bioactive cues further enhanced those results, as detailed later in this section [83].

In the last decade, the question whether the seldom identified donor-derived cells resulted from trans-differentiation events of MSCs into muscle cells or from fusion to host cells raised significant controversy [79]. Today, based on the acquired evidence, the scientific community tends toward the fusion theory. As mentioned earlier, MSCs have been demonstrated to successfully fuse to host cells and contribute with genetic information, leading to the expression of human-derived genes and gene products [2, 79, 88]. Fusion efficiency seems to differ among MSCs sources, and AT-MSCs appear more prone to *in vivo* formation of hybrid myotubes, surpassing BM-MSCs and SM-MSCs [74].

Delivered cells also seem to interact with the resident tissue's satellite pool. Skeletal muscle SCs delivered to a regenerating muscle effectively contribute to the replenishment of the resident satellite pool, migrating to locations far from the lesion site within the muscle. These freshly isolated native skeletal muscle stem cells contribute in high extents to the total Pax7⁺ population (about 38%), unlike expanded MPCs, that only account for about as little as 12% [35], but can still be identified within the muscle tissue and in association to connective structures [31]. Nonmuscular sources seem to behave closer to MPCs in terms of niche replenishment. Based on the location of the remaining delivered cells not associated to hybrid fibers, some authors suggest that these residual donor-derived cells might have also contributed to the resident SCs pool, and that they can effectively contribute to skeletal muscle regeneration in the events of a new injury [2, 35, 76, 88]. Alike exogenous SCs and MPCs integrated into the resident quiescent pool, other sources of engrafted cells are amenable of activation upon reinjury of the muscle [2, 35] and can be reisolated [35] and also originate primary myoblast cultures *in vitro* [2]. These expanded myoblast cultures originating from human-derived isolated SM-MSCs could further engraft a second recipient's regenerating muscle [2].

As suggested by some authors, the different engraftment potential might be due to the distinct MSC sources reported [35, 74], but it may also relate to the chosen disease model: dystrophic muscle models seem to better adopt exogenous cells than non-dystrophic-induced lesion

models [88]. Although some disease models, especially of degenerative nature, seem to display sex-related differences, physical models of skeletal muscle lesions appear to respond similarly, at least in terms of functional recuperation. As for fibrosis development, male specimens might display a decreased tendency for the event [90]. The gender, age, and health status of the MSC donor is also a factor known to influence cell quantity, quality and general performance [65].

In line with the low engraftment potential and low contribution to *de novo* myotube formation and the solid observations that nevertheless MSCs tend to consistently lead to structural and functional improvement in skeletal muscle repair and regeneration models, the paradigm on the mode of action of MSCs started to shift. Evidence supports that their primary mechanism of action may rely on their secretory abilities rather than on their differentiation capacities, as discussed in detail further ahead [89, 90].

3.3. MSC-biomaterial systems for *in vivo* skeletal muscle regeneration

Most of the above-mentioned diseases and disease models involve severe affection of skeletal muscle tissue function (laceration or chemically induced damage) but, generally, the structural integrity of the tissue is maintained, preserving the blood and neural supply to the muscles as well as the resident SCs population [67, 88]. Although skeletal muscle detains a fair capacity for regeneration, severe injuries involving the loss of extensive volumes of muscle, termed as volumetric muscle loss (VML), mostly overwhelm this intrinsic response [12]. To date, these situations pose a relevant therapeutic challenge.

The gold standard for the surgical management is the creation of muscle flaps filling the defective area. However, these autotransplants depend on the maintenance of an adequate blood supply and involve the damage of a neighboring muscle. Therefore, the donor site morbidity and limited success of such approaches push toward the development of new treatment options [92].

The advent of tissue engineering and regenerative medicine research, focusing on both biomaterials, cells, and bioactive molecules, has boosted the search for new possibilities for the development of effective clinical treatment of affected patients [11, 92–94].

These cases are mainly related to traumatic or surgical events and result in complete tear of the myofibers or even significant loss of skeletal muscle tissue portions, VML, in large and relevant active muscle groups. Here, no support structures remain on the lesion site, nor do blood vessels, neural structures, or cell populations with capacity to repair and restore the lost tissue. The loss of 10% to 20% of the mass of a weight-bearing muscle represents a critical loss that will not fully regenerate even after long periods [8] (Figure 3).

The healing of these severe injuries can be improved by the sole administration loose of cells [34], but for the most cases, complete repair of such defects remains dependent on the ability of bridging the gap between the transected muscle segments. For VMLs, this point presents an impending challenge.

The research field of biocompatible biomaterials has opened a possible strategy to address previously irreparable lesions. These materials aim at providing a physical support to the

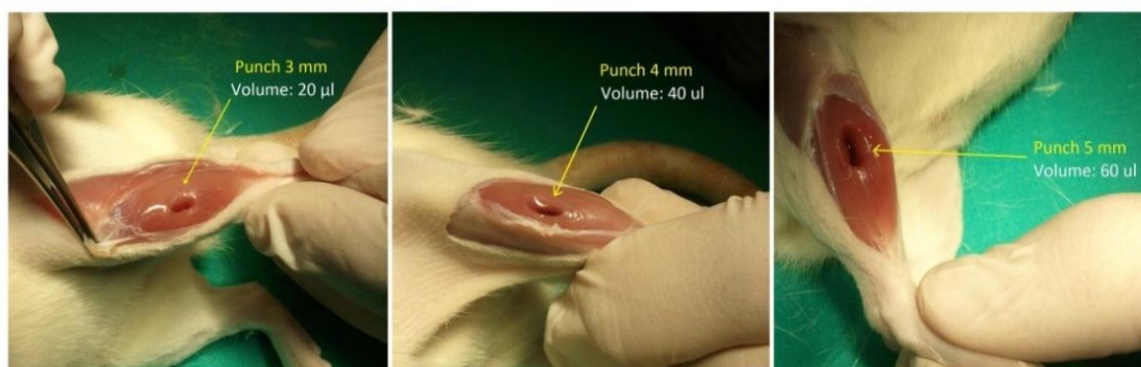


Figure 3. VML lesion model development: myectomy lesions in the tibialis anterior muscle of the rat's hindlimb. Different volumes (20–60 μ l) in the defects produced by biopsy punch blades of different sizes (3–5 mm) [51].

regenerating myofibers on both ends, promoting their development and proper orientation, a key point for structural as well as functional recovery [8]. These biomaterial scaffolds are also valuable in recreating an advantageous mechanical and chemical microenvironment for the proliferation and differentiation of resident or delivered progenitors [8]. In addition, these matrices may act as (as in the case of ECM) [93] or be modified/loaded with bioactive signaling molecules participating in the repair process [83].

Here, decellularized ECMs appear as the most widely explored scaffold material for skeletal muscle tissue engineering, with commercially available products ready for clinical use and amenable of application in skeletal muscle repair and regeneration [33].

Decellularized skeletal muscle ECM has been demonstrated to adequately fill a critical muscle defect, and to benefit structural recovery. Although structural improvement was determined, functional outcomes did not significantly differ between the bridged and the unbridged control defects [8]. Other study reported functional indexes recovery comparing to the unimplanted group, but still insufficient to match undamaged muscles' response [94]. Scaling up to a larger preclinical canine model, these implants were capable of promoting endogenous progenitors migration into the regenerating area [95].

On a clinical setting application, commercially available ECM products have been successfully applied to restore chronic volumetric muscle injuries [11, 94, 96], reporting esthetic and, to some degree, functional improvements.

Besides large voids in the muscle tissue, biomaterials can also be applied in smaller defects. A gelatin-based hydrogel could enhance regeneration after laceration injury. This *in situ* forming gel was loaded with a prosurvival growth factor (bFGF), sustaining controlled release for up to 3 weeks. *In vivo*, the loaded hydrogel significantly improved contractile force of healing muscles, reduced local fibrosis, and increased multinucleated regenerating myofibers and neurovascular structures on site [83].

Stem cell implantation is a possible strategy to enhance the recovery rates whether they are delivered alone or in association to biomaterial scaffolds. Their inclusion has been demonstrated beneficial in skeletal muscle injury.

As a first approach, SCs and other muscle-derived progenitors were investigated toward the optimization of the healing process of biomaterial treated defects [41, 97].

The performance of fibrin-based [31] and hyaluronan-based hydrogels [35] was significantly improved by the association of cellular systems. MPCs were able to reduce inflammatory infiltration and scar formation and regeneration events were improved, with reinnervation and revascularization of the area as well as increasing the regenerating myofibers content. The inclusion of freshly isolated SCs instead of expanded MPCs presented even better results with functional parameters closely meeting up to controls after 6 months of recovery [35].

Given the pros and cons earlier discussed regarding native tissue-derived cells and the effects of undifferentiated nonmuscular MSCs on other lesion models, some focus was given to their potential role in cellular-biomaterial systems.

The benefits of BM-MSCs in a severe VML models through the inclusion of the cell system into a decellularized ECM frame boosted both structural and functional recovery, with increased muscle tissue, blood vessels and nervous supply ingrowth into the defect area and improved muscle functional performance, when compared to the cell-free systems [8, 98].

The potential of AT-MSCs was also tested on a previously described gelatin-based hydrogel vehicle. Although the bFGF-loaded hydrogel alone performed satisfactorily, the addition of hAT-MSCs granted further improvements. The most striking result was the reduction of fibrosis to only roughly 20% and the recovery of functional parameters reaching 89% of uninjured muscles. Indicators of regenerative events (immature myofibers, reinnervation, and neovascularization) were also significantly improved [83]. When associated to Matrigel™, early improvements were observed. However, such differences were no longer evident in a 4-week time point [87].

Besides the obvious impact on the overall regenerative milieu, the exogenous MSCs are also strong modulators of tissue reaction to biomaterials implanted within the muscle tissue.

Using a volumetric loss rat model [51], UC-MSCs demonstrated their potential in modulating early inflammatory responses to a gelatin/thrombin-based matrix [52]. It was further confirmed in response to other biomaterial systems in terms of both the inflammatory response and the collagen type I deposition. The results from the sole application of a good vehicle (fibrin-based) were further improved, and the reaction to vehicles deemed less adequate (gelatin/thrombin-matrix and hyaluronan/alginate-based) was attenuated by the presence of UC-MSCs [54].

As described in an earlier section, one of the challenges faced on the use of cell systems is the success in engrafting the lesion site. The preconditioning of BM-MSCs to the myogenic lineage seems to improve integration in host muscles [61], but the pretreatment of UC-MSCs with SDF-1 does not seem to affect engraftment efficiency, despite the fact that SDF-1 is a known promoter of migration of transplanted and host cells to active lesion sites [76]. By contrast, the combination of bFGF, VEGF, and IGF-1 positively impacted engraftment, with increased donor cells' numbers identified forming mature hybrid myotubes [61].

The delivery of the intended MSCs within a scaffold/vehicle also contributes to prolonged maintenance on site, increasing engrafted cells number comparing to loosely delivered cells

[83]. Hence, the mode of delivery of cells or other regenerative cues is of vital importance [54]. Loosely delivered cells tend to show poor survival and engraftment and inadequate interaction with the host tissue. These drawbacks seem to be counteracted by their association to delivery vehicles [35] that also seem to positively influence cell survival and myodifferentiation, as well as neovascularization of the lesion sites when implanted [83].

In summary, both biomaterials and cells alone can aid the healing process, but their association seems to boost their individual actions. Cells help in functionalizing biomaterials while biomaterial provided beneficial microenvironments for the survival and action of the encapsulated MSCs.

From the currently available data, biomaterials alone are capable of providing fair benefits to volumetric lesions, but longer periods of recovery might be required (over 6 months). The coordination of those with cellular systems is likely to speed up the process, providing evidence of functional recovery earlier after the treatments [97].

The type and the magnitude of the contribution of the seeded population to the regenerative process also seem to relate to their differentiation state. Cells closer to undifferentiated state seem to elicit boosted initial responses, accelerating the onset of the process [87, 97]. By contrast, specialized cells tend to provide a more gradual but sustained response. The combination of the two populations may provide the key for additive effects and magnified recoveries [41].

One of the most striking observations is that the application of lineage committed or undifferentiated cells correlates to increased vascularization (and also innervation) at interface and core areas of implanted materials, which is known to be a vital factor for cell survival and function and tissue regrowth into volumetric matrices [98], since three-dimensional scaffolds easily exceed the diffusion capacity for nutrient and other essential components toward the inner parts of the constructs [99]. Seeding with potentially vasculogenic cells and/or prevascularization of constructs via *in vitro* culture seem viable options for improved results upon implantation into critical defects [100, 101]. The association of seeding strategies to surgical vascularization (i.e., connection to hosts vessels) is also described as boosting regeneration and recovery from large defects [102].

The timing of administration might also impact on MSCs engraftment and function. Most authors describe the existence of delivered cells for weeks following implantation [67, 76, 77, 83], when delivered up to 24 hours after injury. This topic remains highly debatable. The delivery of MSCs into a crush injury model either immediately or 1 week after the event did not lead to significant differences in functional recovery, indicating the possibility of a fairly large time window for the application of these therapies [84]. However, others report that the delivery of MSCs 1 week after injury (in attempt to escape initial inflammatory reaction) seems to impair their engraftment since no trace was detected after only 2 weeks. Most surprisingly, the cells were delivered on a hydrogel vehicle [87] what had been described to positively influence the permanency of delivered cells at the lesion site [83]. It might suggest that the early inflammatory microenvironment modulates MSCs function and maintenance on site or that their modulatory effects on the inflammatory milieu affect their engraftment.

Another critical factor under investigation is the most adequate number of MSCs to be delivered to the defect site, associated or not to a biomaterial vehicle. Winkler and colleagues

demonstrated a dose-dependent response to BM-MSCs administration in functional recovery from 0.1 to 10 million cells administered to a crush injury site [103]. Irrespectively, the number of cells administered regardless of the disease model or cell source or vehicle depicts no consensus among research groups, ranging from few thousands to several millions [52, 54, 67, 76, 77, 88, 98, 103].

Cell Source	Lesion Model	Delivery Mode/ Vehicle	Reference
hBM-MSCs	CTX TA mice	local injection	[88, 74, 79, 61]
rBM-MSCs	VML GTN rat	ECM	[98]
rBM-MSCs	CR SL rat	local injection	[103, 90, 91, 84, 85]
rBM-MSCs	CR SL rat	systemic delivery	[86]
hUC-MSCs	VML TA rat	thrombin-based matrix	[51, 52, 54]
hUC-MSCs	VML TA rat	fibrin-based matrix	[54]
hUC-MSCs	VML TA rat	hyaluronan/ alginate-based hydrogel	[54]
hUC-MSCs (enriched)	BVC TA rat	local injection	[67]
hUC-MSCs	CTX GTN mice	local injection	[76]
hAT-MSCs (+ bFGF)	LAC GTN mice	hyaluronic acid- based hydrogel	[83]
rAT-MSCs	LAC SL rat	Matrigel TM	[87]
hAT-MSCs	CTX TA mice	local injection	[74]
hUCB-MSCs	CTX GTN mice	local injection	[77]
hSM-MSCs	CTX TA mice	local injection systemic delivery	[2]
hSM-MSCs	CTX TA mice	local injection	[74]
hDPSCs	CTX TA mice	local injection	[75]

Table 1. Examples of non-muscular MSCs sources for *in vivo* skeletal muscle repair and regeneration; r: rat or mice derived cells; h: human derived cells; CTX: Cardiotoxin chemical model; VML: Volumetric Muscle Loss model; CR: Crush Injury model; BVC: Bupivacaine chemical model; LAC: Laceration injury model; TA: *Tibialis anterior* muscle; GTN, *Gastrocnemius* muscle; SL: *Soleus* muscle; ECM: Extracellular Matrix.

Since the core topic of this section is *in vivo* skeletal regeneration of acquired lesions models, an important remark must be made. Although the general intrinsic regenerative response of the skeletal muscle tissue to an injury event (approached in detail earlier in this chapter) follows a mostly constant sequence of events, the source and the magnitude of the induced injury lead to distinct native healing efficiencies and consequently distinct responses to implemented therapies [27, 104]. It results into an additional challenge when trying to understand the true magnitude of effects, especially in attempts of comparative analysis on the available literature.

Besides the selected defect model, the animal species/strain also assumes relevance. Different animal strains within the same species depict distinct tissue and systemic response profiles to

a similar injury [105]. Also, the use of immunocompromised [31] or non-immunocompromised [54] animals may also contribute to the inflammatory responses obtained when biomaterials and xenogenic cell sources are applied.

Other consideration possibly precluding the translation of developed therapies to the clinical practice is the character of the lesion site. In research scenarios, therapies are applied to recently injured sites. However, on a clinical setting, the most expectable situation is a chronic irreparable wound that underwent multiple surgical repair attempts though the course of several months or years [11, 94, 96]. Hence, as pointed out by Vigodarzere and Mantero [92], the homeostasis of such extensively injured and remodeled sites is significantly distinct from freshly induced insults; thus, the predictive value of the currently used animal models turns questionable.

4. Secretory potential of MSCs and impact on skeletal muscle regeneration

As evidenced by several authors, the beneficial action of MSCs on regenerating skeletal muscle might not solely depend on their differentiation capabilities, especially in nondegenerative lesion models, where their engraftment capacities seem fairly limited [76, 84, 88–90, 103]. Other proposed action mechanisms involve the secretion power of those cells [42, 76] since relevant growth factors and cytokines have been identified in various MSCs' sources secretome profiles [106, 107].

4.1. The role of growth factors and cytokines in skeletal muscle regeneration

The basis for this approach rests on the evidence that specific growth factors influence skeletal muscle regenerative response [108, 109]. In injured tissues, these factors are secreted into the surrounding microenvironment, exerting effect on, as an example, quiescent progenitor cells or delivered MSCs [78]. Thus, upon injury, the skeletal muscle itself releases a cascade of modulatory and signaling biomolecules, aiming at the recruitment and activation of essential characters to the regenerative process and triggering cell-type-specific programs [15]. These secretory capacities can inclusively modulate *in vitro* cell cultures and induce undifferentiated MSCs toward myoregeneration. In contrast, undamaged muscle seems to remain in a quiescent state, devoid of active stimulatory or differentiating factors [78].

Relevant growth factors are secreted by the remaining tissue but also by the invading immune cells participating in the intrinsic inflammatory response. Some of these molecules act as chemoattractant to additional inflammatory infiltration to the lesion site, such as MCP-1, IL-17, TNF- α , and TGF- β , among many others (a comprehensive table on the normalized nomenclature for some growth factors and cytokines is available as supplementary material on [107]) [110]. Macrophages are a grand character of skeletal muscle inflammatory response and accompany the full process of recovery [111], modulating their phenotype and secretory abilities and interaction with neighboring cells. They are chemoattracted to the site by molecules deriving from damaged muscle cells and other populations, such as neutrophils. They primarily secrete TNF- α and INF- γ then shift to increased levels of IL-4 and IL-10,

promoting initial SCs division in undifferentiated state and, later on, their differentiation toward myogenesis [110]. IL-4 is also actively secreted by eosinophils active in the early stages of response to muscle insult. At this time point, this cytokine is essential to the activation and action of resident cell populations fibro/adipogenic precursors, promoting their proliferation while inhibiting differentiation into adipogenic lineages, contributing to the formation of essential support structures to aid myotube regeneration and to further secretion of bioactive factors [112].

Parallel to the types of growth factors and cytokines involved, it is essential to bear in mind that release/delivery dynamics is also of vital importance [27]. The intrinsic regenerative mechanisms following skeletal muscle damage does rely on the sequential and coordinated interaction of molecules [113], and the key to the development of improved strategies might come from contemplating and replicating these facts. Therefore, besides the growth factors content in a lesion site, the strict patterns of interaction between those play a crucial role in the outcome of the regenerative process. For example, HGF and bFGF activity after crush injury increases during the early regeneration period (first week), while TGF- β 3 only significantly increased later in the process (after 12 days postcrush) [114].

HGF is a potent mitogen for quiescent SCs, inducing their activation and increasing the numbers of proliferating MPCs while preventing their differentiation [115, 116]. The effects of HGF in SCs quiescence appears to be the work of a concentration-dependent negative-feedback mechanism, promoting activation and proliferation at low concentrations, while rebooting SCs to quiescence and promoting muscle-specific proteins expression in increasing concentrations [117]. It is present in the undamaged muscle and is released upon injury [118], mainly of physical/mechanical nature [117], and it is also released from other organs, such as the liver and spleen, acting on skeletal muscle tissue in an endocrine way [119]. Its effects are observed in a restricted time window, peaking for the first days following injury and then decreasing. Given its inhibitory effect in myodifferentiation, its role in later stages of the regenerative process turns deleterious [116], if low expression is maintained [117].

Basic-FGF and IGF-I have also been reported to positively influence muscular cell populations in both *in vitro* and *in vivo* settings. In myoblast cultures, bFGF, IGF-I, and NGF effectively promoted myoblast proliferation and fusion into multinucleated myofibers, while other factors such as PDGF-AA, EGF, TGF- α , and TGF- β s seemed detrimental in that specific setting [120]. *In vivo* application into a laceration injury confirmed the beneficial effects of bFGF and IGF-I [120]. Basic-FGF sustained release at a lesion site (i.e., via hydrogel delivery) also elicits increased revascularization and reinnervation of the regenerated tissue, reducing the development of fibrosis [83]. IGFs are associated to both muscle cell proliferation and differentiation and play a key role in muscle regeneration and hypertrophy, with different isoforms affecting different stages of the process. IGF-IEc/MGF is expressed early in events, associated to SCs and proliferating myoblasts, while IGF-Ia and IGF-II expression occurs later in myogenic differentiation and muscle fiber formation [121]. IGF-I also modulates the local inflammatory response by down-regulating inflammatory cytokines on site, and thus limiting fibrosis development [122]. The combination of anti-fibrotic agents to IGF-I administration exerts

additive effects on muscle regeneration [32]. Sustained IGF-I delivery enhances ischemic muscle fiber regeneration, and beneficial effects are potentiated by combination with other growth factors, such as VEGF, resulting in synchronized angiogenesis, reinnervation, and myogenesis [123].

Other members of the FGF family interfere with skeletal muscle regeneration. FGF-6 is deemed muscle specific and is up-regulated during regenerative events, and its absence has been reported to relate to regenerative defects [124]. High concentrations of FGF-6 stimulate the proliferation of the myogenic stem cells, while lower concentrations regulate muscle differentiation. It is also a determining factor for skeletal muscles' fiber type content [125, 126].

Vascular endothelial growth factor (VEGF) is also an important factor in muscle regeneration. In damaged tissue, VEGF and its receptors are detected in SC and in regenerating muscle fibers, as well as in cultured SC and myoblasts. VEGF acts by stimulating myoblast migration and survival, preventing apoptosis, and promoting myogenic cell growth. Furthermore, VEGF may have a relevant role in the homing of circulating progenitor cells to specific muscle location and/or in regulating the SC pool [127]. The local administration of VEGF has also been associated with reduced scarring and improved muscle regeneration and strength recovery after acute trauma [128]. Sustained VEGF delivery promotes neo-angiogenesis and tissue perfusion recovery, as well as conferring protection from hypoxia and tissue necrosis in ischemic limbs [123], but it may derive into aberrant ECM deposition and undesired fibrosis [129].

Granulocyte colony-stimulating factor (G-CSF) also exerts beneficial effects in skeletal muscle healing, promoting both structural and functional recovery in damaged muscles [130, 131], and is a crucial factor for skeletal muscle development [130]. It promotes myoblasts proliferation *in vitro*, in a dose-dependent manner, and although it is a recruiting factor for hematopoietic stem cells from the bone marrow, it does not influence the recruitment of BM-MSCs for skeletal muscle regeneration purposes [130].

As mentioned before, not all bioactive molecules and interactions have solely positive effects. Increased TGF- β 1 levels are observed at injury sites [132]. This particular growth factor is stimulatory to collagen and ECM deposition that can be detrimental to the skeletal muscle regeneration process, contributing to exacerbated fibrosis and loss of contractile properties [32]. When TGF- β 1 activity is inhibited by the action of decorin, regeneration indexes significantly improve and fibrosis development decreases by 50% in laceration injuries [133], conveying toward *in vitro* observations [132]. TGF- β 1 acts on myoblasts, leading to the overexpression of fibrosis-related proteins and the down-regulation of myogenic proteins (desmin, MyoD, and myogenin). Furthermore, TGF- β 1 released by injured muscle stimulates autocrine expression on surrounding myoblasts and inflammatory cells, amplifying its local fibrogenic effects [132].

These and other growth factors and cytokines are well known to guide and modulate tissue response to damage, and their coordinated actions are essential for the timely activation of myogenic cells, revascularization, and reinnervation of the lesion site and ECM deposition and remodeling.

4.2. MSCs secretome and effects on *in vivo* skeletal muscle regeneration

Most of MSCs secretome components are described to exert regulatory functions in both autocrine and paracrine ways [134], and interact both directly and indirectly with other cells, by triggering direct intracellular signaling pathways or by activating molecules production and release by other targeted cell types [42]. These bioactive molecules are deemed to benefit repair and regeneration processes mostly by inhibiting apoptosis and limiting the extent/propagation of injury, by diminishing fibrotic tissue development, by stimulating angiogenesis and revascularization of the regenerating tissue, and by activating/boosting intrinsic tissue-specific stem cell pools [63].

As disclosed in a previous section, the observations of positive effects upon MSCs application regardless of their presence as differentiated entities on site strongly support the assumption that their actions may alternatively depend on their capacity to produce and secrete compounds when in undifferentiated state [41, 89, 90]. This theory is also supported by the fact that exogenous MSCs mostly position themselves in close vicinity to regenerating myofibers, in native SCs/PICs-like locations, providing controlled release of such components [76]. Caplan and Dennis quite accurately described MSCs as “multi-drug delivery vehicles that are injury-site sensitive and/or responsive” [42], also referring to MSCs “homing” capacity (i.e., their ability to respond to signaling chemokines and preferentially migrate and attach close to lesion sites). Hence, the combination of molecules secreted by MSCs gain interest as modulators of inflammatory, fibrotic, and regenerating events [54].

Since the 1990s, considerable efforts have been made toward the comprehension of the secretion potential of MSCs derived from various tissues and exhaustive studies have focused on the detailed composition of their secretome [107, 135–137] and their actions and functions on the modulation of inflammatory and regenerative events, as thoroughly revised in [42, 63, 134, 138–142].

Briefly, and according to their prospective effects on regenerative processes in general, these factors and chemokines can classically be classified as anti-apoptotic, immunomodulatory, anti-scarring, supportive, angiogenic, and chemoattractant [138]. Factors including HGF, IGFs, FGFs, CSFs, PDGFs, and TGFs as well as cytokines such as IL-6, IL-8, and IL-10 have been identified in different magnitudes in MSCs culture supernatants [106, 107, 142]. Other performers in the skeletal muscle regenerative process seem to be absent, such as IL-4 [107].

The array of secreted molecules is related to the microenvironment accommodating the active MSCs, displaying consistent patterns of secretions in response to their local microenvironment, as well as to their functional status [42]. This responsiveness of MSCs to a variety of microenvironmental cues can be availed as to enhance their therapeutic potential from the amount of secreted factors [134] up to incrementing the engraftment success when implanted at a lesion site [61]. Inflammatory cues alter the expression patterns of MSCs, resulting in increased secretion of selected growth factors and other cytokines [135, 142]. MSCs can be exposed to controlled stimuli before application, such as hypoxia and mechanical stimulation, leading increased expression of growth factors such as bFGF, IGF-I, HGF and, with particular empha-

sis, VEGF [137]. These observations are of particular interest since severely damaged muscles present hypoxic milieus due to the impairment or loss of blood supply.

Nevertheless, most of the knowledge available on the MSC secretome derives from *in vitro* settings, and despite studies focusing on the effects of biomolecules on its profile, it hardly replicates the exact inflammatory scenario within a lesion.

Concurrently to the implantation of undifferentiated MSCs, it has also been hypothesized that the application of secretion products alone (termed as conditioned medium [CM]) display similar if not improved effects on skeletal tissue regeneration (Figure 4) [54], as it does in other damaged or degenerated tissues, such as the central nervous system [141, 143, 144].

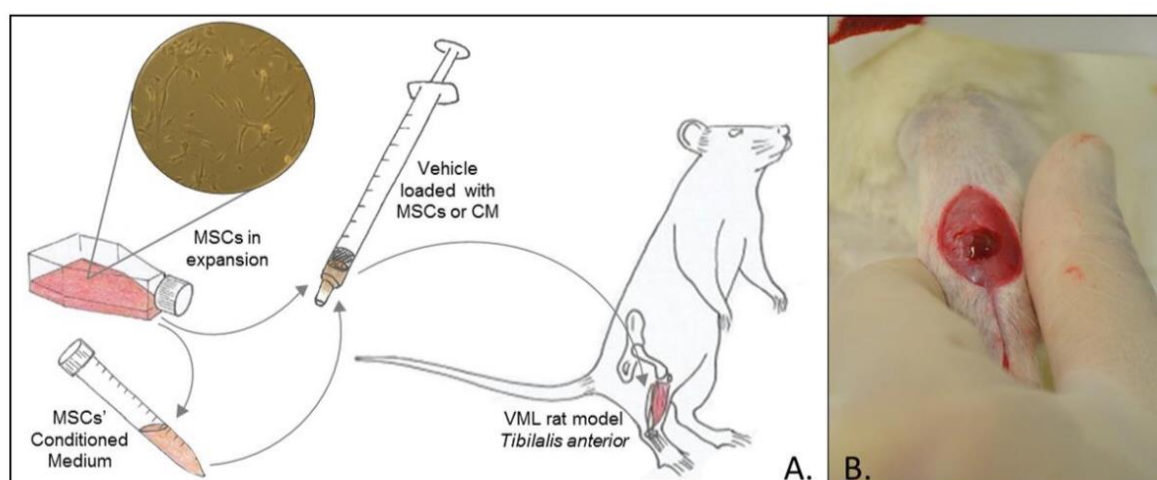


Figure 4. Schematic on the application of MSCs or their CM on hydrogel vehicles for the regeneration of critical muscle defects in a rat model (A) as described in [54]; VML lesion filled with loaded vehicle.

When comparing the skeletal muscle inflammatory response to implanted biomaterials of a severe tissue loss model, the association of either undifferentiated Wharton's jelly MSCs or CM obtained from their *in vitro* culture seems to consistently blunt the inflammatory response at early to medium stage of events (day 15 postlesion) [54]. Besides the reduced afflux of inflammatory cells to the implant sites on these early stages, we have also observed that the presence of MSCs resulted in accelerated progression to chronic inflammatory stages, that would resolve faster than unloaded subcutaneous implants (unpublished data). Therefore, although MSCs secrete a wide range of chemoattractive chemokines, their coordinate effects result in effective immunomodulation and in the control and containment of the local inflammatory response. Similarly, the addition of CM from UC-MSCs significantly improved the local response to different biomaterials as well as promoted improved muscle regeneration, revascularization, and reinnervation at the site of severe VML [54]. These results are supported by the detection of fair levels of immunomodulatory cytokines, such as IL-10 and MCP-1, as well as proregenerative growth factors, such as bFGF and VEGF [107]. Recently, we have observed similar behavior regarding the applications of DPSCs' CM in the same VML model. It similarly reduced the early to medium stages of inflammatory response to implanted

biomaterial, but slight differences were observed. These differences might be attributed to slight differences in the secretome profile of UC-MSCs and DPSCs, and research is ongoing on this topic (unpublished data).

5. Conclusions and final remarks

The following are brief conclusions on the topics discussed along this chapter:

The skeletal muscle is frequently exposed to severe trauma that overwhelms its intrinsic healing mechanisms. To date, conservative and surgical treatment options often fail to restore the structure and function of the affected muscle.

The expansion of the regenerative medicine research field enlightened scientific community on some possible strategies to improve those clinical outcomes. MSCs appear as a promising source for the development of cellular therapies for skeletal muscle and other body systems. Significant achievements have been made toward their isolation from viable tissue sources, with sources like the umbilical cord or adipose tissue gaining ground over the classical bone marrow.

The recognition of nonmuscular MSCs potential for skeletal muscle regeneration lays on the observations that they can (i) assume skeletal muscle cells phenotypes (differentiate) and (ii) fuse to native muscle cells, that (iii) they can integrate living host tissues as differentiated and undifferentiated entities, and finally (iv) that they secrete a wide range of bioactive molecules with impact on the skeletal muscle regeneration milieu.

There is still great ground to cover in search for definitive therapies, but great promise holds on the development and refinement of tissue engineering strategies, combining the use of structural and active biomaterials, nonmuscular MSCs, and their secretion products in order to aid and guide the body's efforts to heal severe volumetric muscle lesion, aiming at the full recovery of the muscles' structure and function that greatly affect patients quality of live and well-being.

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References

- [1] Sicari BM, Dearth CL, Badylak SF. Tissue engineering and regenerative medicine approaches to enhance the functional response to skeletal muscle injury. *Anatomical record* (Hoboken, NJ: 2007). 2014;297(1):51–64.
- [2] De Bari C, Dell’Accio F, Vandenabeele F, Vermeesch JR, Raymackers JM, Luyten FP. Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *Journal of Cell Biology*. 2003;160(6):909–18.
- [3] Corti S, Strazzer S, Del Bo R, Salani S, Bossolasco P, Fortunato F, et al. A Subpopulation of murine bone marrow cells fully differentiates along the myogenic pathway and participates in muscle repair in the mdx dystrophic mouse. *Experimental Cell Research*. 2002;277(1):74–85.
- [4] Kerkis I, Ambrosio CE, Kerkis A, Martins DS, Zucconi E, Fonseca SA, et al. Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: local or systemic? *Journal of Translational Medicine*. 2008;6:35.

- [5] Goncalves MA, de Vries AA, Holkers M, van de Watering MJ, van der Velde I, van Nierop GP, et al. Human mesenchymal stem cells ectopically expressing full-length dystrophin can complement Duchenne muscular dystrophy myotubes by cell fusion. *Human Molecular Genetics*. 2006;15(2):213–21.
- [6] Di Rocco G, Iachininoto MG, Tritarelli A, Straino S, Zacheo A, Germani A, et al. Myogenic potential of adipose-tissue-derived cells. *Journal of Cell Science*. 2006;119(14):2945–52.
- [7] Goudenege S, Pisani DF, Wdziekonski B, Di Santo JP, Bagnis C, Dani C, et al. Enhancement of myogenic and muscle repair capacities of human adipose-derived stem cells with forced expression of MyoD. *Molecular Therapy*. 2009;17(6):1064–72.
- [8] Merritt EK, Hammers DW, Tierney M, Suggs LJ, Walters TJ, Farrar RP. Functional assessment of skeletal muscle regeneration utilizing homologous extracellular matrix as scaffolding. *Tissue Engineering Part A*. 2010;16(4):1395–405.
- [9] Gates C, Huard J. Management of skeletal muscle injuries in military personnel. *Operative Techniques in Sports Medicine*. 2005;13(4):247–56.
- [10] Järvinen TAH, Järvinen TLN, Kääriäinen M, Äärimaa V, Vaittinen S, Kalimo H, et al. Muscle injuries: optimising recovery. *Best Practice and Research Clinical Rheumatology*. 2007;21(2):317–31.
- [11] Mase Jr VJ, Hsu JR, Wolf SE, Wenke JC, Baer DG, Owens J, et al. Clinical application of an acellular biologic scaffold for surgical repair of a large, traumatic quadriceps femoris muscle defect. *Orthopedics*. 2010;33(7):511.
- [12] Grogan BF, Hsu JR, Consortium STR. Volumetric muscle loss. *Journal of the American Academy of Orthopaedic Surgeons*. 2011;19(Suppl 1):S35-S7.
- [13] Jarvinen M, Aho AJ, Lehto M, Toivonen H. Age dependent repair of muscle rupture. A histological and microangiographical study in rats. *Acta Orthopaedica Scandinavica*. 1983;54(1):64–74.
- [14] Gopinath SD, Rando TA. Stem cell review series: aging of the skeletal muscle stem cell niche. *Aging Cell*. 2008;7(4):590–8.
- [15] Giordani L, Puri PL. Epigenetic control of skeletal muscle regeneration: integrating genetic determinants and environmental changes. *FEBS Journal*. 2013;280(17):4014–25.
- [16] Järvinen TAH, Järvinen TLN, Kääriäinen M, Kalimo H, Järvinen M. Muscle injuries. *American Journal of Sports Medicine*. 2005;33(5):745–64.
- [17] MacLean S, Khan W, Malik A, Anand S, Snow M. The potential of stem cells in the treatment of skeletal muscle injury and disease. *Stem Cells International*. 2011;2012.

- [18] Tedesco FS, Dellavalle A, Diaz-Manera J, Messina G, Cossu G. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *Journal of Clinical Investigation*. 2010;120(1):11.
- [19] Borriore P, Di Gianfrancesco A, Pereira MT, Pigozzi F. Platelet-rich plasma in muscle healing. *American Journal of Physical Medicine and Rehabilitation*. 2010;89(10):854–61.
- [20] Tidball JG. Inflammatory processes in muscle injury and repair. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology*. 2005;288(2):R345–53.
- [21] Mauro A. Satellite cell of skeletal muscle fibers. *Journal of Biophysical and Biochemical Cytology*. 1961;9(2):493–5.
- [22] Kuang S, Kuroda K, Le Grand F, Rudnicki MA. Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell*. 2007;129(5):999–1010.
- [23] Mitchell KJ, Pannerec A, Cadot B, Parlakian A, Besson V, Gomes ER, et al. Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development. *Nature Cell Biology*. 2010;12(3):257–66.
- [24] Pannerec A, Formicola L, Besson V, Marazzi G, Sassoon DA. Defining skeletal muscle resident progenitors and their cell fate potentials. *Development*. 2013;140(14):2879–91.
- [25] Pannerec A, Marazzi G, Sassoon D. Stem cells in the hood: the skeletal muscle niche. *Trends in Molecular Medicine*. 2012;18(10):599–606.
- [26] Pannerec A, Sassoon D. PICs (PW1+ interstitial cells), a new muscle stem cell population. *Médecine/Science (Paris)*. 2010;26(10):797–800.
- [27] Charge SBP, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. *Physiological Reviews*. 2004;84(1):209–38.
- [28] Serrano AL, Mann CJ, Vidal B, Ardite E, Perdiguero E, Munoz-Canoves P. Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease. *Current Topics in Developmental Biology*. 2011;96:167–201.
- [29] Serrano AL, Munoz-Canoves P. Regulation and dysregulation of fibrosis in skeletal muscle. *Experimental Cell Research*. 2010;316(18):3050–8.
- [30] Mann CJ, Perdiguero E, Kharraz Y, Aguilar S, Pessina P, Serrano AL, et al. Aberrant repair and fibrosis development in skeletal muscle. *Skeletal Muscle*. 2011;1(1):21.
- [31] Page RL, Malcuit C, Vilner L, Vojtic I, Shaw S, Hedblom E, et al. Restoration of skeletal muscle defects with adult human cells delivered on fibrin microthreads. *Tissue Engineering Part A*. 2011;17(21–22):2629–40.

- [32] Sato K, Li Y, Foster W, Fukushima K, Badlani N, Adachi N, et al. Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis. *Muscle Nerve*. 2003;28(3):365–72.
- [33] Corona BT, Wu X, Ward CL, McDaniel JS, Rathbone CR, Walters TJ. The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. *Biomaterials*. 2013;34(13):3324–35.
- [34] Tamaki T, Uchiyama Y, Okada Y, Ishikawa T, Sato M, Akatsuka A, et al. Functional recovery of damaged skeletal muscle through synchronized vasculogenesis, myogenesis, and neurogenesis by muscle-derived stem cells. *Circulation*. 2005;112(18):2857–66.
- [35] Rossi CA, Flaibani M, Blaauw B, Pozzobon M, Figallo E, Reggiani C, et al. In vivo tissue engineering of functional skeletal muscle by freshly isolated satellite cells embedded in a photopolymerizable hydrogel. *FASEB Journal*. 2011;25(7):2296–304.
- [36] Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, et al. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell*. 2005;122(2):289–301.
- [37] Koning M, Harmsen MC, van Luyn MJA, Werker PMN. Current opportunities and challenges in skeletal muscle tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine*. 2009;3(6):407–15.
- [38] Beier JP, Bitto FF, Lange C, Klumpp D, Arkudas A, Bleiziffer O, et al. Myogenic differentiation of mesenchymal stem cells co-cultured with primary myoblasts. *Cell Biology International*. 2011;35(4):397–406.
- [39] Mizuno H, Zuk PA, Zhu M, Lorenz PH, Benhaim P, Hedrick MH. Myogenic differentiation by human processed lipoaspirate cells. *Plastic and Reconstructive Surgery*. 2002;109(1):199–209.
- [40] Yaffe D. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1968;61(2):477.
- [41] Corona BT, Machingal MA, Criswell T, Vadhavkar M, Dannahower AC, Bergman C, et al. Further development of a tissue engineered muscle repair construct in vitro for enhanced functional recovery following implantation in vivo in a murine model of volumetric muscle loss injury. *Tissue Engineering Part A*. 2012;18(11–12):1213–28.
- [42] Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. *Journal of Cellular Biochemistry*. 2006;98(5):1076–84.
- [43] Alexandre N, Ribeiro J, Gartner A, Pereira T, Amorim I, Fragoso J, et al. Biocompatibility and hemocompatibility of polyvinyl alcohol hydrogel used for vascular grafting- In vitro and in vivo studies. *Journal of Biomedical Materials Research Part A*. 2014;102(12):4262–75.

- [44] Amado S, Rodrigues JM, Luis AL, Armada-da-Silva PA, Vieira M, Gartner A, et al. Effects of collagen membranes enriched with in vitro-differentiated N1E-115 cells on rat sciatic nerve regeneration after end-to-end repair. *Journal of NeuroEngineering and Rehabilitation*. 2010;7:7.
- [45] Gärtner A, Pereira T, Armada-da-Silva P, Amado S, Veloso A, Amorim I, et al. Effects of umbilical cord tissue mesenchymal stem cells (UCX[®]) on rat sciatic nerve regeneration after neurotmesis injuries. *Journal of Stem Cells and Regenerative Medicine*. 2014;Vol.X(1):JSRM Code: 010010300001EPA010214.
- [46] Gärtner A, Pereira T, Armada-da-Silva PA, Amorim I, Gomes R, Ribeiro J, et al. 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. *Differentiation*. 2012;84(5):355–65.
- [47] Gärtner A, Pereira T, Simões MJ, Armada-da-Silva P, França ML, Sousa R, et al. Use of hybrid chitosan membranes and MSC cells for promoting nerve regeneration in an axonotmesis rat model. *Neural Regeneration Research*. 2012;7(29):2247–58.
- [48] Luis AL, Rodrigues JM, Geuna S, Amado S, Shirosaki Y, Lee JM, et al. Use of PLGA 90:10 scaffolds enriched with in vitro-differentiated neural cells for repairing rat sciatic nerve defects. *Tissue Engineering Part A*. 2008;14(6):979–93.
- [49] Luis AL, Rodrigues JM, Geuna S, Amado S, Simoes MJ, Fregnan F, et al. Neural cell transplantation effects on sciatic nerve regeneration after a standardized crush injury in the rat. *Microsurgery*. 2008;28(6):458–70.
- [50] Pereira T, Gartner A, Amorim I, Almeida A, Caseiro AR, Armada-da-Silva PA, et al. Promoting nerve regeneration in a neurotmesis rat model using poly(DL-lactide- ϵ -caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly: in vitro and in vivo analysis. *Biomed Research International*. 2014;2014:302659.
- [51] Pereira T, Gärtner A, Amorim I, Ribeiro J, França M, Armada-da-Silva P, et al., editors. Development of a skeletal muscle injury model in the rat and in vivo evaluation of the use of human mesenchymal stem cells (HMSCs) from the umbilical cord matrix in myectomy injury treatment. 17th annual Congress of the European College of Sport Science; July 4–7, 2012; Bruges, Belgium, 2012.
- [52] Pereira T, Gärtner A, Amorim I, Ribeiro J, França M, Armada-da-Silva P, et al. In vivo evaluation of muscular regeneration treated with human mesenchymal stem cells (HMSCs) from the umbilical cord matrix and different biomaterials in a rat anterior tibial myectomy model. *Journal of Tissue Engineering and Regenerative Medicine*. 2012;6(Suppl. 1):396.
- [53] Ribeiro J, Pereira T, Amorim I, Caseiro AR, Lopes MA, Lima J, et al. Cell therapy with human MSCs isolated from the umbilical cord Wharton jelly associated to a PVA membrane in the treatment of chronic skin wounds. *International Journal of Medical Sciences*. 2014;11(10):979–87.

- [54] Pereira T, Armada-da Silva P, Amorim I, Rêma A, Caseiro A, Gärtner A, et al. Effects of human mesenchymal stem cells isolated from Wharton's jelly of the umbilical cord and conditioned media on skeletal muscle regeneration using a myectomy model. *Stem Cells International*. 2014;2014.
- [55] Friedenstein A, Chailakhjan R, Lalykina K. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Proliferation*. 1970;3(4):393–403.
- [56] Lajtha LG. Stem cells and their properties. *Proceedings Canadian Cancer Conference*. 1967;7:31–9.
- [57] Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation*. 1968;6(2):230–47.
- [58] Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells*. 2007;25(11):2739–49.
- [59] Triffitt JT. Stem cells and the philosopher's stone. *Journal of Cellular Biochemistry. Supplement*. 2002;38:13–9.
- [60] da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *Journal of Cell Science*. 2006;119(11):2204–13.
- [61] Muguruma Y, Reyes M, Nakamura Y, Sato T, Matsuzawa H, Miyatake H, et al. In vivo and in vitro differentiation of myocytes from human bone marrow-derived multipotent progenitor cells. *Experimental Hematology*. 2003;31(12):1323–30.
- [62] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8(4):315–7.
- [63] Caplan AI. Why are MSCs therapeutic? New data: new insight. *Journal of Pathology*. 2009;217(2):318–24.
- [64] Le Blanc K, Ringden O. Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. *Biology of blood and marrow transplantation. Journal of the American Society for Blood and Marrow Transplantation*. 2005;11(5):321–34.
- [65] Fossett E, Khan WS. Optimising human mesenchymal stem cell numbers for clinical application: a literature review. *Stem Cells International*. 2012;2012:465259.
- [66] Kim DW, Staples M, Shinozuka K, Pantcheva P, Kang SD, Borlongan CV. Wharton's jelly-derived mesenchymal stem cells: phenotypic characterization and optimizing

- their therapeutic potential for clinical applications. *International Journal of Molecular Sciences*. 2013;14(6):11692–712.
- [67] Conconi MT, Burra P, Di Liddo R, Calore C, Turetta M, Bellini S, et al. CD105(+) cells from Wharton's jelly show in vitro and in vivo myogenic differentiative potential. *International Journal of Molecular Medicine*. 2006;18(6):1089–96.
- [68] Wu J, Rojas M, Weiss D, Gross G, Häupl T, Almeida-Porada G, et al. Stem cell-dependent therapies mesenchymal stem cells in chronic inflammatory disorders. Berlin: De Gruyter; 2013.
- [69] Kawashima N. Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? *Archives of Oral Biology*. 2012;57(11):1439–58.
- [70] Song M, Kim H, Choi Y, Kim K, Chung C. Skeletal myogenic differentiation of human periodontal ligament stromal cells isolated from orthodontically extracted premolars. *Korean Journal of Orthodontics*. 2012;42(5):249–54.
- [71] Hodgetts S, Stagg K, Sturm M, Edel M, Blancafort P. Long live the stem cell: the use of stem cells isolated from post mortem tissues for translational strategies. *International Journal of Biochemistry and Cell Biology*. 2014;56C:74–81.
- [72] Cheng PH, Snyder B, Fillos D, Ibegbu CC, Huang AH, Chan AW. Postnatal stem/progenitor cells derived from the dental pulp of adult chimpanzee. *BMC Cell Biology*. 2008;9:20.
- [73] De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis and Rheumatism*. 2001;44(8):1928–42.
- [74] de la Garza-Rodea AS, van der Velde-van Dijke I, Boersma H, Gonçalves MA, van Bekkum DW, de Vries AA, et al. Myogenic properties of human mesenchymal stem cells derived from three different sources. *Cell Transplantation*. 2012;21(1):153–73.
- [75] Spath L, Rotilio V, Alessandrini M, Gambarà G, De Angelis L, Mancini M, et al. Explant-derived human dental pulp stem cells enhance differentiation and proliferation potentials. *Journal of Cellular and Molecular Medicine*. 2010;14(6B):1635–44.
- [76] Grabowska I, Brzoska E, Gawrysiak A, Streminska W, Moraczewski J, Polanski Z, et al. Restricted myogenic potential of mesenchymal stromal cells isolated from umbilical cord. *Cell Transplantation*. 2012;21(8):1711–26.
- [77] Grabowska I, Streminska W, Janczyk-Ilach K, Machaj EK, Pojda Z, Hoser G, et al. Myogenic potential of mesenchymal stem cells—the case of adhesive fraction of human umbilical cord blood cells. *Current Stem Cell Research and Therapy*. 2013;8(1):82–90.

- [78] Santa María L, Rojas CV, Minguell JJ. Signals from damaged but not undamaged skeletal muscle induce myogenic differentiation of rat bone-marrow-derived mesenchymal stem cells. *Experimental Cell Research*. 2004;300(2):418–26.
- [79] Shi D, Reinecke H, Murry CE, Torok-Storb B. Myogenic fusion of human bone marrow stromal cells, but not hematopoietic cells. *Blood*. 2004;104(1):290–4.
- [80] Bajek A, Drewa T, Joachimiak R, Spoz Z, Gagat M, Bodnar M, et al. Myogenic differentiation of mesenchymal stem cells is induced by striated muscle influences in vitro. *Current Signal Transduction Therapy*. 2012;7(3):220–7.
- [81] Meligy FY, Shigemura K, Behnsawy HM, Fujisawa M, Kawabata M, Shirakawa T. The efficiency of in vitro isolation and myogenic differentiation of MSCs derived from adipose connective tissue, bone marrow, and skeletal muscle tissue. *In Vitro Cellular and Developmental Biology Animal*. 2012;48(4):203–15.
- [82] Gang E, Jeong J, Hong S, Hwang S, Kim S, Yang I, et al. Skeletal myogenic differentiation of mesenchymal stem cells isolated from human umbilical cord blood. *Stem Cells*. 2004;22:617–24.
- [83] Hwang JH, Kim IG, Piao S, Jung AR, Lee JY, Park KD, et al. Combination therapy of human adipose-derived stem cells and basic fibroblast growth factor hydrogel in muscle regeneration. *Biomaterials*. 2013;34(25):6037–45.
- [84] Winkler T, von Roth P, Radojewski P, Urbanski A, Hahn S, Preininger B, et al. Immediate and delayed transplantation of mesenchymal stem cells improve muscle force after skeletal muscle injury in rats. *Journal of Tissue Engineering and Regenerative Medicine*. 2012;6(Suppl 3):s60–7.
- [85] Winkler T, von Roth P, Schuman MR, Sieland K, Stoltenburg-Didinger G, Taupitz M, et al. In vivo visualization of locally transplanted mesenchymal stem cells in the severely injured muscle in rats. *Tissue Engineering Part A*. 2008;14(7):1149–60.
- [86] Von Roth P, Radojewski P, Matziolis G, Duda G, Perka C, Winkler T. Systemic mesenchymal stem cell transplantation for the treatment of skeletal muscle trauma. *Journal of Bone and Joint Surgery, British Volume*. 2011;93-B(II):134.
- [87] Peçanha R, Bagno LL, Ribeiro MB, Robottom Ferreira AB, Moraes MO, Zapata-Sudo G, et al. Adipose-derived stem-cell treatment of skeletal muscle injury. *Journal of Bone and Joint Surgery, American Volume*. 2012;94(7):609–17.
- [88] de la Garza-Rodea AS, van der Velde I, Boersma H, Gonçalves MA, van Bekkum DW, de Vries AA, et al. Long-term contribution of human bone marrow mesenchymal stromal cells to skeletal muscle regeneration in mice. *Cell Transplantation*. 2011;20(2):217–31.
- [89] Natsu K, Ochi M, Mochizuki Y, Hachisuka H, Yanada S, Yasunaga Y. Allogeneic bone marrow-derived mesenchymal stromal cells promote the regeneration of in-

- jured skeletal muscle without differentiation into myofibers. *Tissue Engineering*. 2004;10(7–8):1093–112.
- [90] von Roth P, Duda GN, Radojewski P, Preininger B, Perka C, Winkler T. Mesenchymal stem cell therapy following muscle trauma leads to improved muscular regeneration in both male and female rats. *Gender Medicine*. 2012;9(2):129–36.
- [91] Matziolis G, Winkler T, Schaser K, Wiemann M, Krockner D, Tuischer J, et al. Autologous bone marrow-derived cells enhance muscle strength following skeletal muscle crush injury in rats. *Tissue Engineering*. 2006;12(2):361–7.
- [92] Cittadella Vigodarzere G, Mantero S. Skeletal muscle tissue engineering: strategies for volumetric constructs. *Frontiers in Physiology*. 2014;5:362.
- [93] Wolf MT, Dearth CL, Sonnenberg SB, Lobo EG, Badylak SF. Naturally derived and synthetic scaffolds for skeletal muscle reconstruction. *Advanced Drug Delivery Reviews*. 2014.
- [94] Sicari BM, Rubin JP, Dearth CL, Wolf MT, Ambrosio F, Boninger M, et al. An acellular biologic scaffold promotes skeletal muscle formation in mice and humans with volumetric muscle loss. *Science Translational Medicine*. 2014;6(234):234ra58.
- [95] Turner NJ, Yates Jr AJ, Weber DJ, Qureshi IR, Stolz DB, Gilbert TW, et al. Xenogeneic extracellular matrix as an inductive scaffold for regeneration of a functioning musculotendinous junction. *Tissue Engineering Part A*. 2010;16(11):3309–17.
- [96] Gentile NE, Stearns KM, Brown EH, Rubin JP, Boninger ML, Dearth CL, et al. Targeted rehabilitation after extracellular matrix scaffold transplantation for the treatment of volumetric muscle loss. *American Journal of Physical Medicine and Rehabilitation*. 2014;93(11):S79–S87.
- [97] Machingal MA, Corona BT, Walters TJ, Kesireddy V, Koval CN, Dannahower A, et al. A tissue-engineered muscle repair construct for functional restoration of an irrecoverable muscle injury in a murine model. *Tissue Engineering Part A*. 2011;17(17–18):2291–303.
- [98] Merritt EK, Cannon MV, Hammers DW, Le LN, Gokhale R, Sarathy A, et al. Repair of traumatic skeletal muscle injury with bone-marrow-derived mesenchymal stem cells seeded on extracellular matrix. *Tissue Engineering Part A*. 2010;16(9):2871–81.
- [99] Klumpp D, Horch RE, Kneser U, Beier JP. Engineering skeletal muscle tissue—new perspectives in vitro and in vivo. *Journal of Cellular and Molecular Medicine*. 2010;14(11):2622–9.
- [100] Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, et al. Engineering vascularized skeletal muscle tissue. *Nature Biotechnology*. 2005;23(7):879–84.
- [101] Koffler J, Kaufman-Francis K, Shandalov Y, Egozi D, Amiad Pavlov D, Landesberg A, et al. Improved vascular organization enhances functional integration of engi-

- neered skeletal muscle grafts. *Proceedings of the National Academy of Sciences*. 2011;108(36):14789–94.
- [102] Shandalov Y, Egozi D, Koffler J, Dado-Rosenfeld D, Ben-Shimol D, Freiman A, et al. An engineered muscle flap for reconstruction of large soft tissue defects. *Proceedings of the National Academy of Sciences*. 2014;111(16):6010–5.
- [103] Winkler T, von Roth P, Matziolis G, Mehta M, Perka C, Duda GN. Dose–response relationship of mesenchymal stem cell transplantation and functional regeneration after severe skeletal muscle injury in rats. *Tissue Engineering Part A*. 2009;15(3):487–92.
- [104] Pereira T, Gärtner A, Amorim I, Armada-da-Silva P, Gomes R, C. P, et al. Biomaterials and stem cell therapies for injuries associated to skeletal muscular tissues. In: Pignatello R, editor. *Advances in Biomaterials Science and Biomedical Applications*: InTech; 2013.
- [105] Anderson JE, Mitchell CM, McGeachie JK, Grounds MD. The time course of basic fibroblast growth factor expression in crush-injured skeletal muscles of SJL/J and BALB/c mice. *Experimental Cell Research*. 1995;216(2):325–34.
- [106] Chen L, Tredget EE, Wu PYG, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PloS One*. 2008;3(4):e1886.
- [107] Pereira T, Ivanova G, Caseiro AR, Barbosa P, Bartolo PJ, Santos JD, et al. MSCs conditioned media and umbilical cord blood plasma metabolomics and composition. *PloS One*. 2014;9(11):e113769.
- [108] Ten Broek RW, Grefte S, Von den Hoff JW. Regulatory factors and cell populations involved in skeletal muscle regeneration. *Journal of Cellular Physiology*. 2010;224(1):7–16.
- [109] Husmann I, Soulet L, Gautron J, Martelly I, Barritault D. Growth factors in skeletal muscle regeneration. *Cytokine and Growth Factor Reviews*. 1996;7(3):249–58.
- [110] Tidball JG, Dorshkind K, Wehling-Henricks M. Shared signaling systems in myeloid cell-mediated muscle regeneration. *Development*. 2014;141(6):1184–96.
- [111] Villalta SA, Nguyen HX, Deng B, Gotoh T, Tidball JG. Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. *Human Molecular Genetics*. 2009;18(3):482–96.
- [112] Heredia JE, Mukundan L, Chen FM, Mueller AA, Deo RC, Locksley RM, et al. Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. *Cell*. 2013;153(2):376–88.

- [113] Hayashi S, Aso H, Watanabe K, Nara H, Rose M, Ohwada S, et al. Sequence of IGF-I, IGF-II, and HGF expression in regenerating skeletal muscle. *Histochemistry and Cell Biology*. 2004;122(5):427–34.
- [114] DO MKQ, Suzuki T, Gerelt B, Sato Y, Mizunoya W, Nakamura M, et al. Time-coordinated prevalence of extracellular HGF, FGF2 and TGF- β 3 in crush-injured skeletal muscle. *Animal Science Journal*. 2012;83(10):712–7.
- [115] Tatsumi R, Anderson JE, Nevoret CJ, Halevy O, Allen RE. HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Developmental Biology*. 1998;194(1):114–28.
- [116] Miller KJ, Thalloor D, Matteson S, Pavlath GK. Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle. *American Journal of Physiology—Cell Physiology*. 2000;278(1):C174–81.
- [117] Yamada M, Tatsumi R, Yamanouchi K, Hosoyama T, Shiratsuchi S-i, Sato A, et al. High concentrations of HGF inhibit skeletal muscle satellite cell proliferation in vitro by inducing expression of myostatin: a possible mechanism for reestablishing satellite cell quiescence in vivo. *American Journal of Physiology—Cell Physiology*. 2010;298(3):C465–C76.
- [118] Tatsumi R, Liu X, Pulido A, Morales M, Sakata T, Dial S, et al. Satellite cell activation in stretched skeletal muscle and the role of nitric oxide and hepatocyte growth factor. *American Journal of Physiology—Cell Physiology*. 2006;290(6):C1487–C94.
- [119] Suzuki S, Yamanouchi K, Soeta C, Katakai Y, Harada R, Naito K, et al. Skeletal muscle injury induces hepatocyte growth factor expression in spleen. *Biochemical and Biophysical Research Communications*. 2002;292(3):709–14.
- [120] Menetrey J, Kasemkijwattana C, Day C, Bosch P, Vogt M, Fu F, et al. Growth factors improve muscle healing in vivo. *Journal of Bone and Joint Surgery, British Volume*. 2000;82(1):131.
- [121] Zanou N, Gailly P. Skeletal muscle hypertrophy and regeneration: interplay between the myogenic regulatory factors (MRFs) and insulin-like growth factors (IGFs) pathways. *Cellular and Molecular Life Sciences*. 2013;70(21):4117–30.
- [122] Pelosi L, Giacinti C, Nardis C, Borsellino G, Rizzuto E, Nicoletti C, et al. Local expression of IGF-1 accelerates muscle regeneration by rapidly modulating inflammatory cytokines and chemokines. *FASEB Journal*. 2007;21(7):1393–402.
- [123] Borselli C, Storrie H, Benesch-Lee F, Shvartsman D, Cezar C, Lichtman JW, et al. Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors. *Proceedings of the National Academy of Sciences*. 2010;107(8):3287–92.
- [124] Floss T, Arnold H-H, Braun T. A role for FGF-6 in skeletal muscle regeneration. *Genes and Development*. 1997;11(16):2040–51.

- [125] Armand AS, Pariset C, Laziz I, Launay T, Fiore F, Della Gaspera B, et al. FGF6 regulates muscle differentiation through a calcineurin-dependent pathway in regenerating soleus of adult mice. *Journal of Cellular Physiology*. 2005;204(1):297–308.
- [126] Armand A-S, Launay T, Pariset C, Della Gaspera B, Charbonnier F, Chanoine C. Injection of FGF6 accelerates regeneration of the soleus muscle in adult mice. *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*. 2003;1642(1–2):97–105.
- [127] Messina S, Mazzeo A, Bitto A, Aguenouz Mh, Migliorato A, De Pasquale MG, et al. VEGF overexpression via adeno-associated virus gene transfer promotes skeletal muscle regeneration and enhances muscle function in mdx mice. *FASEB Journal*. 2007;21(13):3737–46.
- [128] Frey SP, Jansen H, Raschke MJ, Meffert RH, Ochman S. VEGF improves skeletal muscle regeneration after acute trauma and reconstruction of the limb in a rabbit model. *Clinical Orthopaedics and Related Research*. 2012;470(12):3607–14.
- [129] Karvinen H, Pasanen E, Rissanen T, Korpisalo P, Vähäkangas E, Jazwa A, et al. Long-term VEGF-A expression promotes aberrant angiogenesis and fibrosis in skeletal muscle. *Gene Therapy*. 2011;18(12):1166–72.
- [130] Hara M, Yuasa S, Shimoji K, Onizuka T, Hayashiji N, Ohno Y, et al. G-CSF influences mouse skeletal muscle development and regeneration by stimulating myoblast proliferation. *Journal of Experimental Medicine*. 2011;208(4):715–27.
- [131] Stratos I, Rotter R, Eipel C, Mittlmeier T, Vollmar B. Granulocyte-colony stimulating factor enhances muscle proliferation and strength following skeletal muscle injury in rats. *Journal of Applied Physiology*. 2007;103(5):1857–63.
- [132] Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, et al. Transforming growth factor- β 1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis. *American Journal of Pathology*. 2004;164(3):1007–19.
- [133] Hwang JH, Ra Y-J, Lee KM, Lee JY, Ghil SH. Therapeutic effect of passive mobilization exercise on improvement of muscle regeneration and prevention of fibrosis after laceration injury of rat. *Archives of Physical Medicine and Rehabilitation*. 2006;87(1):20–6.
- [134] Baraniak PR, McDevitt TC. Stem cell paracrine actions and tissue regeneration. *Regenerative Medicine*. 2010;5(1):121–43.
- [135] Haynesworth SE, Baber MA, Caplan AI. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 α . *Journal of Cellular Physiology*. 1996;166(3):585–92.
- [136] Chen JS, Wong VW, Gurtner GC. Therapeutic potential of bone marrow-derived mesenchymal stem cells in cutaneous wound healing. *Frontiers in Immunology*. 2012;3.

- [137] Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation*. 2004;109(10):1292–8.
- [138] da Silva Meirelles L, Fontes AM, Covas DT, Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine and Growth Factor Reviews*. 2009;20(5–6):419–27.
- [139] Doorn J, Moll G, Le Blanc K, van Blitterswijk C, de Boer J. Therapeutic applications of mesenchymal stromal cells: paracrine effects and potential improvements. *Tissue Engineering Part B: Reviews*. 2012;18(2):101–15.
- [140] J Salgado A, L Reis R, Sousa N, M Gimble J. Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine. *Current Stem Cell Research and Therapy*. 2010;5(2):103–10.
- [141] Carvalho MM, Teixeira FG, Reis RL, Sousa N, Salgado AJ. Mesenchymal stem cells in the umbilical cord: phenotypic characterization, secretome and applications in central nervous system regenerative medicine. *Current Stem Cell Research and Therapy*. 2011;6(3):221–8.
- [142] Yoo KH, Jang IK, Lee MW, Kim HE, Yang MS, Eom Y, et al. Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. *Cellular Immunology*. 2009;259(2):150–6.
- [143] Teixeira F, Pires A, Fraga J, Carvalho M, Silva N, Panchalingam K, et al. Secretome of mesenchymal stem cells as a new tool for central nervous system regenerative medicine. *Journal of Tissue Engineering and Regenerative Medicine*. 2014;8:298–9.
- [144] Teixeira FG, Carvalho MM, Sousa N, Salgado AJ. Mesenchymal stem cells secretome: a new paradigm for central nervous system regeneration? *Cellular and Molecular Life Sciences*. 2013;70(20):3871–82.

1.2. Mesenchymal Stem/ Stromal Cells application for peripheral nerve repair and regeneration

Neuromuscular Regeneration: Perspective on the Application of Mesenchymal Stem Cells and Their Secretion Products

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Review Article

Neuromuscular Regeneration: Perspective on the Application of Mesenchymal Stem Cells and Their Secretion Products

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Mesenchymal stem cells are posing as a promising character in the most recent therapeutic strategies and, since their discovery, extensive knowledge on their features and functions has been gained. In recent years, innovative sources have been disclosed in alternative to the bone marrow, conveying their associated ethical concerns and ease of harvest, such as the umbilical cord tissue and the dental pulp. These are also amenable of cryopreservation and thawing for desired purposes, in benefit of the donor itself or other patients in pressing need. These sources present promising possibilities in becoming useful cell sources for therapeutic applications in the forthcoming years. Effective and potential applications of these cellular-based strategies for the regeneration of peripheral nerve are overviewed, documenting recent advances and identified issues for this research area in the near future. Finally, besides the differentiation capacities attributed to mesenchymal stem cells, advances in the recognition of their effective mode of action in the regenerative theatre have led to a new area of interest: the mesenchymal stem cells' secretome. The paracrine modulatory pathway appears to be a major mechanism by which these are beneficial to nerve regeneration and comprehension on the specific growth factors, cytokine, and extracellular molecules secretion profiles is therefore of great interest.

1. Introduction

The peripheral nervous system (PNS) is often involved in severe traumatic events which may result in relevant impairment of occupational and everyday life activities performance. The physical disability itself and the associated painful events limit the quality of life of affected patients [1]. Iatrogenic damage related to surgical procedure is also often observed [2]. When compared to the central nervous system (CNS), the PNS depicts a superior capacity for regeneration, although in severe injuries complete repair is not often observed, and functional recovery is poor [3, 4]. Amongst other factors, this capacity is also dependent on the age of

the individual [5], giving the topic additional relevance in an aging world population.

1.1. Peripheral Nerve Lesions' Associated Muscular Atrophy (Neurogenic Muscle Atrophy). Alongside the immediate loss of sensory and voluntary motor functions of the supplied areas and muscle groups, severe nerve injuries are accompanied by atrophy of the latter, resulting from the lack of electrophysiological as well as biochemical communication between the nerve and muscle components [6]. The denervation of a muscle leads to fast progressing muscle mass loss [7, 8], in first instance related to the loss of the contractile machinery, and then to effective loss of muscle

fibres, after prolonged, year lasting, denervation periods [7]. The initial events result from unbalanced protein synthesis and proteolysis [9], while the second stage of muscle mass loss results from the combination of cell death and myonuclei apoptosis with decreased satellite cells responsiveness [10].

The general homeostasis and regenerative capacity of skeletal muscle are under significant neural influence. Denervated muscles' fibre type content suffers significant shifts [7], and muscles lose blood supply over time, with significant degeneration of the whole vascular network [11], impairing chances of recovery of muscle function and strength, even if neural function is restored. The regenerative cells pool within the skeletal muscle also seems sensitive to neural control. The loss of this regulation by means of denervation triggers satellite cells function into repetitive proliferative cycles and differentiation [8], ultimately contributing to its exhaustion and long term regenerative impairment of those muscles [10].

The speed of recovery can be further arrested by delayed surgical repair, as occurs in most clinical cases [4]. Accelerated restoration of the nerve structure and function and consequently its electrophysiological stimulatory capacity are key-points for preventing muscle atrophy and promoting functional recovery. The longer nerve communication remains interrupted, the less effective injury activated Schwann cells will be at stimulating regrowth, and the more severe distal stump degeneration will become [12]. The longer a muscle stands devoid of such stimuli the harsher the alterations to its own structure and contractile capacity, and the harder its recovery is upon reestablishment of electrical communication [7].

1.2. Peripheral Nerve Injuries and Repair Techniques. In the vast list of diseases affecting the nervous system, and specifically the PNS, traumatic events comprise a relevant source of nerve damage [1]. From crush to sectioning or avulsion, such events severely affect peripheral nerve structure and function, conditioning both sensory and motor transmission pathways.

Focal crush injuries (Sunderland type II), termed axonotmesis injuries, cause disruption of axons and involving myelin sheaths, but the connective support structures are maintained [13, 14]. Recovery from this type of injury does not generally require surgical intervention, and axons regenerate along the preserved endoneurial tubes, stimulated by the reactive Schwann cells, ultimately regaining contact with the distal portion of the lesion and finally reinnervating the associated muscle. So, despite being capable of satisfactory self-regeneration, the time-lapse required for the process invariably leads to the atrophy of the formerly supplied muscle groups [15, 16]. Therefore, although no physical reconstruction is necessary for the management of axonotmesis injuries, the development of strategies for an accelerated reconnection process is highly desirable aiming at the optimization of the patient's neuromuscular function recovery.

In the cases of complete nerve severing (Sunderland type V), referred to as neurotmesis injuries, all the nerve fibre structures lose continuity, and spontaneous recuperation becomes more unlikely, demanding important reconstructive

microsurgery techniques and, more recently, new therapeutic approaches based on new biomaterials for guiding conduits and cell-based therapies [14, 16, 17].

Despite the extensive effort towards improved surgical techniques for the repair of severely affected nerves, functional recovery indexes on such injuries remain far from desired [18]. While direct suturing of the sectioned nerve endings (neurorrhaphy) would be the most adequate procedure [19], it turns impractical in cases of nerve tissue loss, which require a more complex approach. Nerve grafts can be utilized to bridge this gap in a tension-free manner, from either autologous or allogeneic source [20], as well as other nonnervous tissues, such as blood vessels. However, these grafting techniques have several limitations, including donor site morbidity and donor-recipient nerve diameter and fibre content mismatch, and hence other possibilities were pursued [19–22].

As alternatives to these techniques, nerve conduits from a diversity of biomaterial types and processing techniques started being explored to substitute and overcome the disadvantages of those “organic” options [22]. Materials such as poly(lactic-co-glycolic acid) [23], chitosan [24], collagen [13, 25], and fibrin [26] amongst many others [14, 15, 22] are under study and some have even reached clinical application approval [14, 27, 28].

Research on the use of these structures as guiding scaffolds for nerve regrowth, termed entubulation or tubulization (Figure 1) [23, 27], is in candid expansion.

Although the biomaterials alone are capable of supporting, guiding, and even promoting the axonal regeneration and restoration of nerve continuity [29], the effectiveness and speed of the process are still up for improvements, especially for long gaps in the nerve structure [30]. The success of therapies under development will rely on the consideration of a set of issues, namely, the speed of axonal regrowth, the chronic denervation changes in neuromuscular cell populations and surrounding support structures, and the deleterious character of the local microenvironment to the regenerative process [18].

Direct delivery of growth factors is a prospective strategy for enhancing the performance of the nerve conduits and answering these identified issues [22]. There are several techniques for associating these factors to the biomaterial conduit and optimizing their bioactivity and release kinetic [31], but some downsides regarding potential side-effects and interactions jeopardize their application [2]. Another option for functionalizing the nerve conduit is the association of cellular systems, which will provide a wider range of signalling molecules, rather than single selected stimuli. Within the nervous tissue, resident and blood borne cell populations act as bioactive molecules sources in response to injury [32]. Hence, the association of neuro- or glial-derived cells that could act as regenerative triggers in the absence of the native population (i.e., in cases of nerve tissue section/loss, neurotmesis [23]) or as additional boosters to the intrinsic ongoing regenerative process (when no gap exists and direct suture is feasible [25] or neural support structures are preserved, axonotmesis/crush injury[13]) has also been explored. The main difficulty of using these tissue specific cell

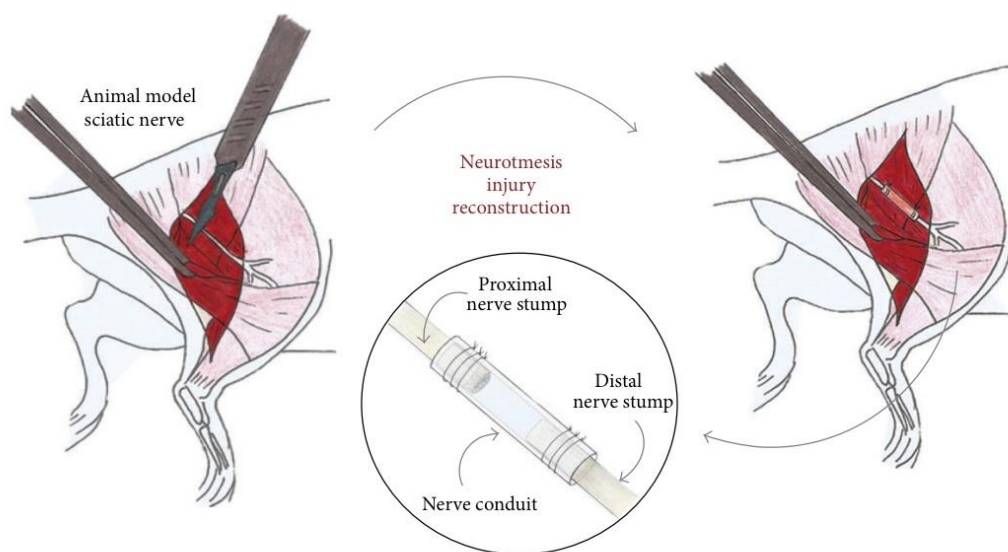


FIGURE 1: Entubulation or tubulization principle for bridging severe peripheral nerve injuries with loss of nerve tissue, preventing tension-free neurorrhaphy. The sectioned nerve stumps are inserted a certain distance into the conduit and microsutured, in a tension-free manner.

sources relates to the procedures required for their isolation, expansion, and differentiation, as in the case of Schwann cells [3].

An interesting alternative to the above-mentioned approaches is the usage of mesenchymal stem cells (MSCs) [33], to be further explored herein. MSCs present precious features, turning them highly fitted candidates to cell therapies development: they can be easily expanded, they have the ability to differentiate into different cell types, and they are immune-privileged and immune-modulatory, as well as preferentially homing to injured sites [34]. MSCs have also been recognized as powerful sources of trophic mediators [35, 36], capable of modulating tissues function, including the central [37] and peripheral nervous [38–40], and musculoskeletal systems [41, 42].

Insight into current and prospective sources for MSCs for regenerative medicine approaches is presented ahead, as well as their role as trophic mediators in the neuromuscular regenerative scenery.

2. Mesenchymal Stem Cells for Peripheral Nerve Repair and Regeneration

Regenerative medicine and tissue engineering are fast expanding scientific research fields, and MSCs are one of the main entities under study, aiming at the development of innovative therapeutic strategies, covering for a number of diseases, affecting multiple body systems [35]. Mesenchymal stem cells were first described as a specific cell population by Friedenstein's research group in the late 1960s [43–45]. At the time, stem cell populations were thought to reside exclusively in organs with observable regenerative capacity, such as the blood, intestine, bone, and skin. As research progressed, we became aware that they are present in virtually all the body

tissues, in variable numbers [46]. These cells generally remain in a quiescent state until activated by significant events, contributing to the efforts of regaining tissue's homeostasis [44, 47, 48].

Through the years, the knowledge on MSCs features and potential has grown exponentially [49, 50] and significant progress has been made towards their better understanding and characterization. In an effort to standardize and unite the scientific community, the Mesenchymal and Tissue Stem Cell Committee, of the International Society for Cellular Therapy (ISCT) gathered a series of recommendations regarding the acceptable criteria for the definition of “mesenchymal stem cell” populations [51]. So, besides their clonogenic and proliferative capacities, while remaining genetically stable and in undifferentiated state, MSCs are also characterized by [51]:

- (i) the plastic adherent ability,
- (ii) the absence of definitive hematopoietic lineage markers, such as CD45, CD34, CD14, CD11b, CD79 α , CD19, and major histocompatibility complex- (MHC-) II/human leukocyte antigen- (HLA-) DR, and expression of nonspecific markers CD105, CD90, and CD73,
- (iii) the ability to differentiate into at least three mesodermal lineage cells: osteocytes, chondrocytes, and adipocytes.

Regarding these differentiation abilities [49, 50, 52, 53], MSCs have also been reported to be capable of differentiating into ectodermal and endodermal cell types [54].

2.1. Impact of MSCs Transplantation on Host Immune System.

The initial notice of MSCs therapeutic potential originated the early concept of “self-therapy” described by Caplan [52], in which immune-rejection was avoided by the possibility

of the donor and host of the expanded cells being the same individual. But, unlike terminally differentiated tissue cells, MSCs are practically devoid of HLA-II, a key player in the body's immune response [55, 56], remaining mostly unnoticed as "foreign" elements, when administered to immunocompetent allorecipients [35, 57, 58]. This nonimmunogenic character of MSCs is an essential factor motivating the research field, considering the difficulty of finding matching donors amongst the human population and the challenges of harvesting sufficient numbers of cells from one patient upon necessity [59]. This is also a relevant topic for the progression of research and the development of new therapies, since it allows for the xenogeneic implantation of human-derived cell in appropriate nonimmunosuppressed animal models [16, 17, 39, 60]. This approach provides valuable information on their behaviour and effect on experimental preclinical models that more closely mimic clinical practice reality [58] and aid the translation of therapies to the clinical ground [32].

MSCs were also found to actively impact on immune events [61]. Significant immune-modulatory actions have been attributed to these cells, mediated by secreted inhibitory and stimulatory molecules, as well as through direct cell-to-cell contact [62].

2.2. MSCs Sources. The bone marrow is without a doubt the most widely explored source of MSCs for therapeutic purposes [46, 50, 63]. The harvesting procedure is however highly invasive and potentially painful [64], motivating the search for more easily accessible sources. Also, the number [53] and "quality" of the isolate cells strongly depends on the age, gender, and health status of the patient or donor [59].

As alternative to the bone marrow, other sources of MSCs are gaining ground [64], for the minimally invasive nature of their harvest as well as for the lesser ethical concerns surrounding their tissues of origin, such as the umbilical cord blood, adipose tissue (AT-MSCs) [64, 65], or the stromal tissue of the umbilical cord (UC-MSCs) [15, 34, 40] and the dental pulp (DPSCs) (Figure 2) [66, 67].

Cells from these alternative sources display comparable phenotypical features and "stemness" potential [51, 54]. Nonetheless, MSCs from distinct sources are not completely identical, differing in, as an example, their differentiation propensity [64, 68–70] and secretory profiles [71].

3. Application of Mesenchymal Stem Cell Based Systems in Neuromuscular Regeneration

Despite all best efforts, available strategies, as discussed in an earlier section, do not seem to meet the healthy nerve's performance, nor the direct end-to-end repair [72], and search towards new methods for improving these outcomes led researchers, including ourselves, to the hypothesis that extraembryonic MSCs would benefit the peripheral nerve regenerative process [40], in both axonotmesis [39, 73] and neurotmesis [16, 17] injury events.

3.1. Delivery Routes for Cellular Systems. Distinct routes could be considered for the delivery of the cellular system. Considering that, on occasion, there can be multiple sites of injury, it has been advocated that systemically administered MSCs could selectively home to those sites and aid recovery. Indeed, these authors demonstrated an active role of MSCs on local inflammatory response as well as accelerated functional recovery after peripheral nerve crushing. This approach also promoted enhanced expression of proregenerative factors at the lesion site [74]. This would be a highly practical route, especially when no surgical access to the site is essential. However, it would ultimately require increased numbers of MSCs upon administration, considering the dilution effect on the total body blood volume and, in the case of venous administration, significant entrapment within the pulmonary capillary network. Intramuscular injection of MSCs in the vicinity of a repaired nerve has also been recently proposed [75, 76]. This way, the cellular system would be "within reach" of the damaged nerve and the related muscle groups. Little attention has been given to this option so far. Direct infiltration of the cellular system into the damaged nerve is another viable option, again as long as support structures are maintained or easily reconstructed, but evidence is that the same cellular system bares improved results in association with a biomaterial membrane or conduit [39]. The biomaterial conduit behaves as a structural selective barrier that provides directed guidance to the growing nerve components and protection against fibrotic tissue ingrowth. It shall also allow for the trading of oxygen, growth factors, and metabolites with the surrounding microenvironment while shielding the area from harsh inflammatory events [27]. As such, it presents as a highly rational strategy to combine the advantages of both approaches into hybrid cellular-biomaterial systems, seeking for major advancements in the peripheral nerve regeneration field. It appears as the most appropriate method to maintain the cells in their intended action site. This foreseen concentration effect would in turn lead to an inferior requirement on the administered cell numbers, in comparison to formerly mentioned strategies.

Roughly, there can be considered two methods of associating such systems with the biomaterial, regarding the timing of surgical implantation. Cells can be injected directly into the scaffold upon its implantation [77] or they can be added prior to contact with the damaged nerve. In this latter situation, cells can additionally be allowed to proliferate for variable periods of time, forming adherent monolayers in the interior of the conduit [17, 26, 39].

3.2. Effects of MSCs Transplantation on Nerve Regeneration. Several studies have reported beneficial effects of MSCs' systems on nerve injury models (Table I) that can be overall listed as follows: (i) modulation of the inflammatory environment on the site; (ii) modulation of the Wallerian degeneration stage; (iii) increased thickness of the myelin sheaths; (iv) accelerated fibre regeneration and in increased numbers; (v) improved fibre organization; (vi) enhanced vascularization of the regenerating site; and (vii) reduction of fibrotic scarring.

TABLE 1: Examples of MSCs systems applications in peripheral nerve injury models.

	Cellular system	MSC's differentiation status	Delivery mode	References
Axonotmesis lesions	rAF-MSCs	Undifferentiated	Fibrin glue + cellulose derivate membrane	[78]
	mAT-MSCs	Undifferentiated	Matrigel	[38]
	hAT-MSCs	Undifferentiated	Systemic intravenous delivery	[74]
	hUC-MSCs	Undifferentiated	Local injection/chitosan III membranes	[73]
	hUC-MSCs	Undifferentiated & neuroglial differentiated	Poly(DL-lactide-ε-caprolactone) (PLC) membranes	[39]
	hAF-MSCs	Undifferentiated	Fibrin glue + cellulose derivate membrane	[79]
	rAT-MSCs	Undifferentiated	Fibrin + PHB (poly-3-hydroxybutyrate) conduit	[80]
	rAT-MSCs	Neuroglial differentiated	Fibrin conduit	[26]
	hAT-MSCs	Neuroglial differentiated	Chitosan coated silicon tube/silicon tube	[81]
	rAT-MSCs	Neuroglial differentiated	PCL (polycaprolactone) conduit	[82]
Neurotmesis lesions	rAT-MSCs	Neuroglial differentiated	Tethered type I collagen gel + collagen membrane	[83]
	rAT-MSCs	Neuroglial differentiated	Collagen gel + xenogeneic acellular nerve matrix	[84]
	hAT-MSCs	Undifferentiated & growth factor stimulated	Fibrin conduit	[85]
	hAT-MSCs	Undifferentiated & neuroglial differentiated	Fibrin conduit	[86]
	rAT-MSCs	Undifferentiated & neuroglial differentiated	Fibrin conduit	[86]
	hAT-MSCs	Undifferentiated & neuroglial differentiated	Silastic conduit	[87]
	rAT-MSCs	Undifferentiated & neuroglial differentiated	Local intramuscular delivery	[76]
	mBM-MSCs	Undifferentiated	Resorbable collagen conduit	[3]
	rBM-MSCs	Undifferentiated	Laminin-modified chitosan + silicon conduit	[88]
	mBM-MSCs	Undifferentiated	PCL (polycaprolactone) conduit	[77]
	rBM-MSCs	Undifferentiated	Fibrin + PCL (polycaprolactone) conduit	[89]
	rBM-MSCs	Undifferentiated	Systemic intravenous or local intramuscular delivery	[75]
	rBM-MSCs	Neuroglial differentiated	Fibrin conduit	[26]
	rBM-MSCs	Undifferentiated & neuroglial differentiated	Matrigel graft	[90]
	rDP-MSCs	Undifferentiated	Type I collagen gel + silicone conduit	[91]
	rDP-MSCs	Undifferentiated	Type I collagen gel + silicone conduit	[92]
	rDP-MSCs	Undifferentiated	Type I collagen gel + poly-DL-lactide-co-glycolide (PLGA) conduit	[93]
	hUC-MSCs	Undifferentiated	Injected/gelatin-thrombin matrix	[16]
	hUC-MSCs	Undifferentiated	Polyvinyl alcohol (PVA), PVA-carbon nanotubes (CNTs) conduits	[94]
	hUC-MSCs	Undifferentiated & neuroglial differentiated	Poly(DL-lactide-ε-caprolactone) (PLC) membranes	[17]

h (human), m (mouse), r (rat); AF (amniotic fluid), AT (adipose tissue), BM (bone marrow), DP (dental pulp), and UC (umbilical cord tissue) derived MSCs; neuroglial differentiation includes the application of differentiation/induction protocol towards neuronal or glial phenotypes.

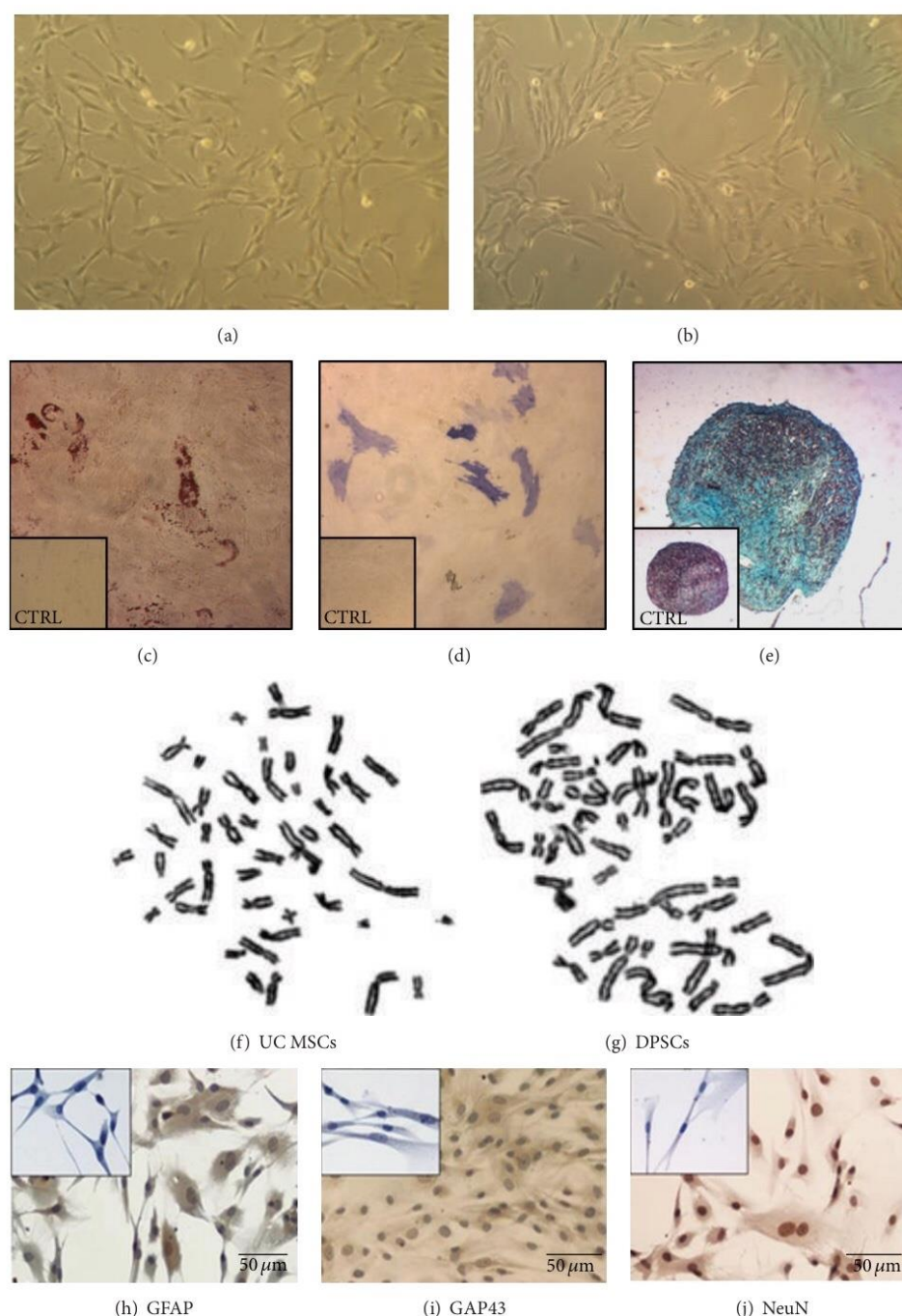


FIGURE 2: Morphological similarities between DPSCs (a) and UC-MSCs (b) (magnification: 100x), in [41]; qualitative analysis of the tridifferentiation potential of umbilical cord stroma derived MSCs by histological staining methods: adipogenic differentiation [Oil Red O staining (c)]; osteogenic differentiation [Alkaline Phosphatase staining (d)]; and chondrogenic differentiation [Alcian Blue staining (e)], in [16]; UC MSCs (f) and DPSCs (g) karyotype, to assess for chromosomal stability in terms of structure and number of chromosomes and the absence of neoplastic characteristics, demonstrating the stability and safety of the cells in usage; positive immunochemical staining of UC-MSCs for neural markers following *in vitro* culture in neurogenic differentiation medium. Cultured cells stained positive for (h) GFAP which is a glial cell marker; (i) GAP-43 which is related to axonal outgrowth; and (j) NeuN which is a marker for nucleus of neurons. Undifferentiated MSCs cells from Wharton's jelly presenting a negative staining for (small panel inserted in (h)) GFAP; (small panel inserted in (i)) GAP-43, and (small panel inserted in (j)) NeuN (magnification: 200x), in [17].

The specific mechanisms by which MSCs impact on peripheral nerve regeneration still care irrefutable evidence, but scientific community strongly leans towards paracrine modulation role, on basis of a number of observations. MSCs can be identified near the injury site, generally in association with the *vasa nervora* [95], but not generally penetrating the regenerating nerve, although their presence relates to stronger expression of Schwann and neuronal cell markers on the nerve itself [78]. Indeed MSCs colocalize with increased neurotrophic expression, demonstrating their role as paracrine stimulators for the resident population [89]. Therefore, evidence suggests that they may mostly act directly on the Schwann cells population, increasing the expression of myelinisation and Schwann cells activity-related genes and trophic factors production [89, 95], depicted by increased myelin sheath thickness, therefore modulating the events of Wallerian degeneration and axonal regeneration [16, 39]. Parallel to direct stimulatory effects on Schwann cells (boosting their own expression of neuromodulatory factors, e.g., CNTF and GDNF [95] that act on axonal growth), the array of molecules provided by MSCs includes strong angiogenic promoters, which are associated with improved outcomes, as detailed ahead. The antifibrotic effect attributed to MSCs is also described in peripheral nerve studies [16, 78]. This effect is possibly associated with modulation of the immune response exerted by the MSCs on active inflammatory sites [62] and in association with biomaterial implants [42], as observed for other tissues and applications. Besides the effects on Schwann cells and axonal growth on the site of injury, the association of MSCs with therapeutic reconstruction has also been demonstrated to protect from neuronal cell death, associated with the axonal retrograde transportation of neurotrophic factors [82].

The need for predifferentiation of delivered MSCs towards neuron related phenotypes (Figures 2(h), 2(i), and 2(j)) is also still unanswered [4, 39]. On one hand, the differentiation process seems to boost neurotrophins secretion [38, 96, 97]. This observation is dominantly supported by *in vivo* data but, on the other hand, controversial reports can also be found in the available literature (references cited in Table 1). Also the process of predifferentiation is time-consuming, affecting the availability of cellular therapies for prompt and speedy reconstructions of affected nerves [80]. It is still not clear whether the differentiation process interferes with postimplantation survival and thus with the window of action of the system [4]. The effects of exogenous manipulation on cells' viability and features and the variations in applied protocols and biomaterials might reason the lack of consensus on the matter. Also, as no definitive data is available, it should be left under consideration that differentiated MSCs may affect neuromuscular regeneration in a more targeted way, while undifferentiated MSCs may play a greater role in supportive functions, such as neoangiogenesis and inflammatory modulation.

3.3. Association of MSCs with Biomaterial Nerve Conduits. After peripheral nerve crush, the deposition of AT-MSCs-biomaterial system onto the nerve lesion accelerated

both sensory and function recovery [38]. Alternatively, the crushed nerve can be wrapped around by the system in a membrane form [39, 73]. Amniotic fluid derived- (AF-) MSCs promoted function recovery, as wells as Schwann cells activity, and limited fibrosis on site following envelopment with a biomaterial membrane [78].

In complete section situations, even when the damaged nerve is amenable of tension-free suturing, the association of a UC-MSCs system (by local deposition) with the surgical therapy has proved advantageous to the overall regenerative process. Modulation of the Wallerian degeneration phase and improved fibre organization could be attributed to MSCs' action, and thicker myelin sheaths as well as increased numbers of regenerating fibres could be observed for the long term recovery, correlating with positive function recovery indicators [16].

Entubulation or complete wrapping with a MSCs-biomaterial system can also benefit direct end-to-end repair, as well as in graft repaired nerves. Biomaterial conduits associated with undifferentiated and neuroglial differentiated UC-MSCs bared slight contributions to motor deficit recovery. At a histological level, both cell systems seemed to improve microfasciculation observed in surgically reconstructed groups [17].

In patent nerve gaps, AT- and BM-MSCs-loaded conduits contribute to an accelerated kick-off of the regenerative response, promoting enhanced axonal ingrowth into the conduit in the first weeks after surgery [26, 80, 85], almost reaching the effectiveness of Schwann cells-loaded ones [26]. To a longer term, BM-MSCs-loaded conduits also resulted in regenerating nerves with overall superior organization and vascularization, and significant increase in the number of myelinated nerve fibres, and thickness of the myelin sheaths [3, 77], correlating with improved functional indexes [77]. AF-MSCs systems also ensured bridging of the nerve gap with properly oriented, larger axonal fibres and diminished surrounding inflammation, accompanied by regain of electrophysiological conduction and motor function [79]. More recently, Georgiou et al. functionalized a commercially available conduit with an inner type I collagen membrane with self-aligned glial-like differentiated AT-MSCs, and although no function indicators are presented, these approaches granted a 3,5-fold increase in the axons reaching the distal stump and increased myelin sheath thickness, comparing to the conduit alone [83]. Along the UC-, BM-, and AT-MSCs detailed herein, many other examples and applications can be found in hybrid cellular-biomaterial systems, such as skin-derived MSCs [28]. DPSCs have also been suggested to elicit superior regenerative response in tubularized nerve injuries [91, 92]. DPSCs granted early connection of regenerating fibres to the distal nerve stump (100% versus 50% of untreated controls, after only 5 days) [93].

Considering the electrophysiology of the neuromuscular tissue, and the recognition that it influences its homeostasis and regeneration, electrofunctionalised biomaterials may also play a relevant role. Low frequency stimulation implemented shortly after injury seems to promote improved electrophysiological recovery and myelinated fibres content in reconstructed nerves [98, 99]. Nerve conduits functionalized

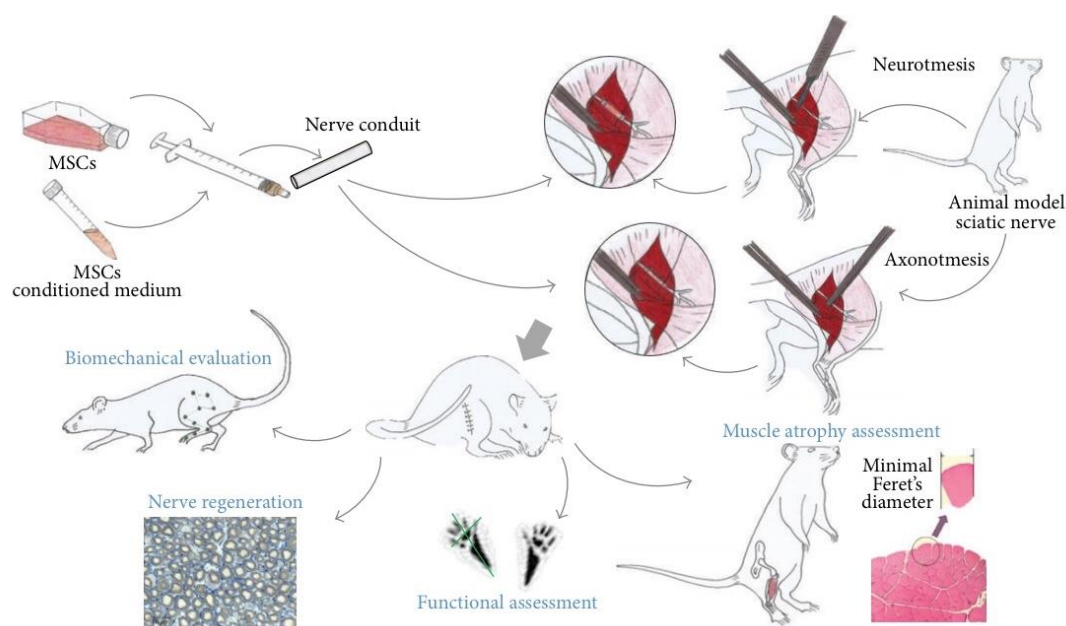


FIGURE 3: Proposed strategies for *in vivo* preclinical assessment of MSCs and MSCs' CM-based strategies for PNS regeneration in axonotmesis and neurotmesis injuries.

with electroconductive agents, such as carbon nanotubes and polypyrrole, boosted axon regeneration and neuromuscular recovery in a neurotmesis lesion with tissue loss, achieving comparable results to end-to-end repaired nerves. The actions of the nanotubes seemed further promoted by the MSCs association, in terms of nerve regeneration indexes and functional assessment [94]. The observed effects may relate to the direct effect on nerve structures stimulation, as well as to their influence on the grafted cells activity. Carbon nanotubes are capable of modulating MSCs activity, enhancing the expression of neuronal markers and selected neurotrophic growth factors (NGF and BDNF) in cultured BM-MSCs [100].

These and other examples of MSCs-based systems intended for PNS lesions regeneration are summarized in Table 1.

Evidence supports that the addition of either differentiated or undifferentiated MSCs to regenerative medicine reconstructive approaches boosts healing through structural and functional responses in relevant animal models, opening the possibilities for the translation of such experimental therapies for clinical practice. Regarding animal models, all of the above-mentioned studies utilised rodents as experimental subjects. However, the proportion and effective size of the lesions observed do not exactly replicate the clinical reality in human patients. Further advancement of these trialled therapies will certainly require the scale-up to larger models, approaching the clinical settings, such as the ovine model proposed by Casañas et al. [101].

So, in neuromuscular regeneration, stem cells' major action is possibly through their fine secretory activities, exerting paracrine modulatory actions in the target tissue.

Consequently, some groups investigated the extent to which the presence of the cells themselves was absolutely essential to the observation of beneficial effects (Figure 3). Some results demonstrate that the application of the secretion products alone (in the form of conditioned medium (CM)) displayed similar if not improved results, as demonstrated for volumetric skeletal muscle injuries [42], central nervous system [102], skin wounds [103], fulminating hepatic failure [104] and hepatic transplant [105], chronic kidney disease [106], and acute lung injury [107, 108].

3.4. Impact of MSCs Therapy on Regional Muscle Atrophy. Bearing in mind the importance of the muscular component for full function regain, strategic actions towards the prevention of it atrophy and/or its speeded recovery should also be considered, as it has been demonstrated that AT-MSCs differentiated towards Schwann phenotype positively affect muscular activity restoration after sectioned nerve repair [76]. Neurosphere induced AT-MSCs are also described to provide increased muscle fibre diameter and muscle weight comparing to empty nerve conduits [81].

BM-MSCs systemic (IV), regional (IM) [75], or biomaterial associated delivery [77] also depicted positive results in terms of counteracting neurogenic atrophy. The MSCs-biomaterial system was also associated with diminished decrease of creatine phosphokinase levels in muscle, as well as improving functional recovery in mice [77].

Although the specific evaluation of skeletal muscle macroscopic and microscopic features is not always employed, significant information on its status can be taken from their performance, more frequently assessed in PNS studies

through functional testing, as has been highlighted throughout the previous sections.

4. Mesenchymal Stem Cell's Secretome: The Potential for Neuromuscular Regeneration

Earlier in the application of MSCs as therapeutic agents, they were hypothesized to contribute to the healing process by effectively differentiating into the required cell types and replacing the damaged cells in their functions. However, as research progressed, evidence arose in that this mode of action might not be universal to every body tissue. It was observed that in some instances MSCs remained in their undifferentiated state at the lesion site or in its vicinity [78], or that could only be identified for a short period of time, or even that only minimal percentages of the MSCs would effectively differentiate and integrate host tissues [63, 80]. Nevertheless, better outcomes were consistently observed whenever MSCs were added to the therapeutic system.

Although MSCs can acquire neuron-like appearance and phenotypical profiles and even display electrical activity under specific inducing stimuli, effective differentiation into active neuronal cells is yet to be demonstrated [63]. As previously mentioned, these observations further support the belief that MSCs benefits to tissue regeneration reside in mechanisms alternative to differentiation and were attributed to the secretion products of those MSCs [17, 78]. In recent years, research has focused on deepening the knowledge on the effective composition of the MSCs secretome. A wide range of growth factors, cytokines, chemokines, and extracellular matrix components have already been identified [36, 109–111]; many of them are known to impact on neuromuscular tissues structure, function, and regeneration.

4.1. The Role of Growth Factors in Neuromuscular Regeneration. Peripheral nerve injury triggers a complex cascade of events, as detailed elsewhere [2, 32], where the glial population of Schwann cells assume a primary role, at a structural level, but also as major paracrine modulators, through the secretion of key bioactive factor that will determine axonal regeneration.

CNS neuroprotection and neuroregeneration functions have been attributed to a group of growth factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin- (NT-) 3, NT-4/5, ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), and erythropoietin (EPO) [112]. A similar arrangement of growth factors seems to modulate peripheral nerve growth and regeneration [32, 78], with additional relevance to vascular endothelial growth factor (VEGF) and insulin-like growth factor- (IGF-) 1 [31, 113].

Neurotrophic factors can be classified into three major groups, regarding their targeted receptors, as neurotrophins (NGF, BDNF, NT-3, and NT-4/5), neurokinins (CNTF and leukemia inhibitory factor (LIF)), and the transforming growth factor- (TGF-) β family (TGF- β 1, TGF- β 2, TGF- β 3, and glial cell-derived neurotrophic factor (GDNF)) [78, 114]. Through diverse pathways, these molecules majorly act

in guiding axonal growth, in apoptosis prevention, and in promoting Schwann cells activity [38, 114]. Schwann cells action modulation and potentiation might come as one of the main actions of these targeted factors [78], since axonal growth and differential sensory and motor neurons development strongly depend on the regulatory “cocktail” to which they are exposed, which is *in situ* provided by the Schwann cells population [78, 113].

Some specific roles and functions have already been identified for many of these growth factors, enlightening on the mechanisms by which MSCs specifically contribute to the observed benefits in neuromuscular regeneration. These and other factors directly impact on axonal extension but the observed effects upon application of MSCs are assumingly related to their parallel action on the glial population. This results in the amplification of delivered factors' effect by the intrinsic regenerative cocktail provided by the Schwann cells. Detailed molecular pathways analysis on axonal growth and growth factor interaction [114–116] escapes the scope of the present review, but some relevant observations on the impact of key factors are commented herein.

BDNF assumes an important role since its ablation from the MSCs/biomaterial system significantly impairs nerve length [38]. This event naturally occurs in chronic injuries due to a decreased production by endogenous cells. However, its benefit is dose-dependent and may become deleterious for axonal growth in high concentration [117].

IGF-1 seems to exert relevant protection against apoptosis in CNS populations [118], and similar neuroprotection and supportive functions are associated with the PNS.

NGF's importance against neuronal apoptosis is debatable, since its neutralization does not significantly affect neuroprotective actions [118]. However, for PNS, NGF delivery in different situations seems to improve myelination and neural following complete transection and reconstruction [31].

CNTF and NT-3 are overexpressed in MSCs treated nerves and correlate with improved structural and functional findings [78]. Besides its attributed neuroprotective actions, CNTF has also been recognized as myotrophic, contributing to the attenuation of the morphological and functional changes resultant from skeletal muscle denervation [6]. Despite their individual functions, the key to successful neuroregeneration is in their synergistic action. The combined actions of NGF, CNTF, and GDNF were proven to override their individual benefits in nervous function recovery [119].

A neurotrophic and neuroprotective effect has also been attributed to cerebral dopamine neurotrophic factor (CDNF) in CNS and PNS injuries. It inhibits the production of proinflammatory cytokines and promotes nerve regrowth after sectioning and function recuperation, as demonstrated by the administration of CDNF enhanced MSCs (transfected). This growth factor also exerted a positive influence on the muscle atrophy associated with the nerve lesion [120].

VEGFs also assumes a preponderant role in peripheral nerve regeneration, although seemingly to be in an indirect manner, by boosting the proliferation of vascular cells which are in turn additional sources of neurotrophins [38]. Impaired VEGF expression in response to injury, as it occurs

in aged individuals, appears to relate to poorer outcomes in peripheral nerve regeneration [5]. The strict relationship between vascular and neural events is evident in almost any body tissue, and such structures are commonly referred to as “neurovascular” units. Furthermore, the loss of the vascular supply to denervated muscles and consequent hypoxic and underperfused environment is deemed to be a significant factor impairing its recovery after long term denervation [11]. Hence, VEGF is crucial to both the reinnervation process and the recovery from the associated skeletal muscle atrophy.

Additional detail and summarized evidence on the importance of these and other neurotrophic factors for peripheral nerve regeneration can be found in [22]. Many other neurotrophins and neuroregulins are known to be expressed by MSCs, as well as molecules related to extracellular matrix (ECM) components, neurite guidance, and myelination, possibly impacting on MSCs actions at lesion sites [38].

4.2. Neurotrophic Factors Secretion by MSCs. Several publications are dedicated to the detailed analysis of the composition and methods of analysis of the bioactive factors composition of diverse MSCs populations [36, 109, 110, 121–123]. Interestingly, one work reported that the major part of molecules secreted by BM-MSCs accounts for ECM components, while only minor proportions include growth factors and inflammatory regulators [124]. Noteworthy, some of these secreted bioactive factors are not only found in soluble forms, but rather associated with exocytic vesicles (exosomes) transporting additional agents and genetic data (mRNA and miRNA) to the system [125–127]. Exosomes have been demonstrated to interact with neural cells populations [128] and promote neurite outgrowth *in vitro* [127].

Most of the neurotrophic factor previously detailed have been associated with MSCs sources applied in cellular or MSCs-biomaterial therapeutic strategies (Table 2).

MSCs from the UC and the BM are described to secrete significant levels of several well-described proliferative, chemotactic, and immune-modulatory molecules, such as TGF- β , G-colony stimulating factor (CSF), GM-CSF, monocyte chemotactic protein- (MCP-) 1, interleukin- (IL-) 6 and IL-8 [111, 129]. Other major vasculogenic factors have also been detected in MSCs populations, and BM- and AF-MSCs depict interesting values of these, in addition to proliferative and chemotactic ones [103, 130].

BM-MSCs, AT-MSCs, and to a greater extent UC-MSCs have all been demonstrated capable of secreting significant neuroregulatory factors to its surrounding medium, such as bFGF, NGF, NT-3, NT-4, and GDNF [70]. Differentiation of UC-MSCs towards glial phenotypes seems to further enhance the already interesting basal undifferentiated state secretion levels of BDNF, NGF, and NT-3 [97]. Undifferentiated DPSCs express interesting amounts of bFGF, TGF- β s, and VEGF [71], and also specific neurotrophic factors, such as BDNF, NGF, NT-3, and GDNF [96].

Although one could consider that these molecules are reportedly secreted in small amounts, it is essential to notice that the therapeutic doses of growth factors in a delivery

TABLE 2: Secretion of major neurotrophic and support factors by MSCs from different tissue sources.

	BDNF	bFGF	GDNF	IGF	NGF	NT-3	NT-4/5	VEGF
AF	•	◦	•	•	•	•	•	•
AT	•	◦	◦	•	•	•	•	•
BM	•	◦	•	•	◦	•	•	•
DP	•	•	•	•	•	•	•	•
UC	•	•	•	—	•	•	•	•

AF (amniotic fluid), AT (adipose tissue), BM (bone marrow), DP (dental pulp), and UC (umbilical cord tissue) derived MSCs; •: detected in cell culture supernatant/conditioned medium; ◦: conflicting reports on the detection of the specific factor in cell culture supernatant/conditioned medium; —: not disclosed in presented literature; and references: [38, 70, 71, 74, 78, 96, 97, 103, 111, 131–136].

system generally stand at considerable low ranges, from nano- to microgram amounts [31].

From the analysis of the available literature, there seems to be little agreement on exact content of the MSCs secretome [62]. A number of factors that can be responsible for these differences, such as the different assays employed and their respective sensitivities, the tissue source from where the MSCs were harvested, as well as the standard and conditioning culture conditions to which the MSCs are subjected [62, 109, 123]. Another important observation is that conditioning time influences the content and quantity of present factors and other proteins [132, 137], as does the passage number of the cultured cells [132].

Those last points are worth some consideration since, beyond modulating their surrounding environment, MSCs are sensitive themselves to signalling factors, altering their secretory profile in response to their surroundings [62] which can be explored to our advantage, modulating the secretion activities of the MSCs to better fit the therapeutic intents. Modulating agents can be, as an example, the presence of inflammatory cytokines and other stimuli [138–140], growth factors medium supplementation [139, 141, 142], or reduced oxygen tension [38, 103, 107].

Hypoxic environments during conditioning seem to be a determining factor in enhancing provasculogenic factors secretion by MSCs populations [103, 131, 143], as well as neuroprotective/neuroregenerative factors expression, such as NGF and BDNF [38]. The induction of MSCs towards phenotypes through differentiation medium [38, 96, 139] is also an interesting tool to boost target growth factor secretion, such as BDNF [38], NGF, NT-3, and GDNF [96]. Neurotrophic factors secretion can also be boosted by EGF/bFGF stimulation during expansion culture [142].

Much more emphasis has been given to this cellular product on CNS models and applications [37, 112, 132, 137, 144], rather than to PNS. Paring up the contents of MSCs secretome and the biomolecular players of neuromuscular regeneration and considering that the native developmental and regenerative environment of neural tissues involves multiple growth factors, in diverse and dynamic combinations, the potential benefits of the association of MSCs and their

products to nerve conduits (Figure 3) and other therapies turn evident [96].

Besides the obviously essential role of the secreted growth factor fraction of MSCs conditioned medium, other components can be associated to vehicles, such as a great variety and amount of metabolic substrates, as demonstrated in [111], aiding the synthetic activities of delivered and native cells. Amongst other metabolite fractions, amino acids assume a preponderant role, providing basic molecules for further growth factors production by active cell populations.

MSCs' products, and more specifically MSCs' CM, may rise as a relevant tool for the regenerative medicine tool. It would grant the ready availability of biomolecule cocktails in therapeutic dosages from a small number of cells, amenable of preservation (frozen or lyophilized) for their timely application, the factors' concentration, and even content manipulation. By associating this with appropriate biomaterials, sustained delivery could be achieved and assure proregenerative environments on neuromuscular injuries, speeding up and optimizing their recovery.

5. Conclusions

The present review summarises the vision on why and how MSCs have been contributing and may hereafter aid the search for improved therapy methods for neuromuscular regeneration. MSCs present interesting features for the task, and similar characteristics have been attributed to other recently appealing sources, such as the umbilical cord stroma and the dental pulp. These cell populations may become privileged sources of stem cells for multiple therapeutic purposes, given their accessibility and ease of banking.

Some other issues rather than the cell source itself still require thorough addressing, in search for optimised strategies. Which route is more suited for the administration of MSCs' systems? Which would be the most suitable biomaterials used as nerve conduits for optimizing stem cells' transplants effectiveness? Is it more advantageous to induce them toward neural lineages prior to application or should we opt for undifferentiated cells? How does the local neuromuscular environment affect delivered MSCs performance and action?

As literature demonstrates, MSCs contribute to improved axonal ingrowth and myelination, resulting in electrophysiological and functional advantage over conventional grafting strategies and biomaterial nerve conduits alone. Further, considering the dominant trophic function attributed to these cells, details on the current knowledge of the MSCs secretory capacities and their secretome characterization are analysed, sustaining its potential as a new interesting strategy to explore. Successful application of such therapies will bring tremendous advantages in terms of therapy availability, expansion, and cell numbers requirement and also the controlled administration of "naturally" occurring growth factors cocktails. As observed for other tissue systems, we propose that MSCs cultures conditioned medium can turn up as a mode of delivery of proregenerative biomolecules to injured peripheral nerves, in search for the potentiation of regenerative medicine approaches.

Abbreviations

AF-MSCs:	Amniotic fluid derived mesenchymal stem cells
AT-MSCs:	Adipose tissue derived mesenchymal stem cells
BDNF:	Brain-derived neurotrophic factor
bFGF:	Basic fibroblast growth factors or fibroblast growth factors -2
BM-MSCs:	Bone marrow derived mesenchymal stem cells
CDNF:	Cerebral dopamine neurotrophic factor
CM:	Condition medium
CNS:	Central nervous system
CNTF:	Ciliary neurotrophic factor
CSF:	Colony stimulating factor
DPSCs:	Dental pulp derived stem cells
ECM:	Extracellular matrix
EPO:	Erythropoietin
GDNF:	Glial cell-derived neurotrophic factor
HLA:	Human leukocyte antigen
IGF:	Insulin-like growth factor
IL:	Interleukin
ISCT:	International Society for Cellular Therapy
LIF:	Leukemia inhibitory factor
LPS:	Lipopolysaccharide
MCP:	Monocyte chemotactic protein 1/chemokine (C-C motif) ligand 2 (CCL2)
MHC:	Major histocompatibility complex
MSCs:	Mesenchymal stem cells
NGF:	Nerve growth factor
NT:	Neurotrophin
PNS:	Peripheral nervous system
TGF:	Transforming growth factor
TGF:	Transforming growth factor
TNF:	Tumoral necrosis factor
UC-MSCs:	Umbilical cord stroma/Wharton's jelly derived mesenchymal stem cells
VEGF:	Vascular endothelial growth factor.

Disclosure

Ana Rita Caseiro and Tiago Pereira are both considered as first authors.

Conflict of Interests

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

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References

- [1] P. Ciaramitaro, M. Mondelli, F. Logullo et al., "Traumatic peripheral nerve injuries: epidemiological findings, neuropathic pain and quality of life in 158 patients," *Journal of the Peripheral Nervous System*, vol. 15, no. 2, pp. 120–127, 2010.
- [2] A. Faroni, S. A. Mobasser, P. J. Kingham, and A. J. Reid, "Peripheral nerve regeneration: experimental strategies and future perspectives," *Advanced Drug Delivery Reviews*, vol. 82, pp. 160–167, 2015.
- [3] F. R. Pereira Lopes, L. C. de Moura Campos, J. Dias Corrêa Jr. et al., "Bone marrow stromal cells and resorbable collagen guidance tubes enhance sciatic nerve regeneration in mice," *Experimental Neurology*, vol. 198, no. 2, pp. 457–468, 2006.
- [4] S. Walsh and R. Midha, "Practical considerations concerning the use of stem cells for peripheral nerve repair," *Neurosurgical Focus*, vol. 26, no. 2, article E2, 2009.
- [5] R. Pola, T. R. Aprahamian, M. Bosch-Marcé et al., "Age-dependent VEGF expression and intraneural neovascularization during regeneration of peripheral nerves," *Neurobiology of Aging*, vol. 25, no. 10, pp. 1361–1368, 2004.
- [6] M. E. Helgren, S. P. Squinto, H. L. Davis et al., "Trophic effect of ciliary neurotrophic factor on denervated skeletal muscle," *Cell*, vol. 76, no. 3, pp. 493–504, 1994.
- [7] N. Adami, H. Kern, W. Mayr, U. Carraro, D. Biral, and Z. Sandra, "Permanent denervation of rat Tibialis Anterior after bilateral sciactomy: determination of chronaxie by surface electrode stimulation during progression of atrophy up to one year," *Basic and Applied Myology*, vol. 17, no. 6, pp. 237–243, 2007.
- [8] J.-P. K. Hyatt, R. R. Roy, K. M. Baldwin, and V. R. Edgerton, "Nerve activity-independent regulation of skeletal muscle atrophy: role of MyoD and myogenin in satellite cells and myonuclei," *The American Journal of Physiology—Cell Physiology*, vol. 285, no. 5, pp. C1161–C1173, 2003.
- [9] R. W. Jackman and S. C. Kandarian, "The molecular basis of skeletal muscle atrophy," *The American Journal of Physiology—Cell Physiology*, vol. 287, no. 4, pp. C834–C843, 2004.
- [10] E. I. Dedkov, T. Y. Kostrominova, A. B. Borisov, and B. M. Carlson, "Reparative myogenesis in long-term denervated skeletal muscles of adult rats results in a reduction of the satellite cell population," *Anatomical Record*, vol. 263, no. 2, pp. 139–154, 2001.
- [11] A. B. Borisov, E. I. Dedkov, and B. M. Carlson, "Abortive myogenesis in denervated skeletal muscle: differentiative properties of satellite cells, their migration, and block of terminal differentiation," *Anatomy and Embryology*, vol. 209, no. 4, pp. 269–279, 2005.
- [12] S. K. Walsh, T. Gordon, B. M. J. Addas, S. W. P. Kemp, and R. Midha, "Skin-derived precursor cells enhance peripheral nerve regeneration following chronic denervation," *Experimental Neurology*, vol. 223, no. 1, pp. 221–228, 2010.
- [13] A. L. Luís, J. M. Rodrigues, S. Geuna et al., "Neural cell transplantation effects on sciatic nerve regeneration after a standardized crush injury in the rat," *Microsurgery*, vol. 28, no. 6, pp. 458–470, 2008.
- [14] B. J. Pfister, T. Gordon, J. R. Loverde, A. S. Kochar, S. E. Mackinnon, and D. Kacy Cullen, "Biomedical engineering strategies for peripheral nerve repair: surgical applications, state of the art, and future challenges," *Critical Reviews in Biomedical Engineering*, vol. 39, no. 2, pp. 81–124, 2011.
- [15] J. Ribeiro, A. Gartner, T. Pereira et al., "Perspectives of employing mesenchymal stem cells from the Wharton's jelly of the umbilical cord for peripheral nerve repair," *International Review of Neurobiology*, vol. 108, pp. 79–120, 2013.
- [16] A. Gärtner, T. Pereira, P. A. S. Armada-da-Silva et al., "Effects of umbilical cord tissue mesenchymal stem cells (UCX) on rat sciatic nerve regeneration after neurotmesis injuries," *Journal of Stem Cells and Regenerative Medicine*, vol. 10, no. 1, pp. 14–26, 2014.
- [17] T. Pereira, A. Gärtner, I. Amorim et al., "Promoting nerve regeneration in a neurotmesis rat model using poly(DL-lactide-epsilon-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly: *in vitro* and *in vivo* analysis," *BioMed Research International*, vol. 2014, Article ID 302659, 17 pages, 2014.
- [18] A. Höke, "Mechanisms of Disease: what factors limit the success of peripheral nerve regeneration in humans?" *Nature Clinical Practice Neurology*, vol. 2, no. 8, pp. 448–454, 2006.
- [19] B. Battiston, S. Geuna, M. Ferrero, and P. Tos, "Nerve repair by means of tubulization: literature review and personal clinical experience comparing biological and synthetic conduits for sensory nerve repair," *Microsurgery*, vol. 25, no. 4, pp. 258–267, 2005.
- [20] T. Matsuyama, M. Mackay, and R. Midha, "Peripheral nerve repair and grafting techniques: a review," *Neurologia Medico-Chirurgica*, vol. 40, no. 4, pp. 187–199, 2000.
- [21] G. Lundborg, "Alternatives to autologous nerve grafts," *Handchirurgie Mikrochirurgie Plastische Chirurgie*, vol. 36, no. 1, pp. 1–7, 2004.
- [22] X. Jiang, S. H. Lim, H.-Q. Mao Hai-Quan, and S. Y. Chew, "Current applications and future perspectives of artificial nerve conduits," *Experimental Neurology*, vol. 223, no. 1, pp. 86–101, 2010.
- [23] A. L. Luís, J. M. Rodrigues, S. Geuna et al., "Use of PLGA 90:10 scaffolds enriched with *in vitro*-differentiated neural cells for repairing rat sciatic nerve defects," *Tissue Engineering A*, vol. 14, no. 6, pp. 979–993, 2008.
- [24] S. Amado, M. J. Simões, P. A. S. Armada da Silva et al., "Use of hybrid chitosan membranes and N1E-115 cells for promoting nerve regeneration in an axonotmesis rat model," *Biomaterials*, vol. 29, no. 33, pp. 4409–4419, 2008.
- [25] S. Amado, J. M. Rodrigues, A. L. Luís et al., "Effects of collagen membranes enriched with *in vitro*-differentiated N1E-115 cells on rat sciatic nerve regeneration after end-to-end repair," *Journal of NeuroEngineering and Rehabilitation*, vol. 7, article 7, 2010.
- [26] P. G. di Summa, P. J. Kingham, W. Raffoul, M. Wiberg, G. Terenghi, and D. F. Kalbermatten, "Adipose-derived stem cells enhance peripheral nerve regeneration," *Journal of Plastic, Reconstructive & Aesthetic Surgery*, vol. 63, no. 9, pp. 1544–1552, 2010.
- [27] S. Kehoe, X. F. Zhang, and D. Boyd, "FDA approved guidance conduits and wraps for peripheral nerve injury: a review of materials and efficacy," *Injury*, vol. 43, no. 5, pp. 553–572, 2012.

- [28] X. Gu, F. Ding, and D. F. Williams, "Neural tissue engineering options for peripheral nerve regeneration," *Biomaterials*, vol. 35, no. 24, pp. 6143–6156, 2014.
- [29] M. J. Simões, S. Amado, A. Gärtner et al., "Use of chitosan scaffolds for repairing rat sciatic nerve defects," *Italian Journal of Anatomy and Embryology*, vol. 115, no. 3, pp. 190–210, 2010.
- [30] J. Pettersson, A. McGrath, D. F. Kalbermatten et al., "Muscle recovery after repair of short and long peripheral nerve gaps using fibrin conduits," *Neuroscience Letters*, vol. 500, no. 1, pp. 41–46, 2011.
- [31] L. A. Pfister, M. Papaloizos, H. P. Merkle, and B. Gander, "Nerve conduits and growth factor delivery in peripheral nerve repair," *Journal of the Peripheral Nervous System*, vol. 12, no. 2, pp. 65–82, 2007.
- [32] P. A. S. Armada-da-Silva, C. Pereira, S. Amado, A. Luís, and A. C. Mauricio, "Activity-based strategies in the rehabilitation of peripheral nerve injuries," in *Peripheral Neuropathy*, P. Armada-Da-Silva, Ed., chapter 3, InTech, Rijeka, Croatia, 2014.
- [33] A. C. Mauricio, A. Gärtner, P. Armada-da-Silva et al., "Cellular systems and biomaterials for nerve regeneration in neurotmesis injuries," in *Biomaterials Applications for Nanomedicine*, R. Pignatello, Ed., chapter 20, InTech, Rijeka, Croatia, 2011.
- [34] S. Frausin, S. Viventi, L. Verga Falzacappa et al., "Wharton's jelly derived mesenchymal stromal cells: biological properties, induction of neuronal phenotype and current applications in neurodegeneration research," *Acta Histochemica*, vol. 117, no. 4–5, pp. 329–338, 2015.
- [35] A. I. Caplan and J. E. Dennis, "Mesenchymal stem cells as trophic mediators," *Journal of Cellular Biochemistry*, vol. 98, no. 5, pp. 1076–1084, 2006.
- [36] L. da Silva Meirelles, A. M. Fontes, D. T. Covas, and A. I. Caplan, "Mechanisms involved in the therapeutic properties of mesenchymal stem cells," *Cytokine & Growth Factor Reviews*, vol. 20, no. 5–6, pp. 419–427, 2009.
- [37] F. G. Teixeira, M. M. Carvalho, N. Sousa, and A. J. Salgado, "Mesenchymal stem cells secretome: a new paradigm for central nervous system regeneration?" *Cellular and Molecular Life Sciences*, vol. 70, no. 20, pp. 3871–3882, 2013.
- [38] T. Lopatina, N. Kalinina, M. Karagyaour et al., "Adipose-derived stem cells stimulate regeneration of peripheral nerves. BDNF secreted by these cells promotes nerve healing and axon growth *De Novo*," *PLoS ONE*, vol. 6, no. 3, Article ID e17899, 2011.
- [39] A. Gärtner, T. Pereira, P. A. S. Armada-da-Silva et al., "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," *Differentiation*, vol. 84, no. 5, pp. 355–365, 2012.
- [40] A. Gärtner, T. Pereira, R. Gomes et al., "Mesenchymal stem cells from extra-embryonic tissues for tissue engineering—regeneration of the peripheral nerve," in *Advances in Biomaterials Science and Biomedical Applications*, R. Pignatello, Ed., InTech, 2013.
- [41] A. R. Caseiro, T. Pereira, P. Bártolo, J. Domingos, A. L. Luís, and A. C. Mauricio, "Chapter—trends in mesenchymal stem cells applications for skeletal muscle repair and regeneration," in *Progress in Stem Cell Transplantation*, T. Demirel, Ed., 2015.
- [42] T. Pereira, P. A. S. Armada-Da Silva, I. Amorim et al., "Effects of human mesenchymal stem cells isolated from Wharton's jelly of the umbilical cord and conditioned media on skeletal muscle regeneration using a myectomy model," *Stem Cells International*, vol. 2014, Article ID 376918, 16 pages, 2014.
- [43] A. J. Friedenstein, R. K. Chailakhjan, and K. S. Lalykina, "The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells," *Cell and Tissue Kinetics*, vol. 3, no. 4, pp. 393–403, 1970.
- [44] L. G. Lajtha, "Stem cells and their properties," *Proceedings. Canadian Cancer Conference*, vol. 7, pp. 31–39, 1967.
- [45] A. J. Friedenstein, K. V. Petrakova, A. I. Kurolesova, and G. P. Frolova, "Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues," *Transplantation*, vol. 6, no. 2, pp. 230–247, 1968.
- [46] B. M. Abdallah and M. Kassem, "Human mesenchymal stem cells: from basic biology to clinical applications," *Gene Therapy*, vol. 15, no. 2, pp. 109–116, 2008.
- [47] J. T. Triffitt, "Stem cells and the philosopher's stone," *Journal of Cellular Biochemistry. Supplement*, vol. 38, pp. 13–19, 2002.
- [48] L. da Silva Meirelles, P. C. Chagastelles, and N. B. Nardi, "Mesenchymal stem cells reside in virtually all post-natal organs and tissues," *Journal of Cell Science*, vol. 119, no. 11, pp. 2204–2213, 2006.
- [49] G. Chamberlain, J. Fox, B. Ashton, and J. Middleton, "Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing," *Stem Cells*, vol. 25, no. 11, pp. 2739–2749, 2007.
- [50] M. Ohishi and E. Schipani, "Bone marrow mesenchymal stem cells," *Journal of Cellular Biochemistry*, vol. 109, no. 2, pp. 277–282, 2010.
- [51] M. Dominici, K. Le Blanc, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.
- [52] A. I. Caplan, "Mesenchymal stem cells," *Journal of Orthopaedic Research*, vol. 9, no. 5, pp. 641–650, 1991.
- [53] M. F. Pittenger, A. M. Mackay, S. C. Beck et al., "Multilineage potential of adult human mesenchymal stem cells," *Science*, vol. 284, no. 5411, pp. 143–147, 1999.
- [54] Y. Muguruma, M. Reyes, Y. Nakamura et al., "In vivo and in vitro differentiation of myocytes from human bone marrow-derived multipotent progenitor cells," *Experimental Hematology*, vol. 31, no. 12, pp. 1323–1330, 2003.
- [55] K. Le Blanc and O. Ringdén, "Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation," *Biology of Blood and Marrow Transplantation*, vol. 11, no. 5, pp. 321–334, 2005.
- [56] N. Wada, D. Menicanin, S. Shi, P. M. Bartold, and S. Gronthos, "Immunomodulatory properties of human periodontal ligament stem cells," *Journal of Cellular Physiology*, vol. 219, no. 3, pp. 667–676, 2009.
- [57] D.-W. Kim, M. Staples, K. Shinozuka, P. Pantcheva, S.-D. Kang, and C. V. Borlongan, "Wharton's jelly-derived mesenchymal stem cells: phenotypic characterization and optimizing their therapeutic potential for clinical applications," *International Journal of Molecular Sciences*, vol. 14, no. 6, pp. 11692–11712, 2013.
- [58] A. I. Caplan, "Why are MSCs therapeutic? New data: new insight," *The Journal of Pathology*, vol. 217, no. 2, pp. 318–324, 2009.
- [59] E. Fossett and W. S. Khan, "Optimising human mesenchymal stem cell numbers for clinical application: a literature review," *Stem Cells International*, vol. 2012, Article ID 465259, 5 pages, 2012.
- [60] D. J. Maltman, S. A. Hardy, and S. A. Przyborski, "Role of mesenchymal stem cells in neurogenesis and nervous system

- repair," *Neurochemistry International*, vol. 59, no. 3, pp. 347–356, 2011.
- [61] S. Ghannam, C. Bouffi, F. Djouad, C. Jorgensen, and D. Noël, "Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications," *Stem Cell Research and Therapy*, vol. 1, no. 1, article 2, 2010.
- [62] J. R. Lavoie and M. Rosu-Myles, "Uncovering the secrets of mesenchymal stem cells," *Biochimie*, vol. 95, no. 12, pp. 2212–2221, 2013.
- [63] D. G. Phinney and D. J. Prockop, "Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views," *Stem Cells*, vol. 25, no. 11, pp. 2896–2902, 2007.
- [64] S. Kern, H. Eichler, J. Stoeve, H. Klüter, and K. Bieback, "Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue," *STEM CELLS*, vol. 24, no. 5, pp. 1294–1301, 2006.
- [65] M. H. Lim, W. K. Ong, and S. Sugii, "The current landscape of adipose-derived stem cells in clinical applications," *Expert reviews in Molecular Medicine*, vol. 16, article e8, 2014.
- [66] S. Gronthos, M. Mankani, J. Brahimi, P. G. Robey, and S. Shi, "Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13625–13630, 2000.
- [67] M. Miura, S. Gronthos, M. Zhao et al., "SHED: stem cells from human exfoliated deciduous teeth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 5807–5812, 2003.
- [68] W. Wagner, F. Wein, A. Seckinger et al., "Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood," *Experimental Hematology*, vol. 33, no. 11, pp. 1402–1416, 2005.
- [69] M. Al-Nbaheen, R. Vishnubalaji, D. Ali et al., "Human stromal (mesenchymal) stem cells from bone marrow, adipose tissue and skin exhibit differences in molecular phenotype and differentiation potential," *Stem Cell Reviews and Reports*, vol. 9, no. 1, pp. 32–43, 2013.
- [70] S. Balasubramanian, C. Thej, P. Venugopal et al., "Higher propensity of Wharton's jelly derived mesenchymal stromal cells towards neuronal lineage in comparison to those derived from adipose and bone marrow," *Cell Biology International*, vol. 37, no. 5, pp. 507–515, 2013.
- [71] A. Caseiro, T. Pereira, P. Bartolo, J. Santos, A. Luís, and A. Mauricio, "Mesenchymal stem cells and biomaterials systems—perspectives for skeletal muscle tissue repair and regeneration," *Procedia Engineering*, vol. 110, pp. 90–97, 2015.
- [72] A. L. Luis, J. M. Rodrigues, S. Amado et al., "PLGA 90/10 and caprolactone biodegradable nerve guides for the reconstruction of the rat sciatic nerve," *Microsurgery*, vol. 27, no. 2, pp. 125–137, 2007.
- [73] A. Gärtner, T. Pereira, M. J. Simões et al., "Use of hybrid chitosan membranes and MSC cells for promoting nerve regeneration in an axonotmesis rat model," *Neural Regeneration Research*, vol. 7, no. 29, pp. 2247–2258, 2012.
- [74] S. Marconi, G. Castiglione, E. Turano et al., "Human adipose-derived mesenchymal stem cells systemically injected promote peripheral nerve regeneration in the mouse model of sciatic crush," *Tissue Engineering—Part A*, vol. 18, no. 11–12, pp. 1264–1272, 2012.
- [75] P. Wang, Y. Zhang, J. Zhao, and B. Jiang, "Intramuscular injection of bone marrow mesenchymal stem cells with small gap neurorrhaphy for peripheral nerve repair," *Neuroscience Letters*, vol. 585, pp. 119–125, 2015.
- [76] D. Schaakxs, D. F. Kalbermatten, W. Raffoul, M. Wiberg, and P. J. Kingham, "Regenerative cell injection in denervated muscle reduces atrophy and enhances recovery following nerve repair," *Muscle and Nerve*, vol. 47, no. 5, pp. 691–701, 2013.
- [77] J. T. Oliveira, F. M. Almeida, A. Biancalana et al., "Mesenchymal stem cells in a polycaprolactone conduit enhance median-nerve regeneration, prevent decrease of creatine phosphokinase levels in muscle, and improve functional recovery in mice," *Neuroscience*, vol. 170, no. 4, pp. 1295–1303, 2010.
- [78] H.-C. Pan, F.-C. Cheng, C.-J. Chen et al., "Post-injury regeneration in rat sciatic nerve facilitated by neurotrophic factors secreted by amniotic fluid mesenchymal stem cells," *Journal of Clinical Neuroscience*, vol. 14, no. 11, pp. 1089–1098, 2007.
- [79] H.-C. Pan, D.-Y. Yang, Y.-T. Chiu et al., "Enhanced regeneration in injured sciatic nerve by human amniotic mesenchymal stem cell," *Journal of Clinical Neuroscience*, vol. 13, no. 5, pp. 570–575, 2006.
- [80] P. Erba, C. Mantovani, D. F. Kalbermatten, G. Pierer, G. Terenghi, and P. J. Kingham, "Regeneration potential and survival of transplanted undifferentiated adipose tissue-derived stem cells in peripheral nerve conduits," *Journal of Plastic, Reconstructive and Aesthetic Surgery*, vol. 63, no. 12, pp. e811–e817, 2010.
- [81] Y.-Y. Hsueh, Y.-J. Chang, T.-C. Huang et al., "Functional recoveries of sciatic nerve regeneration by combining chitosan-coated conduit and neurosphere cells induced from adipose-derived stem cells," *Biomaterials*, vol. 35, no. 7, pp. 2234–2244, 2014.
- [82] A. J. Reid, M. Sun, M. Wiberg, S. Downes, G. Terenghi, and P. J. Kingham, "Nerve repair with adipose-derived stem cells protects dorsal root ganglia neurons from apoptosis," *Neuroscience*, vol. 199, pp. 515–522, 2011.
- [83] M. Georgiou, J. P. Golding, A. J. Loughlin, P. J. Kingham, and J. B. Phillips, "Engineered neural tissue with aligned, differentiated adipose-derived stem cells promotes peripheral nerve regeneration across a critical sized defect in rat sciatic nerve," *Biomaterials*, vol. 37, pp. 242–251, 2015.
- [84] Y. Zhang, H. Luo, Z. Zhang et al., "A nerve graft constructed with xenogeneic acellular nerve matrix and autologous adipose-derived mesenchymal stem cells," *Biomaterials*, vol. 31, no. 20, pp. 5312–5324, 2010.
- [85] P. J. Kingham, M. K. Kolar, L. N. Novikova, L. N. Novikov, and M. Wiberg, "Stimulating the neurotrophic and angiogenic properties of human adipose-derived stem cells enhances nerve repair," *Stem Cells and Development*, vol. 23, no. 7, pp. 741–754, 2014.
- [86] M. Tremp, M. M. Zu Schwabedissen, E. A. Kappos et al., "The regeneration potential after human and autologous stem cell transplantation in a rat sciatic nerve injury model can be monitored by MRI," *Cell Transplantation*, vol. 24, no. 2, pp. 203–211, 2015.
- [87] T. Scholz, A. Sumarto, A. Krichevsky, and G. R. D. Evans, "Neuronal differentiation of human adipose tissue-derived stem cells for peripheral nerve regeneration in vivo," *Archives of Surgery*, vol. 146, no. 6, pp. 666–674, 2011.
- [88] S.-H. Hsu, W.-C. Kuo, Y.-T. Chen et al., "New nerve regeneration strategy combining laminin-coated chitosan conduits and stem cell therapy," *Acta Biomaterialia*, vol. 9, no. 5, pp. 6606–6615, 2013.

- [89] L. P. Cartarozzi, A. B. Spejo, R. S. Ferreira et al., "Mesenchymal stem cells engrafted in a fibrin scaffold stimulate Schwann cell reactivity and axonal regeneration following sciatic nerve tubulization," *Brain Research Bulletin*, vol. 112, pp. 14–24, 2015.
- [90] M. Dezawa, I. Takahashi, M. Esaki, M. Takano, and H. Sawada, "Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells," *European Journal of Neuroscience*, vol. 14, no. 11, pp. 1771–1776, 2001.
- [91] R. Sasaki, "Regeneration of facial nerve defects using a silicone tube filled with dental pulp cells," *Journal of Oral and Maxillofacial Surgery*, vol. 65, no. 9, pp. 40.e1–40.e2, 2007.
- [92] R. Sasaki, S. Aoki, M. Yamato et al., "P418 Dental pulp cells for facial nerve regeneration," *Journal of Cranio-Maxillofacial Surgery*, vol. 36, supplement 1, pp. S271–S272, 2008.
- [93] R. Sasaki, S. Aoki, M. Yamato et al., "PLGA artificial nerve conduits with dental pulp cells promote facial nerve regeneration," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 5, no. 10, pp. 823–830, 2011.
- [94] J. Ribeiro, T. Pereira, A. R. Caseiro et al., "Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells," *World Journal of Stem Cells*, vol. 7, no. 6, pp. 956–975, 2015.
- [95] J. W. Han, D. Choi, M. Y. Lee, Y. H. Huh, and Y.-S. Yoon, "Bone marrow-derived mesenchymal stem cells improve diabetic neuropathy by direct modulation of both angiogenesis and myelination in peripheral nerves," *Cell Transplantation*, 2015.
- [96] W. Martens, K. Sanen, M. Georgiou et al., "Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro," *The FASEB Journal*, vol. 28, no. 4, pp. 1634–1643, 2014.
- [97] J. Peng, Y. Wang, L. Zhang et al., "Human umbilical cord Wharton's jelly-derived mesenchymal stem cells differentiate into a Schwann-cell phenotype and promote neurite outgrowth in vitro," *Brain Research Bulletin*, vol. 84, no. 3, pp. 235–243, 2011.
- [98] M.-C. Lu, C.-Y. Ho, S.-F. Hsu et al., "Effects of electrical stimulation at different frequencies on regeneration of transected peripheral nerve," *Neurorehabilitation and Neural Repair*, vol. 22, no. 4, pp. 367–373, 2008.
- [99] C.-C. Yeh, Y.-C. Lin, F.-J. Tsai, C.-Y. Huang, C.-H. Yao, and Y.-S. Chen, "Timing of applying electrical stimulation is an important factor deciding the success rate and maturity of regenerating rat sciatic nerves," *Neurorehabilitation and Neural Repair*, vol. 24, no. 8, pp. 730–735, 2010.
- [100] J. H. Lee, J.-Y. Lee, S. H. Yang, E.-J. Lee, and H.-W. Kim, "Carbon nanotube-collagen three-dimensional culture of mesenchymal stem cells promotes expression of neural phenotypes and secretion of neurotrophic factors," *Acta Biomaterialia*, vol. 10, no. 10, pp. 4425–4436, 2014.
- [101] J. Casañas, J. da la Torre, F. Soler et al., "Peripheral nerve regeneration after experimental section in ovine radial and tibial nerves using synthetic nerve grafts, including expanded bone marrow mesenchymal cells: morphological and neurophysiological results," *Injury*, vol. 45, pp. S2–S6, 2014.
- [102] F. Teixeira, A. Pires, J. Fraga et al., "Secretome of mesenchymal stem cells as a new tool for central nervous system regenerative medicine," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 8, pp. 298–299, 2014.
- [103] L. Chen, E. E. Tredget, P. Y. G. Wu, Y. Wu, and Y. Wu, "Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing," *PLoS ONE*, vol. 3, no. 4, Article ID e1886, 2008.
- [104] D. van Poll, B. Parekkadan, C. H. Cho et al., "Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo," *Hepatology*, vol. 47, no. 5, pp. 1634–1643, 2008.
- [105] Z. Du, C. Wei, K. Cheng et al., "Mesenchymal stem cell-conditioned medium reduces liver injury and enhances regeneration in reduced-size rat liver transplantation," *Journal of Surgical Research*, vol. 183, no. 2, pp. 907–915, 2013.
- [106] A. van Koppen, J. A. Joles, B. W. M. van Balkom et al., "Human embryonic mesenchymal stem cell-derived conditioned medium rescues kidney function in rats with established chronic kidney disease," *PLoS ONE*, vol. 7, no. 6, Article ID e38746, 2012.
- [107] P. Waszak, R. Alphonse, A. Vadivel, L. Ionescu, F. Eaton, and B. Thébaud, "Preconditioning enhances the paracrine effect of mesenchymal stem cells in preventing oxygen-induced neonatal lung injury in rats," *Stem Cells and Development*, vol. 21, no. 15, pp. 2789–2797, 2012.
- [108] L. Ionescu, R. N. Byrne, T. van Haaften et al., "Stem cell conditioned medium improves acute lung injury in mice: in vivo evidence for stem cell paracrine action," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 303, no. 11, pp. L967–L977, 2012.
- [109] H. Skalnikova, J. Motlik, S. J. Gadher, and H. Kovarova, "Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines," *Proteomics*, vol. 11, no. 4, pp. 691–708, 2011.
- [110] A. J. B. O. G. Salgado, R. L. G. Reis, N. J. C. Sousa, and J. M. Gimble, "Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine," *Current Stem Cell Research and Therapy*, vol. 5, no. 2, pp. 103–110, 2010.
- [111] T. Pereira, G. Ivanova, A. R. Caseiro et al., "MSCs conditioned media and umbilical cord blood plasma metabolomics and composition," *PLoS ONE*, vol. 9, no. 11, Article ID e113769, 2014.
- [112] A. J. Salgado, J. S. Fraga, A. R. Mesquita, N. M. Neves, R. L. Reis, and N. Sousa, "Role of human umbilical cord mesenchymal progenitors conditioned media in neuronal/glia cell densities, viability, and proliferation," *Stem Cells and Development*, vol. 19, no. 7, pp. 1067–1074, 2010.
- [113] A. Höke, R. Redett, H. Hameed et al., "Schwann cells express motor and sensory phenotypes that regulate axon regeneration," *The Journal of Neuroscience*, vol. 26, no. 38, pp. 9646–9655, 2006.
- [114] A. Markus, T. D. Patel, and W. D. Snider, "Neurotrophic factors and axonal growth," *Current Opinion in Neurobiology*, vol. 12, no. 5, pp. 523–531, 2002.
- [115] A. B. Huber, A. L. Kolodkin, D. D. Ginty, and J.-F. Cloutier, "Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance," *Annual Review of Neuroscience*, vol. 26, pp. 509–563, 2003.
- [116] G. Corfas, M. O. Velardez, C.-P. Ko, N. Ratner, and E. Peles, "Mechanisms and roles of axon-Schwann cell interactions," *The Journal of Neuroscience*, vol. 24, no. 42, pp. 9250–9260, 2004.
- [117] T. Gordon, "The physiology of neural injury and regeneration: the role of neurotrophic factors," *Journal of Communication Disorders*, vol. 43, no. 4, pp. 265–273, 2010.
- [118] X. Wei, L. Zhao, J. Zhong et al., "Adipose stromal cells-secreted neuroprotective media against neuronal apoptosis," *Neuroscience Letters*, vol. 462, no. 1, pp. 76–79, 2009.
- [119] J. Chen, Y. F. Chu, J. M. Chen, and B. C. Li, "Synergistic effects of NGF, CNTF and GDNF on functional recovery following sciatic

- nerve injury in rats," *Advances in Medical Sciences*, vol. 55, no. 1, pp. 32–42, 2010.
- [120] Y. Liu, L. Nie, H. Zhao et al., "Conserved dopamine neurotrophic factor-transduced mesenchymal stem cells promote axon regeneration and functional recovery of injured sciatic nerve," *PLoS ONE*, vol. 9, no. 10, Article ID e110993, 2014.
- [121] P. R. Baraniak and T. C. McDevitt, "Stem cell paracrine actions and tissue regeneration," *Regenerative Medicine*, vol. 5, no. 1, pp. 121–143, 2010.
- [122] J. Doorn, G. Moll, K. Le Blanc, C. Van Blitterswijk, and J. De Boer, "Therapeutic applications of mesenchymal stromal cells: paracrine effects and potential improvements," *Tissue Engineering—Part B: Reviews*, vol. 18, no. 2, pp. 101–115, 2012.
- [123] H. K. Skalnikova, "Proteomic techniques for characterisation of mesenchymal stem cell secretome," *Biochimie*, vol. 95, no. 12, pp. 2196–2211, 2013.
- [124] M. Polacek, J.-A. Bruun, J. Elvenes, Y. Figenschau, and I. Martinez, "The secretory profiles of cultured human articular chondrocytes and mesenchymal stem cells: implications for autologous cell transplantation strategies," *Cell Transplantation*, vol. 20, no. 9, pp. 1381–1393, 2011.
- [125] R. C. Lai, R. W. Y. Yeo, K. H. Tan, and S. K. Lim, "Exosomes for drug delivery—a novel application for the mesenchymal stem cell," *Biotechnology Advances*, vol. 31, no. 5, pp. 543–551, 2013.
- [126] L. Biancone, S. Bruno, M. C. Derigibus, C. Tetta, and G. Camussi, "Therapeutic potential of mesenchymal stem cell-derived microvesicles," *Nephrology Dialysis Transplantation*, vol. 27, no. 8, pp. 3037–3042, 2012.
- [127] H. Xin, Y. Li, Z. Liu et al., "MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles," *Stem Cells*, vol. 31, no. 12, pp. 2737–2746, 2013.
- [128] A. Farinazzo, E. Turano, S. Marconi, E. Bistaffa, E. Bazzoli, and B. Bonetti, "Murine adipose-derived mesenchymal stromal cell vesicles: in vitro clues for neuroprotective and neuroregenerative approaches," *Cytotherapy*, vol. 17, no. 5, pp. 571–578, 2015.
- [129] M. N. M. Walter, K. T. Wright, H. R. Fuller, S. MacNeil, and W. E. B. Johnson, "Mesenchymal stem cell-conditioned medium accelerates skin wound healing: an *in vitro* study of fibroblast and keratinocyte scratch assays," *Experimental Cell Research*, vol. 316, no. 7, pp. 1271–1281, 2010.
- [130] T. Mirabellaa, M. Cilli, S. Carlone, R. Cancedda, and C. Gentilia, "Amniotic liquid derived stem cells as reservoir of secreted angiogenic factors capable of stimulating neo-arteriogenesis in an ischemic model," *Biomaterials*, vol. 32, no. 15, pp. 3689–3699, 2011.
- [131] C.-P. Chang, C.-C. Chio, C.-U. Cheong, C.-M. Chao, B.-C. Cheng, and M.-T. Lin, "Hypoxic preconditioning enhances the therapeutic potential of the secretome from cultured human mesenchymal stem cells in experimental traumatic brain injury," *Clinical Science*, vol. 124, no. 3, pp. 165–176, 2013.
- [132] C. A. Ribeiro, J. S. Fraga, M. Grãos et al., "The secretome of stem cells isolated from the adipose tissue and Wharton jelly acts differently on central nervous system derived cell populations," *Stem Cell Research and Therapy*, vol. 3, article 18, 2012.
- [133] C. Rota, B. Imberti, M. Pozzobon et al., "Human amniotic fluid stem cell preconditioning improves their regenerative potential," *Stem Cells and Development*, vol. 21, no. 11, pp. 1911–1923, 2012.
- [134] A. Skardal, D. Mack, E. Kapetanovic et al., "Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds," *Stem Cells Translational Medicine*, vol. 1, no. 11, pp. 792–802, 2012.
- [135] F. M. de Almeida, S. A. Marques, B. D. S. Ramalho et al., "Human dental pulp cells: a new source of cell therapy in a mouse model of compressive spinal cord injury," *Journal of Neurotrauma*, vol. 28, no. 9, pp. 1939–1949, 2011.
- [136] C. Gandia, A. N. A. Armiñan, J. M. García-Verdugo et al., "Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction," *Stem Cells*, vol. 26, no. 3, pp. 638–645, 2008.
- [137] J. S. Fraga, N. A. Silva, A. S. Lourenço et al., "Unveiling the effects of the secretome of mesenchymal progenitors from the umbilical cord in different neuronal cell populations," *Biochimie*, vol. 95, no. 12, pp. 2297–2303, 2013.
- [138] M. J. Lee, J. Kim, M. Y. Kim et al., "Proteomic analysis of tumor necrosis factor- α -induced secretome of human adipose tissue-derived mesenchymal stem cells," *Journal of Proteome Research*, vol. 9, no. 4, pp. 1754–1762, 2010.
- [139] G. E. Kilroy, S. J. Foster, X. Wu et al., "Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors," *Journal of Cellular Physiology*, vol. 212, no. 3, pp. 702–709, 2007.
- [140] M. Wang, P. R. Crisostomo, C. Herring, K. K. Meldrum, and D. R. Meldrum, "Human progenitor cells from bone marrow or adipose tissue produce VEGF, HGF, and IGF-I in response to TNF by a p38 MAPK-dependent mechanism," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 291, no. 4, pp. R880–R884, 2006.
- [141] R. Tasso, M. Gaetani, E. Molino et al., "The role of bFGF on the ability of MSC to activate endogenous regenerative mechanisms in an ectopic bone formation model," *Biomaterials*, vol. 33, no. 7, pp. 2086–2096, 2012.
- [142] M. K. McCoy, T. N. Martinez, K. A. Ruhn et al., "Autologous transplants of Adipose-Derived Adult Stromal (ADAS) cells afford dopaminergic neuroprotection in a model of Parkinson's disease," *Experimental Neurology*, vol. 210, no. 1, pp. 14–29, 2008.
- [143] J. Rehman, D. Traktuev, J. Li et al., "Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells," *Circulation*, vol. 109, no. 10, pp. 1292–1298, 2004.
- [144] X. Wei, Z. Du, L. Zhao et al., "IFATS collection: the conditioned media of adipose stromal cells protect against hypoxia-ischemia-induced brain damage in neonatal rats," *Stem Cells*, vol. 27, no. 2, pp. 478–488, 2009.

1.3. References

- Armada-da-Silva, P. A., C. Pereira, S. Amado, A. Luís and A. Maurício (2014). Activity-Based Strategies in the Rehabilitation of Peripheral Nerve Injuries.
- Caplan, A. I. and J. E. Dennis (2006). Mesenchymal stem cells as trophic mediators. Journal of Cellular Biochemistry **98**(5): 1076-1084.
- da Silva Meirelles, L., A. M. Fontes, D. T. Covas and A. I. Caplan (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. Cytokine & Growth Factor Reviews **20**(5–6): 419-427.
- Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop and E. Horwitz (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy **8**(4): 315-317.
- Giordani, L. and P. L. Puri (2013). Epigenetic control of skeletal muscle regeneration: Integrating genetic determinants and environmental changes. FEBS J **280**(17): 4014-4025.
- Gopinath, S. D. and T. A. Rando (2008). Stem Cell Review Series: Aging of the skeletal muscle stem cell niche. Aging Cell **7**(4): 590-598.
- Hoke, A. (2006). Mechanisms of Disease: what factors limit the success of peripheral nerve regeneration in humans? Nat Clin Pract Neurol **2**(8): 448-454.
- Jarvinen, M., A. J. Aho, M. Lehto and H. Toivonen (1983). Age dependent repair of muscle rupture. A histological and microangiographical study in rats. Acta Orthop Scand **54**(1): 64-74.
- Järvinen, T. A. H., T. L. N. Järvinen, M. Kääriäinen, V. Äärimaa, S. Vaittinen, H. Kalimo and M. Järvinen (2007). Muscle injuries: optimising recovery. Best Practice & Research Clinical Rheumatology **21**(2): 317-331.
- Järvinen, T. A. H., T. L. N. Järvinen, M. Kääriäinen, H. Kalimo and M. Järvinen (2005). Muscle Injuries. The American Journal of Sports Medicine **33**(5): 745-764.
- Koning, M., M. C. Harmsen, M. J. A. van Luyn and P. M. N. Werker (2009). Current opportunities and challenges in skeletal muscle tissue engineering. Journal of Tissue Engineering and Regenerative Medicine **3**(6): 407-415.
- Maurício, A. C., A. Gärtner, P. Armada-da-Silva, S. Amado, T. Pereira, A. P. Veloso, A. Varejão, A. L. Luís and S. Geuna (2011). Cellular Systems and Biomaterials for Nerve Regeneration in Neurotmesis Injuries. Biomaterials Applications for Nanomedicine ISBN: 978-953-307-661-4, , Available from: . R. Pignatello, InTech.

- Merritt, E. K., D. W. Hammers, M. Tierney, L. J. Suggs, T. J. Walters and R. P. Farrar (2010). Functional Assessment of Skeletal Muscle Regeneration Utilizing Homologous Extracellular Matrix as Scaffolding. Tissue Engineering Part A **16**(4): 1395-1405.
- Page, R. L., C. Malcuit, L. Vilner, I. Vojtic, S. Shaw, E. Hedblom, J. Hu, G. D. Pins, M. W. Rolle and T. Dominko (2011). Restoration of Skeletal Muscle Defects with Adult Human Cells Delivered on Fibrin Microthreads. Tissue Engineering Part A **17**(21-22): 2629-2640.
- Pereira Lopes, F. R., L. Camargo de Moura Campos, J. Dias Correa, Jr., A. Balduino, S. Lora, F. Langone, R. Borojevic and A. M. Blanco Martinez (2006). Bone marrow stromal cells and resorbable collagen guidance tubes enhance sciatic nerve regeneration in mice. Exp Neurol **198**(2): 457-468.
- Pereira, T., P. Armada-da Silva, I. Amorim, A. Rêma, A. Caseiro, A. Gärtner, M. Rodrigues, M. Lopes, P. Bártolo and J. Santos (2014). Effects of Human Mesenchymal Stem Cells Isolated from Wharton's Jelly of the Umbilical Cord and Conditioned Media on Skeletal Muscle Regeneration Using a Myectomy Model. Stem Cells International **2014**.
- Pola, R., T. R. Aprahamian, M. Bosch-Marce, C. Curry, E. Gaetani, A. Flex, R. C. Smith, J. M. Isner and D. W. Losordo (2004). Age-dependent VEGF expression and intraneural neovascularization during regeneration of peripheral nerves. Neurobiol Aging **25**(10): 1361-1368.
- Prockop, D. J. and J. Y. Oh (2012). Medical therapies with adult stem/progenitor cells (MSCs): a backward journey from dramatic results in vivo to the cellular and molecular explanations. J Cell Biochem **113**(5): 1460-1469.
- Tamaki, T., Y. Uchiyama, Y. Okada, T. Ishikawa, M. Sato, A. Akatsuka and T. Asahara (2005). Functional recovery of damaged skeletal muscle through synchronized vasculogenesis, myogenesis, and neurogenesis by muscle-derived stem cells. Circulation **112**(18): 2857-2866.
- Tedesco, F. S., A. Dellavalle, J. Diaz-Manera, G. Messina and G. Cossu (2010). Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. Journal of Clinical Investigation **120**(1): 11.
- Walsh, S. and R. Midha (2009). Practical considerations concerning the use of stem cells for peripheral nerve repair. Neurosurg Focus **26**(2): E2.
- Yaffe, D. (1968). Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proceedings of the National Academy of Sciences of the United States of America **61**(2): 477.

Chapter II

Dental Pulp Stem/ Stromal cells as an alternative
system for regenerative therapies development

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Introductory note

From the earliest descriptions of MSCs populations (Lajtha, 1967, Friedenstein et al., 1968, Friedenstein et al., 1970), numerous features have been debated and attributed to this particular cell family, from their proliferative capacities and multilineage differentiation (Chamberlain et al., 2007) to their immunologic and immunomodulatory profiles (Le Blanc and Ringden, 2005, Caplan and Dennis, 2006, Caplan, 2009, Kim et al., 2013).

These quiescent populations can be identified in virtually all the body tissues (Lajtha, 1967, Triffitt, 2002, da Silva Meirelles et al., 2006), but the bone marrow remains the 'gold standard' for stem/ stromal cell research and clinical translation of disclosed advancements. However, the bone marrow donation process is painful, and resulting cellular isolates vary widely in numbers and 'quality', reflecting the age, health and other characteristics of the donors (Fossett and Khan, 2012). These and other concerns preclude the development and clinical application of MSCs-based therapies and motivate the search for alternative sources, that implicate less invasive collection methods, as well as ensuring ethical considerations on the process of donation. Amongst these conscious sources, the umbilical cord stroma (Gärtner et al., 2013) and the dental pulp (Kawashima, 2012) are worth highlighting.

The umbilical cord stromal tissue as a source of MSCs bares advantages for the non-invasive nature of its collection and its classification as medical waste. In the last decade, cryopreservation services have expanded and, to date, millions of infants have viable samples of umbilical cord tissue and cells safely preserved and available for therapeutic usage if necessity arises. The research group framing the present work has strenuously dedicated to the development and validation of protocols for the isolation and culturing of MSCs from this tissue, and demonstrated its potential on tissue engineering applications for neuromuscular regeneration (Gartner et al., 2012, Ribeiro et al., 2013, Pereira et al., 2014, Pereira et al., 2014, Pereira et al., 2014, Ribeiro et al., 2014, Ribeiro et al., 2015, Caseiro et al., 2016, Silva et al., 2016).

Populations isolated from dental tissues present similar advantages, and cryopreservation options for infants and adult individuals are increasingly available (Perry et al., 2008, Woods et al., 2009). This option widens the possibility for the cryopreservation of MSCs baring tissues for an extended age group that did not have the possibility to benefit from umbilical cord preservation services.

The first part of this Chapter presents an extensive literature review on dental tissue-derived stem/ stromal cells. Primarily, it focuses in detail on these MSCs features and capacities, as well as on the isolation and culturing techniques reported.

Throughout the research on the topic, an evident aptitude of these populations towards mineralised tissue regeneration was substantiated, which was deemed worth summarising, and is well patent on the title of the resulting final publication. Therefore, and although the drive for the currently presented work is the development of advanced therapeutic systems for soft tissue (skeletal muscle and peripheral nerve) regeneration, the disclosure of their recognised potential for mineralised tissue regeneration presented itself appropriate.

From the scrutiny of the available literature, protocols for the isolation and culturing of dental pulp derived populations were designed and are herewith presented, in the second section of the Chapter. The MSCs character of the isolated cells was then validated, according to the criteria defined by the international scientific community.

2.1. Dental Pulp Stem/ Stromal Cells as an innovative source for therapies development

The potential clinical application of mesenchymal stem cells from the dental pulp (DPSCs) for bone regeneration

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CHAPTER 18

The Potential Clinical Application of Mesenchymal Stem Cells from the Dental Pulp (DPSCs) for Bone Regeneration

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Abstract: The skeleton has the vital role of providing support and protection to all body organs, as well as to function as storage system for ions and other components essential to its homeostasis, also presenting essential function in the movement and posture of the individual. Bone fractures are a fairly common situation affecting individuals of all ages, but gain importance when concomitant pathologies are present or when the bone lesions' extension exceeds the tissues' intrinsic healing capabilities. As such, biomedical research has invested in unveiling adequate therapies to aid in those cases. The tissue engineering field has therefore evolved in the direction of

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developing biomaterials and scaffolds to structure, support and promote bone ingrowth, and in developing strategies to optimize these biomaterials *in vivo* performance, by including cell-based therapies and growth factors. Herein, we discuss one promising strategy for the optimization of these hybrid systems, through the association of biomaterials to a specific source of mesenchymal stem cells: the dental pulp stem cells. Dental pulp stem cells can be found in individuals of any age, and can be easily isolated from deciduous and definitive teeth, expanded and cryopreserved for further use. These cells are capable of differentiating towards multiple lineages, presenting great potential for osteo-differentiation. Dental pulp stem cells have been demonstrated to incorporate diverse biomaterial systems and promote mineral deposition both *in vitro* and *in vivo*, aiming at the reconstruction of osseous defects, in either experimental or clinical situations. The mesenchymal stem cells from the dental pulp can also be found and isolated from many species other than humans, granting them potential to be implemented not only in human medicine but also in veterinary care practices, and in regenerative strategies for other organs and tissues, such as dental reconstruction and nervous system regeneration.

Keywords: Adult teeth, Animal models, Biomaterials, Bone regeneration, Cell isolation, Cell sources, Ceramic biomaterials, Deciduous teeth, Dental cells, Dental pulp, Dental regeneration, Differentiation, Growth factors, *In vitro*, *In vivo*, Mesenchymal stem cells, Nerve regeneration, Stem cells, Tissue regeneration, Tooth.

INTRODUCTION

Nearly 30 years ago, Vacanti [1] described the transplantation of cells using bio-absorbable polymer matrices. That was the beginning of tissue engineering, then described as “an interdisciplinary field that applies principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function”. The fundamentals of tissue engineering are based on three major pillars: scaffolds, cells and regeneration factors, all deemed essential for the success of stem cells based therapies (Fig. 1) [2 - 4].

Stem cells are responsive undifferentiated cells with varying degrees of self-proliferation and differentiation plasticity. These characteristics makes stem cells attractive as tissue regeneration motors [5]. Although the number of stem cells is higher before birth, in the adult there are still several “niches” with a significant

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number of stem cells [6].

The stem cells “niches” in adults include the skin, adipose tissue, peripheral blood, bone marrow, pancreas, intestine, brain, hair follicles, and others, as well as in the dental pulp [7]. In this review we focus on dental pulp derived stem cell population. Dental stem cells (DPSCs) are one good alternative to other sources’ cells due to their easy collection from healthy donors and high proliferation and differentiation ability [8 - 11], with the advantage of being collect from both infants and adults, which are frequently subjected to dental orthodontic treatments that imply the removal of healthy teeth.

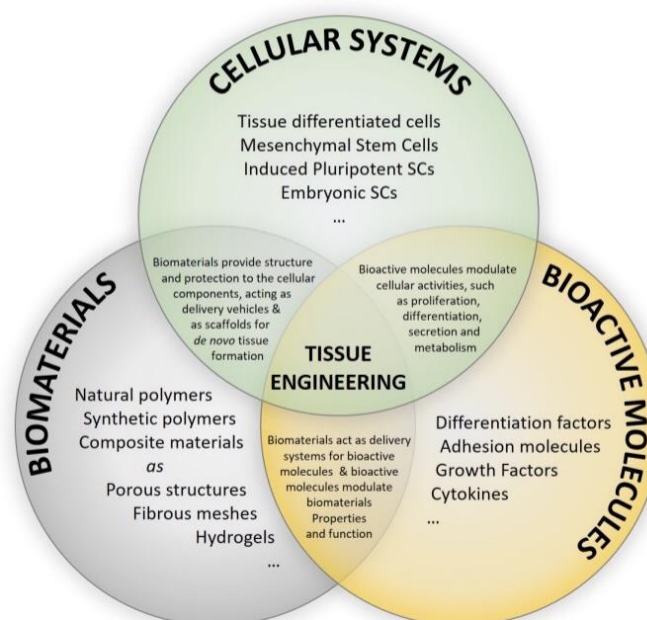


Fig. (1). The three pillars of tissue engineering – the biomaterial scaffolds that provide structure and a protective environment for the tissue regeneration; the cells that motor the regeneration itself; and the regeneration factors that promote and enhance cell growth and differentiation.

DENTAL STEM CELLS

Tooth Anatomy and the DPSCs Niche

Human teeth are an incredibly specialized organ composed of the crown (visible part that protrudes from the gingiva), the neck (the transition area), and the root

(anchored to the alveolar bone). The external layer of the tooth is composed by enamel that covers and provides protection to the intermediate dentine part, and the inner pulp. Throughout life, human exhibit two series of teeth: the initial deciduous teeth (that start sequentially erupting around six months of age), followed by permanent / term or definitive teeth. The latest set of dentition starts developing from the tooth germ at approximately six years of age and sequentially ‘pushes out’ the deciduous dentition, in approximately six years (with exception for the 3rd molars or ‘wisdom teeth’ that, when present, arise around the twentieth year of age) [12].

The dental pulp itself is composed of two regions: a central vascular-nervous trunk and external odontogenic region that in turn can be organized in four layers with distinct features and functions. The inner layer is the pulpal core, with an extensive vascular supply, that interfaces with a similar cell rich zone, where fibroblastic and mesenchymal stem cells (MSCs) populations reside. The outer layers are the cell free zone (also designated as zone of Weil), supporting capillary and nervous structures, and finally the odontoblastic layer, that interfaces with the dentin [13].

This particular structure of the tooth creates a well-defined regulatory environment that harbors the valuable population of stem/ progenitor cells, commonly addressed as the ‘stem cell niche’ [14]. Specific interactions with the surrounding environment (neighboring cells, extracellular matrix, and soluble signaling molecule, among other factors) modulate cell proliferation, survival, migration, fate, and aging, in order to maintain tissue homeostasis [15]. This mostly quiescent cell population is recruited in response to stimuli, such as injury or disease, to regenerate the damaged tissue and assure the host organ functions. The amount of stem cells in their niche and their regeneration potential is highly dependent on the age of the organism: cells from younger individuals and/or from more immature tissues (the dental follicle, for example) have higher proliferative capacity and tend to display different differentiation capabilities, when compared to adults and/or fully developed organs (such as permanent teeth) [15 - 18]. The pulp structure itself evolves as age progresses: young rats pulp contains higher cell/blood vessels ration than older ones, increased proliferation and colony forming units (CFU) capacity, but decreased mineralization potential [17]. In fact,

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the introduction of these young cells conditioned medium enhanced adult DPSCs proliferation and diminished its osteo-differentiation capacity, while adult cells conditioned medium had the opposite effects on young DPSCs, reinforcing the idea of niche modulation on stem cells activities, properties and potential [17]. Nevertheless, for the human species, some authors report no significant differences in expansion and bone differentiation potential of young (under 30 years old) and aged individuals (from 30 to 45 years old) [19].

Based on the expression of cell surface markers [endothelial and smooth muscle markers, such as Vascular cell adhesion protein 1 (VCAM-1), Cluster of differentiation 146 (CD146), and Alpha-smooth muscle actin (α -SMA), and pericyte and mesenchymal stem cell markers 3G5 ganglioside antigen and Stromal Cell Precursor Surface Antigen (STRO-1)], it was proposed that DPSCs may majorly originate from the perivascular niche [14, 20]. The major perivascular origin of DPSCs is also supported by a consistent Ca^{2+} release in response to Endotelin-1, an endothelium-related peptide sequence [21].

Alternative sources for the stem cells within the dental pulp have been demonstrated, as DPSCs were isolated from both the perivascular and the sub-odontoblastic compartments (inner surface of tooth and outer part of the pulp tissue), by separate digestion of the tooth and extracted pulp tissue. Both populations presented identical cell size, doubling times and karyotype stability, differing only in morphology with rounder cells in the sub - odontoblastic compartment versus spindle shaped cells with long processes in the perivascular one [22].

The assumption of multiple sources or niches for the multipotent DPSCs is further supported by *in situ* markers expression profiles. Besides the classically considered perivascular niche, cells with multiple lineage differentiation associated markers were identified intra - nerve, within the pulp connective tissue (pulpocytes) as well as in a perineural location, considering evidence that CD146 and α -SMA might not be putative markers for dental cells with stem cell characteristics [23]

Stem Cells form Human Permanent Teeth Dental Pulp

DPSCs were first described upon enzymatic isolation from impacted third molars [20], and are reported to be established with approximately 80% success rate from freshly extracted teeth [24]. Immediate isolation seems not to be an essential prerequisite to adequate cell yields, since satisfactory numbers can still be recovered after 5 days of tooth extraction, if kept in adequate refrigerated transport medium [24].

DPSCs are commonly isolated from pre-molars (wisdom teeth), but supernumerary teeth are another possible source for populations with stem cell characteristics [25]. Dental pulp can also be retrieved without tooth extraction, following for example traumatic crown fractures [26]. It has however been observed that inflammatory environments affect isolated cells proliferation and differentiation capacities [27].

Several authors report heterogeneous populations of isolated DPSCs [10, 18, 22, 28] differing in clonogenic, proliferation and differentiation capacities. The existence of at least two different morphologies within isolates cells is also stated [22, 29]: spindle shaped cells with long processes (associated with perivascular location) and more rounded cells (associated with the outer area of the pulp) [22]. The presence of multiple stem cell populations within the tooth is attributed to its embryonic origins, as teeth originate from two distinct embryonic layers (ectomesenchyme of neural crest and ectoderm of dental lamina) [15, 22], and to the fact that enzymatically obtained isolated are not pure stem cell cultures, but rather contain different types of stem/progenitor cells, as well as more committed fibroblast-like cells [29].

DPSCs usually perform the asymmetric cell division, originating daughter cells with different potentials: ones tend to retain a stem-cell fate and others tend to generate differentiated progenies. As such, serial passaging of DPSCs results in loss of self-renewal capabilities and the deviation to self-differentiation. As proliferation arrests in routine expansion medium, the expression of differentiation marker genes and proteins increases [Alkaline phosphatase (ALP),

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Osteopontin (OPN), Osterix (OSX), Bone sialoprotein (BSP), Collagen type II (Coll II), Dentin sialophosphoprotein (DSPP)] as does the response of those cells to osteoinducing medium. *In vivo*, implanted undifferentiated cells at late passages present tendency for bone production, with decreased dentin and cartilage production potential [30].

Phenotypic Features

When compared to the ‘gold standard’ of MSCs populations (bone marrow MSCs or BM-MSCs), DPSCs generally present increased proliferation rates. Gene expression analysis agrees with such observation, since among 4400 genes commonly expressed by DPSCs and BM-MSCs, Insulin-like growth factor 2 (IGF-2, cell growth stimulation properties) and Insulin-like growth factor-binding protein 7 (IGFBP-7, cell proliferation inhibitor) are up- and down-regulated in dental cells [31]. DPSCs high proliferation and population-doubling times are reported to be quite stable up until the 40th population-doubling (12-50 hours), increasing then to 60-90 hours. Their chromosomic stability and undifferentiated state is maintained beyond the Hayflicks limit (approximately 50 population doublings), for at least 60 population doublings [22].

Additionally, these cells expressed common phenotypic markers with BM isolates, recognized for their stemness potential, while absent of major hematopoietic markers (CD14, CD34 and CD45) [20]. Isolates displayed minimal stem cells characteristics, as all DPSCs cultures exhibited $\geq 95\%$ expression of CD73, CD90, and CD105, with $\leq 5\%$ expression of CD34, CD45, CD11b, CD19, and HLA-DR, as well as trilineage (osteogenic, adipogenic, and chondrogenic) differentiation [24]. DPSCs are also reported positive for CD44 (Cell surface markers associated with stromal cells, like CD90), CD166 (Cell surface markers associated with stromal cells and endothelial cells, like CD105), CD146 (Cell surface marker associated with stromal cells, endothelial cells and perivascular cells), but negative for CD40, CD80, and CD86 (Cell surface markers of hematopoietic cells) [32]. HLA-ABC (MHC class I antigen) high expression is also in accordance to BM isolates [32].

Although DPSCs are classically compared to BM-MSCs, they also reveal close

similarities with other emerging stem cell sources, such as the stromal tissue from the umbilical cord, named Wharton's jelly. Phenotyping revealed a similar expression pattern of antigens characteristic of MSCs including CD105, CD29, CD44, CD146, and STRO-1 on both cell types. DPSCs revealed to be less inclined to adipogenic lineages, but were superior in terms of osteogenic and chondrogenic potential [33].

STRO-1 is a mesenchymal stem cell marker and its expression can be detected is about 9 to 23% of the isolated cells [14, 29, 34, 35], with most of this fraction displaying colony-forming capacity [14]. However, low expression STRO-1 populations do display several stemness attributes and differentiation capacity [36]. CD146 is deemed a perivascular marker, associated with DPSCs CFU capacity [14], although it appears not to be an essential requisite to multilineage differentiation [23, 36].

Despite being a reference marker for the classification of mesenchymal stem cells [37], the expression of CD34 (expressed in primitive pluripotent stem cells, of both stromal and hematopoietic origin) by DPSCs is controversial: + [29], - [12, 20, 24, 35, 38] since some authors report CD34⁺ DPSCs sorted isolates retaining all the remaining mesenchymal stem cell features and even enhanced *in vitro* and *in vivo* bone formation capacity [19, 39]. CD34 is regarded as a cell surface marker expressed by the most primitive stromal other than hematopoietic stem cells, before lineage commitment, and is expressed by adherent dental pulp cells, representing about 10% of the population [19]. Most often, CD34⁺ cells were reported to differentiate into bone producing cells *in vivo* [40]. An array of pluripotency/ embryonic stem cell markers can also be identified in DPSC isolates [36].

Differentiation Capacities

Cells resulting from the first isolation procedures were shown to differentiated into adipocytes and odontoblasts and regenerate dentin-like tissues *in vivo* [10, 20]. To date, DPSCs have demonstrated differentiation capacity towards multiple other mesodermal and endodermal lineages, under appropriate conditions: adipogenic, osteo/dentinogenic, chondrogenic, neurogenic, endothelial,

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myofibroblastic [23] and hepatocytes [36].

Their differentiation potential is not identical to other possible sources of MSCs, for their apparently diminished ability towards adipogenesis and tendency towards mineral tissue formation [33], as primary cultures already mark positive to bone-related markers [20] and indications of spontaneous differentiation have been observed [29]. Other reports also show that DPSCs marker expression profile indicates a tendency of these towards the neuroectodermal lineage differentiation [16], and that successful neuronal differentiation occurs [41].

Stem Cells from Human Deciduous Teeth Dental Pulp

Deciduous teeth, whether they exfoliate naturally [42] or are extracted (SHEDs - stem cells from human exfoliated deciduous teeth; or DTSCs – deciduous teeth stem cells) [43, 44] also harbor stem cell populations. These increased proliferative capacity [16, 42, 44, 45], and increased expression of stemness markers, suggesting a more primitive cell population [16].

Deciduous teeth derived cells revealed major up-regulation of cell proliferation and extracellular matrix production genes (which impact on cells proliferative capacities) [44].

Surface markers expression resembles DPSCs' [42, 46 - 48], as does the multilineage differentiation capacity *in vitro* (osteogenic, adipogenic, chondrogenic, myogenic and neurogenic lineages), although slightly distinct from that of DPSCs and BM-MSCs [42, 43, 45, 47]. SHEDs seem to differentiate towards osteogenic, chondrogenic and adipogenic lineages more effectively than DPSCs [45, 49]. SHEDs are also reported to have stronger expression of CD146 and CD105 than DPSCs [45, 49], and controversy surrounding CD34 expression persists as does in DPSCs [42, 45, 46, 48].

A tendency to neuronal differentiation can also be suggested, since expanded SHEDs inherently express several neural cell markers [42]. However, not only of glial and neuronal markers can be found in undifferentiated cells, but also myogenic, osteogenic and chondrogenic differentiation markers are present [47].

Stem Cells from Other Dental-Related Tissues

Stem Cells from Human Permanent Teeth Apical Dental Pulp (APDCs/SCAPs)

The apical papilla is a smooth-surfaced soft tissue at the apex of permanent immature teeth that can be easily detached from the apex, exposing the pulp tissue present in the canal space. Constitutively, the apical papilla contains less blood vessels and cellular components than the adjacent dental pulp [50]. Although having a smaller cellular component, apical papilla's cells undergo improved proliferation [29, 50], and proliferation rates tend to be superior to those of DPSCs [51], forming earlier colonies and reaching confluency faster than tooth matched DPSCs after isolation [29]. APDCs present a phenotype similar to BM-MSCs and DPSCs [29, 50], with exception for the expression on CD49d (or $\alpha 4$ -integrin), absent in the latter [52]. They were also capable of multilineage differentiation [51, 52]. Osteogenic and dentinogenic potential of apical papilla stem cells seem comparable to that of BM-MSCs, although weaker adipogenic differentiation is patent [29, 50].

Stem Cells from Human Dental Follicle (DF-MSCs)

The tooth germ consists of the enamel organ, dental papilla, and dental follicle, and is the primordial structure from which a tooth is formed. Dental follicles' MSCs have been isolated and originated a population with stem cell characteristics [18, 53]. When compared to DPSCs, DF-MSCs present different differentiation capacity, being more prone to adipogenesis than to chondrogenesis or osteogenesis. Similarly to DPSCs, they comprise of a heterogeneous cell population, but of larger diameter. DF-MSCs fulfill the criteria for MSCs classification [37], and their increased proliferation rates and surface markers expression suggest that DF-MSCs constitute a more undifferentiated cell population than DPSCs [18].

Stem Cells from Human Periodontal Ligament (PDLSCs)

The periodontium is composed of the periodontal ligament (PDL), alveolar bone and the mineralized bone-like cementum covering the tooth root surfaces that arises from the dental follicle progenitor cells and functions as the supporting tissue of the tooth. The PDL represents a specialized connective tissue whose

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fibers are embedded in the cementum and the alveolar bone is ensuring the attachment of the teeth [53]. Besides its support functions, PLD also contributes to tooth nutrition, homeostasis and damaged tissue repair [54].

Cells isolated from the periodontal ligament present similar proliferation rates to DPSCs and comparable adipogenic differentiation [32, 51, 54], but contradictory mineralization potential was evidenced, when cultured in osteo/odontoiducing medium, being enhanced [51, 55] or diminished [32, 54] when compared to DPSCs and BM-MSCs.

Comparably DPSCs, PDLSCs are positive for STRO3, CD44, CD90, CD166, CD105, CD146 and HLA-ABC while being negative for CD40, CD80, CD86, CD106 and HLA-DR [32].

PDLSCs express a particular transcription factor, scleraxis, and a tendon/ligament specific transcription factor [54]. Gene expression profiles between PDLSCs and DPSCs display slight differences, that become further evident when undergoing differentiation, as changes in gene expression profiles are more pronounced in PDLSCs than in DPSCs, suggesting a more complex regulatory process [55].

DPSCs from Other Species

The use of animal models in studies of structural and functional regeneration of dental pulp is essential [56]. Given this premise, there are several studies that have culminated with the isolation and characterization of DPSCs in different animal species such as dog, pig, sheep, mouse, rat, ferret, rabbit, cattle and chimpanzee [57].

In general, larger animals are considered best models in dental and orofacial investigation than smaller animals [58], but small animals are often chosen due to costs, breeding and ethical issues [59]. Nevertheless, even if it may be preferable to use small animals such as rats, hamsters, rabbits or ferrets, their physiopathology is not as similar as humans and so, experimental models need to be reproducible in order to obtain relevant results [57].

Other emerging and growing field of application is also the Veterinary Cellular Therapy, further boosting for the isolation and investigation into domestic and

production animals MSCs populations.

Small rodent are the smallest species to be explored. Murine DPSCs (mDPSCs) are amenable of isolation and multilineage differentiation and have been explored and characterized by several research groups [60 - 65]. Mouse DPSCs present appreciative expression of STRO-1, CD31, CD73, CD90 and Sca-1, low expression of CD117 and are negative for CD11b, CD34 and CD45 [66]. Rat DPSCs (rDPSCs) have also been successfully isolated and differentiated [8, 17, 28, 67, 68]. Comparing to rat BM-MSCs, DPSCs presented comparable markers profiles ($\geq 95\%$ positive for CD29, CD59, CD90 and CD106, and $\leq 5\%$ positive CD45 and CD11b) and trilineage differentiation capacity (osteogenic, chondrogenic and adipogenic). DPSCs showed remarkably enhanced proliferation and colony-forming capacity. Indeed, isolates from one tooth and one femur (approximately 1 and 100×10^6 cells, respectively) would reach confluency at the same time. ALP activity also indicated and increased osteogenic potential of DPSCs over BM-MSCs [8]. Conversely, other authors reported reduced attachment by rat DPSCs and *in vivo* hard tissue formation inferior to BM-MSCs [69]. Adult and juvenile rat DPSCs display distinct proliferation and differentiation capacities, whom can be modulated through the application of differently aged conditioned medium to the cultures [17]. They have also shown *in vivo* self-differentiation capacity, by forming bone, dentin and cartilage like tissues when implanted in rat renal capsules [30].

Using rabbit derived cells, rabbit dental stem cells (raDPSCs) were assessed in scaffolds of different porosities for their potential to regenerate dentine/pulp tissue [70]. Rabbits have also been described as a preclinical animal model to test the regeneration of critical-size alveolar bone defects using raDPSCs, ceramic/polymer composite materials and recombinant rhBMP-2 [71].

One very recent report describes the isolation and characterization of ferret DPSCs [72].

Cells from canine teeth have also been isolated (cDPSCs) [56, 73] and their full characterization has been attempted [56]. These have been assessed for their regenerative capacity at the level of dentin [73], dental pulp [74] and bone [75,

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76]. Similarly to human-derived DPSCs, cDPSCs also present the capacity to adhere to plastic surfaces and extensively proliferate and display fibroblast-like morphology, CFU formation and multilineage differentiation potential. However, phenotypical characterization remains challenging, due to the limited availability of species-specific antibodies. In fact, the lack of expression of CD90 and CD105 and reduced expression of STRO-1 may be caused by the use of anti-human surface markers, which have very limited cross-reactivity with canine MSC markers [56].

Both DPSCs and PDLSCs have been isolated from ovine teeth [77, 78]. Both populations expressed markers CD44, CD106, and CD166 and lacked expression of the hematopoietic markers CD14 and CD45 and the endothelial marker CD31. CD106⁺ sorted populations of these isolates exhibited fair clonogenic capacity and demonstrated a capacity to form mineralized deposits *in vitro*, with slight superiority for DPSCs. When implanted into immunocompromised mice in association with Hydroxyapatite/ tricalcium phosphate (HA/TCP) carriers, ovine PDLSCs (oPDLSCs) could produce both mineralized and soft connective tissues with many morphological features of original periodontal ligament. On the other hand, DPSCs transplants produced dentin/pulp-like structures along the HA/TCP surfaces, resembling dentin-like matrix [77]. oDPSCs and oPDLSCs have been further compared to BM-MSCs from a paired donor [78].

Porcine dental stem cells have been described [73, 79]. Swine cells isolated from minipig incisor teeth also form adherent clonogenic cell clusters of fibroblast-like cell morphology, forming an heterogeneous spindle-shaped cell population, comprising of 10.9% STRO-1⁺ cells [79].

Chimpanzees DPSCs, collected post-mortem, produced stem cell cultures remarkably similar to those of human BM-MSC and DPSCs. They displayed an initial high rate of proliferation, which tended to moderate after about 15 passages, accompanied by a slight reduction telomeres length, although it still remains within the length characteristic of stem cells (10-20 kb). Chimpanzee's DPSCs expressed stem cell markers (Nanog, Sox-2, Rex-1 and Oct-4) and underwent multilineage differentiation (osteogenic, adipogenic and chondrogenic). Surface markers revealed 99% of the cells expressed CD29,

CD44, CD59, CD73, CD90, CD105, CD150 and CD166, and no expression of specific hematopoietic cells, including CD14, CD34, and CD45 [80].

Isolation and Culture Methods

Regardless of the dentition and species or origin, the isolation of MSCs populations from pulpal tissue is reported through three methods, either alone or combined. Simple mechanical dissociation has been described to effectively isolate DPSCs [81, 82], but explant culture and enzymatic digestion of the connective pulp tissue are by far the most widely applied methods. In Table 1 we summarize the techniques – mechanical and enzymatic – for isolation of dental derived stem cells.

Explant isolation requires for an extended isolation period, since DPSCs take 7-15 days to initiate migration from the seeded tissue into the culture surface, where they then expand and quickly provide numerous cells [33, 83 - 85].

Table 1. Representative examples of Human derived mesenchymal stem cells populations' isolation from dental related tissues.

Isolation technique		Dental cells population	Refs
Mechanical		DPSCs	[81, 82, 100]
Explants	without pre-digestion	DPSCs	[33, 83 - 85]
		PLDSCs	[53, 83, 101]
		APDCs	[52]
		DTSCs/SHEDS	[29, 46, 48]
		DPapillaSCs	[102]
	With pre-digestion (0,2% Trip, 5 min)	DPSCs	[21]

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(Table 1) contd....

Isolation technique			Dental cells population	Refs
Enzymatic digestion	Enzyme	Incubation time		
		30 min	SHEDs	[103]
			SCAPs	[50]
		1h	DPSCs	[5, 14, 20, 25 - 29, 35, 38 - 42, 44 - 46, 49, 51, 53, 56, 80, 93 - 97, 101, 104 - 106]
			SHEDs	[19, 29, 41, 46]
			PDLSCs	[54]
		70 min	DPSCs	[22, 23]
			SHEDS	[22]
		2h	DPSCs	[32, 77]
			PDLCs	[32, 77]
	Coll I	30 min	DPSCs	(Iida et al., 2010),
		40 min	DPSCs	[16]
			SHEDs	[16]
		60 min	DPSCs	[12, 36, 69, 88 - 91, 107]
			SHEDs	[88]
	Coll. I +DNase,	1h	DPSCs	[18]
			DF MSCs	[18]
	Coll I+ Coll II+ Thermolysin	15-30 min	DPSCs	[99]
		40 min	DPSCs	[8, 24, 98]

It is also possible to apply a pre-digestion step to the explant procedure [21]. Dental pulp cell yield with explant isolation can be largely increased by mechanically transferring the explant tissue to new culture surfaces every 3-4 days. The process can be extended for at least 6 months, continuously assuring early passage cells without jeopardizing cell morphology, expression of stem cells markers, chromosome stability, or differentiation potential, even after cryopreservation [86].

Enzymatic digestion can be achieved using a primarily type I Collagenase, alone [12, 16, 36, 87 - 92] or in combination to other dissociation agents, such as Dispase [5, 14, 19, 20, 22, 23, 25 - 28, 32, 34, 35, 38 - 42, 44, 45, 49, 51, 53, 56, 77, 80, 93 - 97], DNase [18] or type II Collagenase and Thermolysin [24, 98, 99].

DPSCs do not seem to present specific culture requirements, although a comparative study of 10% FBS supplemented α MEM and DMEM for culture expansion suggest that the first is more adequate for DPSCs [68].

Classic dental pulp stem cells culture medium contains serum from xenogenic origin, however, its use rises several key issues. It potentiates the occurrence of immune reactions when implanted (due to the presence of immunogens or toxic components) and increases the risk of pathogen transference of animal origin. Additionally, the detailed composition of serum is still unknown and prone to relevant batch-to-batch variation, adding difficulty to its use for research and clinical application purposes [48, 88]. Some observations suggest that medium formulations containing serum may predispose to malignant transformation in latter population doublings [23]. Therefore, low-serum and serum free formulations have been explored, for both isolation and expansion, as well as for differentiation protocols.

Low-serum medium (2% fetal calf serum - FCS) supplemented with 10 ng/ml EGF (Epidermal growth factor) and 10 ng/ml PDGF-BB (Platelet derived growth factor-BB), originally formulated for the expansion of Mesenchymal progenitor cell from the Bone-Marrow, is able to sustain the proliferation [22] while preserving the stem cell phenotype of DPSCs [23] and differentiation capacity into multiple lineages. Major differences may be found in terms of initial cell adhesion and CD146 and α -SMA markers expression that might be related to an initial selection on the adherent cells, imposed by the serum level [23]. The addition of insulin-transferrin-sodium selenite (ITS) supplement to the low-serum medium improves population doubling times [22].

The replacement of animal origin serum by human sources is also possible. Partial substitution of fetal bovine serum (FBS) content for human umbilical cord blood (UCB) derived platelet rich plasma (PRP) resulted in favored proliferation of DPSCs, SHEDs and PDLSCs [38]. Phenotypical profiles of mesenchymal and embryonic markers were maintained or even optimized and osteogenic differentiation capacity was unaffected in cells expanded in human serum containing medium [48]. Completely serum-free conditions appear to sustain acceptable 84%–90% viability (although significantly inferior to 90%–93% in

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10% FBS medium) and stem cell markers expression was maintained [88].

Hypoxic conditions (3% oxygen - O₂) seem to enhance the proliferation of DPSCs, in approximately 2-fold when compared to classically defined normoxic O₂ tension (21%). The proliferation enhancement occurs even in late passages, especially when seeded at lower densities [87].

Still regarding culture settings of DPSCs, a sticking limitation in DPSCs isolation and maintenance can be fungal (yeast) contamination that can reportedly affect in approximately 18% of cultures, despite the presence of amphotericin B in the culture medium and thorough decontamination procedures preceding pulp exposure [24].

The number of isolated cells and the speed at which significant numbers can be achieved depend on a variety of limiting factors, which in turn affect their availability and applicability in therapeutic approaches.

Firstly, the isolation method selected affects the initial MSCs' population numbers. Enzymatic isolation rapidly provides DPSCs populations, while explant isolations may take as long as 3 weeks to display isolated cells. As so, enzymatic isolation may be considered as methods most indicated for fast isolation and expansion of DPSCs. On the other hand, explants can be maintained for a long period of time, providing periodic transfer for new culture plates, granting extended supply of primary and early passage cells for long periods of time (up to six months, as described by Lizier et al., 2012, <http://dx.doi.org/10.1371/journal.pone.0039885>). Other methods can be hypothesised for DPSCs scale-up expansion, such as growth factor supplemented low-density seeded cultures (described for umbilical cord matrix stem cells by Kekanti et al., 2010 [108] or the usage of microcarrier-based stirred culture (as described for bone-marrow mesenchymal stem cells, by Eibes et al., 2010 [109]. To the authors knowledge little has been explored regarding the application of such techniques to DPSCs culturing, but microcarrier-based stirred culture is currently being addressed by our research group.

Secondly, the isolation/ expansion medium employed influence on efficiency and speed high DPSCs' numbers achievement. Xeno-free medium are highly desirable

for clinical applications given the risks associated to the use of animal derived culturing supplements [48] generally applied in standardized protocols. Human peripheral blood serum has been demonstrated as viable alternative to FBS supplementation for the isolation and expansion of dental cells populations [48]. Chemically defined serum-free media [88], and platelet rich plasma from the umbilical cord blood are other described possibilities [38, 106].

Nevertheless, providing large numbers of readily available cells is a critical factor for the dissemination of cell-based therapies, and suffers further limitation from regulatory and legal constraints applicable. In some countries, such as in Portugal, it is not possible to isolate and expand donor cells prior to banking (as it occurs in the umbilical cord matrix stem cells and dental pulp stem cells) and one is only allowed to cryopreserve cord and pulp tissue. Samples are only allowed to be manipulated upon necessity from de donor (in private bank) or from a matched patient (for the public bank). Research purposes are excluded from such limitations, but these circumstances limit the dissemination of such innovative therapeutic approaches.

Immunomodulatory Properties

Cells isolated from dental pulp tissue possess a privileged immunophenotype, allowing for allo-transplantation without host rejection. SHEDs can be systemically and locally administered to immunocompetent mice and dogs, engrafting host tissues without giving raise to immune rejection [110]. Dental pulp cells Implantation in bone defects also elicits no rejection response and cells integrate and contribute to *de novo* tissue formation [43]. Comparing to BM-MSCs, DPSCs present potentially superior immunosuppressive/ immunomodulatory properties [84]. Also, *in vitro* results demonstrate that SHEDS are able to reduce interleukin (IL) 17 production by challenged lymphocytes T in co-culture to a greater extent than BM-MSCs [111]. In a murine model of systemic lupus erythematosus, the positive effects of SHEDS administration were comparable or even superior to those of BM-MSCs [27, 111].

However, the immunosuppressive role of MSCs may be modulated by the immune stimulation they suffer as well as by their tissue source [18, 32]. The

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application of conditioned medium from stimulated or unstimulated populations' results in differential responses in immune cell populations, suggesting the inflammatory stimulation is also of utmost importance to the level and type of immunoactive soluble factors secretion [32].

Pro-Angiogenic Properties

From the regenerative perspective, angiogenesis is a critical event for repairing damaged tissues, through the assurance of adequate nutrients and oxygen supply, as well as other significant regenerative cues [112].

The effects on DPSCs on angiogenic events are primarily attributed to their paracrine secretion capacities. Dental derived MSCs populations are capable of secreting angiogenic factors (such as angiopoietin-1, IGFBP-3 or VEGF), as detected by conditioned medium analysis, and are further able to interact with endothelial cells, and thus impact on tissue vascularization. *In vitro* assays suggest that these seem to promote endothelial migration and tubulogenesis, although no relevant impact on endothelial cells proliferation [113]. Exposure to hypoxic conditions is described as a potentiating factor for these features [114].

In vivo, type I Collagen and Matrigel® scaffolds subcutaneously implanted in nude mice presented improved angiogenesis when leaded with DPSCs [90, 112]. The presence of DPSCs connects to increased numbers of vessels in the regenerating tissues, both capillary and of bigger caliber than 20 µm [115]. Furthermore, DPSCs are able to differentiate *in situ* and physically contribute to the newly formed vasculature [115].

This later topics were thoroughly approached in a recent review by Ratajczak et al., 2016, providing insight on the angiogenic immunomodulatory and angiogenic of dental derived stem cell populations [116].

Cryopreservation of Pulp Tissues and Cells

A vital point of considering and exploring a cell source for clinical application purposes is the possibility of cell preservation for future use [24, 91]. As such cryopreservation of pulp tissues / cells is crucial, and few key parameters must be thoroughly considered: freezing medium composition, cell density, and freezing

temperature. Tuning of these parameters is essential for optimal cell viability upon thawing, and for the preservation of their relevant features [91].

Dimethyl Sulfoxide (DMSO) has been determined the best cryoprotector, and as for cell concentration, post thaw viability is maintained up to a concentration of 2×10^6 cells/ml. The frozen vial can be maintained at -85°C or -169°C , for at least 6 months, with no major impact on cell viability, growth rate, specific marker levels or differentiation capacity [91, 99], even for long-time preservation (2 years) [39].

Minimal manipulation of the tissues before preservation would be highly desirable and it is possible to isolate viable DPSCs from cryopreserved teeth [24, 34, 99]. Intact teeth cryopreservation is not a viable methods, due to the poor freezing medium penetration, neither is the cryopreservation of pre-digested pulp tissue, due to cumulative cell damage from enzyme and freezing process on the cell membrane [99]. Alternatively, the creation of micro-channels at the level of the crown-root connection enables the cryoprotector to effectively penetrate into the pulp tissue, enabling the recovery of comparable cell numbers to those obtained from fresh teeth, preserving proliferation and phenotype of the DPSCs [12].

DPSCS Potential in Tissue Regeneration Applications

Stem cell-based therapy is a promising fields in tissue engineering and regenerative medicine in general. DPSCs are relatively abundant cells and easily recovered and demonstrated high differentiation ability and therefore are excellent candidates for therapeutic applications.

DPSCs have been mostly hypothesized for mineralized tissue regeneration, namely dentin/dentin-pulp complexes [6, 10, 20, 117 - 125] and bone structures [5, 94, 126]. Some works focus solely on the mineralization potential, disregarding the specifics of dentin and bone tissues [127]. In Table 2 we summarize the studies regarding dental pulp stem cells applications in tissue regeneration.

***IN VITRO* INDUCTION OF MINERALIZED TISSUE FORMATION**

As previously disclosed, dental tissue derived MSCs populations present a

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competence, and possibly an intrinsic tendency, to the formation of mineralized tissues. This potential is however related to the source and isolation method and, most importantly, the standard culturing and osteoinducing conditions, as different dental cell sources appear to respond differently to supplements, with distinct mineralization profiles [128]:

Regarding isolation, and comparing explant culture to enzymatic isolation, the latter seems to provide a population with higher osteogenic differentiation capacity, with culture mineralization rate differing in about 40 to 50% at 3 weeks in culture [46].

For differentiation purposes, normoxic conditions (21% O₂ tension) seem to be more adequate than hypoxic ones, which tend to promote proliferation, as DPSCs osteogenic activity decreased at 3% O₂ culture conditions [87].

Dexamethasone is classically added to osteoinductive medium, as its presence leads to increased osteogenic (ALP) activity [85, 129] as well as it promotes the formation of larger mineralized nodules by DPSCs [67]. However, dexamethasone is described to decrease cell proliferation [85, 129]. Conversely, both SHEDs and DPSCs displayed enhanced proliferation in dexamethasone and β -glycerophosphate containing inducing medium over control medium for up to 28 days, in a 3D hydrogel matrix [128].

Osteogenic differentiation of DPSCs/SHEDs can be achieved without the addition of the traditional mineralization-inducing supplements (ex. Dexamethasone - Dex), by increasing FBS concentration in the medium to 20% [5, 19, 39, 94, 105]. Cell pre-differentiated in high serum medium can even be cryopreserved, retaining their acquired phenotype and *in vitro* woven bone formation capacity, that rapidly remodels into mature lamellar bone when applied *in vivo* [39, 105].

In the run for animal free culture approaches, partial substitution of FBS for human UCB-PRP (5% FBS + 1-2% UCB-PRP) positively affects ALP and calcium deposition of DPSCs, SHEDs and PDLSCs [38].

Table 2. Examples of dental tissue derived cell-based therapies associated with biomaterials for mineralized tissue regeneration applications.

Application	Model	Cells Origin	Biomaterial/Scaffold	Regenerative factor(s)	Refs.
Mineralized tissue regeneration - BONE	Human	Impacted 3 rd molars	Spongy collagen, a porous ceramic, and a fibrous titanium mesh	-	[107]
		Mandible 3 rd molars	Collagen	-	[82, 100]
		Mandible 3 rd molars	Collagen	-	[157]
	Human	Non-specified	Collagen	-	[115]
		3 rd molars	Hyaluronic acid	-	[159]
		Deciduous & permanent teeth	Granular deproteinized bovine bone (GDPB) & β -tricalcium phosphate (β -TCP)	-	[198]
	Human	Molars	Hyaluronic acid & Hydroxyapatite/tri-calcium-phosphate	-	[199]
		3 rd molars	Hyaluronic acid	-	[159]
		Deciduous & permanent teeth	Granular deproteinized bovine bone (GDPB) & β -tricalcium phosphate (β -TCP)	-	[198]
	Human	Molars	Hyaluronic acid & Hydroxyapatite/tri-calcium-phosphate	-	[199]
		Non-specified	Collagen	-	[115]
		Deciduous teeth	β -Tricalcium Phosphate	-	[79]
	Swine	Non specified	Nano- hydroxiapatite/collagen/poli-L-lactic acid (nHAC/PLA)	rhBMP-2	[71]
		Molars & deciduous teeth	Platelets enriched plasma	platelet-derived — growth — factor (PDGF) — Transforming — growth factor — beta (TGF- β) and — insulin-like growth factor — I (IGF-I)	[75, 76]
		Molars	Platelets enriched plasma	-	[161]
	Macaca nemestrina	Upper and lower first incisive	Hydroxyapatite/tricalcium-phosphate/chitosan (HA/TCP/Ch)	-	[150]
		Pre-molars	Polycaprolactone (PCL)- hyaluronic acid- tricalcium phosphate (HT-PCL)	-	[160, 200]
		Upper and lower incisors	Collagen type I & II	-	[156]

(Table 2) *contd.....*

Table 2/3 (contd.....)							
Application		Model	Cells Origin	Biomaterial/Scaffold	Regenerative factor(s)	Refs.	
Mineralized tissue regeneration - DENTAL	Dental pulp repair	Human	3 rd molars	Alginate Hydrogel	Transforming growth factor (TGF)-β1	[142]	
	dental tissue regeneration	Human	3 rd molars	Collagen	Bone morphogenetic proteins (BMP)	[124]	
			Exfoliated deciduous teeth & 3 rd molars	PLA	-	[172, 176, 200]	
			impacted 3 rd molar	PLGA scaffold inserted in canal space of human root fragment	-	[163]	
			3 rd molars & exfoliated deciduous teeth	Peptide-Amphiphilic	Cell adhesion sequence RGD and an enzyme-cleavable site	[128, 154]	
			3 rd molars	Sylgard 184 silicone elastomer & canal of the tooth root segment	-	[201]	
			Non specified	Puramatrix™	-	[202]	
			3 rd molars	Nano-hydroxyapatite (nHA) & silk	-	[203]	
			3 rd molars	Chitosan hydrogel	-	[204]	
		Human & Bovine	Human 3 rd molars & bovine incisor roots	Bovine roots & horizontally cut human root canal discs	-	[205 - 207]	
	Canine	Non specified	Collagen	Mobilized dental pulp stem cells (MDPSCs) induced by Granulocyte-colony stimulating factor (G-CSF)	[179]		

(Table 2) *contd....*

Application	Model	Cells Origin	Biomaterial/Scaffold	Regenerative factor(s)	Refs.
Other tissue regeneration applications	Human	3 rd molars	-	-	[196]
	Rat	Incisors	-	-	[65]
	Swine	Deciduous teeth	-	-	[208]
	Human	Non specified	-	-	[209]
	Mouse	Exfoliated deciduous teeth	-	-	[180]
	Human	3 rd molars	Collagen conduits	-	[191]
		3 rd molars	Chitosan	-	[188]
		3 rd molars	-	DPSCs transfected with human OLIG2 gene	[189]
	Rat	Incisors	PLA nerve conduits	-	[190, 210]
		Incisors	-	-	[194, 195]
		Incisors	PLGA nerve conduits and collagen hydrogel	-	[185, 186]
	Swine	Incisors	Collagen type I membrane	-	[187]

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The addition of Bone morphogenetic protein 2 (BMP-2) to a standard osteo/odontogenic induction medium (basal culture medium supplemented with β -glycerophosphate, dexamethasone and ascorbic acid) significantly increased its effect on DPSCs. Both *in vitro* ALP activity and ribonucleic acid (RNA) expression and *in vivo* ectopic bone-like tissue formation (in association to a hydroxyapatite granular vehicle or porous scaffold) were potentiated by BMP-2 [83, 129]. This effect occurred in a dose dependent manner, with BMP-2 concentrations up to 300 ng/mL [83]. Stem cells derived from the periodontal ligament displayed and enhanced osteogenic potential, when compared to DPSCs [83].

Alternatively, Retinoic Acid (RA) has been shown to effectively induce osteogenic differentiation in both SHEDS and PDLSCs. It significantly increases ALP, Runx2 and OPN expression, in a dose-dependent manner, as well as mineral deposition for up to 14 days, when compared to Dexamethasone. Moreover, supplementation of RA containing serum-free medium with ITS further potentiates its effect. In terms of cell proliferation, RA alone has drastic inhibitory effects, especially on SHEDS, but it is counteracted by the ITS supplementation [130].

As known, Vitamin D(3) metabolites regulate the bone metabolism, researchers have investigated the effect of 1 α ,25-Dihydroxyvitamin D3 (1 α ,25(OH)(2)D(3)) and 25-hydroxyvitamin D(3) (25OHD(3)) on proliferation and osteogenic differentiation of human dental pulp (hDPCs) and dental follicle cells (hDFCs) *in vitro* [131]. Also, 1,25(OH)2D3 – an active metabolite of vitamin D – has been described to promote osteoblastic differentiation in hMSCs [132 - 134]. Researchers [135] have demonstrated the osteoblastic differentiation effect in Dental Bud Stem Cells by the expression of RUNX2 and Collagen I (Coll I) and production of mineralized matrix nodules. Moreover, 1 α ,25(OH)(2)D(3) (10 and 100 nM) and 25OHD(3) (500 nM) act synergistically as osteogenic inducers.

Studies have demonstrated that lithium chloride effect on osteogenesis promotion and inhibition of adipogenesis in human BMSCs by synergistically regulating the Wnt and Hedgehog pathways [136]. Regarding DPSCs, researchers [137] have investigated the effect of lithium chloride on odontoblastic differentiation, through

β -catenin. After up to 14 days, there was enhanced calcium nodule formation and matrix mineralization and mRNA levels of DSPP, DMP-1, ALP, BSP and OCN were upregulated.

The topology of the surface culture has also been demonstrated to be a key-parameter to guide DPSCs osteoinduction. Dual-acid-etched titanium surfaces (average roughness 100 times higher than polystyrene culture surfaces) up-regulated the expression of bone-related genes (Coll I, OPN, and Osteocalcin - OCN) early in the culture and the mineralized tissue displayed improved mechanical properties, up to values comparable to those of human trabecular bone [67]. In a similar approach [138], researchers found that nanotube titanium disks stimulates osteoblast differentiation in adipose tissue-derived stem cells and DPSCs. The expression of bone-related genes - RUNX2, COL1A1 and FOSL1 in the first 15 days and ALPL, SPP1 and collagens after 30 days – was up-regulated. Also, after 30 days there was an increase in RUNX2 and COL3A1 gene expression. Additionally, the type of surface irregularity also impacts on the DPSCs performance on mineral tissue formation. Microconcave surfaces supplanted smooth and convex surfaces in terms of osteogenic cell activity and tissue formation, in a 20% FBS supplemented medium (no other induction supplements added). The microconcave material displayed primary and secondary microcavities (80-120 μ m diameter/ 40-100 μ m depth, and 10-20 μ m/ 3-10 μ m depth, respectively), that closely resemble native bone trabecular spaces. These promoted earlier and enhanced osteo - differentiation and mineralization by seeded DPSCs, with cells adopting differentiating phenotype and nuclear polarity (regarded as an index of secretion). Newly formed tissue thickness was also potentiated, reaching 1 mm, versus 0.3 mm obtained on smooth surface and virtually no tissue formation on convex cultures [104].

Mechanical stimulation also acts as a modulator of cell behavior. Since pathological changes in force transmission to teeth lead to pulp calcifications, the effects of dynamic hydrostatic pressure applied to DPSCs was examined. DPSCs subjected to 2.5 MPa for 2h at 0.5 Hz displayed reversible impairment in cell adhesion and viability but enhanced response to BMP-2 stimulation [96]. Hsu and Chang, 2010 [139] demonstrated that static magnetic field (SMF) also stimulates rat DPSC differentiation into osteoblast *in vitro*. Cells were exposed continuously,

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to the SMF with and without osteogenic promoters (dexamethasone and β -glycerophosphate). Results revealed that cells exposed to the combination of SMF and osteogenic induction significantly increase expression of mRNA osteogenic genes, ALP activity and extracellular calcium concentration. Also, SMF exposure increased expression of other osteogenic markers (ERK1/2, Cbfa1 and mRNA osteogenic transcription factor). Therefore, SMF could effectively stimulate osteogenic differentiation of DPSCs.

In vitro stimulation can be applied with intention other than inducing direct and immediate differentiation, since it can be employed as priming stimuli to boost *in vivo* mineralization responses. Pre-culture of rat STRO-1 selected DPSCs in osteo/odontogenic inducing medium prior to implantation also impacts further *in vivo* tissue development. By pre-culturing for 1-4 days in classical osteoinducing medium, with or without supplementation of BMP-2, increased initial ALP activity and gene expression profiles (OCN, BSP, DSPP, and Dentin matrix acidic phosphoprotein 1 - DMP-1) and *in vivo* ectopic bone formation are attained. Results with 8 days pre-culture show better results for the not supplemented (control) samples, however still lower than supplemented mediums with 4 days pre-culture [129].

The differentiation of DPSCs into mineralized tissue productive cell carries major changes in their gene and protein expression profiles. Several TGF- β related genes and VEGF show major up-regulation upon culture in differentiation medium. ALP activity also increases in the early stages of differentiation, but is reported to decrease upon confluence and terminal differentiation. Matrix Extracellular Phosphoglycoprotein (MEPE) expression showed sustained down-regulation, suggesting although it might have a role in the proliferative and early maturation stages it may pose an inhibitory role in later differentiation and mineralization of DPSCs [140].

Notch receptor mediated signaling pathways seem to play a relevant role in undifferentiated state maintenance by DPSCs. Notch receptors bind to specific ligands, both expressed at the cell surface, initiating a cascade leading to the induction of targeted genes. Over-expression of the Notch ligand Jagged-1 (by retroviral transfection) inhibited mineralization of DPSCs in an inducing medium,

both *in vitro* and *in vivo* (subcutaneously implanted in nude mice, on HA/TCP carriers). This effect is hypothesized to rely on the inhibition of Runx2-dependent gene transcription by the Notch signaling pathway, an essential step to induction osteoblastic/odontoblastic phenotypes [92].

Differentiating DPSCs suffer gene modulation, and the expression of JunB (a proto-oncogene, regulates bone remodeling and directs osteoblasts towards bone formation), IGFBP-5 (stimulates osteoblast proliferation and activity), and nuclear receptor related 1 protein (NURR1; a parathyroid hormone induced orphan nuclear receptor, transactivates OC and OPN expression) progressively and significantly increase during osteogenic differentiation [95].

There are no exclusive markers to odontogenic differentiation, due to the close similarities between dentin and bone, but DSPP is considered as an important factor in dentinogenesis, presenting only residual detection in bone [52, 91]. DMP-1 is also an odontogenic marker, but its expression is also encountered in bone tissue [91].

Association to Biomaterials for Mineralized Tissue Regeneration

Tissue engineering strategies' success is greatly influenced by the biomaterial design and composition. Scaffolds are three-dimensional structures that should allow cells to attach, grow and differentiate and after its initial function, should degrade into biocompatible metabolites. Also, scaffolds can be used as drug delivery system in the controlled release of bioactive molecules [6, 7, 141]. Intelligent scaffolds are designed to mimic the extracellular matrix (ECM) and the physiological environment of the stem cells niche and target host tissues. Stem cells can recognize specific materials or material properties and launch biochemical signs that will affect its regenerative fate. Hence, scaffold design regarding its composition, topography, porosity, interconnectivity, biocompatibility and biodegradability greatly influences its competence as a regenerative tool [4, 142]. Several biomaterial and dental tissue derived applications are herein described (Table 1).

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Bone Tissue Regeneration

Synthetic or natural polymers have been extensively used in tissue engineering through the years. Lactic acid and glycolic acid derived polymers are biomaterials with interest in the tissue engineering field due to controlled hydrolytic degradation, biocompatible metabolites and production of porous scaffolds may be achieved by several different methodologies [143]. Poly-lactic-glycolic acid (PLGA) 85:15 presenting topographic microncave features was demonstrated advantageous over convex HA coated titanium surfaces in terms of osteo-differentiation and mineralization of DPSCs [104]. Un-expanded CD34⁺ sorted cells (about 2x10⁴ cells per implant) were directly loaded onto PLGA membranes and implanted subcutaneously on immunosuppressed rats. Two bone formation steps were clear, at 30 and 60 days post-implantation. Firstly, fibrous bone was observed, that then remodeled into mature bone tissue by day 60, surrounded by a periosteal layer. The PLGA membrane was almost completely re-absorbed. This bone tissue was of determined of human origin, as was the extensive neo-angiogenesis observed. Control membranes with CD34⁻ cells showed no presence of bone nodules [40].

STRO-1 selected DPSCs population was seeded onto Polycaprolactone (PCL)/ Gelatin electrospun meshes, with or without nHA. Both situations resulted in *in vivo* ectopic new bone-like hard tissues with entrapped osteocyte-like cells and osteoblast-like cells lying orderly arranged at the surface of the implants, but enhanced gene expression was observed on the nano HA containing scaffolds [144].

Other studies evaluated the reaction of DPSC on three kinds of scaffolds materials: a spongeous collagen, a porous sintered HA/TCP, and a fibrous titanium mesh. They revealed that all three types of materials comparably supported the attachment, growth and differentiation of human DPSCs *in vitro*. The *in vivo* analysis illustrated that cell loading impacted on the amount of newly formed tissue and on its vascularization. However, on all the tested materials only little amounts of hard tissue formed following 12 weeks of *in vivo* implantation on immunocompromised mice [107].

For mineralized tissue engineering, hydroxyapatite based material are a recurrent choice. DPSCs seeded onto a highly porous natural hydroxyapatite scaffold (Biocoral®) are able to migrate and adhere inside the structure and differentiate and produce osseous matrix. The produced matrix completely integrated with the scaffold. Osteoblast-related gene expression (OC, OPN and BSP) was highly up-regulated in mineralized tissues and culture medium analyses also revealed enhanced bone- and angiogenic-related growth factor production (Bone morphogenetic protein 2 - BMP-2 and Vascular endothelial growth factor - VEGF) production by the differentiating cells [94]. APDCs seeded on similar structures and cultured in osteoinducing medium (with BMP-2) also displayed good cell adhesion and dissemination through the structure, and progressive increment in ALP activity. When implanted *in vivo* (subcutaneously in nude rat), bone-like tissue was patent after 3 weeks, containing osteocyte-like cells embedded within the calcified matrix, and osteoblast-like cells along the lining of the bone surface [52].

Ex vivo expanded-SHED seeded onto HA/TCP carriers were implanted in the same model and developed comparable amounts of mineralized tissue to BM-MSCs [145]. An identical formulation implanted in critical size calvarial defects (2.7 mm diameter) resulted in long term formation of mineralized tissue, and the newly formed tissue derived from SHEDs' effects on recruitment and induction of host cells, as well as from their own osteogenic differentiation and matrix production [103]. However, reports state that SHED-mediated bone lacked hematopoietic marrow elements, unlike BM-MSCs derived bone tissue [45, 103]. Conversely, rat DPSCs on HA/TCP porous structures are reported to give rise bone marrow-like hematopoietic tissue, within distinct concentric lamellae with entrapped osteocyte-like cells and osteoblast-like cells, after SC implantation on immunocompromised mice [146].

In a different approach, reconstitution of alveolar bone defects in New Zealand rabbits was achieved with nano-hydroxyapatite/collagen/poli-L-lactic (nHAC/PLA) acid scaffold and recombinant human BMP-2. The complex nHAC/PLA with BMP-2 showed support, proliferation and differentiation ability towards DPSC representing a viable alternative to autologous bone transplantation in the bone tissue regeneration [71].

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Autologous DTSCs have also successfully restored critical size mandibular defects in a swine model. Cells seeded on a β -TCP scaffold provided nearly complete regeneration of initial defect sites (83% of the defect), which were filled with newly formed lamellar bone and degraded β -TCP scaffold. Cell loaded scaffolds resulted in improved regeneration when compared to the scaffold alone and the untreated group, with moderate to minimal bone formation within the defect, which was mainly filled by abundant unmineralized connective tissue in the latter case. The seeded cells were shown to remain in the implant and differentiate, and actively contribute to bone formation [79].

Several other *in vitro* studies have demonstrated the osteogenic DPSC potential [10, 53, 101, 147, 148]. Some authors [149] have compared *in vitro* DPSC proliferation, differentiation and attachment to β -TCP, freeze-dried bone allograft (FDBA), and deproteinized bovine bone mineral (DBBM), as the most clinically used substitutes. β -TCP showed highest proliferation, while FDBA induced more significant osteoblastic differentiation.

Recent studies were performed in male *Macaca nemestrina* to evaluate the effectiveness of HA/TCP/chitosan scaffolds for osteogenic regeneration in mandibular defects. DPSC enriched scaffolds were transplanted into mandibular defects and active bone and blood vessel formation occurred [150].

ECM is largely comprised by fibrillary proteins that *in vivo* surround cells in a well-organized manner providing physical support and enabling cell adhesion and migration. Softer material like hydrogels and natural polymer derived mesh structures intent to mimic ECM characteristic have also been reported.

Peptide-amphiphilic can also be modified to enhance mineral deposition [151], ectopic bone formation [152] and to optimize cell adhesion [153]. The amphiphilic material self-assembles in aqueous environment into three-dimensional structures forming a gel matrix [154, 155]. Studies showed that cells are able to move, proliferate, differentiate and grow within the hydrogel matrix. Comparing the mineralization of SHEDs and DPSCs on self-assembling biodegradable peptide amphiphilic hydrogel, the first ones showed a tendency for soft tissue formation, while DPSCs developed more maturely mineralized tissues.

SHED displayed enhanced ECM molecules expression (Coll I and Col III), especially in β -glycerophosphate/ dexamethasone medium, with only limited mineralization observed in KH_2PO_4 / dexamethasone medium. On the other hand, DPSCs seemed more prone to osteogenic differentiation, with increased mineralization indicators in on potassium dihydrogen phosphate (KH_2PO_4)/ dexamethasone medium, suggested a further advanced lineage commitment. Results ensured the biocompatibility of the hydrogel and demonstrate cell proliferation, differentiation and subsequent mineralization with specific osteogenic supplementation [128].

The application of DTSCs loaded collagen membranes to calvarial defects in immunocompetent rats promoted enhanced defect filling and bone regeneration. At 30 days, the defects were completely filled with maturing bone, while in unloaded collagen membranes the tissue was more immature and had a higher granulation tissue component [43].

Collagen membranes were also loaded with autologous expanded DPSCs and applied clinically to patients undergoing bilateral third molar extractions. The treated sites showed accelerated radiographic improvements as demonstrated in Fig. (2). At 3 months post intervention, tissue samples were collected, revealing a complete re-sorption of the collagen sponges, well organized and well vascularized bone with a lamellar architecture surrounding the Haversian channels in the treated sites while control sites had a more immature bone (Fig. 2), with fibrous bone entrapped among new lamellae, incomplete and large Haversian channels and evidence of bone reabsorption.

Although all samples expressed bone proteins (ON, OC) a different distribution was noted, as well as increased expression of BMP-2 and VEGF in the DPSCs treated sites [82]. Collagen type I and II scaffolds they were able to support the differentiation of DPSCs into the osteogenic lineage *in vivo*. Histological and immunohistochemically results showed significant expression of ALP, BSP and OPN in transplants DPSCs in collagen scaffolds [156]. In recent work, a follow-up [157] was performed 3 years after the intervention. In these work they assessed the stability and quality of the regenerated bone and vessel network after the grafting intervention, with conventional procedures and recently and more

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advanced methodologies, such as in-line holotomography - an advanced phase-imaging method using synchrotron radiation that offers improved sensitivity toward low-absorbing structures. Results showed the regenerated sites were composed by compact bone with a higher matrix density than control human alveolar spongy bone. Although the bone regenerated at the graft sites is not of the proper type found in the mandible, it does seem to create steadier mandibles that may well increase implant stability, and, additionally, may improve resistance to mechanical, physical, chemical, and pharmacological agents.

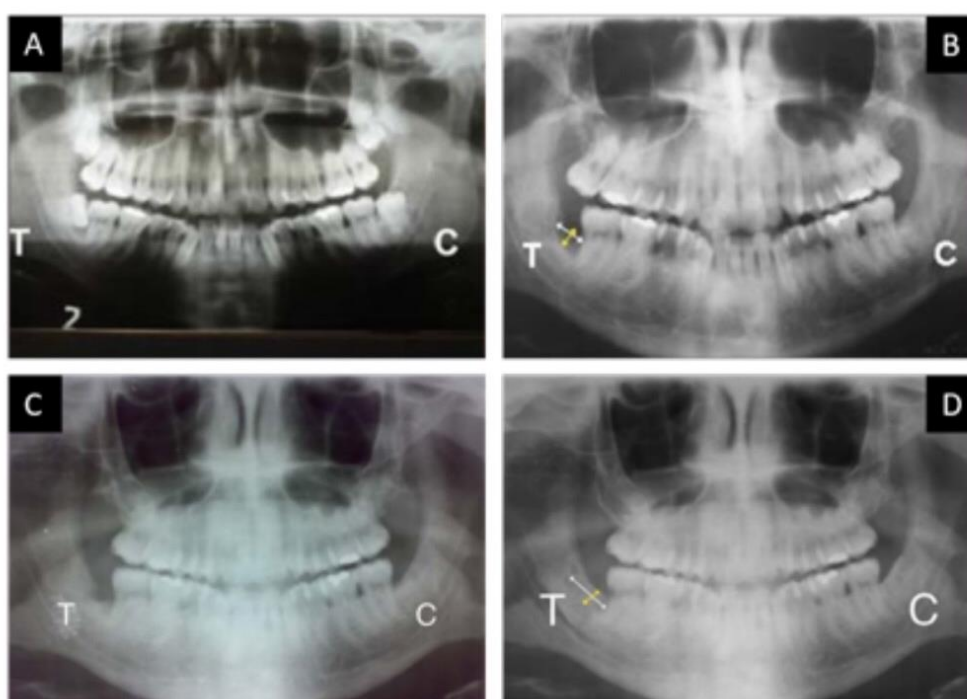


Fig. (2). Clinical application of a Collagen sponge and DPSCs in oro-maxillo-facial (OMF) bone tissue repair: (A) Pre operative X-ray. In this representative X-ray it is possible to see how the lower right third molar is in close contact with the second molar root; (B) X-ray control 7 days after surgical intervention. At the T site it is possible to observe the wide gap behind the distal roots of the right second molar. The yellow double-headed arrow evidences the vertical gap and the white double-headed arrow indicates the horizontal gap; (C) X-ray control 3 months after grafting; (D) X-ray control 1 year after graft, evidencing the optimal regeneration at the Test site. The yellow double-headed arrow indicates the gain in vertical bone height and the white double-headed arrow indicates the anteroposterior gain. (T: test site; C: control site). Adapted from [82].

Back to the immunocompromised mice model, silk fibroin scaffolds with osteogenically driven human DPSC successfully regenerated calvarial critical defects. At 30 days post implantation the treated defects were vascularized and filled with bone tissue. The internal and external tables were almost completely reconstructed and areas of spongy bone evidenced between them, intersperse with resorbing fibroin remnants. Implanted cells were still patent, in osteocyte and osteoblast typical location within the forming tissue, suggesting complete differentiation and a critical role for the effective bone regeneration [158].

Self-assembling nano-fiber hydrogels can also successfully support mineralized tissue formation. Subcutaneous implantation of those scaffolds resulted in vascularized mineral tissue formation, expressing both collagenous (Coll I) and non-collagenous (OPN, OC and osteonectin - ON) bone matrix proteins, depicting different stages of bone formation [89].

In similar approach, hyaluronic acid three dimensional scaffolds were used implanted into rat calvarial critical-size defects [159]. In these study they proved commitment differentiation to neuronal, glial, endothelial and osteogenic phenotypes, of DPSCS-scaffold complex *in vitro*. *In vivo* tests regarding DPSCs application in calvaria rat defects revealed potential as regenerative tissue engineering biocomplex. Other researchers have developed three-dimensional scaffolds composed PCL – hyaluronic acid – tricalcium phosphate for the transplantation of DPSCs to osteogenic repair [160]. Pig calvaria critical-size bone defects were implanted with these stem cells enriched scaffold and comparative results showed DPSCs exhibited a higher osteogenic potential compared with BM-MSCs both *in vitro* and *in vivo*.

Implants vascularization is a limiting factor for the success of many bone regeneration attempted strategies and complementary development of mineralized tissue and respective blood vessel supply is essential. Selected DPSCs populations (CD117⁺/ CD34⁺/flk-1⁺/ STRO-1⁺) give rise do two distinctive progenitor populations, in high serum osteoinducing medium. As cells differentiate, they lose their stem cell phenotype (CD117, CD34, CD90, CD133) and acquire osteoblastic (VEGFR-2⁺/ STRO-1⁻/ CD44⁺/ RUNX-2⁺, 70% of the population) and endothelial phenotypes (VEGFR-2⁺/ STRO-1⁻/ CD44⁺/ CD45⁺, 30% of the population). *In*

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in vitro spontaneously formed bone chips displayed blood vessel structures within the mineralized matrix and *in vivo* implantation of those resulted in mature vascularized bone. Both osteocytes and endotheliocytes were mainly of human origin, containing host blood cells [93].

In critical size bone defects, DPSCs granted healing capacity to an implanted collagen scaffold, presenting newly formed bone after up to 8 weeks. Highly vascularized areas were present in the still unmineralized areas of the implant. Cell-seeded groups presented a higher number of vessels and these were demonstrated to be composed of implanted DPSCs that differentiated toward endothelial identity [115].

These observations further support the potential of MSCs, and particularly DPSCs, for the regeneration of critical or non-healing bone fractures, commonly characterized by diminished vascularization and hostile microenvironment [115].

Studies performed in canine model with cBM-MSCs, cDPSCs and cDTSCs with PRP as scaffold was assessed as regenerative alternative in mandibular bone defects, demonstrating a positive effect of the MSCs populations on the overall mandibular bone and peri-implant bone formation and vascularization, comparing to empty defect controls [75, 76]. New bone formation was detectable in the presence of DPSCs after 4 weeks (active osteocytes) and after 8 weeks mature bone structures with lamellar tissue and abundant medullar cavity [75]. The comparison of the performance of DPSCs- and BM-MSCs-PRP demonstrated increased bone formation in treated defects. Controls presented fibrous tissue filling whilst BM-MSCs and DPSCs treated sites depicted trabecular and lamellar bone formation, respectively. All treated defects presented increased bone-implant contact, not differing significantly amongst each other [76, 161], although the DTSCs populations appear to present enhanced osteogenic potential [75].

DPSCs for Dental Tissue Regeneration

Original *in vivo* studies involving DPSCs didn't contemplate scaffolds or restraining biomaterial and focused on the regeneration of dentin-like tissue [20]. When administered, DPSCs formed dentin-like structure with odontoblasts-like cells surrounded by pulp-like tissue.

Since then, DPSCs have been employed in the regeneration of tooth root defects with promising results [162]. However, it is in dentin-pulp tissue regeneration that DPSCs, SHEDs and SCAPs have demonstrated their regenerative potential. In a study using human tooth root fragments, DPSCs were transplanted into empty root canal space filled with a poly-D,L-lactide/glycolide. Regeneration of vascularized dentin-pulp-like tissue was achieved [163]. Also, DPSCs generated pulp-like structures lined with odontoblast-like cells when transplanted into immunocompromised mice [106]. Puramatrix™ is a commercial synthetic peptide based hydrogel with numerous applications in tissue engineering and has been demonstrated has a potential scaffold for DPSCs and results have been promising regarding its application on dental tissue regeneration [164 - 170].

Xenogenic transplantation of SHEDs with HA/TCP scaffolds generated dentin-pulp-like tissues with distinct odontoblast layers lining the mineralized dentin-matrix [171]. SHEDs prepared from human tooth slices have demonstrated differentiation ability towards odontoblast-like cells on the dentin surface, representing a viable source of cells for dental pulp tissue engineering [172]. Also, SHEDs seeded onto polylactic acid scaffolds with the addition of BMP-2 and TGF- β 1 generated pulp tissue [173].

In another field of dental tissue regeneration there is periodontal therapy. Due to their periodontal ligament derivation and their capacity to differentiate into osteoblasts, cementoblasts, and fibroblasts, PDLSCs may be the first and fittest candidate cellular source for PDL regeneration [174, 175].

Currently, several clinical trials regarding DPSCs application in dental tissue regeneration are ongoing or were recently completed, as described in Table 3. Some envisage the application of allogeneic human dental pulp stem cell as treatment for chronic periodontal disease (NCT02523651). Other researchers' focus on the autotransplantation method, and the evaluation of its efficacy (NCT02464202 and NCT02842515). Some clinical trials (NCT00595595 and NCT01814436) also specifically focused on young immature teeth revitalizing resourcing to regenerative medicine and DPSCs.

In ongoing clinical trial researchers are studying *osseous* integration using Allogenic

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Mesenchymal Stem cells while placing dental implants (NCT02731586).

Table 3. Clinical application of dental pulp derived stem cells.

Study Number	Application	Dental cell Population	Country	Start Date	Estimated Enrollment	Status
NCT01814436	Revitalization of necrotic pulp	Autologous SHEDs	China	February 2013	80	Recruiting
NCT02464202	Dental Pulp Regeneration	Autologous PDLSCs	Belgium	May 2013	100	Recruiting
NCT01932164	Bone Regeneration in Cleft Lip and Palate	Autologous SHEDs	Spain	May 2013	5	Unknown
NCT02523651	Chronic Peridontal Disease	Allogenic DPSCs	China	December 2014	40	Recruiting
NCT02731586	Dental Implantation	Allogenic DPSCs	India	January 2016	10	Recruiting
NCT02728115	Huntington's Disease	Allogenic iDPSCs	Brazil	July 2016	6	Waiting
NCT00595595	Dental Pulp Regeneration	Allogenic DPSCs	USA	February 2007	1080	Completed
NCT02842515	Dental Pulp Regeneration	Autologous DPSCs	France	January 2015	30	Completed

Other Tissue Regeneration Applications

Several authors have reported proangiogenic and antiapoptotic effects and promotion of new lymphatic vessels of DPSCs [74, 176, 177]. Endothelial differentiation of DPSC is thought to be mediated by VEGF-A and other angiogenic factors secreted by these cells [176, 177]. Hence, it is speculated that DPSCs can potentially be significant in lymphatic regeneration either through direct differentiation into lymphogenic lineage or through the production of lymphogenic cytokines [178]. Therefore, researchers have investigated, therapeutic potential in the repair of myocardial infarction (MI) by administration of DPSCs intra-myocardially in nude rats induced with myocardial infarction by coronary artery ligation. After four weeks, animals showed an improvement in cardiac function, reduction of left ventricular wall thickness, in parallel with a reduction in infarct size.

Iohara' research team, 2009 [74] used Endothelial Progenitor cells (EPCs) markers – AC133, CD34, VGFR2, CXCR4 and c-kit and CD31⁺, CD146⁺ - from porcine dental pulp tissue. Also, they successfully isolated EPCs cells from canine primary pulp tissue and transplanted them with collagen type I and II scaffolds into partial removed canine dental pulp model. They were able to demonstrate for the first time that dental pulp cells population CD31⁺/CD146⁺ have high vasculogenic potential and induce angiogenesis and therefore have great potential in orthodontics and dentistry but also in other tissue engineering fields.

Granulocyte-colony stimulating factor (G-CSF) induces dental pulp stem cells to form mobilized dental pulp stem cells (MDPSCs) [179]. This subset of DPSCs are stem cells with high level of trophic factors production that ensure high proliferation, migration and antiapoptotic effects. Further, preclinical autologous transplants in dogs assured safety, efficacy and reproducibility of MDPSCs [179].

In a different approach [180] DPSCs have demonstrated beneficial effects in skin regeneration, with effects attributed to the anigiogenic regeneration ability.

Muscle regeneration can also be modulated by DPSCs. Co-culture with myoblast and direct injection into injured muscles resulted in effective fusion of the stem cells to resident myotubes, leading to the expression of the human MyoD muscle-specific transcription factor by murine muscle cells. This suggests an activation of dormant, but already existing, genes in the DPSCs by environmental factors [21].

Interestingly, the transplantation of tissue-engineered DTSCs sheets was helpful for the reconstruction of corneal epithelium in the laprine animal model of total limbal stem cell deficiency (LSCD) [181].

As mentioned before, benefits of stem cells transplantation might be related to their secretion abilities. The secretion of trophic and immunomodulatory cytokines, designed secretome is hypothesized to have regenerative potential in neural trauma or degeneration [182]. Further investigation must be made to better understand the importance and the regenerative potential by itself of these molecules in tissue engineering.

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One of the most recent clinical trials (NCT02728115) comprehends the application of Cellavita HD – a DPSCs based therapy - in the treatment of Huntington's disease. This is a first-in-human, dose escalation study in which participants with Huntington's disease will receive 3 intravenous injections and will be followed for 5 years to evaluate safety and preliminary evidence of effectiveness.

Researchers have also focused their attention on DPSCs applied to nerve regeneration. Successful neuronal differentiation has been reported, based on morphological, phenotypical and genotypical as well as electrophysiological evidences [41] and DPSCs have been demonstrated to act *in vivo* as central axonal migration modulators via secretion of growth factors [183].

Sasaki et al. [184 - 186] developed PLGA nerve conduits containing DPSCs embedded in collagen hydrogel for facial nerve regeneration in rat model. As expected PLGA tubes were resorbed and after 2 months of implantation, no mineralization signs were detected and tubes contained myelinated fibers and nerves were effectively re-connected in the presence of DPSCs. Also, DPSCs were used in autologous transplantation in pigs envisaging peripheral nerve recovery [187]. Other authors [188] have used three-dimensional chitosan scaffolds for the attachment, growth and neural differentiation of DPSCs with success.

Alternative strategies regarding myelin injuries repair in the Peripheral nervous system are also employing DPSCs neurogenic differentiation capacity. *In vivo* studies with local demyelination induced in the mouse sciatic nerve by lysolecithin was treated with human OLIG2 gene transfected DPSCs. OLIG2 expression was able to induce oligodendrocyte differentiation in DPSCs *in vivo* [189]. In another approach, PLA nerve conduits were employed to concomitant transplantation of a Schwann cells (SC) adult stem cells and DPSC. Results revealed that nerve conduction velocity and new blood vessels in SCs and DPSCs treated animals was better than with DPSC alone [190]. Mobilized dental pulp stem cells (MDPSCs) induced by granulocyte-colony stimulating factor (G-CSF) gradient demonstrated neurotrophic/angiogenic potential in peripheral nerve regeneration. Further *in vitro* and *in vivo* assays demonstrated MDPSCs axon

regeneration through trophic functions, acting on Schwann cells, and promoting angiogenesis [191].

Other Neural Damage Models have also been Explored

Retinal ganglion cells and their axon may also suffer damage and benefit from the transplantation of DPSCs. In a recent study in adult rats [192] [193] intravitreal optic nerve transection was treated with DPSCs, bone marrow-derived mesenchymal stem cells (BM-MSC) and adipose-derived stem cells (AMSC). In conclusion they reported that DPSCs in particular showed better neuroprotective/axonogenic results.

Another approach to nerve regeneration efficacy of DPSCs, cells transplanted into diabetic rats were able to modulate M1/M2 macrophages in diabetic peripheral nerves, demonstrating anti-inflammatory ability. The anti-inflammatory effect may be modulated by the extensive immunomodulatory cytokines known to be produced by DPSCs. These promising results in diabetic patients' makes authors suggest cryopreserved DPSCs - collected from young patients before onset of diabetes - for the treatment of diabetes polyneuropathy [194]. In fact, in more recent work [195], they demonstrated that cryopreservation of DPSCs didn't affect effectiveness of the treatment in diabetes neuropathy.

In a very interesting study, human DPSCs were transplanted intracerebral into focal cerebral ischemia rodent model [196]. Although minimal cell survival 4 weeks after stroke was observed it also visible cells migrated towards stroke lesion. DPSCs derived into astrocytes, known to be important in restoration of neurological function. Also, as described by others [176, 177] it seemed angiogenic effect was achieved by the secretion of VEGF and other angiogenic factors. In a similar approach researchers [65] have assessed neurogenic potential of autologous murine model transplantation with DPSC. Neuronal-like cells were successfully generated although in an immature stage of development.

Recent work suggest the application of DPSC in the treatment of neurodegenerative diseases like Alzheimer's disease and temporal lobe epilepsy [197] Table 4.

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Application	Model	Cells Origin	Biomaterial/Scaffold	Regenerative factor(s)	Refs.
Mineralized tissue regeneration - BONE	Human	Impacted 3 rd molars	Spongy collagen, a porous ceramic, and a fibrous titanium mesh	-	[107]
		Mandible 3 rd molars	Collagen	-	[82, 100]
	Human	Mandible 3 rd molars	Collagen	-	[157]
		Non-specified	Collagen	-	[115]
	Human	3 rd molars	Hyaluronic acid	-	[159]
		Deciduous & permanent teeth	Granular deproteinized bovine bone (GDPB) & β -tricalcium phosphate (β -TCP)	-	[198]
		Molars	Hyaluronic acid & Hydroxyapatite/tri-calcium-phosphate	-	[199]
		3 rd molars	Hyaluronic acid	-	[159]
		Deciduous & permanent teeth	Granular deproteinized bovine bone (GDPB) & β -tricalcium phosphate (β -TCP)	-	[198]
	Swine Rabbit Canine	Molars	Hyaluronic acid & Hydroxyapatite/tri-calcium-phosphate	-	[199]
		Non-specified	Collagen	-	[115]
		Deciduous teeth	β -Tricalcium Phosphate	-	[79]
		Non specified	Nano- hydroxyapatite/collagen/poli-L-lactic acid (nHAC/PLA)	rhBMP-2	[71]
		Molars & deciduous teeth	Platelets enriched plasma	Platelet-derived—growth—factor (PDGF), Transforming—growth factor—beta-(TGF- β) and Insulin-like-growth-factor-1 (IGF-1)	[75, 76]
Osteogenic regeneration in pigs calvaria	<i>Macaca nemestrina</i>	Molars	Platelets enriched plasma	-	[161]
		Upper and lower first incisive	Hydroxyapatite/tricalcium-phosphate/chitosan (HA/TCP/Ch)	-	[150]
	Swine	Pre-molars	Polycaprolactone (PCL)- hyaluronic acid- tricalcium phosphate (HT-PCL)	-	[160, 200]
Osteogenesis	Rat	Upper and lower incisors	Collagen type I & II	-	[156]

contd.....		Application	Model	Cells Origin	Biomaterial/Scaffold	Regenerative factor(s)	Refs.
<i>Mineralized tissue regeneration - DENTAL</i>	Dental pulp repair	Human	3 rd molars	Alginate Hydrogel		Transforming growth factor (TGF)- β 1	[142]
	dental regeneration	Human	3 rd molars	Collagen		Bone morphogenetic proteins (BMP)	[124]
			Exfoliated deciduous teeth & 3 rd molars	PLA		-	[172, 200]
			impacted 3 rd molar	PLGA scaffold inserted in canal space of human root fragment		-	[163]
			3 rd molars & exfoliated deciduous teeth	Peptide-Amphiphilic		Cell adhesion sequence RGD and an enzyme-cleavable site	[128, 154]
			3 rd molars	Sylgard 184 silicone elastomer & canal of the tooth root segment		-	[201]
			Non specified	Puramatrix™		-	[202]
			3 rd molars	Nano-hydroxyapatite (nHA) & silk		-	[203]
			3 rd molars	Chitosan hydrogel		-	[204]
		Human & Bovine	Human 3 rd molars & bovine incisor roots	Bovine roots & horizontally cut human root canal discs		-	[205 - 207]
		Canine	Non specified	Collagen		Mobilized dental pulp stem cells (MDPSCs) induced by Granulocyte-colony stimulating factor (G-CSF)	[179]

contd.....		Application	Model	Cells Origin	Biomaterial/Scaffold	Regenerative factor(s)	Refs.
<i>Other tissue regeneration applications</i>	Post-stroke functional recovery	Human	3 rd molars	-	-	-	[196]
		Rat	Incisors	-	-	-	[65]
		Swine	Deciduous teeth	-	-	-	[208]
	Angiogenic in mouse model	Human	Non specified	-	-	-	[209]
		Mouse	Exfoliated deciduous teeth	-	-	-	[180]
		Human	3 rd molars	Collagen conduits	-	-	[191]
	Nerve regeneration		3 rd molars	Chitosan	-	-	[188]
			3 rd molars	-	-	DPSCs transfected with human OLIG2 gene	[189]
		Rat	Incisors	PLA nerve conduits	-	-	[190, 210]
			Incisors	-	-	-	[194, 195]
			Incisors	PLGA nerve conduits and collagen hydrogel	-	-	[185, 186]
		Swine	Incisors	Collagen type I membrane	-	-	[187]

FINAL REMARKS

The dental tissue is an important tissue source to obtain MSCs to be used in multiple trialed and potential human and veterinary clinical applications, since these cells have been isolated in several different mammal species. These cell-based therapies can be associated with several natural and synthetic biomaterials, and the scaffolds can be used to promote bone regeneration, including critical bone defects, and can also be used in other tissues and organs regeneration, like peripheral nerve regeneration. The MSC populations can be isolated from different anatomical regions of healthy teeth, from young and adult individuals (from deciduous and definitive dentition) and can be considered as interesting alternatives to bone marrow, adipose tissue and umbilical cord matrix as sources of MSCs, that are as or more easily isolated, *in vitro* expanded and cryopreserved for future clinical application.

Nonetheless, several challenges remain for stem cells based therapies regarding its clinical application. As expected, there are safety issues associated with the clinical application of *in vitro* expanded cells. Xenogenic components of cell culturing medium must be optimized to not include components of animal origin. Also, purification of stem cells must be considered to overcome the heterogeneity of the cell culture, through well-established cellular markers. Most of these aspects have already been the focus of stem cell researchers and progresses have been made regarding some of these aspects.

For the successful clinical application of these stem cell based therapies, specific protocols regarding selection of ideal cellular source and *in vivo* intervention protocols must be developed and established. Due to innate immune response, in clinical application, stem cell therapies should always be elected from autologous sources. However, availability of biological substrate from debilitated patients – for example restenosis and cancer patients - is sometimes limited. In these cases, a combination of adjuvants (bioactive molecules, such as growth factors) and autologous sources or allogenic stem cells are the answer. The establishment of allogenic stem cells bank with all possible immunohistocompatibility sources available to clinicians entails a fascinating future that may not be so unattainable. Moreover, investing in research and translation to clinical application and also,

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the overcome of legal aspects regarding these advanced therapies crucial.

LIST OF ABBREVIATIONS

1α,25(OH)(2)D(3)	1 α ,25-Dihydroxyvitamin D3
ALP	Alkaline phosphatase
ALPL	Alkaline Phosphatase, Liver/Bone/Kidney
AMSC	Adipose-derived stem cells
APDCs/SCAPs	Stem cells from human permanent teeth apical dental pulp
BM-MSCs	Bone marrow derived macrophages
BMP-2	Bone morphogenetic protein 2
BSP	Bone sialoprotein
Ca²⁺	Calcium ion
CD	Cluster of differentiation
CFU	Colony Forming Units
COL1A1	Collagen Type I Alpha 1 Chain
COL3A1	Collagen Type III Alpha 1 Chain
Coll	Collagen type
Coll I	Collagen Type I
CXCR	Chemokine receptor
DBBM	Deproteinized bovine bone mineral
Dex	Dexamethasone
DF-MSCs	Stem cells from human dental follicle
DMP-1	Dentin matrix acidic phosphoprotein 1
DMSO	Dimethyl Sulfoxide
DPSCs	Dental Pulp Stem cells
DSPP	Dentin sialophosphoprotein
DTSCs	Deciduous teeth stem cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDBA	Freeze-dried bone allograft
FOSL1	FOS Like 1
G-CSF	Granulocyte-colony stimulating factor
GDPB	Granular deproteinized bovine bone

HA	Hydroxyapatite
HA/TCP/Ch	Hydroxyapatite/tricalcium-phosphate/chitosan
hDPSC	Human Dental Pulp Stem cells
hMSC	Human Mesenchymal Stem cells
HT-PCL	Hyaluronic acid- tricalcium phosphate - Polycaprolactone
IGF	Insulin-like growth factor
IGFBP-7	Insulin-like growth factor-binding protein 7
IL	Interleukin
ITS	Insulin-transferrin-sodium selenite
KH₂PO₄	Potassium dihydrogen phosphate
LSCD	Limbal stem cell deficiency
MDPSCs	Mobilized dental pulp stem cells
MEPE	Matrix Extracellular Phosphoglycoprotein
MI	Myocardial infarction
MSCs	Mesenchymal Stem Cells
nHA	nano Hydroxyapatite
nHAC/PLA	nano-Hydroxiapatite/collagen/poli-L-lactic acid
NURR1	Nuclear receptor related 1 protein
O₂	Oxygen
OCN	Osteocalcin
OLIG2	Oligodendrocyte transcription factor
ON	Osteonectin
OPN	Osteopontin
OSX	Osterix
PCL	Polycaprolactone
PDGF	Platelet derived growth factor
PDL	Periodontal ligament
PDLSCs	Stem cells from human Periodontal Ligament
PLA	Poly Lactic acid
PLGA	Poly-lactic-glycolic acid
PRP	Platelet rich plasma
RA	Retinoic acid
RGD	Arginylglycylaspartic acid
rhBMP-2	Recombinant human Bone morphogenetic protein 2
RNA	Ribonucleic acid

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RUNX2	Runt Related Transcription Factor 2
SC	Schwann cells
SHEDs	Stem cells from human exfoliated deciduous teeth
SMF	Static magnetic field
SPP1	Secreted Phosphoprotein 1
STRO-1	Stromal Cell Precursor Surface Antigen
TCP	Tricalcium phosphate
TGF-β	Transforming growth factor beta
UCB	Human umbilical cord blood
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VGFR	VEGF receptor
α-SMA	Alfa smooth muscle actin

CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest to declare for this publication.

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REFERENCES

- [1] Vacanti JP, Morse MA, Saltzman WM, Domb AJ, Perez-Atayde A, Langer R. Selective cell transplantation using bioabsorbable artificial polymers as matrices. *J Pediatr Surg* 1988; 23(1 Pt 2): 3-9.
[http://dx.doi.org/10.1016/S0022-3468(88)80529-3] [PMID: 2895175]
- [2] Langer R, Vacanti J. Advances in tissue engineering. *J Pediatr Surg* 2016; 51(1): 8-12.
[http://dx.doi.org/10.1016/j.jpedsurg.2015.10.022] [PMID: 26711689]
- [3] Janjić K, Cvikić B, Moritz A, Agis H. Dental pulp regeneration. *Int J Stomatol Occlusion Med* 2016; 8(1): 1-9.
[http://dx.doi.org/10.1007/s12548-015-0139-1]
- [4] Muschler GF, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. *J Bone Joint Surg Am* 2004; 86-A(7): 1541-58.
[http://dx.doi.org/10.2106/00004623-200407000-00029] [PMID: 15252108]
- [5] Graziano A, d'Aquino R, Laino G, Papaccio G. Dental pulp stem cells: a promising tool for bone regeneration. *Stem Cell Rev* 2008; 4(1): 21-6.
[http://dx.doi.org/10.1007/s12015-008-9015-3] [PMID: 18300003]
- [6] Demarco FF, Conde MC, Cavalcanti BN, Casagrande L, Sakai VT, Nör JE. Dental pulp tissue engineering. *Braz Dent J* 2011; 22(1): 3-13.
[PMID: 21519641]
- [7] Potdar PD, Jethmalani YD. Human dental pulp stem cells: Applications in future regenerative medicine. *World J Stem Cells* 2015; 7(5): 839-51.
[http://dx.doi.org/10.4252/wjsc.v7.i5.839] [PMID: 26131314]
- [8] Alge DL, Zhou D, Adams LL, *et al.* Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. *J Tissue Eng Regen Med* 2010; 4(1): 73-81.
[PMID: 19842108]
- [9] Caseiro AR, Pereira T, Bártolo PJ, Santos JD, Luís AL, Mauricio AC. Mesenchymal Stem Cells and Biomaterials Systems – Perspectives for Skeletal Muscle Tissue Repair and Regeneration. *Procedia Eng* 2015; 110: 90-7.
[http://dx.doi.org/10.1016/j.proeng.2015.07.014]
- [10] Gronthos S, Brahimi J, Li W, *et al.* Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002; 81(8): 531-5.
[http://dx.doi.org/10.1177/154405910208100806] [PMID: 12147742]
- [11] Isobe Y, Koyama N, Nakao K, *et al.* Comparison of human mesenchymal stem cells derived from bone marrow, synovial fluid, adult dental pulp, and exfoliated deciduous tooth pulp. *Int J Oral Maxillofac Surg* 2016; 45(1): 124-31.
[http://dx.doi.org/10.1016/j.ijom.2015.06.022] [PMID: 26235629]
- [12] Gioventù S, Andriolo G, Bonino F, *et al.* A novel method for banking dental pulp stem cells. *Transfus Apheresis Sci* 2012; 47(2): 199-206.
[http://dx.doi.org/10.1016/j.transci.2012.06.005] [PMID: 22795998]

The Potential Clinical Application Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4 51

- [13] Nanci A. Ten cate's oral histology-pageburst on vitalsource: development, structure, and function. Elsevier Health Sciences 2007.
- [14] Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* 2003; 18(4): 696-704.
[http://dx.doi.org/10.1359/jbmr.2003.18.4.696] [PMID: 12674330]
- [15] Kerkis I, Caplan AI. Stem cells in dental pulp of deciduous teeth. *Tissue Eng Part B Rev* 2012; 18(2): 129-38.
[http://dx.doi.org/10.1089/ten.teb.2011.0327] [PMID: 22032258]
- [16] Govindasamy V, Abdullah AN, Ronald VS, *et al.* Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. *J Endod* 2010; 36(9): 1504-15.
[http://dx.doi.org/10.1016/j.joen.2010.05.006] [PMID: 20728718]
- [17] Ma D, Ma Z, Zhang X, *et al.* Effect of age and extrinsic microenvironment on the proliferation and osteogenic differentiation of rat dental pulp stem cells in vitro. *J Endod* 2009; 35(11): 1546-53.
[http://dx.doi.org/10.1016/j.joen.2009.07.016] [PMID: 19840645]
- [18] Tomic S, Djokic J, Vasilijic S, *et al.* Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists. *Stem Cells Dev* 2011; 20(4): 695-708.
[http://dx.doi.org/10.1089/scd.2010.0145] [PMID: 20731536]
- [19] Laino G, Graziano A, dAquino R, *et al.* An approachable human adult stem cell source for hard-tissue engineering. *J Cell Physiol* 2006; 206(3): 693-701.
[http://dx.doi.org/10.1002/jcp.20526] [PMID: 16222704]
- [20] Gronthos S, Mankani M, Brahimi J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci USA* 2000; 97(25): 13625-30.
[http://dx.doi.org/10.1073/pnas.240309797] [PMID: 11087820]
- [21] Spath L, Rotilio V, Alessandrini M, *et al.* Explant-derived human dental pulp stem cells enhance differentiation and proliferation potentials. *J Cell Mol Med* 2010; 14(6B): 1635-44.
[http://dx.doi.org/10.1111/j.1582-4934.2009.00848.x] [PMID: 19602052]
- [22] Suchánek J, Soukup T, Ivancaková R, *et al.* Human dental pulp stem cells isolation and long term cultivation. *Acta Med (Hradec Kralove)* 2007; 50(3): 195-201.
[PMID: 18254273]
- [23] Karbanová J, Soukup T, Suchánek J, Pytlík R, Corbeil D, Mokry J. Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium. *Cells Tissues Organs (Print)* 2011; 193(6): 344-65.
[http://dx.doi.org/10.1159/000321160] [PMID: 21071916]
- [24] Perry BC, Zhou D, Wu X, *et al.* Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. *Tissue Eng Part C Methods* 2008; 14(2): 149-56.
[http://dx.doi.org/10.1089/ten.tec.2008.0031] [PMID: 18489245]
- [25] Huang AH, Chen YK, Lin LM, Shieh TY, Chan AW. Isolation and characterization of dental pulp stem cells from a supernumerary tooth. *J Oral Pathol Med* 2008; 37(9): 571-4.

- [http://dx.doi.org/10.1111/j.1600-0714.2008.00654.x] [PMID: 18331285]
- [26] Huang AH, Chen YK, Chan AW, Shieh TY, Lin LM. Isolation and characterization of human dental pulp stem/stromal cells from nonextracted crown-fractured teeth requiring root canal therapy. *J Endod* 2009; 35(5): 673-81.
[http://dx.doi.org/10.1016/j.joen.2009.01.019] [PMID: 19410081]
- [27] Alongi DJ, Yamaza T, Song Y, *et al.* Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. *Regen Med* 2010; 5(4): 617-31.
[http://dx.doi.org/10.2217/rme.10.30] [PMID: 20465527]
- [28] Waddington RJ, Youde SJ, Lee CP, Sloan AJ. Isolation of distinct progenitor stem cell populations from dental pulp. *Cells Tissues Organs* (Print) 2009; 189(1-4): 268-74.
[http://dx.doi.org/10.1159/000151447] [PMID: 18701814]
- [29] Bakopoulou A, Leyhausen G, Volk J, *et al.* Comparative analysis of in vitro osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP). *Arch Oral Biol* 2011; 56(7): 709-21.
[http://dx.doi.org/10.1016/j.archoralbio.2010.12.008] [PMID: 21227403]
- [30] Yu J, He H, Tang C, *et al.* Differentiation potential of STRO-1+ dental pulp stem cells changes during cell passaging. *BMC Cell Biol* 2010; 11: 32.
[http://dx.doi.org/10.1186/1471-2121-11-32] [PMID: 20459680]
- [31] Shi S, Robey PG, Gronthos S. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. *Bone* 2001; 29(6): 532-9.
[http://dx.doi.org/10.1016/S8756-3282(01)00612-3] [PMID: 11728923]
- [32] Wada N, Menicanin D, Shi S, Bartold PM, Gronthos S. Immunomodulatory properties of human periodontal ligament stem cells. *J Cell Physiol* 2009; 219(3): 667-76.
[http://dx.doi.org/10.1002/jcp.21710] [PMID: 19160415]
- [33] Struys T, Moreels M, Martens W, Donders R, Wolfs E, Lambrechts I. Ultrastructural and immunocytochemical analysis of multilineage differentiated human dental pulp- and umbilical cord-derived mesenchymal stem cells. *Cells Tissues Organs* (Print) 2011; 193(6): 366-78.
[http://dx.doi.org/10.1159/000321400] [PMID: 21124001]
- [34] Lee SY, Chiang PC, Tsai YH, *et al.* Effects of cryopreservation of intact teeth on the isolated dental pulp stem cells. *J Endod* 2010; 36(8): 1336-40.
[http://dx.doi.org/10.1016/j.joen.2010.04.015] [PMID: 20647092]
- [35] Wei X, Ling J, Wu L, Liu L, Xiao Y. Expression of mineralization markers in dental pulp cells. *J Endod* 2007; 33(6): 703-8.
[http://dx.doi.org/10.1016/j.joen.2007.02.009] [PMID: 17509410]
- [36] Atari M, Barajas M, Hernández-Alfaro F, *et al.* Isolation of pluripotent stem cells from human third molar dental pulp. *Histol Histopathol* 2011; 26(8): 1057-70.
[PMID: 21692038]
- [37] Dominici M, Le Blanc K, Mueller I, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; 8(4): 315-7.

The Potential Clinical Application Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4 53

- [http://dx.doi.org/10.1080/14653240600855905] [PMID: 16923606]
- [38] Lee JY, Nam H, Park YJ, *et al.* The effects of platelet-rich plasma derived from human umbilical cord blood on the osteogenic differentiation of human dental stem cells. *In Vitro Cell Dev Biol Anim* 2011; 47(2): 157-64.
[http://dx.doi.org/10.1007/s11626-010-9364-5] [PMID: 21082281]
- [39] Papaccio G, Graziano A, d'Aquino R, *et al.* Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. *J Cell Physiol* 2006; 208(2): 319-25.
[http://dx.doi.org/10.1002/jcp.20667] [PMID: 16622855]
- [40] Graziano A, d'Aquino R, Laino G, *et al.* Human CD34+ stem cells produce bone nodules in vivo. *Cell Prolif* 2008; 41(1): 1-11.
[http://dx.doi.org/10.1111/j.1365-2184.2007.00497.x] [PMID: 18211282]
- [41] Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. *Stem Cells* 2008; 26(7): 1787-95.
[http://dx.doi.org/10.1634/stemcells.2007-0979] [PMID: 18499892]
- [42] Miura M, Gronthos S, Zhao M, *et al.* SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* 2003; 100(10): 5807-12.
[http://dx.doi.org/10.1073/pnas.0937635100] [PMID: 12716973]
- [43] de Mendonça Costa A, Bueno DF, Martins MT, *et al.* Reconstruction of large cranial defects in nonimmunosuppressed experimental design with human dental pulp stem cells. *J Craniofac Surg* 2008; 19(1): 204-10.
[http://dx.doi.org/10.1097/scs.0b013e31815c8a54] [PMID: 18216690]
- [44] Nakamura S, Yamada Y, Katagiri W, Sugito T, Ito K, Ueda M. Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene expression profile from promising dental pulp. *J Endod* 2009; 35(11): 1536-42.
[http://dx.doi.org/10.1016/j.joen.2009.07.024] [PMID: 19840643]
- [45] Wang X, Sha XJ, Li GH, *et al.* Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells. *Arch Oral Biol* 2012; 57(9): 1231-40.
[http://dx.doi.org/10.1016/j.archoralbio.2012.02.014] [PMID: 22455989]
- [46] Bakopoulou A, Leyhausen G, Volk J, *et al.* Assessment of the impact of two different isolation methods on the osteo/odontogenic differentiation potential of human dental stem cells derived from deciduous teeth. *Calcif Tissue Int* 2011; 88(2): 130-41.
[http://dx.doi.org/10.1007/s00223-010-9438-0] [PMID: 21153807]
- [47] Karaöz E, Doğan BN, Aksoy A, *et al.* Isolation and in vitro characterisation of dental pulp stem cells from natal teeth. *Histochem Cell Biol* 2010; 133(1): 95-112.
[http://dx.doi.org/10.1007/s00418-009-0646-5] [PMID: 19816704]
- [48] Ferro F, Spelat R, Beltrami AP, Cesselli D, Curcio F. Isolation and characterization of human dental pulp derived stem cells by using media containing low human serum percentage as clinical grade substitutes for bovine serum. *PLoS One* 2012; 7(11): e48945.
[http://dx.doi.org/10.1371/journal.pone.0048945] [PMID: 23155430]

- [49] Koyama N, Okubo Y, Nakao K, Bessho K. Evaluation of pluripotency in human dental pulp cells. *J Oral Maxillofac Surg* 2009; 67(3): 501-6.
[http://dx.doi.org/10.1016/j.joms.2008.09.011] [PMID: 19231772]
- [50] Sonoyama W, Liu Y, Yamaza T, *et al.* Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod* 2008; 34(2): 166-71.
[http://dx.doi.org/10.1016/j.joen.2007.11.021] [PMID: 18215674]
- [51] Jo YY, Lee HJ, Kook SY, *et al.* Isolation and characterization of postnatal stem cells from human dental tissues. *Tissue Eng* 2007; 13(4): 767-73.
[http://dx.doi.org/10.1089/ten.2006.0192] [PMID: 17432951]
- [52] Abe S, Yamaguchi S, Amagasa T. Multilineage cells from apical pulp of human tooth with immature apex. *Oral Science Int* 2007; 4(1): 45-58.
[http://dx.doi.org/10.1016/S1348-8643(07)80011-5]
- [53] Morsezeck C, Götz W, Schierholz J, *et al.* Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biol* 2005; 24(2): 155-65.
[http://dx.doi.org/10.1016/j.matbio.2004.12.004] [PMID: 15890265]
- [54] Seo B-M, Miura M, Gronthos S, *et al.* Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004; 364(9429): 149-55.
[http://dx.doi.org/10.1016/S0140-6736(04)16627-0] [PMID: 15246727]
- [55] Liu L, Ling J, Wei X, Wu L, Xiao Y. Stem cell regulatory gene expression in human adult dental pulp and periodontal ligament cells undergoing odontogenic/osteogenic differentiation. *J Endod* 2009; 35(10): 1368-76.
[http://dx.doi.org/10.1016/j.joen.2009.07.005] [PMID: 19801232]
- [56] Dissanayaka WL, Zhu X, Zhang C, Jin L. Characterization of dental pulp stem cells isolated from canine premolars. *J Endod* 2011; 37(8): 1074-80.
[http://dx.doi.org/10.1016/j.joen.2011.04.004] [PMID: 21763897]
- [57] Struillou X, Boutigny H, Soueidan A, Layrolle P. Experimental animal models in periodontology: a review. *Open Dent J* 2010; 4(1): 37-47.
[http://dx.doi.org/10.2174/1874210601004010037] [PMID: 20556202]
- [58] Harding J, Roberts RM, Mirochnitchenko O. Large animal models for stem cell therapy. *Stem Cell Res Ther* 2013; 4(2): 23.
[http://dx.doi.org/10.1186/scrt171] [PMID: 23672797]
- [59] Kim S, Shin S-J, Song Y, Kim E. In Vivo Experiments with Dental Pulp Stem Cells for Pulp-Dentin Complex Regeneration. *Mediators Inflamm* 2015.
- [60] Grottkau BE, Purudappa PP, Lin YF. Multilineage differentiation of dental pulp stem cells from green fluorescent protein transgenic mice. *Int J Oral Sci* 2010; 2(1): 21-7.
[http://dx.doi.org/10.4248/IJOS10015] [PMID: 20690415]
- [61] Balic A, Aguila HL, Caimano MJ, Francone VP, Mina M. Characterization of stem and progenitor cells in the dental pulp of erupted and unerupted murine molars. *Bone* 2010; 46(6): 1639-51.
[http://dx.doi.org/10.1016/j.bone.2010.02.019] [PMID: 20193787]

The Potential Clinical Application Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4 55

- [62] Balic A, Mina M. Characterization of progenitor cells in pulps of murine incisors. *J Dent Res* 2010; 89(11): 1287-92.
[http://dx.doi.org/10.1177/0022034510375828] [PMID: 20739699]
- [63] Kermani S, Megat Abdul Wahab R, Zarina Zainol Abidin I, Zainal Ariffin Z, Senafi S, Hisham Zainal Ariffin S. Differentiation capacity of mouse dental pulp stem cells into osteoblasts and osteoclasts. *Cell J* 2014; 16(1): 31-42.
[PMID: 24518973]
- [64] Akmal MN, Zarina ZI, Rohaya M, Sahidan S, Zaidah Z, Hisham ZS. Isolation and characterization of dental pulp stem cells from murine incisors. *J Biol Sci* 2014; 14(4): 327.
[http://dx.doi.org/10.3923/jbs.2014.327.331]
- [65] Ellis KM, OCarroll DC, Lewis MD, Rychkov GY, Koblar SA. Neurogenic potential of dental pulp stem cells isolated from murine incisors. *Stem Cell Res Ther* 2014; 5(1): 30.
[http://dx.doi.org/10.1186/scrt419] [PMID: 24572146]
- [66] Guimarães ET, Cruz GS, de Jesus AA, *et al.* Mesenchymal and embryonic characteristics of stem cells obtained from mouse dental pulp. *Arch Oral Biol* 2011; 56(11): 1247-55.
[http://dx.doi.org/10.1016/j.archoralbio.2011.05.008] [PMID: 21683341]
- [67] Nakamura H, Saruwatari L, Aita H, Takeuchi K, Ogawa T. Molecular and biomechanical characterization of mineralized tissue by dental pulp cells on titanium. *J Dent Res* 2005; 84(6): 515-20.
[http://dx.doi.org/10.1177/154405910508400606] [PMID: 15914587]
- [68] Patel M, Smith AJ, Sloan AJ, Smith G, Cooper PR. Phenotype and behaviour of dental pulp cells during expansion culture. *Arch Oral Biol* 2009; 54(10): 898-908.
[http://dx.doi.org/10.1016/j.archoralbio.2009.06.008] [PMID: 19619870]
- [69] Zhang W, Walboomers XF, van Osch GJ, van den Dolder J, Jansen JA. Hard tissue formation in a porous HA/TCP ceramic scaffold loaded with stromal cells derived from dental pulp and bone marrow. *Tissue Eng Part A* 2008; 14(2): 285-94.
[http://dx.doi.org/10.1089/tea.2007.0146] [PMID: 18333781]
- [70] El-Backly RM, Massoud AG, El-Badry AM, Sherif RA, Marei MK. Regeneration of dentine/pulp-like tissue using a dental pulp stem cell/poly(lactic-co-glycolic) acid scaffold construct in New Zealand white rabbits. *Aust Endod J* 2008; 34(2): 52-67.
[http://dx.doi.org/10.1111/j.1747-4477.2008.00139.x] [PMID: 18666990]
- [71] Liu HC, e LL, Wang DS, *et al.* Reconstruction of alveolar bone defects using bone morphogenetic protein 2 mediated rabbit dental pulp stem cells seeded on nano-hydroxyapatite/collagen/poly(-lactide). *Tissue Eng Part A* 2011; 17(19-20): 2417-33.
[http://dx.doi.org/10.1089/ten.tea.2010.0620] [PMID: 21563858]
- [72] Homayounfar N, Verma P, Nosrat A, *et al.* Isolation, Characterization, and Differentiation of Dental Pulp Stem Cells in Ferrets. *J Endod* 2016; 42(3): 418-24.
[http://dx.doi.org/10.1016/j.joen.2015.12.002] [PMID: 26794343]
- [73] Iohara K, Zheng L, Ito M, Tomokiyo A, Matsushita K, Nakashima M. Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis. *Stem Cells* 2006; 24(11): 2493-503.

- [http://dx.doi.org/10.1634/stemcells.2006-0161] [PMID: 16873765]
- [74] Iohara K, Zheng L, Ito M, *et al.* Regeneration of dental pulp after pulpotomy by transplantation of CD31(-)/CD146(-) side population cells from a canine tooth. *Regen Med* 2009; 4(3): 377-85.
[http://dx.doi.org/10.2217/rme.09.5] [PMID: 19438313]
- [75] Yamada Y, Ito K, Nakamura S, Ueda M, Nagasaka T. Promising cell-based therapy for bone regeneration using stem cells from deciduous teeth, dental pulp, and bone marrow. *Cell Transplant* 2011; 20(7): 1003-13.
[http://dx.doi.org/10.3727/096368910X539128] [PMID: 21054950]
- [76] Yamada Y, Nakamura S, Ito K, *et al.* A feasibility of useful cell-based therapy by bone regeneration with deciduous tooth stem cells, dental pulp stem cells, or bone-marrow-derived mesenchymal stem cells for clinical study using tissue engineering technology. *Tissue Eng Part A* 2010; 16(6): 1891-900.
[http://dx.doi.org/10.1089/ten.tea.2009.0732] [PMID: 20067397]
- [77] Gronthos S, Mrozik K, Shi S, Bartold PM. Ovine periodontal ligament stem cells: isolation, characterization, and differentiation potential. *Calcif Tissue Int* 2006; 79(5): 310-7.
[http://dx.doi.org/10.1007/s00223-006-0040-4] [PMID: 17033723]
- [78] Mrozik KM, Zilm PS, Bagley CJ, *et al.* Proteomic characterization of mesenchymal stem cell-like populations derived from ovine periodontal ligament, dental pulp, and bone marrow: analysis of differentially expressed proteins. *Stem Cells Dev* 2010; 19(10): 1485-99.
[http://dx.doi.org/10.1089/scd.2009.0446] [PMID: 20050811]
- [79] Zheng Y, Liu Y, Zhang CM, *et al.* Stem cells from deciduous tooth repair mandibular defect in swine. *J Dent Res* 2009; 88(3): 249-54.
[http://dx.doi.org/10.1177/0022034509333804] [PMID: 19329459]
- [80] Cheng PH, Snyder B, Fillos D, Ibegbu CC, Huang AH, Chan AW. Postnatal stem/progenitor cells derived from the dental pulp of adult chimpanzee. *BMC Cell Biol* 2008; 9: 20.
[http://dx.doi.org/10.1186/1471-2121-9-20] [PMID: 18430234]
- [81] Sakdee JB, White RR, Pagonis TC, Hauschka PV. Hypoxia-amplified proliferation of human dental pulp cells. *J Endod* 2009; 35(6): 818-23.
[http://dx.doi.org/10.1016/j.joen.2009.03.001] [PMID: 19482178]
- [82] dAquino R, De Rosa A, Lanza V, *et al.* Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. *Eur Cell Mater* 2009; 18: 75-83.
[http://dx.doi.org/10.22203/eCM.v018a07] [PMID: 19908196]
- [83] Ikeda H, Sumita Y, Ikeda M, *et al.* Engineering bone formation from human dental pulp- and periodontal ligament-derived cells. *Ann Biomed Eng* 2011; 39(1): 26-34.
[http://dx.doi.org/10.1007/s10439-010-0115-2] [PMID: 20614244]
- [84] Pierdomenico L, Bonsi L, Calvitti M, *et al.* Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. *Transplantation* 2005; 80(6): 836-42.
[http://dx.doi.org/10.1097/01.tp.0000173794.72151.88] [PMID: 16210973]
- [85] Alliot-Licht B, Bluteau G, Magne D, *et al.* Dexamethasone stimulates differentiation of odontoblast-like cells in human dental pulp cultures. *Cell Tissue Res* 2005; 321(3): 391-400.

The Potential Clinical Application Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4 57

- [http://dx.doi.org/10.1007/s00441-005-1115-7] [PMID: 15988617]
- [86] Lizier NF, Kerkis A, Gomes CM, *et al.* Scaling-up of dental pulp stem cells isolated from multiple niches. PLoS One 2012; 7(6): e39885.
[http://dx.doi.org/10.1371/journal.pone.0039885] [PMID: 22768154]
- [87] Iida K, Takeda-Kawaguchi T, Tezuka Y, Kunisada T, Shibata T, Tezuka K. Hypoxia enhances colony formation and proliferation but inhibits differentiation of human dental pulp cells. Arch Oral Biol 2010; 55(9): 648-54.
[http://dx.doi.org/10.1016/j.archoralbio.2010.06.005] [PMID: 20630496]
- [88] Hirata TM, Ishkitiev N, Yaegaki K, *et al.* Expression of multiple stem cell markers in dental pulp cells cultured in serum-free media. J Endod 2010; 36(7): 1139-44.
[http://dx.doi.org/10.1016/j.joen.2010.03.002] [PMID: 20630286]
- [89] Chan B, Wong RW, Rabie B. In vivo production of mineralised tissue pieces for clinical use: a qualitative pilot study using human dental pulp cell. Int J Oral Maxillofac Surg 2011; 40(6): 612-20.
[http://dx.doi.org/10.1016/j.ijom.2011.01.008] [PMID: 21353764]
- [90] Zhang W, Walboomers XF, Van Kuppevelt TH, *et al.* In vivo evaluation of human dental pulp stem cells differentiated towards multiple lineages. J Tissue Eng Regen Med 2008; 2(2-3): 117-25.
[http://dx.doi.org/10.1002/term.71] [PMID: 18338838]
- [91] Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA. Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. Tissue Eng 2006; 12(10): 2813-23.
[http://dx.doi.org/10.1089/ten.2006.12.2813] [PMID: 17518650]
- [92] Zhang C, Chang J, Sonoyama W, Shi S, Wang C-Y. Inhibition of human dental pulp stem cell differentiation by Notch signaling. J Dent Res 2008; 87(3): 250-5.
[http://dx.doi.org/10.1177/154405910808700312] [PMID: 18296609]
- [93] dAquino R, Graziano A, Sampaioles M, *et al.* Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. Cell Death Differ 2007; 14(6): 1162-71.
[http://dx.doi.org/10.1038/sj.cdd.4402121] [PMID: 17347663]
- [94] Mangano C, Paino F, dAquino R, *et al.* Human dental pulp stem cells hook into biocoral scaffold forming an engineered biocomplex. PLoS One 2011; 6(4): e18721.
[http://dx.doi.org/10.1371/journal.pone.0018721] [PMID: 21494568]
- [95] Mori G, Brunetti G, Oranger A, *et al.* Dental pulp stem cells: osteogenic differentiation and gene expression. Ann N Y Acad Sci 2011; 1237: 47-52.
[http://dx.doi.org/10.1111/j.1749-6632.2011.06234.x] [PMID: 22082364]
- [96] Yu V, Damek-Poprawa M, Nicoll SB, Akintoye SO. Dynamic hydrostatic pressure promotes differentiation of human dental pulp stem cells. Biochem Biophys Res Commun 2009; 386(4): 661-5.
[http://dx.doi.org/10.1016/j.bbrc.2009.06.106] [PMID: 19555657]
- [97] Batouli S, Miura M, Brahim J, *et al.* Comparison of stem-cell-mediated osteogenesis and dentinogenesis. J Dent Res 2003; 82(12): 976-81.
[http://dx.doi.org/10.1177/154405910308201208] [PMID: 14630898]
- [98] Gandavarapu NR, Alge DL, Anseth KS. Osteogenic differentiation of human mesenchymal stem cells

- on $\alpha 5$ integrin binding peptide hydrogels is dependent on substrate elasticity. *Biomater Sci* 2014; 2(3): 352-61.
[http://dx.doi.org/10.1039/C3BM60149H] [PMID: 24660057]
- [99] Woods EJ, Perry BC, Hockema JJ, Larson L, Zhou D, Goebel WS. Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. *Cryobiology* 2009; 59(2): 150-7.
[http://dx.doi.org/10.1016/j.cryobiol.2009.06.005] [PMID: 19538953]
- [100] dAquino R, De Rosa A, Laino G, *et al.* Human dental pulp stem cells: from biology to clinical applications. *J Exp Zool B Mol Dev Evol* 2009; 312B(5): 408-15.
[http://dx.doi.org/10.1002/jez.b.21263] [PMID: 19065566]
- [101] Morsczeck C, Moehl C, Götz W, *et al.* In vitro differentiation of human dental follicle cells with dexamethasone and insulin. *Cell Biol Int* 2005; 29(7): 567-75.
[http://dx.doi.org/10.1016/j.cellbi.2005.03.020] [PMID: 15951208]
- [102] Park BW, Hah YS, Choi MJ, *et al.* In vitro osteogenic differentiation of cultured human dental papilla-derived cells. *J Oral Maxillofac Surg* 2009; 67(3): 507-14.
[http://dx.doi.org/10.1016/j.joms.2008.08.037] [PMID: 19231773]
- [103] Seo BM, Sonoyama W, Yamaza T, *et al.* SHED repair critical-size calvarial defects in mice. *Oral Dis* 2008; 14(5): 428-34.
[http://dx.doi.org/10.1111/j.1601-0825.2007.01396.x] [PMID: 18938268]
- [104] Graziano A, dAquino R, Cusella-De Angelis MG, *et al.* Scaffolds surface geometry significantly affects human stem cell bone tissue engineering. *J Cell Physiol* 2008; 214(1): 166-72.
[http://dx.doi.org/10.1002/jcp.21175] [PMID: 17565721]
- [105] Laino G, dAquino R, Graziano A, *et al.* A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). *J Bone Miner Res* 2005; 20(8): 1394-402.
[http://dx.doi.org/10.1359/JBMR.050325] [PMID: 16007337]
- [106] Lee JH, Lee DS, Choung HW, *et al.* Odontogenic differentiation of human dental pulp stem cells induced by preameloblast-derived factors. *Biomaterials* 2011; 32(36): 9696-706.
[http://dx.doi.org/10.1016/j.biomaterials.2011.09.007] [PMID: 21925730]
- [107] Zhang W, Walboomers XF, van Kuppevelt TH, Daamen WF, Bian Z, Jansen JA. The performance of human dental pulp stem cells on different three-dimensional scaffold materials. *Biomaterials* 2006; 27(33): 5658-68.
[http://dx.doi.org/10.1016/j.biomaterials.2006.07.013] [PMID: 16916542]
- [108] Nekanti U, Mohanty L, Venugopal P, Balasubramanian S, Totey S, Ta M. Optimization and scale-up of Whartons jelly-derived mesenchymal stem cells for clinical applications. *Stem Cell Res (Amst)* 2010; 5(3): 244-54.
[http://dx.doi.org/10.1016/j.scr.2010.08.005] [PMID: 20880767]
- [109] Eibes G, dos Santos F, Andrade PZ, *et al.* Maximizing the ex vivo expansion of human mesenchymal stem cells using a microcarrier-based stirred culture system. *J Biotechnol* 2010; 146(4): 194-7.
[http://dx.doi.org/10.1016/j.jbiotec.2010.02.015] [PMID: 20188771]

The Potential Clinical Application Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4 59

- [110] Kerkis I, Ambrosio CE, Kerkis A, *et al.* Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: Local or systemic? *J Transl Med* 2008; 6: 35.
[http://dx.doi.org/10.1186/1479-5876-6-35] [PMID: 18598348]
- [111] Akiyama K, Chen C, Wang D, *et al.* Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. *Cell Stem Cell* 2012; 10(5): 544-55.
[http://dx.doi.org/10.1016/j.stem.2012.03.007] [PMID: 22542159]
- [112] Yindeedej W, Manotham K, Chattong S, Kiatsomchai P, Ploypradith D. Angiogenesis Induction using Dental Pulp Stem Cells, A Collagen Scaffold Seeded in Pulp Chamber of Endodontically Treated Teeth and Subcutaneously Transplanted into Immunocompromised Mice. *Int J Stem Cell Res Transplant* 2016; 4(9): 251-5.
- [113] Hilkens P, Fanton Y, Martens W, *et al.* Pro-angiogenic impact of dental stem cells in vitro and in vivo. *Stem Cell Res (Amst)* 2014; 12(3): 778-90.
[http://dx.doi.org/10.1016/j.scr.2014.03.008] [PMID: 24747218]
- [114] Aranha AM, Zhang Z, Neiva KG, Costa CA, Hebling J, Nör JE. Hypoxia enhances the angiogenic potential of human dental pulp cells. *J Endod* 2010; 36(10): 1633-7.
[http://dx.doi.org/10.1016/j.joen.2010.05.013] [PMID: 20850667]
- [115] Maraldi T, Riccio M, Pisciotta A, *et al.* Human amniotic fluid-derived and dental pulp-derived stem cells seeded into collagen scaffold repair critical-size bone defects promoting vascularization. *Stem Cell Res Ther* 2013; 4(3): 53.
[http://dx.doi.org/10.1186/scrt203] [PMID: 23688855]
- [116] Ratajczak J, Bronckaers A, Dillen Y, Gervois P, Vangansewinkel T, Driesen RB, *et al.* The Neurovascular Properties of Dental Stem Cells and Their Importance in Dental Tissue Engineering. *Stem Cells Int* 2016; 2016
[http://dx.doi.org/10.1155/2016/9762871]
- [117] Wei X, Wu LP, Ling JQ, Liu L. [A proteomic analysis of human dental pulp cells undergoing odontoblast differentiation]. *Zhonghua Kou Qiang Yi Xue Za Zhi* 2009; 44(2): 85-6. [A proteomic analysis of human dental pulp cells undergoing odontoblast differentiation].
[PMID: 19563040]
- [118] Wei X, Wu LP, Ling JQ, Liu L. [Expression of matrix extracellular phosphoglycoprotein in human dental pulp cells undergoing odontoblastic differentiation]. *Zhonghua Kou Qiang Yi Xue Za Zhi* 2009; 44(9): 524-8. [Expression of matrix extracellular phosphoglycoprotein in human dental pulp cells undergoing odontoblastic differentiation].
[PMID: 20079249]
- [119] Wei X, Wu LP, Ling JQ, Liu L. [Multilineage differentiation of human dental pulp cells and periodontal ligament cells in vitro]. *Zhonghua Kou Qiang Yi Xue Za Zhi* 2008; 43(8): 495-9. [Multilineage differentiation of human dental pulp cells and periodontal ligament cells in vitro].
[PMID: 19087591]
- [120] Wei QW, Dong YM, Chen XF, Li YL, Miao GH. [Growth ability of human dental pulp cells on three bioactive scaffolds]. *Beijing Da Xue Xue Bao* 2013; 45(3): 484-8. [Growth ability of human dental pulp cells on three bioactive scaffolds].

- [PMID: 23774933]
- [121] Wei FL, Geng J, Guo J, *et al.* Metabolic changes of human dental pulp after rapid palatal expansion. *Orthod Craniofac Res* 2013; 16(3): 185-92.
[http://dx.doi.org/10.1111/ocr.12021] [PMID: 23458154]
- [122] Liu J, Jin T, Chang S, Ritchie HH, Smith AJ, Clarkson BH. Matrix and TGF-beta-related gene expression during human dental pulp stem cell (DPSC) mineralization. *In Vitro Cell Dev Biol Anim* 2007; 43(3-4): 120-8.
[http://dx.doi.org/10.1007/s11626-007-9022-8] [PMID: 17516126]
- [123] Liu CH, Hung CJ, Huang TH, Lin CC, Kao CT, Shie MY. Odontogenic differentiation of human dental pulp cells by calcium silicate materials stimulating via FGFR/ERK signaling pathway. *Mater Sci Eng C Mater Biol Appl* 2014; 43: 359-66.
[http://dx.doi.org/10.1016/j.msec.2014.06.025] [PMID: 25175224]
- [124] Prescott RS, Alsanea R, Fayad MI, *et al.* In vivo generation of dental pulp-like tissue by using dental pulp stem cells, a collagen scaffold, and dentin matrix protein 1 after subcutaneous transplantation in mice. *J Endod* 2008; 34(4): 421-6.
[http://dx.doi.org/10.1016/j.joen.2008.02.005] [PMID: 18358888]
- [125] Demarco FF, Casagrande L, Zhang Z, *et al.* Effects of morphogen and scaffold porogen on the differentiation of dental pulp stem cells. *J Endod* 2010; 36(11): 1805-11.
[http://dx.doi.org/10.1016/j.joen.2010.08.031] [PMID: 20951292]
- [126] Paino F, La Noce M, Tirino V, *et al.* Histone deacetylase inhibition with valproic acid downregulates osteocalcin gene expression in human dental pulp stem cells and osteoblasts: evidence for HDAC2 involvement. *Stem Cells* 2014; 32(1): 279-89.
[http://dx.doi.org/10.1002/stem.1544] [PMID: 24105979]
- [127] Hara ES, Ono M, Eguchi T, *et al.* miRNA-720 controls stem cell phenotype, proliferation and differentiation of human dental pulp cells. *PLoS One* 2013; 8(12): e83545.
[http://dx.doi.org/10.1371/journal.pone.0083545] [PMID: 24386225]
- [128] Galler KM, Cavender A, Yuwono V, *et al.* Self-assembling peptide amphiphile nanofibers as a scaffold for dental stem cells. *Tissue Eng Part A* 2008; 14(12): 2051-8.
[http://dx.doi.org/10.1089/ten.tea.2007.0413] [PMID: 18636949]
- [129] Yang X, Walboomers XF, van den Beucken JJ, Bian Z, Fan M, Jansen JA. Hard tissue formation of STRO-1-selected rat dental pulp stem cells in vivo. *Tissue Eng Part A* 2009; 15(2): 367-75.
[http://dx.doi.org/10.1089/ten.tea.2008.0133] [PMID: 18652538]
- [130] Chadipiralla K, Yochim JM, Bahuleyan B, *et al.* Osteogenic differentiation of stem cells derived from human periodontal ligaments and pulp of human exfoliated deciduous teeth. *Cell Tissue Res* 2010; 340(2): 323-33.
[http://dx.doi.org/10.1007/s00441-010-0953-0] [PMID: 20309582]
- [131] Khanna-Jain R, Vuorinen A, Sándor GK, Suuronen R, Miettinen S. Vitamin D(3) metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells. *J Steroid Biochem Mol Biol* 2010; 122(4): 133-41.
[http://dx.doi.org/10.1016/j.jsbmb.2010.08.001] [PMID: 20723601]

The Potential Clinical Application Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4 61

- [132] van Driel M, van Leeuwen JP. Vitamin D endocrine system and osteoblasts. *Bonekey Rep* 2014; 3: 493.
[http://dx.doi.org/10.1038/bonekey.2013.227] [PMID: 24605210]
- [133] Zhou S, Glowacki J, Kim SW, *et al.* Clinical characteristics influence in vitro action of 1,25-dihydroxyvitamin D(3) in human marrow stromal cells. *J Bone Miner Res* 2012; 27(9): 1992-2000.
[http://dx.doi.org/10.1002/jbmr.1655] [PMID: 22576852]
- [134] Piek E, Sleumer LS, van Someren EP, *et al.* Osteo-transcriptomics of human mesenchymal stem cells: accelerated gene expression and osteoblast differentiation induced by vitamin D reveals c-MYC as an enhancer of BMP2-induced osteogenesis. *Bone* 2010; 46(3): 613-27.
[http://dx.doi.org/10.1016/j.bone.2009.10.024] [PMID: 19857615]
- [135] Posa F, Di Benedetto A, Colaianni G, Cavalcanti-Adam EA, Brunetti G, Porro C, *et al.* Vitamin D Effects on Osteoblastic Differentiation of Mesenchymal Stem Cells from Dental Tissues. *Stem Cells Int.* 2016; 2016: 9150819.
- [136] Tang L, Chen Y, Pei F, Zhang H. Lithium Chloride Modulates Adipogenesis and Osteogenesis of Human Bone Marrow-Derived Mesenchymal Stem Cells. *Cell Physiol Biochem* 2015; 37(1): 143-52.
[http://dx.doi.org/10.1159/000430340] [PMID: 26303458]
- [137] Han N, Zheng Y, Li R, *et al.* β -catenin enhances odontoblastic differentiation of dental pulp cells through activation of Runx2. *PLoS One* 2014; 9(2): e88890.
[http://dx.doi.org/10.1371/journal.pone.0088890] [PMID: 24520423]
- [138] Pozio A, Palmieri A, Girardi A, Cura F, Carinci F. Titanium nanotubes stimulate osteoblast differentiation of stem cells from pulp and adipose tissue. *Dent Res J (Isfahan)* 2012; 9 (Suppl. 2): S169-74.
[PMID: 23814578]
- [139] Hsu SH, Chang JC. The static magnetic field accelerates the osteogenic differentiation and mineralization of dental pulp cells. *Cytotechnology* 2010; 62(2): 143-55.
[http://dx.doi.org/10.1007/s10616-010-9271-3] [PMID: 20464482]
- [140] Liu H, Li W, Shi S, Habelitz S, Gao C, Denbesten P. MEPE is downregulated as dental pulp stem cells differentiate. *Arch Oral Biol* 2005; 50(11): 923-8.
[http://dx.doi.org/10.1016/j.archoralbio.2005.03.003] [PMID: 16183369]
- [141] Barnes CP, Sell SA, Boland ED, Simpson DG, Bowlin GL. Nanofiber technology: designing the next generation of tissue engineering scaffolds. *Adv Drug Deliv Rev* 2007; 59(14): 1413-33.
[http://dx.doi.org/10.1016/j.addr.2007.04.022] [PMID: 17916396]
- [142] Dobie K, Smith G, Sloan AJ, Smith AJ. Effects of alginate hydrogels and TGF-beta 1 on human dental pulp repair in vitro. *Connect Tissue Res* 2002; 43(2-3): 387-90.
[http://dx.doi.org/10.1080/03008200290000574] [PMID: 12489186]
- [143] Murphy WL, Peters MC, Kohn DH, Mooney DJ. Sustained release of vascular endothelial growth factor from mineralized poly(lactide-co-glycolide) scaffolds for tissue engineering. *Biomaterials* 2000; 21(24): 2521-7.
[http://dx.doi.org/10.1016/S0142-9612(00)00120-4] [PMID: 11071602]
- [144] Yang X, Yang F, Walboomers XF, Bian Z, Fan M, Jansen JA. The performance of dental pulp stem

62 *Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4*

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- cells on nanofibrous PCL/gelatin/nHA scaffolds. *J Biomed Mater Res A* 2010; 93(1): 247-57.
[PMID: 19557787]
- [145] Yamaza T, Kentaro A, Chen C, *et al.* Immunomodulatory properties of stem cells from human exfoliated deciduous teeth. *Stem Cell Res Ther* 2010; 1(1): 5.
[http://dx.doi.org/10.1186/sert5] [PMID: 20504286]
- [146] Yang X, van der Kraan PM, Bian Z, Fan M, Walboomers XF, Jansen JA. Mineralized tissue formation by BMP2-transfected pulp stem cells. *J Dent Res* 2009; 88(11): 1020-5.
[http://dx.doi.org/10.1177/0022034509346258] [PMID: 19828890]
- [147] Gronthos S. The therapeutic potential of dental pulp cells: more than pulp fiction? *Cytotherapy* 2011; 13(10): 1162-3.
[http://dx.doi.org/10.3109/14653249.2011.623827] [PMID: 21999372]
- [148] Okamoto Y, Sonoyama W, Ono M, *et al.* Simvastatin induces the odontogenic differentiation of human dental pulp stem cells in vitro and in vivo. *J Endod* 2009; 35(3): 367-72.
[http://dx.doi.org/10.1016/j.joen.2008.11.024] [PMID: 19249597]
- [149] Motamedian SR, Tabatabaei FS, Akhlaghi F, Torshabi M, Gholamin P, Khojasteh A. Response of Dental Pulp Stem Cells to Synthetic, Allograft, and Xenograft Bone Scaffolds. *Int J Periodontics Restorative Dent* 2016.
- [150] Rinanda A, SuhardiPradono, AbasBasril. Scaffold degradation during bone tissue reconstruction in *Macaca nemestrina* mandible. *Interv Med Appl Sci* 2016; 8(2): 77-81.
[http://dx.doi.org/10.1556/1646.8.2016.2.5]
- [151] Hartgerink JD. Covalent capture: a natural complement to self-assembly. *Curr Opin Chem Biol* 2004; 8(6): 604-9.
[http://dx.doi.org/10.1016/j.cbpa.2004.10.001] [PMID: 15556403]
- [152] Hosseinkhani H, Hosseinkhani M, Tian F, Kobayashi H, Tabata Y. Osteogenic differentiation of mesenchymal stem cells in self-assembled peptide-amphiphile nanofibers. *Biomaterials* 2006; 27(22): 4079-86.
[http://dx.doi.org/10.1016/j.biomaterials.2006.03.030] [PMID: 16600365]
- [153] Guler MO, Hsu L, Soukasene S, Harrington DA, Hulvat JF, Stupp SI. Presentation of RGDS epitopes on self-assembled nanofibers of branched peptide amphiphiles. *Biomacromolecules* 2006; 7(6): 1855-63.
[http://dx.doi.org/10.1021/bm060161g] [PMID: 16768407]
- [154] Galler KM, Hartgerink JD, Cavender AC, Schmalz G, DSouza RN. A customized self-assembling peptide hydrogel for dental pulp tissue engineering. *Tissue Eng Part A* 2012; 18(1-2): 176-84.
[http://dx.doi.org/10.1089/ten.tea.2011.0222] [PMID: 21827280]
- [155] Galler KM, DSouza RN, Hartgerink JD, Schmalz G. Scaffolds for dental pulp tissue engineering. *Adv Dent Res* 2011; 23(3): 333-9.
[http://dx.doi.org/10.1177/0022034511405326] [PMID: 21677088]
- [156] Khan SZ, Kokubu E, Matsuzaka K, Inoue T. Behaviour of rat-cultured dental pulp cells in three-dimensional collagen type-I gel in vitro and in vivo. *Aust Endod J* 2013; 39(3): 137-45.
[http://dx.doi.org/10.1111/j.1747-4477.2012.00351.x] [PMID: 24279661]

The Potential Clinical Application Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4 63

- [157] Giuliani A, Manescu A, Langer M, *et al.* Three years after transplants in human mandibles, histological and in-line holotomography revealed that stem cells regenerated a compact rather than a spongy bone: biological and clinical implications. *Stem Cells Transl Med* 2013; 2(4): 316-24. [http://dx.doi.org/10.5966/sctm.2012-0136] [PMID: 23502599]
- [158] Riccio M, Maraldi T, Pisciotta A, *et al.* Fibroin scaffold repairs critical-size bone defects in vivo supported by human amniotic fluid and dental pulp stem cells. *Tissue Eng Part A* 2012; 18(9-10): 1006-13. [http://dx.doi.org/10.1089/ten.tea.2011.0542] [PMID: 22166080]
- [159] Ferroni L, Gardin C, Sivoletta S, *et al.* A hyaluronan-based scaffold for the in vitro construction of dental pulp-like tissue. *Int J Mol Sci* 2015; 16(3): 4666-81. [http://dx.doi.org/10.3390/ijms16034666] [PMID: 25739081]
- [160] Jensen J, Tvedesøe C, Rølfing JH, *et al.* Dental pulp-derived stromal cells exhibit a higher osteogenic potency than bone marrow-derived stromal cells in vitro and in a porcine critical-size bone defect model. *SICOT J* 2016; 2: 16. [http://dx.doi.org/10.1051/sicotj/2016004] [PMID: 27163105]
- [161] Ito K, Yamada Y, Nakamura S, Ueda M. Osteogenic potential of effective bone engineering using dental pulp stem cells, bone marrow stem cells, and periosteal cells for osseointegration of dental implants. *Int J Oral Maxillofac Implants* 2011; 26(5): 947-54. [PMID: 22010075]
- [162] Chen G, Chen J, Yang B, *et al.* Combination of aligned PLGA/Gelatin electrospun sheets, native dental pulp extracellular matrix and treated dentin matrix as substrates for tooth root regeneration. *Biomaterials* 2015; 52: 56-70. [http://dx.doi.org/10.1016/j.biomaterials.2015.02.011] [PMID: 25818413]
- [163] Huang GT, Yamaza T, Shea LD, *et al.* Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. *Tissue Eng Part A* 2010; 16(2): 605-15. [http://dx.doi.org/10.1089/ten.tea.2009.0518] [PMID: 19737072]
- [164] Akiyama N, Yamamoto-Fukuda T, Takahashi H, Koji T. In situ tissue engineering with synthetic self-assembling peptide nanofiber scaffolds, PuraMatrix, for mucosal regeneration in the rat middle-ear. *Int J Nanomedicine* 2013; 8: 2629-40. [http://dx.doi.org/10.2147/IJN.S47279] [PMID: 23926427]
- [165] Allen P, Kang KT, Bischoff J. Rapid onset of perfused blood vessels after implantation of ECFCs and MPCs in collagen, PuraMatrix and fibrin provisional matrices. *J Tissue Eng Regen Med* 2015; 9(5): 632-6. [http://dx.doi.org/10.1002/term.1803] [PMID: 23955835]
- [166] Allen P, Melero-Martin J, Bischoff J. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. *J Tissue Eng Regen Med* 2011; 5(4): e74-86. [http://dx.doi.org/10.1002/term.389] [PMID: 21413157]
- [167] McGrath AM, Novikova LN, Novikov LN, Wiberg M. BD™ PuraMatrix™ peptide hydrogel seeded with Schwann cells for peripheral nerve regeneration. *Brain Res Bull* 2010; 83(5): 207-13.

- [http://dx.doi.org/10.1016/j.brainresbull.2010.07.001] [PMID: 20633614]
- [168] Misawa H, Kobayashi N, Soto-Gutierrez A, *et al.* PuraMatrix facilitates bone regeneration in bone defects of calvaria in mice. *Cell Transplant* 2006; 15(10): 903-10.
[http://dx.doi.org/10.3727/000000006783981369] [PMID: 17299995]
- [169] Moradi F, Bahktiari M, Joghataei MT, *et al.* BD PuraMatrix peptide hydrogel as a culture system for human fetal Schwann cells in spinal cord regeneration. *J Neurosci Res* 2012; 90(12): 2335-48.
[http://dx.doi.org/10.1002/jnr.23120] [PMID: 22996688]
- [170] Nakahara H, Misawa H, Yoshida A, *et al.* Bone repair using a hybrid scaffold of self-assembling peptide PuraMatrix and polyetheretherketone cage in rats. *Cell Transplant* 2010; 19(6): 791-7.
[http://dx.doi.org/10.3727/096368910X508906] [PMID: 20573298]
- [171] Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S. The efficacy of mesenchymal stem cells to regenerate and repair dental structures. *Orthod Craniofac Res* 2005; 8(3): 191-9.
[http://dx.doi.org/10.1111/j.1601-6343.2005.00331.x] [PMID: 16022721]
- [172] Cordeiro MM, Dong Z, Kaneko T, *et al.* Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. *J Endod* 2008; 34(8): 962-9.
[http://dx.doi.org/10.1016/j.joen.2008.04.009] [PMID: 18634928]
- [173] Gotlieb EL, Murray PE, Namerow KN, Kuttler S, Garcia-Godoy F. An ultrastructural investigation of tissue-engineered pulp constructs implanted within endodontically treated teeth. *J Am Dent Assoc* 2008; 139(4): 457-65.
[http://dx.doi.org/10.14219/jada.archive.2008.0189] [PMID: 18385030]
- [174] Akiyama K, Chen C, Gronthos S, Shi S. Lineage differentiation of mesenchymal stem cells from dental pulp, apical papilla, and periodontal ligament. *Methods Mol Biol* 2012; 887: 111-21.
[http://dx.doi.org/10.1007/978-1-61779-860-3_11] [PMID: 22566051]
- [175] Andreasen JO. Pulp and periodontal tissue repair - regeneration or tissue metaplasia after dental trauma. A review. *Dent Traumatol* 2012; 28(1): 19-24.
[http://dx.doi.org/10.1111/j.1600-9657.2011.01058.x] [PMID: 21933343]
- [176] Sakai VT, Zhang Z, Dong Z, *et al.* SHED differentiate into functional odontoblasts and endothelium. *J Dent Res* 2010; 89(8): 791-6.
[http://dx.doi.org/10.1177/0022034510368647] [PMID: 20395410]
- [177] Marchionni C, Bonsi L, Alviano F, *et al.* Angiogenic potential of human dental pulp stromal (stem) cells. *Int J Immunopathol Pharmacol* 2009; 22(3): 699-706.
[http://dx.doi.org/10.1177/039463200902200315] [PMID: 19822086]
- [178] Qi S, Pan J. Cell-based therapy for therapeutic lymphangiogenesis. *Stem Cells Dev* 2015; 24(3): 271-83.
[http://dx.doi.org/10.1089/scd.2014.0390] [PMID: 25329370]
- [179] Nakashima M, Iohara K. Mobilized dental pulp stem cells for pulp regeneration: initiation of clinical trial. *J Endod* 2014; 40(4) (Suppl.): S26-32.
[http://dx.doi.org/10.1016/j.joen.2014.01.020] [PMID: 24698690]
- [180] Nishino Y, Ebisawa K, Yamada Y, Okabe K, Kamei Y, Ueda M. Human deciduous teeth dental pulp cells with basic fibroblast growth factor enhance wound healing of skin defect. *J Craniofac Surg* 2011;

The Potential Clinical Application Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4 65

- 22(2): 438-42.
[http://dx.doi.org/10.1097/SCS.0b013e318207b507] [PMID: 21403563]
- [181] Gomes JA, Geraldes Monteiro B, Melo GB, *et al.* Corneal reconstruction with tissue-engineered cell sheets composed of human immature dental pulp stem cells. *Invest Ophthalmol Vis Sci* 2010; 51(3): 1408-14.
[http://dx.doi.org/10.1167/iops.09-4029] [PMID: 19892864]
- [182] Kumar A, Kumar V, Rattan V, Jha V, Bhattacharyya S. Secretome Cues Modulate the Neurogenic Potential of Bone Marrow and Dental Stem Cells. *Mol Neurobiol* 2016; ••: 1-11.
[PMID: 27422132]
- [183] Arthur A, Shi S, Zannettino AC, Fujii N, Gronthos S, Koblar SA. Implanted adult human dental pulp stem cells induce endogenous axon guidance. *Stem Cells* 2009; 27(9): 2229-37.
[http://dx.doi.org/10.1002/stem.138] [PMID: 19544412]
- [184] Sasaki R, Aoki S, Yamato M, *et al.* PLGA artificial nerve conduits with dental pulp cells promote facial nerve regeneration. *J Tissue Eng Regen Med* 2011; 5(10): 823-30.
[http://dx.doi.org/10.1002/term.387] [PMID: 22002926]
- [185] Sasaki R, Matsumine H, Watanabe Y, *et al.* Electrophysiologic and functional evaluations of regenerated facial nerve defects with a tube containing dental pulp cells in rats. *Plast Reconstr Surg* 2014; 134(5): 970-8.
[http://dx.doi.org/10.1097/PRS.0000000000000602] [PMID: 25347632]
- [186] Sasaki R, Matsumine H, Watanabe Y, Yamato M, Ando T. Surgical procedure of extracting teeth for obtaining dental pulp for regenerative medicine in swine. *Lab Anim* 2015; 49(2): 172-6.
[http://dx.doi.org/10.1177/0023677214560002] [PMID: 25448868]
- [187] Spyridopoulos T, Lambropoulou M, Pagonopoulou O, *et al.* Regenerated Nerve Defects with a Nerve Conduit Containing Dental Pulp Stem Cells in Pigs: An Immunohistochemical and Electrophysiological Evaluation. *J Reconstr Microsurg* 2015; 31(7): 516-26.
[http://dx.doi.org/10.1055/s-0035-1555751] [PMID: 26125150]
- [188] Feng X, Lu X, Huang D, *et al.* 3D porous chitosan scaffolds suit survival and neural differentiation of dental pulp stem cells. *Cell Mol Neurobiol* 2014; 34(6): 859-70.
[http://dx.doi.org/10.1007/s10571-014-0063-8] [PMID: 24789753]
- [189] Askari N, Yaghoobi MM, Shamsara M, Esmaeili-Mahani S. Tetracycline-regulated expression of OLIG2 gene in human dental pulp stem cells lead to mouse sciatic nerve regeneration upon transplantation. *Neuroscience* 2015; 305: 197-208.
[http://dx.doi.org/10.1016/j.neuroscience.2015.07.088] [PMID: 26254831]
- [190] Dai LG, Huang GS, Hsu SH. Sciatic nerve regeneration by cocultured Schwann cells and stem cells on microporous nerve conduits. *Cell Transplant* 2013; 22(11): 2029-39.
[http://dx.doi.org/10.3727/096368912X658953] [PMID: 23192007]
- [191] Yamamoto T, Osako Y, Ito M, *et al.* Trophic Effects of Dental Pulp Stem Cells on Schwann Cells in Peripheral Nerve Regeneration. *Cell Transplant* 2016; 25(1): 183-93.
[http://dx.doi.org/10.3727/096368915X688074] [PMID: 25903498]
- [192] Mead B, Logan A, Berry M, Leadbeater W, Scheven BA. Intravitreally transplanted dental pulp stem

- cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. *Invest Ophthalmol Vis Sci* 2013; 54(12): 7544-56.
[http://dx.doi.org/10.1167/iops.13-13045] [PMID: 24150755]
- [193] Mead B, Logan A, Berry M, Leadbeater W, Scheven BA. Dental pulp stem cells, a paracrine-mediated therapy for the retina. *Neural Regen Res* 2014; 9(6): 577-8.
[http://dx.doi.org/10.4103/1673-5374.130089] [PMID: 25206857]
- [194] Omi M, Hata M, Nakamura N, *et al.* Transplantation of dental pulp stem cells suppressed inflammation in sciatic nerves by promoting macrophage polarization towards anti-inflammation phenotypes and ameliorated diabetic polyneuropathy. *J Diabetes Investig* 2016; 7(4): 485-96.
[http://dx.doi.org/10.1111/jdi.12452] [PMID: 27181261]
- [195] Hata M, Omi M, Kobayashi Y, *et al.* Transplantation of cultured dental pulp stem cells into the skeletal muscles ameliorated diabetic polyneuropathy: therapeutic plausibility of freshly isolated and cryopreserved dental pulp stem cells. *Stem Cell Res Ther* 2015; 6: 162.
[http://dx.doi.org/10.1186/s13287-015-0156-4] [PMID: 26345292]
- [196] Leong WK, Henshall TL, Arthur A, *et al.* Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms. *Stem Cells Transl Med* 2012; 1(3): 177-87.
[http://dx.doi.org/10.5966/sctm.2011-0039] [PMID: 23197777]
- [197] Shamir C, Venugopal C, Dhanushkodi A. Dental pulp stem cells for treating neurodegenerative diseases. *Neural Regen Res* 2015; 10(12): 1910-1.
[http://dx.doi.org/10.4103/1673-5374.169629] [PMID: 26889163]
- [198] Annibali S, Bellavia D, Ottolenghi L, *et al.* Micro-CT and PET analysis of bone regeneration induced by biodegradable scaffolds as carriers for dental pulp stem cells in a rat model of calvarial critical size defect: Preliminary data. *J Biomed Mater Res B Appl Biomater* 2014; 102(4): 815-25.
[http://dx.doi.org/10.1002/jbm.b.33064] [PMID: 24142538]
- [199] Asutay F, Polat S, Gül M, Subaşı C, Kahraman SA, Karaöz E. The effects of dental pulp stem cells on bone regeneration in rat calvarial defect model: micro-computed tomography and histomorphometric analysis. *Arch Oral Biol* 2015; 60(12): 1729-35.
[http://dx.doi.org/10.1016/j.archoralbio.2015.09.002] [PMID: 26433189]
- [200] Wang J, Liu X, Jin X, *et al.* The odontogenic differentiation of human dental pulp stem cells on nanofibrous poly(L-lactic acid) scaffolds in vitro and in vivo. *Acta Biomater* 2010; 6(10): 3856-63.
[http://dx.doi.org/10.1016/j.actbio.2010.04.009] [PMID: 20406702]
- [201] Syed-Picard FN, Ray HL Jr, Kumta PN, Sfeir C. Scaffoldless tissue-engineered dental pulp cell constructs for endodontic therapy. *J Dent Res* 2014; 93(3): 250-5.
[http://dx.doi.org/10.1177/0022034513517901] [PMID: 24401375]
- [202] Cavalcanti BN, Zeitlin BD, Nör JE. A hydrogel scaffold that maintains viability and supports differentiation of dental pulp stem cells. *Dent Mater* 2013; 29(1): 97-102.
[http://dx.doi.org/10.1016/j.dental.2012.08.002] [PMID: 22901827]
- [203] Yang C, Lee JS, Jung UW, Seo YK, Park JK, Choi SH. Periodontal regeneration with nano-hydroxyapatite-coated silk scaffolds in dogs. *J Periodontal Implant Sci* 2013; 43(6): 315-22.
[http://dx.doi.org/10.5051/jpis.2013.43.6.315] [PMID: 24455445]

The Potential Clinical Application Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 4 67

- [204] Soares DG, Rosseto HL, Basso FG, Scheffel DS, Hebling J, Costa CA. Chitosan-collagen biomembrane embedded with calcium-aluminate enhances dentinogenic potential of pulp cells. *Braz Oral Res* 2016; 30(1): e54.
[<http://dx.doi.org/10.1590/1807-3107BOR-2016.vol30.0054>] [PMID: 27119587]
- [205] Neunzehn J, Weber M-T, Wittenburg G, Lauer G, Hannig C, Wiesmann H-P. Dentin-like tissue formation and biomineralization by multicellular human pulp cell spheres in vitro. *Head Face Med* 2014; 10(1): 25.
[<http://dx.doi.org/10.1186/1746-160X-10-25>] [PMID: 24946771]
- [206] Schmalz G, Schuster U, Thonemann B, Barth M, Esterbauer S. Dentin barrier test with transfected bovine pulp-derived cells. *J Endod* 2001; 27(2): 96-102.
[<http://dx.doi.org/10.1097/00004770-200102000-00009>] [PMID: 11491647]
- [207] Shao MY, Fu ZS, Cheng R, *et al.* The presence of open dentinal tubules affects the biological properties of dental pulp cells ex vivo. *Mol Cells* 2011; 31(1): 65-71.
[<http://dx.doi.org/10.1007/s10059-011-0010-1>] [PMID: 21120627]
- [208] Iohara K, Zheng L, Wake H, *et al.* A novel stem cell source for vasculogenesis in ischemia: subfraction of side population cells from dental pulp. *Stem Cells* 2008; 26(9): 2408-18.
[<http://dx.doi.org/10.1634/stemcells.2008-0393>] [PMID: 18583536]
- [209] Gandia C, Armiñan A, García-Verdugo JM, *et al.* Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. *Stem Cells* 2008; 26(3): 638-45.
[<http://dx.doi.org/10.1634/stemcells.2007-0484>] [PMID: 18079433]
- [210] Al-Zer H, Kalbounh H. Dental pulp stem cells-derived schwann cells for peripheral nerve injury regeneration. *Neural Regen Res* 2015; 10(12): 1945-6.
[<http://dx.doi.org/10.4103/1673-5374.172309>] [PMID: 26889180]

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2.2. Dental Pulp Stem/ Stromal Cells

Protocol selection and validation

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1. Introduction

To develop the DPSCs-based cellular system for tissue regeneration application basic protocols were designed, relying on available literature revised and detailed in the previous section, and validated for the isolation of this cellular population (**Figure 1**).

Further, the isolated cells were characterised regarding the minimal criteria for the acceptance as a mesenchymal stem cell population, as recommended by the ISCT (Dominici et al., 2006). Extensive MSCs characterisation was performed in cellular populations from healthy teeth from two randomly selected donors at Passage 5.

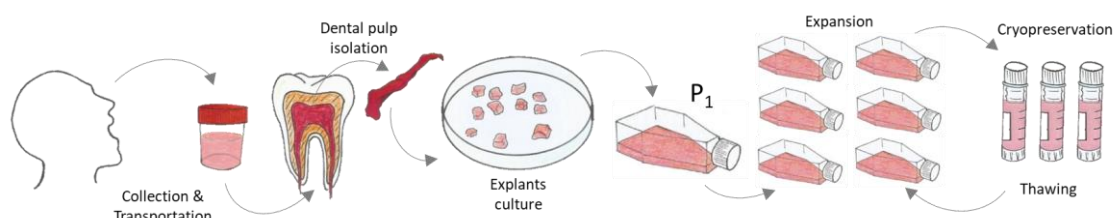


Figure 1: Schematic of the procedure for the isolation and maintenance of DPSCs.

2. Materials and Methods

Selected protocols for the isolation and culture of dental derived Stem/ Stromal Cells

2.1. Selection criteria

Tooth donations were accepted upon informed consent from the patients or legal tutors. Selected donor were healthy individuals, aged 17-30 years of age (considered young adults), irrespective of gender. Samples of healthy and unhealthy teeth (mostly presenting with caries) were equally processed. However, the later were maintained in a quarantine incubator and were not cryopreserved or further manipulated (after isolation).

2.2. Sample transportation

Following exodontia procedure, performed by partnering Medical Dentists, teeth were collected and placed individually in a sterile container, with transportation medium. The transportation medium was composed of DMEM + GlutaMAX™ (21885-025, Gibco®) supplemented penicillin (300 U/mL) - streptomycin (300 µg/mL)(151140-122, Gibco®), and amphotericin B (5 µg/mL)(15290-026, Gibco®). The collected samples were then stored at 4°C for approximately 24 hours (maximum storage time of approximately 48 hours).

2.3. Decontamination and microbiological analysis

Upon arrival to the laboratory, each tooth was individually transferred into a 100 mm glass petri dish with 10 mL DPBS (14190-086, Gibco®) and primarily washed. Remaining debris and the periodontal ligament were thoroughly removed using a periodontal scaler, scrapping towards the root tip (away from the dentin-enamel junction). The tooth was then washed with DPBS and its outside surfaces wiped with 70% ethanol swabs.

For the microbiological analysis, the external surface of the tooth was swabbed with a flexible stainless-steel file (K-files 25mm 3525010, Sendoline®). The file was placed in Brain-Heart Infusion Broth (BHI-T, 42081, BioMérieux SA), hermetically sealed and maintained at room temperature (**Figure 2**). Samples were shipped and tested by a credited laboratory (LabCo, Portugal) within 24 hours.

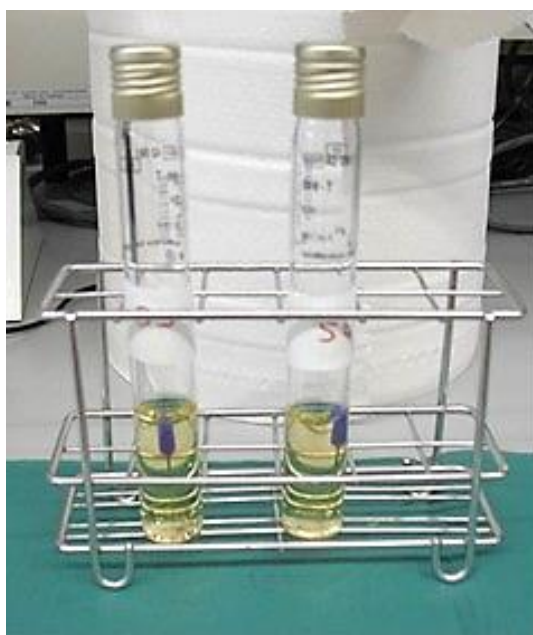


Figure 2: Samples for microbiological analysis: Brain-Heart Infusion Broth (BHI-T) containing the flexible stainless-steel file used to swab the external surface of the tooth.

2.4. Isolation of dental pulp tissue from fresh teeth

Once decontaminated, the teeth were manipulated in a sterile environment, in a laminar flow hood (**Figure 3A**). The tooth was held by the crown with a large haemostat. Several perforations were carefully performed right below the dentin-enamel junction using a fissure burr, avoiding cutting into the pulp tissue (**Figure 3B**). Overheating of the tissue was avoided by keeping the surface wet in ice-cold DPBS. The perforations were then joined together, forming a ridge along the perimeter of the tooth (**Figure 3C**). With the aid of a second haemostat (holding the root), the two portions of the tooth were separated with a twisting movement. The exposed pulp (**Figure 3D**) was removed from its cavity with a 24G needle and placed onto a sterile petri dish (**Figure 3E**). Tissue desiccation was prevented by immersing in sterile DPBS (**Figure 3F**).

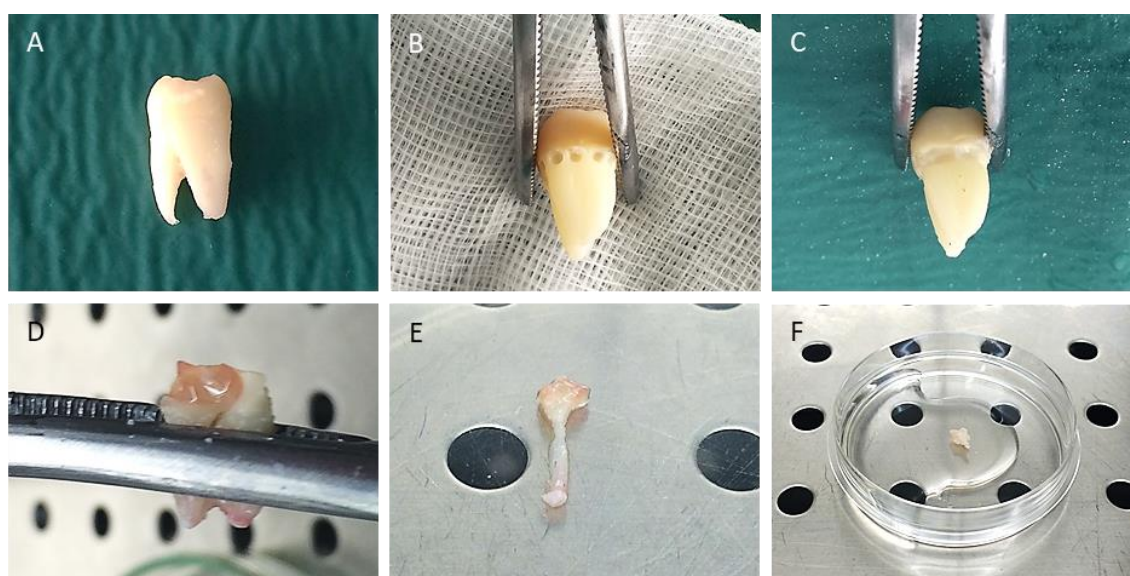


Figure 3: Cleaned and decontaminated tooth (A); perforations right below the dentin-enamel junction (B) that are joined together, forming a ridge along the perimeter of the tooth (C); exposed pulp cavity and pulp tissue (D); isolated dental pulp (E) immersed in DPBS to prevent desiccation (F).

2.5. Explant isolation of Dental Pulp Stem/ Stromal Cells

The isolated pulp tissue was cut into small fragments (1-2 mm²) and transferred to a 6 well tissue culture treated plate (10062-892, VWR®)(or equivalent), 1 to 2 fragments per well. Fragments were allowed to adhere to the well surface for 5-10 minutes, air dry.

Once attached, fragments were covered with culture medium [MEM- α + GlutaMAX™ (32561-029, Gibco®) supplemented with FBS (20% (v/v))(A31608-01, Gibco®), HEPES buffer (1% (v/v))(15630-049, Gibco®), penicillin (100 U/mL) - streptomycin (100 μ g/mL) and amphotericin B (2,5 μ g/mL)].

Explants were incubated at 37°C, 5% CO₂ humidified atmosphere.

2.6. Cell passaging

Cells were allowed to migrate into the culture plate and proliferate until 80-90% confluence (covered culture surface). In order to detach the migrating cells from the current culture vessel, the culture medium was removed, and the cells were gently washed two (2) times, by adding and discarding DPBS (at room temperature). The DPBS was removed, and Trypsin (Trypsin-EDTA 0.25%, 25200-072, Gibco®) was added and incubated for 3-4 minutes, at 37°C. Cell detachment was promoted by gentle tapping of the culture vessel and visually confirmed in the inverted phase-contrast microscope (Axiovert 40 CFL, Zeiss).

Culture medium was added to arrest the enzymatic process, and detached cells were transferred into a 15mL centrifuge tube. Cellular suspension was centrifuged at 275 xg for 7 minutes, to promote the formation of a cell pellet at the base of the tube. The supernatant was discarded, and the remaining cell pellet resuspended in fresh culture medium. Cells could then be cryopreserved or re-plated.

2.7. Cryopreservation

To cryopreserve the cells, an appropriate number of 2 mL cryovials were labelled [indicating species source, cellular population, passage, date, cell numbers and cryopreservation media]. Cellular concentration in the suspension was determined using Trypan Blue exclusion dye (10 μ L Trypan Blue (T10282, Invitrogen®): 10 μ L of cell suspension) and cell counting was performed by the Countess II FL Automated Cell Counter (Thermo-Fisher Scientific®).

Cellular concentration was tuned to 1 to 2 x10⁶ cells/mL, and a volume of 900 μ L of cell suspension added to each cryovial. A volume of 100 μ L of DMSO (D2650, Sigma®) was slowly added to each vial (10%(v/v)). Vials were capped and transferred into the designated places on the freezing container (Mr Frosty™, Thermo Scientific™), and placed at -80°C, for 3 days. For longtime storage, vials were transferred to liquid nitrogen storage (-195.79 °C) and maintained until necessary.

2.8. Thawing

To thaw cryopreserved cells, the culture medium was warmed, and a new vessel prepared. The vial was selected from the nitrogen container and quickly placed in a 37°C water bath, care being taken not to submerge the cap. The cellular suspension should thaw within 1 minute until only a small crystal of ice remains. The tube was wiped sterile, placed into the laminar flow hood and opened. The contents were carefully transferred to a 15mL centrifuge tube, and warm culture medium slowly added (drop-by-drop). Cellular suspension was centrifuged at 275 $\times g$ for 7 minutes, and the supernatant was discarded. Cells were re-suspended and counted or directly plated onto the new culture vessel.

Validation of isolated populations as dental derived Stem/ Stromal Cells

2.9. Growth dynamics and Population Doubling Times

Isolated cells at Passage 5 were expanded in standard culture conditions in a T75 flask (initial seeding of 8000 cells/cm²), using MEM- α + GlutaMAX™ (32561-029, Gibco®) supplemented with FBS (10% (v/v)), HEPES buffer (1% (v/v)), penicillin (100 U/mL) - streptomycin (100 μ g/mL), and amphotericin B (2.5 μ g/mL). Cells were expanded at a temperature of 37 °C in a 5% CO₂ humidified atmosphere until approximately 80% confluence was reached. Then, cells were passed and seeded onto 24 well tissue culture-treated plates at 6.000 cells/cm². Standard culture media was changed every 3 days. Cells were harvested and counted daily, in duplicates, for 10 days (initial set up of 20 wells).

For daily recovery, duplicate wells were washed with DPBS once and incubated with Trypsin for 3-4 minutes. Culture media was added and transferred to 2mL tubes. Final volume was recorded, and cells counted using Trypan Blue exclusion dye assay (10 μ L Trypan Blue: 10 μ L of cell suspension). Triplicate counting was performed by the Countess II FL Automated Cell Counter.

Results were plotted using the GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla California USA). Obtained results were represented as Mean $\pm$ Standard Deviation (M $\pm$ SD). Groups were compared using two-tailed unpaired T-test

(IC95%), and differences were deemed as statistically significant when $P \leq 0.05$. Significant results between groups were indicated using one, two, three or four of the symbols (*) which correspond to $0.01 < P \leq 0.05$, $0.001 < P \leq 0.01$, $0.0001 < P \leq 0.001$ e $P \leq 0.0001$, respectively.

The population doubling time (PDT), or generation time, is the average time required for a cellular population to double in number. This assessment is recommended the exponential growth phase, through the formula:

$$PDT = \frac{t \ln 2}{\ln \left(\frac{n_f}{n_i} \right)}$$

Where 't' is the incubation time; 'nf' is the number of cells at the end of the incubation period, and 'ni' is the number of cells at the beginning of the incubation period.

2.10. Karyotype analysis

Chromosome analysis was carried out for the assessment of the numeric and structural stability of the chromosomes, through karyotype analysis G-band staining.

Cells were expanded and cultured in a humidified atmosphere of 5% CO₂ at 37°C. Once in the exponential growth phase and approaching 80% confluency, the cells were exposed to colcemid solution (0.4 µg/mL, 15212-012, Gibco®) for 2-4 hours at 37°C for cell cycle arrest at metaphase. Cells were then collected and suspended in hypotonic treatment (KCl 0.75M, 15 min at 37°C). Following centrifugation (270xg, 7 minutes), the cells were fixated (6:1 methanol to acetic acid), centrifuged and resuspended in 3:1 methanol to acetic acid. Cell smears were prepared onto a glass slide, air-dried and stained with Giemsa (4%, 6 minutes) using a routine laboratory procedure. Karyotype analysis was performed as recommended by the International System for Human Cytogenetic Nomenclature (2016).

2.11. Mesenchymal Stem/ Stromal Cells Phenotypic identity

2.11.1. Flow cytometry for surface markers expression

Cultured cells were detached using a variation of the previously detailed protocol, as the enzymatic detachment solution classically utilised (Trypsin) was replaced by Accutase™ Cell detachment solution (561527, BD Biosciences®) and suspended in Stain Buffer (554676, BD Biosciences®). Cellular suspensions were then incubated with anti-negative marker (CD34, CD11b, CD19, CD45, MHC class II) and anti-positive (CD90, CD105, CD44) antibodies, as per manufacturer's instructions (Human MSC Analysis Kit, 562245, BD Biosciences®). Data were acquired using BD FACSCalibur™ 3 CA Becton Dickinson, BD Biosciences®, and processed using FlowJo Engine X10.4 (v3.05478, LLC).

2.11.2. Immunocytochemistry for 'stemness' markers

Expanding cells were routinely detached and suspended in warm DPBS. The suspension was centrifuged, the supernatant discarded and Shandon™ Cytoblock™ Cell Block Preparation System (Thermo Scientific, USA) was added, aiming at a concentration of 1×10^5 cells/mL. The formed gel cone (shaped by the bottom of the 50mL centrifuged tube) was collected and processed for immunocytochemistry. Antigen retrieval was completed on deparaffinised sections by immersion in Target Retrieval Solution (Dako™, Glostrup, Denmark) in a water bath for 20 minutes. The Novolink™ Max-Polymer detection system (Novocastra, UK) was used for visualisation as per manufacturer's instructions. Appropriate controls were performed for each antibody. The prepared slides were incubated for 2 hours, at room temperature, with listed monoclonal antisera (**Table 1**).

Table 1: Monoclonal antibodies for the Immunochemical staining of 'stemness' markers in the isolated populations

<i>Antigen/ Marker</i>	<i>Antibody Clone (Manufacturer)</i>	<i>Dilution</i>
STRO-1	STRO-1 clone (Invitrogen, Zymed laboratories)	1:200
vimentin	clone V9 (Dako)	1:500
CD117 (c-Kit)	A4502 (DakoCytomtion)	1:450
NANOG-1	clone MAB (ABGent)	1:50
CD31	clone JC70A (Dako)	1:50

2.12. Multilineage differentiation

Osteogenic differentiation: Cells were harvested and re-plated onto 24-well plates (8000 viable cells/cm²) and allowed to grow in standard culture media until achieving 70-80% confluence. Wells were then transitioned to osteogenic medium (StemPro™ Osteogenesis Differentiation Kit, A1007201, Gibco®). Controls were maintained in expansion culture media. Culture media was replaced every 3 days.

Von Kossa staining was performed to assess for osteogenic differentiation and mineral matrix deposition after 21 days. Cells were fixated, dehydrated (increasing ethanol concentrations) and air-dried. Upon re-hydration, wells were incubated with 2% Silver nitrate solution (85193, Sigma Aldrich®), while exposed to Ultra-violet light. Afterwards, wells were incubated for 3 minutes in Sodium thiosulfate 5% (72049, Sigma Aldrich®). Wells were thoroughly rinsed, and photographic record obtained.

Adipogenic differentiation: As for the osteogenic lineage differentiation, cells were harvested and re-plated onto 24-well plates (8000 viable cells/cm²) and allowed to grow in standard culture media until achieving 70-80% confluence. At that moment, expansion media was replaced by adipogenic medium (StemPro® Adipogenesis Differentiation kit, A10070-01, Gibco®). Differentiation and control media were refreshed every 3 days.

After 14 days in culture, cells under adipogenic differentiation were fixated using 4% paraformaldehyde (3.7-4% buffered to pH7, 252931.1315, Panreac AppliChem®) and stained with Oil Red O (ORO)(O0625, Sigma-Aldrich®) working solution for 10-20 minutes. Wells were thoroughly washed to remove excess dye. Lipid droplet formation was observed in differentiated cells under the inverted microscope.

Chondrogenic differentiation: Chondrogenic differentiation was induced in micromass cultures, by seeding at 2x10⁴ viable cells/well at the centre the well on a 96-well plate. Cells were allowed to adhere and expanded for 48 hours before replacing expansion medium for chondrogenic specific culture medium (StemPro® Chondrogenesis Differentiation kit, A10071-01, Gibco®). Differentiation and control media were replaced every other day.

After 14 days, Alcian Blue stain was used to assess for proteoglycan's synthesis, indicative of chondrogenic matrix deposition. Cells were fixated and stained with Alcian Blue (A9186, Sigma®) solution. The dye was removed, and wells were repeatedly rinsed with acetic acid at 3% (v/v)(A6289, Sigma Aldrich®). Distilled water was added to neutralise acidity, and stained matrix was observed under the inverted microscope.

3. Results

3.1. Isolation of dental pulp tissue from fresh teeth (from sample transportation to Dental Pulp Stem/ Stromal Cells isolation and culturing)

A total of forty (40) tooth samples were donated and received at the laboratory and proposed for isolation, which included eighteen (18) incisor, nine (9) pre-molar, eight (8) molar and five (5) canine teeth.

Five (5) potential donors were excluded due to existing systemic diseases and five (5) due to extended transportation periods, exceeding 72 hours post collection. Further, incisor teeth were excluded due to the minimal amount of dental pulp amenable for collection for cellular isolation. Ten (10) samples of molar and pre-molar teeth were therefore processed.

Microbiological analysis of the tooth surface swabs after the decontamination protocol indicated two (2) positive (contaminated) samples. One (1) sample presented aerobic contamination (*Streptococcus mitis* / *Streptococcus oralis* and *Staphylococcus warmer*) and the second resulted from anaerobic organism (*Lactobacillus gasseri*), indicating a decontamination success of 80%.

Since microbiological analysis does not provide immediate results, all the samples were processed for dental pulp isolation through tissue explants culture. All but one sample resulted in successful migration of cells from the explant, presenting fibroblast-like shape, and rapidly proliferating through the culturing surface (**Figure 4**). The first cells migrating from the explant were recorded between the 10th and 15th days in culture (13.20 ± 2.10 days, $M \pm SD$). The sample that did not result in adherent cells isolation was cultured for a maximum of 25 days, with frequent medium refreshment. Cells were further allowed to migrate/ expand for additional 7-10 days (depending on the number of tissue fragments in the culture surface) and were then passaged for the first time, for further expansion. Cells directly resulting from explant migration were considered Passage 0 populations (P_0).

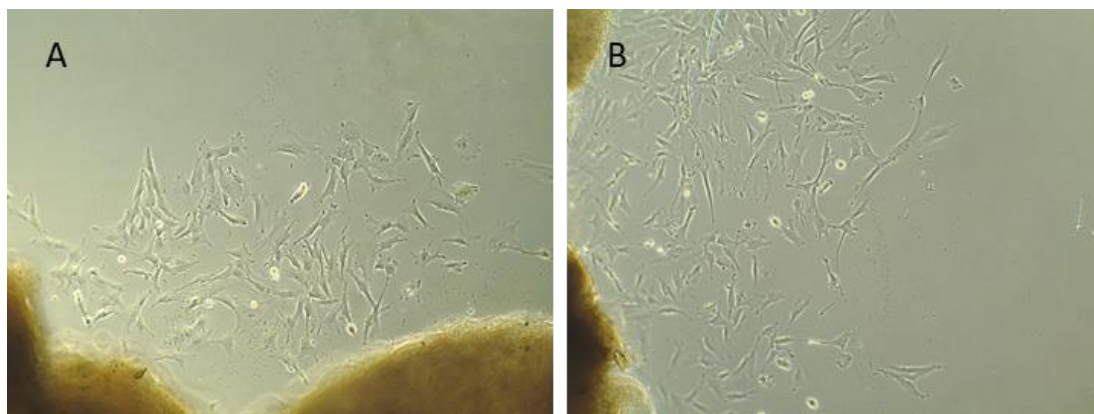


Figure 4: Explant isolation of adherent cells from dental pulp tissue fragments, after 17 days in culture (first evidence of adherent cells at the edge of the explant at day 15), Magnification 40x.

The sample that did not result in successful cell isolation presented signs of enamel damage and dental perforation and was later determined as positive in microbiologic control. Nevertheless, no signs of contamination in the explant culture were observed.

On the other hand, the remaining sample that was determined as positive in the microbiologic control did result in successful isolation of adherent and proliferative populations and did not display signs of culture contamination.

All samples were successfully detached and passaged to a larger vessel for further expansion (Passage 1).

3.2. Validation of isolated populations as dental derived Stem/ Stromal Cells

3.2.1. Growth dynamics and Population doubling times

Upon the first passage, two (2) samples were randomly selected from the samples presenting negative microbiologic control and expanded for further characterisation.

Cells at Passage 5 were individually counted and plated and counted daily, and their growth curves were plotted.

Both donors revealed very similar growth dynamics for up to 10 days. Culture observation indicated that complete confluence was reached after 7 days in expansion, although a slight increase in cell numbers was still accounted until the 10th day of counting (**Figure 5**). No statistical difference was identified in the cell yield from each

donor.

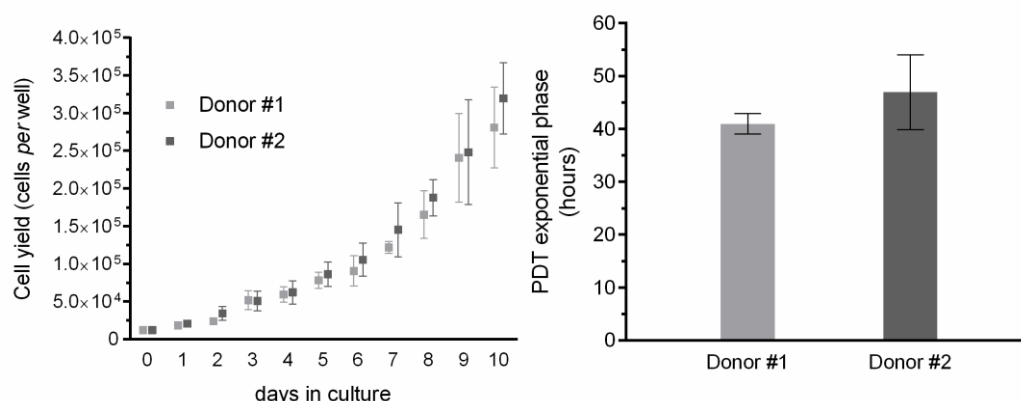


Figure 5: Growth curve of isolated cells at Passage 5, for up to 10 days of culture (left panel); Population Doubling Time of the exponential phase of cellular growth (2nd – 7th day) (right panel); results presented as Mean $\pm$ SD.

Population doubling times were calculated on the cell yield numbers for each day (**Table 2**). Between days 2 and 7 (the exponential phase of cellular growth), Donor #1 presented a PDT of 40.96 ± 1.91 hours, and Donor #2 presented PDT of 46.95 ± 7.06 hours. Additionally, the global doubling time of the exponential phase of cellular growth was calculated for each donor. No statistical difference was encountered on the PDT from each donor.

Table 2: Population Doubling Times of the 10 days of expansion of isolated cellular populations.

<i>Population Doubling Time (hours)</i>	day 1	day 2	day 3	day 4	day 5
Donor #1	51.12 ± 29.18	92.11 ± 92.27	25.12 ± 10.13	164.59 ± 145.81	87.64 ± 55.45
Donor #2	35.78 ± 14.49	96.04 ± 159.99	46.44 ± 17.57	96.50 ± 40.08	74.06 ± 50.35
<i>Population Doubling Time (hours)</i>	day 6	day 7	day 8	day 9	day 10
Donor #1	104.58 ± 129.92	68.39 ± 29.29	129.87 ± 135.77	101.46 ± 106.85	217.33 ± 227.06
Donor #2	91.97 ± 157.95	78.96 ± 70.79	109.07 ± 81.05	302.38 ± 509.45	171.84 ± 239.08

3.2.2. Karyotype analysis

In the chromosome analysis, a total of 17 metaphases were assessed. They all presented a total of 46 chromosomes (2n), and no numerical abnormalities were identified. Gender chromosomes indicated that the donors were male (XY) (**Figure 6**). No other alterations were identified in the addressed metaphases.

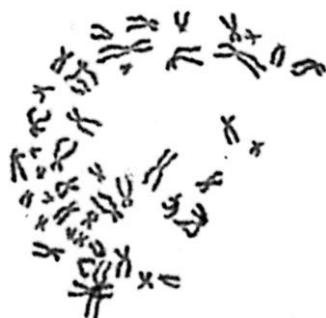


Figure 6: Chromosome analysis of isolated cells from Donor #2, depicting characteristic human karyotype (2n=46), of male gender (XY).

3.3. Mesenchymal Stem/ Stromal Cells Phenotypic identity

3.3.1. Flow cytometry for 'stemness' surface markers

Isolated cells were assessed for characteristic MSCs phenotypical markers, based on the recommendations of the ISCT (Dominici et al., 2006). Assessed populations presented $\geq 99\%$ positive cells for CD105 and CD44, $\geq 90\%$ positive staining for CD90, and $\leq 1\%$ global negative staining for CD34, CD11b, CD19, CD45 and MHC II (cocktail)(**Figure 7**).

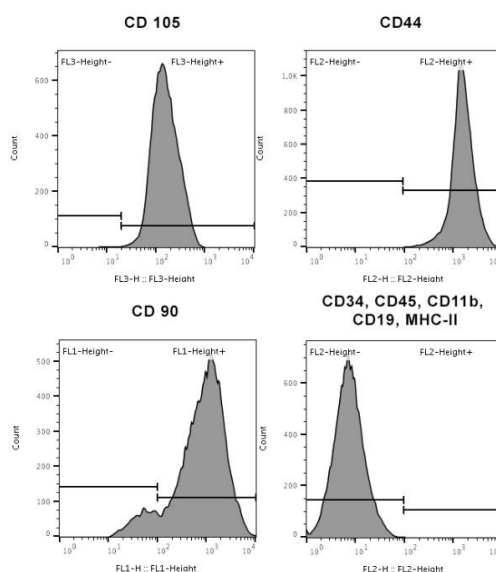


Figure 7: Surface marker expression for characteristic phenotypical markers of MSCs in the isolated population from Donor #1, assessed by Flow cytometry.

3.3.2. Immunocytochemistry for 'stemness' markers

The CytoBlock™ technique was used to suspend detached cells in a three-dimensional jellified matrix for the handling and staining of the cells through conventional immunocytochemical procedures. The isolated cells presented intense staining (indicated by the brown immunostaining) for vimentin and STRO-1, moderate to intense staining to NANOG-1, and weaker immunostaining for c-kit. As expected, the hematopoietic/ endothelial marker CD31 was not evidenced in the isolated populations (Figure 8).

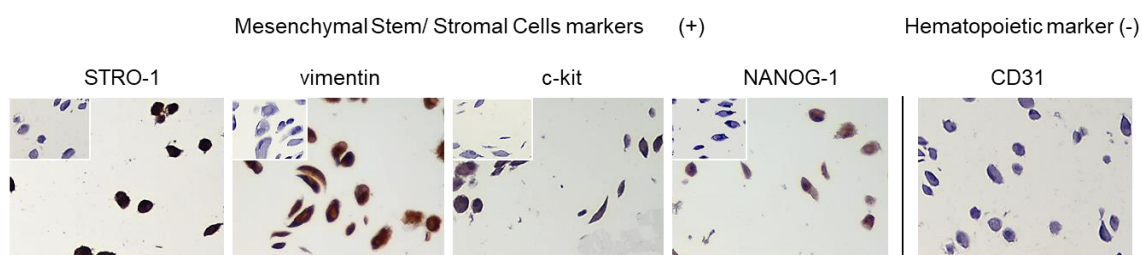


Figure 8: Immunocytochemical staining of isolated cells processed using the CytoBlock™ technique, for the detection of characteristic mesenchymal and 'stemness' markers (STRO-1, vimentin, c-kit, NANOG-1; negative controls in the upper left panel of each figure), and for the endothelial marker CD31; 200x magnification.

3.4. Multilineage differentiation

Differentiation capacity of the isolated cells was evaluated towards the three mesodermal lineages (Figure 9).

After 21 days in osteogenic medium, the culture populations demonstrated mineral matrix deposition, evidenced by the dark pigment that stained the cellular surface following the Von Kossa protocol. Macroscopic observation suggested no noteworthy staining of undifferentiated controls. Microscopic assessment confirmed the staining of mineralised matrix in the differentiated wells. The pigment was deposited within and above the cellular monolayer, formed by the continued proliferation (until fully confluent) of the differentiating cell verified after transition to the differentiation medium. Control wells presented complete coverage of the culture surface, with cellular staking on more than one layer. No evidence of dark pigment was confirmed in the undifferentiated controls. Macroscopic assessment did not indicate evident uptake of ORO by the cultured cells in either differentiated or control groups. However, microscopic amplification denoted occasional populations of cells depicting intracellular red-stained

vacuole. Suspended droplets of unwashed dye were still apparent but could be distinguished from the intracellularly fixed dye as they floated when hydrating medium was agitated. In the differentiated wells, cell confluency was of about 50%, indicating an arrest on cellular proliferation upon differentiation medium addition. Control wells presented the same multi-layered cellular coverage as the controls for the osteogenic differentiation, and no intracellular vacuole were observable.

In the chondrogenic differentiation wells, micromass cultures in the centre of the well further aggregated along the 14 days of differentiating culture. The micromass margins retracted, forming 'mountain-like' cellular masses that remained attached to the bottom of the well. In the control group, retraction and elevation phenomena were less pronounced. Indeed, cells proliferated slightly towards the margins of the well but did not reach to cover the culture surface. Microscopic observation of the Alcian Blue stained wells indicated intense staining of the differentiated wells, more pronounced towards the centre of the micromass, where cells assumed a more polygonal shape. In the controls, slight Alcian Blue retention was observed in the centre of the micromass, but cell retained an elongated spindle shape, stacked on countless superimposed layers.

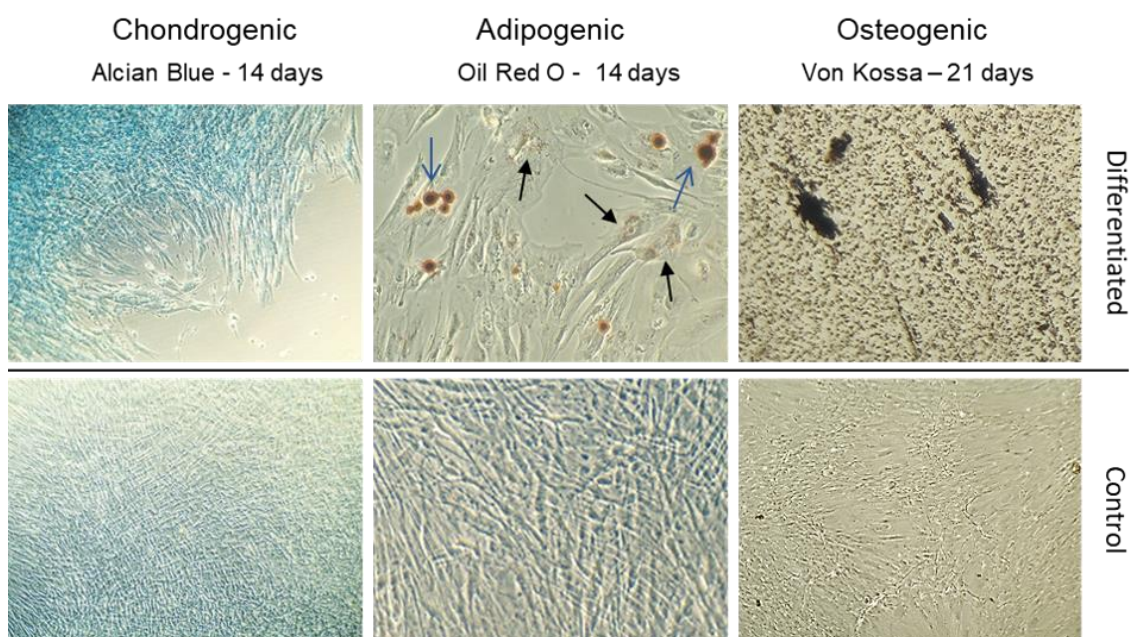


Figure 9: Multilineage differentiation of isolated cells into mesodermal lineages following culture in Chondrogenic (upper left panel), Adipogenic (upper entre panel) and Osteogenic (upper right panel) differentiating and control (lower panels, respectively) media; blue open arrow: Oil Red O droplets in suspension, black closed arrow: Oil Red O dye retention by intracellular lipid droplets; Magnification: 40x (Chondrogenic and Osteogenic) and 120x (Adipogenic).

4. Discussion and Conclusions

The currently presented protocols were developed aiming at the isolation of DPSCs, for the application in tissue regeneration strategies. The protocols were designed on the basis of available literature, in which interesting research has already been endured concerning isolation techniques, expansion media and supplementation, or even cryopreservation strategies.

The prepared protocols were successfully employed for the isolation of adherent fast proliferating population, that were later confirmed to display features characteristic of MSCs.

The selection criteria defined for patient selection resulted in ten (10) samples adequate for cellular isolation.

Due to the native commensal populations of the oral cavity, collected tooth samples are assumed as highly contaminated and transported accordingly. As recommended in the literature (Yildirim, 2013), the first decontamination step can be started on the transport media, adjusting the antibiotic and antimycotic components to 3% (regular supplementation is 1% for culturing purposes, when employed). Secondly, the thorough stripping of any tissue debris remaining from the extraction process should be scrapped down and, finally, the smooth surface wiped with disinfecting solution (70% ethanol or other aseptic solution). By combining the three-step decontamination, an 80% success rate was achieved, as only 2 (two) of the 10 (ten) samples assessed presented microbial growth. Remarkably, none of the isolated populations displayed sign of contamination while in culture, although this is regarded as a limitation of DPSCs isolation and culture, even when employing standard antibiotic and antimycotic concentrations (1%)(Perry et al., 2008).

Although teeth with the most robust anatomy (molar and pre-molar teeth) were selected, the amount of pulp tissue isolated from each sample was very limited, which led to the selection of the outgrowth (explant) as the method of isolation. Although specific literature is dominated by the enzymatic isolation protocols (as summarised in the first part of this Chapter), the choice of isolation method does not impact on the isolated DPSCs features (Hilkens et al., 2013), and the explant method proved to be adequate for the defined purpose.

As described, the major downside is the extended time required for the detection of first adherent cells and subsequent proliferation, which in turn bares increased contamination risks. The first cells were observed in the explant's borders between the 10th and 15th days, which is consistent with the lowest limits of the times reported in literature (Alliot-Licht et al., 2005, Pierdomenico et al., 2005, Struys et al., 2010, Ikeda et al., 2011), that can go as long as 21 days (Lizier et al., 2012). Only one (1) of the ten (10) samples did not successfully result in cell isolation, resulting in a 90% success rate of the proposed protocol. The sample that did not result in cell isolation presented signs of enamel damage and dental perforation and later came as positive in microbiologic control. Nevertheless, no signs of contamination in the explant culture were observed.

The isolated populations were further expanded and characterised and demonstrated to comply with the reported features of DPSCs isolated by other research groups. They depicted the classical 'fibroblast-like' shape (although narrower and more elongated than the UC-MSCs usually handled in the laboratory), and were firmly adherent to the culture surface, resisting to mechanical stress (from the culture washing with DPBS) and trypsin-induced detachment. It was experienced the need to perform two (2) to three (3) washes with DPBS prior to trypsin addition (versus the one (1) wash generally required for cell detachment) to maintain the incubation time within 3 minutes and avoid cellular membrane damage and loss of cell viability.

The isolated cells also displayed the expected proliferative dynamics, with PDT under 50 hours at P5. Additionally, chromosomal analysis confirmed the stability of the isolated cells, presenting the 46 chromosomes (2n) characteristic of the human species, absent of any numerical or structural abnormalities (Suchánek et al., 2007).

Isolated cells were assessed for characteristic MSCs phenotypical markers, based on the recommendations issued by the experts panel of the ISCT (Dominici et al., 2006) which proposed that populations had to be adherent to plastic substrates when maintained in standard culture conditions using tissue culture flasks, to express specific surface antigen (Ag) identity, namely, be mostly positive for CD105, CD73 and CD90, and lack major expression of CD45, CD34,

CD14/ CD11b, CD79a/ CD19 and HLA class II, assessed by flow cytometry. Additionally, these cells should be capable of at least undergo trilineage differentiation into osteogenic, adipogenic and chondrogenic phenotypes.

The populations herein isolated did majorly comply with the listed requisites and can, therefore, be classified as MSCs. The isolated cells exceeded $\geq 99\%$ population expression for CD105 and CD44 (an alternative to CD73), $\geq 90\%$ expression for CD90, and $\leq 1\%$ global negative expression of CD34, CD11b, CD19, CD45 and MHC II (negative identity cocktail).

In parallel, an immunocytochemical study of the isolated cells was conducted, where the mesenchymal nature of the isolates (staining positive for vimentin) and the absence of staining for the hematopoietic marker CD31 (which assumes great relevance in the profiling of blood or marrow-derived isolates) were confirmed.

The populations also presented positivity to additional markers often associated with MSCs pluripotency: STRO-1, NANOG-1 and c-kit. NANOG-1 expression in MSCs is associated with (although not directly regulating) the transition from *in vivo* quiescence to adaptation to *in vitro* growth condition, being detected only in proliferating cells, and not in MSCs induced to differentiate (Pierantozzi et al., 2011). STRO-1 expression has been reported in 9 to 23% of isolated DPSCs (Shi and Gronthos, 2003, Wei et al., 2007, Lee et al., 2010, Bakopoulou et al., 2011), considerably relating to their colony-forming capacity (Shi and Gronthos, 2003). A weaker positive expression of c-kit was also observed. This gene is associated with high proliferative profiles as well as to multilineage differentiation capacity (Blazquez-Martinez et al., 2014).

Finally, the populations demonstrated to differentiate towards the three (3) representative mesodermal lineages. The DPSCs are described as particularly prone to osteogenic differentiation (Gronthos et al., 2000, Struys et al., 2010). Indeed, Bakopoulou and colleagues reported spontaneous differentiation of cultured DPSCs in standard culture (control) conditions (Bakopoulou et al., 2011), which was not observed in the isolated populations. The very discrete development of intracellular lipidic vesicles herein observed upon adipogenic differentiation suggests that, in line with the other reported works, DPSCs do

differentiate toward adipocyte, but might do so with limited efficiency (Struys et al., 2010).

The isolated and characterised cells have been utilised in routine laboratory assays, presenting >90% viability upon thawing, and maintaining their proliferative, phenotypic and differentiation capacities throughout.

Therefore, the proposed protocols were herein validated for the isolation of MSCs populations from fresh adult dental pulp tissue, which were further explored for their potential towards the clinical application in advanced cell-based regenerative techniques.

2.3. References

- Alliot-Licht, B., G. Bluteau, D. Magne, S. Lopez-Cazaux, B. Lieubeau, G. Daculsi and J. Guicheux (2005). Dexamethasone stimulates differentiation of odontoblast-like cells in human dental pulp cultures. Cell Tissue Res **321**(3): 391-400.
- Bakopoulou, A., G. Leyhausen, J. Volk, A. Tsiftoglou, P. Garefis, P. Koidis and W. Geurtsen (2011). Comparative analysis of in vitro osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP). Arch Oral Biol **56**(7): 709-721.
- Blazquez-Martinez, A., M. Chiesa, F. Arnalich, J. Fernandez-Delgado, M. Nistal and M. P. De Miguel (2014). c-Kit identifies a subpopulation of mesenchymal stem cells in adipose tissue with higher telomerase expression and differentiation potential. Differentiation **87**(3-4): 147-160.
- Caplan, A. I. (2009). Why are MSCs therapeutic? New data: new insight. The Journal of Pathology **217**(2): 318-324.
- Caplan, A. I. and J. E. Dennis (2006). Mesenchymal stem cells as trophic mediators. Journal of Cellular Biochemistry **98**(5): 1076-1084.
- Caseiro, A. R., T. Pereira, G. Ivanova, A. L. Luis and A. C. Mauricio (2016). Neuromuscular Regeneration: Perspective on the Application of Mesenchymal Stem Cells and Their Secretion Products. Stem Cells Int **2016**: 9756973.
- Chamberlain, G., J. Fox, B. Ashton and J. Middleton (2007). Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells **25**(11): 2739-2749.
- da Silva Meirelles, L., P. C. Chagastelles and N. B. Nardi (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. Journal of Cell Science **119**(11): 2204-2213.
- Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop and E. Horwitz (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy **8**(4): 315-317.
- Fossett, E. and W. S. Khan (2012). Optimising human mesenchymal stem cell numbers for clinical application: a literature review. Stem Cells International **2012**: 465259.
- Friedenstein, A., R. Chailakhjan and K. Lalykina (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Proliferation **3**(4): 393-403.

- Friedenstein, A. J., K. V. Petrakova, A. I. Kurolesova and G. P. Frolova (1968). Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation **6**(2): 230-247.
- Gärtner, A., T. Pereira, R. Gomes, A. L. Luís, M. França, S. Geuna, P. A. S. Armada-da-Silva and A. C. Maurício (2013). Mesenchymal Stem Cells from Extra-Embryonic Tissues for Tissue Engineering – Regeneration of the Peripheral Nerve. Advances in Biomaterials Science and Biomedical Applications. R. Pignatello, InTech.
- Gartner, A., T. Pereira, M. J. Simoes, P. A. Armada-da-Silva, M. L. Franca, R. Sousa, S. Bompasso, S. Raimondo, Y. Shirosaki, Y. Nakamura, S. Hayakawa, A. Osakah, B. Porto, A. L. Luis, A. S. Varejao and A. C. Mauricio (2012). Use of hybrid chitosan membranes and human mesenchymal stem cells from the Wharton jelly of umbilical cord for promoting nerve regeneration in an axonotmesis rat model. Neural Regen Res **7**(29): 2247-2258.
- Gronthos, S., M. Mankani, J. Brahimi, P. G. Robey and S. Shi (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A **97**(25): 13625-13630.
- Hilkens, P., P. Gervois, Y. Fanton, J. Vanormelingen, W. Martens, T. Struys, C. Politis, I. Lambrechts and A. Bronckaers (2013). Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells. Cell and tissue research **353**(1): 65-78.
- Ikeda, H., Y. Sumita, M. Ikeda, H. Ikeda, T. Okumura, E. Sakai, M. Nishimura and I. Asahina (2011). Engineering bone formation from human dental pulp- and periodontal ligament-derived cells. Ann Biomed Eng **39**(1): 26-34.
- Kawashima, N. (2012). Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? Archives of Oral Biology **57**(11): 1439-1458.
- Kim, D. W., M. Staples, K. Shinozuka, P. Pantcheva, S. D. Kang and C. V. Borlongan (2013). Wharton's Jelly-Derived Mesenchymal Stem Cells: Phenotypic Characterization and Optimizing Their Therapeutic Potential for Clinical Applications. International Journal of Molecular Sciences **14**(6): 11692-11712.
- Lajtha, L. G. (1967). Stem cells and their properties. Proc Can Cancer Conf **7**: 31-39.
- Le Blanc, K. and O. Ringden (2005). Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant **11**(5): 321-334.
- Lee, S. Y., P. C. Chiang, Y. H. Tsai, S. Y. Tsai, J. H. Jeng, T. Kawata and H. M. Huang (2010). Effects of cryopreservation of intact teeth on the isolated dental pulp stem cells. J Endod **36**(8): 1336-1340.

- Lizier, N. F., A. Kerkis, C. M. Gomes, J. Hebling, C. F. Oliveira, A. I. Caplan and I. Kerkis (2012). Scaling-up of dental pulp stem cells isolated from multiple niches. PLoS One **7**(6): e39885.
- Pereira, T., P. A. Armada-da Silva, I. Amorim, A. Rema, A. R. Caseiro, A. Gartner, M. Rodrigues, M. A. Lopes, P. J. Bartolo, J. D. Santos, A. L. Luis and A. C. Mauricio (2014). Effects of Human Mesenchymal Stem Cells Isolated from Wharton's Jelly of the Umbilical Cord and Conditioned Media on Skeletal Muscle Regeneration Using a Myectomy Model. Stem Cells Int **2014**: 376918.
- Pereira, T., A. Gartner, I. Amorim, A. Almeida, A. R. Caseiro, P. A. Armada-da-Silva, S. Amado, F. Fregnan, A. S. Varejao, J. D. Santos, P. J. Bartolo, S. Geuna, A. L. Luis and A. C. Mauricio (2014). Promoting nerve regeneration in a neurotmesis rat model using poly(DL-lactide-epsilon-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly: in vitro and in vivo analysis. Biomed Res Int **2014**: 302659.
- Pereira, T., G. Ivanova, A. R. Caseiro, P. Barbosa, P. J. Bartolo, J. D. Santos, A. L. Luis and A. C. Mauricio (2014). MSCs Conditioned Media and Umbilical Cord Blood Plasma Metabolomics and Composition. PLoS One **9**(11): e113769.
- Perry, B. C., D. Zhou, X. Wu, F. C. Yang, M. A. Byers, T. M. Chu, J. J. Hockema, E. J. Woods and W. S. Goebel (2008). Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. Tissue Eng Part C Methods **14**(2): 149-156.
- Pierantozzi, E., B. Gava, I. Manini, F. Roviello, G. Marotta, M. Chiavarelli and V. Sorrentino (2011). Pluripotency Regulators in Human Mesenchymal Stem Cells: Expression of NANOG But Not of OCT-4 and SOX-2. Stem Cells and Development **20**(5): 915-923.
- Pierdomenico, L., L. Bonsi, M. Calvitti, D. Rondelli, M. Arpinati, G. Chirumbolo, E. Becchetti, C. Marchionni, F. Alviano and V. Fossati (2005). Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation **80**(6): 836-842.
- Ribeiro, J., A. Gartner, T. Pereira, R. Gomes, M. A. Lopes, C. Goncalves, A. Varejao, A. L. Luis and A. C. Mauricio (2013). Perspectives of employing mesenchymal stem cells from the Wharton's jelly of the umbilical cord for peripheral nerve repair. Int Rev Neurobiol **108**: 79-120.
- Ribeiro, J., T. Pereira, I. Amorim, A. R. Caseiro, M. A. Lopes, J. Lima, A. Gartner, J. D. Santos, P. J. Bartolo, J. M. Rodrigues, A. C. Mauricio and A. L. Luis (2014). Cell therapy with human MSCs isolated from the umbilical cord Wharton jelly associated

- to a PVA membrane in the treatment of chronic skin wounds. Int J Med Sci **11**(10): 979-987.
- Ribeiro, J., T. Pereira, A. R. Caseiro, P. Armada-da-Silva, I. Pires, J. Prada, I. Amorim, S. Amado, M. Franca, C. Goncalves, M. A. Lopes, J. D. Santos, D. M. Silva, S. Geuna, A. L. Luis and A. C. Mauricio (2015). Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells. World J Stem Cells **7**(6): 956-975.
- Shi, S. and S. Gronthos (2003). Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. Journal of bone and mineral research **18**(4): 696-704.
- Silva, D. M., A. R. Caseiro, I. Amorim, I. Pereira, F. Faria, T. Pereira, J. D. Santos, F. M. Gama and A. C. Mauricio (2016). Inflammatory response to dextrin-based hydrogel associated with human mesenchymal stem cells, urinary bladder matrix and Bonelike(R) granules in rat subcutaneous implants. Biomed Mater **11**(6): 065004.
- Struys, T., M. Moreels, W. Martens, R. Donders, E. Wolfs and I. Lambrichts (2010). Ultrastructural and immunocytochemical analysis of multilineage differentiated human dental pulp- and umbilical cord-derived mesenchymal stem cells. Cells Tissues Organs **193**(6): 366-378.
- Suchánek, J., T. Soukup, R. Ivancáková, J. Karbanová, V. Hubková, R. Pytlík and L. Kucerová (2007). Human dental pulp stem cells--isolation and long term cultivation. Acta medica (Hradec Králové)/Universitas Carolina, Facultas Medica Hradec Králové(50): 195-201.
- Triffitt, J. T. (2002). Stem cells and the philosopher's stone. Journal of Cellular Biochemistry. Supplement **38**: 13-19.
- Wei, X., J. Ling, L. Wu, L. Liu and Y. Xiao (2007). Expression of mineralization markers in dental pulp cells. J Endod **33**(6): 703-708.
- Woods, E. J., B. C. Perry, J. J. Hockema, L. Larson, D. Zhou and W. S. Goebel (2009). Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. Cryobiology **59**(2): 150-157.
- Yildirim, S. (2013). Isolation methods of dental pulp stem cells. Dental pulp stem cells, Springer: 41-51.

Chapter III

Xeno-free cellular systems for clinical application in Regenerative Medicine

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Introductory note

The emergence of MSCs as one of the pillars for advanced therapeutic systems entailed the necessity to develop and optimise isolation, expansion, and cryopreservation protocols. The MSCs-based systems are perceived by the European Medicines Agency (EMA) and European Commission regulation [EC 1394/2007 of the European Commission] as medicinal products, and must therefore comply with good manufacturing practices (GMP), amongst other requisites (Fekete et al., 2012), implying the utilization of fully traceable clinical grade and xeno-free reagents (Bieback et al., 2009, Pereira et al., 2014, Pham et al., 2014). The guidelines that apply to human-derived cellular products are clearly defined, but the standards applicable to veterinary products still care for definition by the international regulatory agencies. The EMA's Committee for Medicinal Products for Veterinary Use (CVMP) has recently created the Ad Hoc Expert Group on Veterinary Novel Therapies (ADVENT), whose purpose is to defined adequate regulatory references for the market approval of *nouvel* therapeutic approaches, in which Veterinary MSCs systems are included.

Widespread methods for mammalian cell culture in research rely on Foetal Bovine Serum (FBS) supplementation, which however bare noteworthy concerns when aiming at clinical application, namely the potential immunogenicity and infectiousness (Friedenstein et al., 1970, Phinney, 2007, Phinney and Prockop, 2007, Simonetti et al., 2007, Tekkatte et al., 2011, Pereira et al., 2014, Pereira et al., 2014), as well as the heavy ethical burden on animal welfare (Pereira et al., 2014). Completely serum-free culturing conditions and methods are being explored, but the general unawareness of the detailed composition of FBS makes it extremely challenging to develop synthetic versions containing each particular compound in suitable concentrations (Van der Valk et al., 2010).

These concerns foster the search for substituent cell culture medium supplements, such as the umbilical cord blood serum or plasma (UCBS or UCBP, respectively). These haemoderivate products are suggested to provide the essential factors for MSCs culturing (Bieback et al., 2009, Huang et al., 2011, Hong et al., 2014), addressing many of the disadvantages of the FBS. Additionally, the umbilical cord blood (UCB) can be safely collected from both human and veterinary species upon birth (Bartholomew et al., 2009, Sultana et al., 2018, Roberts et al., 2019), deeming it an interesting option for the development of xeno-free systems for the translation into clinical application in both medical fields.

In this Chapter, the potential of the human UCBP for the development of MSCs-based therapeutic systems is addressed. Particularly, the metabolite content of hUCBP is investigated through Nuclear Magnetic Resonance (NMR). Further, the FBS substitution by hUCBP is explored for UC-MSCs and DPSCs culturing, enlightening on its impact on cell viability and senescence, surface marker and gene expression, and tri-lineage differentiation capacity. An additional study on the detailed bioactive factors content of the proposed supplement is presented, to further comprehend the growth factors and cytokines combination provided to culturing cells.

3.1. Metabolomic profile of human Umbilical Cord Blood Plasma and Foetal Bovine Serum, and their effects on Mesenchymal Stem/ Stromal Cells populations

**Human Umbilical Cord Blood Plasma as an Alternative to Animal Sera for
Mesenchymal Stromal Cells *In vitro* Expansion for Clinical Applications –
a Multicomponent Metabolomic Analysis**

by AR Caseiro, G Ivanova, SS Pedrosa, MV Branquinho, P Georgieva, PP Barbosa,
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RESEARCH ARTICLE

Human umbilical cord blood plasma as an alternative to animal sera for mesenchymal stromal cells *in vitro* expansion – A multicomponent metabolomic analysis

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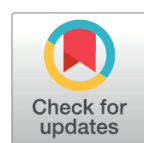
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Abstract

Mesenchymal Stromal cells (MSCs) have a potential role in cell-based therapies. Foetal bovine serum (FBS) is used to supplement the basal cell culture medium but presents several disadvantages and risks. Other alternatives have been studied, including human umbilical cord blood plasma (hUCBP), aiming at the development of xeno-free culturing protocols. A comparative characterization of multicomponent metabolic composition of hUCBP and commercial FBS based on Nuclear Magnetic Resonance (NMR) spectroscopy and multivariate statistical analysis was performed. The analysis of ¹H-NMR spectra revealed both similarities and differences between the two proposed supplements. Similar metabolites (amino acids, glucose, lipids and nucleotides) were found in the hUCBP and FBS NMR spectra. The results show that the major difference between the metabolic profiles of the two proposed supplements are due to the significantly higher levels of glucose and lower levels of lactate, glutamate, alanine and branched chain amino acids in hUCBP. Similar or slightly different levels of important proteinogenic amino acids, as well as of nucleotides, lipids were found in the hUCBP and FBS. In order to validate its suitability for cell culture, umbilical cord-MSCs (UC-MSCs) and dental pulp stem cells (DPSCs) were expanded using hUCBP. In both hMSCs, *in vitro* culture with hUCBP supplementation presented similar to improved metabolic performances when compared to FBS. The two cell types tested expressed different optimum hUCBP percentage content. For DPSCs, the optimum hUCBP content was 6%



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and for UC-MSCs, 4%. Cultured hMSCs displayed no changes in senescence indicators, as well as maintained characteristic surface marker's expression. FBS substitution was associated with an increase in early apoptosis events, in a dose dependent manner, as well as to slight up- and down-regulation of targeted gene's expression. Tri-lineage differentiation capacity was also influenced by the substitution of FBS by hUCBP.

1. Introduction

Mesenchymal Stem / Stromal Cells are gaining ground as a source of innovative advanced therapeutic strategies for a number of diseases, with hundreds of ongoing or recruiting trials involving application of Human Mesenchymal Stromal Cells (hMSCs) (www.clinicaltrials.gov; database accessed on January 2018). As such, the scientific community has focused on developing and optimising protocols for their isolation, expansion, and cryopreservation aiming at their effective application in state of the art therapeutic approaches to relevant clinical situations. MSCs can be isolated from many tissue sources, such as the bone marrow, peripheral blood, dental pulp, umbilical cord, and amniotic fluid [1]. Human MSCs characteristics have been defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [2] as being plastic-adherent when maintained in standard culture conditions, expressing cluster of differentiation (CD) 105, CD73 and CD90, and lacking expression of CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR surface molecules. Finally, the hMSCs are able to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*, in the presence of adequate differentiation media [3].

The umbilical cord tissue or matrix (Wharton jelly–UCT) is an important source of MSCs, baring advantages over other sources for being non-invasively collected (previously considered as medical waste). Also, increasing numbers of high quality cryopreserved samples worldwide, in private and public cryopreservation banks, and the ready availability for therapeutic usage [4–12] adds a further advantage. Dental derived stem cells are other population with increasing relevance, mostly due to the increasing availability of cryopreservation options, for autologous and allogeneic applications, in both infants and adult individuals.

According to the European Medicines Agency (EMA) and European Commission regulation [EC 1394/2007 of the European Commission], hMSCs are considered as medicinal products and must be produced in compliance with good manufacturing practices (GMP) [13]. For that purpose, stem cells from either source must be cultured on large scale, and isolated, processed and cryopreserved according to GMP conditions. This implies the usage of xeno-free medium and reagents of clinical grade for the cell culture, for quality assurance and control, and long term rastreability of the samples [8, 14, 15].

Currently available hMSCs expansion protocols use foetal bovine serum (FBS) and other animal sera for culture media supplementation, providing vital nutrients and growth factors. These also act as a pH buffering agent, and provide protection against cytotoxic components, playing a crucial role in hMSCs survival. Nonetheless, there are several concerns on the use of FBS for clinical applications, due to the potential immunogenicity of FBS cultured cells and of xenogeneic proteins present in the cell culture, capable of inducing anaphylactic or *arthus*-like immune reactions, which have been reported. Notwithstanding this, FBS-cultured hMSCs have been approved by the US Food and Drug Administration (FDA) for medical applications [16, 17]. Complications of using animal sera for *in vitro* cell culture also include high batch-to-batch variability, unexpected cell growth characteristics, cytotoxicity of uncharacterized factors

in the serum, and the risk of possible contamination with virus, prions, bacteria, nanobacteria, mycoplasma, yeast, *fungi*, and endotoxins [1, 7, 8, 18–21]. The use of animal sera also implies the sacrifice of a huge number of young animals for blood collection, entailing severe economic, ethical and scientific issues on animal welfare [8].

These issues reinforce the intensive search for new alternatives to animal sera to better comply with GMP and FDA regulations (EMA CPMP/BWP/1793/02; 2003; EMA/410/01 rev. 2; 2004) [22, 23]. The substituent cell culture medium supplement must match the animal sera characteristics crucial for *ex vivo* cell culture, while maintaining a fair cost, being suitable for safety and quality control, and readily/easily available. A variety of human blood products have been already considered as animal sera substitutes [15, 24–26]. Amongst them, human umbilical cord blood serum or plasma (hUCBS or hUCBP, respectively), due to its rich soluble growth factors' content. These soluble factors support the growth, proliferation, and differentiation of hMSCs and HSCs [15, 26, 27], with potential pre-clinical and clinical applications [6–8, 14, 28]. The implementation of hUCBS or hUCBP could solve important economic, ethical and scientific issues associated with FBS supplementation in cell-based therapies development. Moreover, the hUCBS and hUCBP are attractive alternatives to FBS due to the worldwide increase number of cryopreserved UCB units in Public and Private Cord Blood Banks, following high quality standards imposed by the international and national authorities [4–9, 29].

The successful employment of hUCBP can result in the preparation of fully autologous cellular therapies, for patients with cryopreserved serum/plasma and cells or tissue. Alternatively, once the regulatory requisites are established, it can be envisioned for the preparation of allogeneic cellular therapies. This would bring increased benefits for a larger number of patients, and turning cellular therapies promptly available, allowing medical action in optimal 'windows of therapeutic opportunity' for numerous diseases. Additionally, it would contribute to the broadening of possible beneficiaries for such therapies, including people that might have not had the chance to preserve autologous samples; and that may be in need of these advanced therapeutic strategies (for example, suffering from age-related or degenerative diseases).

In a previous study, we have identified specific components and factors (proliferative and anti-apoptotic growth factors, immunomodulatory and immunosuppressive cytokines and chemokines) in hUCBP that could have promoting effects on hMSCs *in vitro* expansion. The results strongly suggest that hUCBP might be an alternative for the FBS supplemented culture medium used in hMSCs *in vitro* isolation, expansion and cryopreservation [4, 8]. This assumption converges with the recently published results on the effect of haemoderived supplements on the hMSCs growth rate, viability and differentiation properties [15, 26, 30–33]. For this purpose, a detailed analysis of the hUCBP components is crucial to validate this human haemoderivate by-product as a supplement for *ex vivo* expansion and processing of hMSCs for clinical applications.

Metabolomics is an important and well-established analytical approach for the comprehensive and systematic determination of metabolite levels in biological samples. Nuclear magnetic resonance (NMR) spectroscopy is one of the main tools for metabolic profiling, identification and quantification of known and unknown metabolites in bio-fluids, tissues and cells. [34–36]. All metabolomics studies including NMR-metabolomics, result in a large number of complex multivariate data sets that require chemometric and bio-informatics methods for accurate data interpretation, visualization and analysis [37, 38]. Data-reduction and pattern recognition techniques such as principal component analysis (PCA) allow for analysis, interpretation and classification of the large number of complex NMR spectroscopic data usually acquired for metabolic profiling of biological samples [34–36, 39, 40].

The first part of this study aims at the characterization of the metabolite composition of hUCBP, to address its capacity as a culture medium supplement and FBS substituent to

support xeno-free *in vitro* proliferation of hMSCs. The second part of the study aimed at the validation of hUCBP substitution for the expansion of umbilical cord (UC-MSCs) and dental pulp- derived mesenchymal stem cells (DPSCs), and its effects on cell viability and senescence, surface marker- and gene expression, as well as on their tri-lineage differentiation capacity.

2. Material and methods

The hUCB samples included in the present study were processed and cryopreserved by Biosskin, Molecular and Cell Therapies S.A. (Maia, Portugal), which is an authorized Portuguese Private Cord Blood Bank for UCB processing and cryopreservation by the Direção-Geral de Saúde (DGS), and certified for ISO9001 and for NP4457 (Authorization from DGS in attachment). The hUCB was collected from healthy donors that signed a contract with Biosskin, Molecular and Cell Therapies S.A. for processing and cryopreserved the buffy coat of the collected umbilical cord blood where the hematopoietic stem cells are included. On the other hand, the hUCB plasma is not cryopreserved and it is a by-product which is not cryopreserved and it is usually destroyed. The present experimental work used those hUCB plasmas and it should be emphasized that the donors that have this contract with Biosskin, Molecular and Cell Therapies S.A. authorize the use of these by-products for research. Patient records used in our retrospective study were fully anonymized before the authors accessed the data for the present study and the ethics committee waived the requirement for informed consent. Also, there is a scientific protocol / contract between the University of Porto and the Biosskin, Molecular and Cell Therapies S.A., which includes I&D projects, PhD and master supervision of students, among other training and teaching activities. For that reason, it was not necessary to submit this study to approval of the ethical commission.

2.1. Study design

In the first part of the study, appropriate 1D and 2D NMR techniques have been used to identify metabolites in the hUCBP (a total number of 13 samples collected from healthy donors) and two commercial FBS products (FBS_I and FBS_II), and to define their relative quantitative distribution. Principal component analysis has been used to interpret and classify the NMR spectral data and to evaluate the main factors contributing to the discrimination between these two groups of samples. Prior to NMR analysis, a complete characterization of the hUCB samples was performed (haematology for total nucleated cells (TNC) and white blood cells (WBC) count, flow cytometry for CD45⁺ / CD34⁺ cell counts, and cell viability), accompanied by pre-birth and immediate after-birth maternal screening for relevant transmittable pathogens, including Cytomegalovirus, Venereal disease/ Syphilis, Hepatitis B and C, HIV-I and II, Toxoplasmosis (and Human T-Cell Lymphotropic Virus Types I and II (HTLV-I/-II), if relevant). Microbiological analysis was performed to hUCB units after volume reduction using an automated blood culture system for aerobic and anaerobic microorganisms and *fungi*.

In the second part of the study, the capacity of hUCBP to sustain both hMSCs expansion was assessed with PrestoBlue[®] viability assay at different time points [24h, 72h, 120h, 168h, 216h], using umbilical cord-MSCs (UC-MSCs) and dental pulp stem cells (DPSCs). Crescent concentrations of hUCBP were added as a supplement of the basal culture medium [2%, 4%, 6%, 8%], and compared with FBS_II at 10%, generally used standard culturing protocols. The effects of the supplement substitution on hMSCs cultures were assessed regarding senescence and apoptosis events, hMSCs' phenotypical identity/ surface marker and gene expression, as well as tri-lineage differentiation capacity.

2.2. Ethics and regulation

The hUCB included in the present study were processed and cryopreserved by Biosskin, Molecular and cell Therapies S.A. (Maia, Portugal), an authorized Portuguese Private Cord Blood Bank for UCB processing and cryopreservation by the Direcção-Geral de Saúde (DGS), and certified for ISO9001 and for NP4457. The hUCB was collected from healthy donors, according to Netcord guidelines and following Portuguese laws N° 12/2009 and N° 1/2015 (Diário da República, lei 12/2009 de 26 de Março de 2009 and Diário da República, lei 1/2015 de 8 de Janeiro de 2015), with written informed consents according to Directive 2004/23/EC, which sets the standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells.

2.3. Preparation of umbilical cord plasma

Thirteen samples of hUCBP collected from different healthy donors were used for ¹H-NMR analysis (N = 13). The hUCBP samples were analysed by haematology auto-analyser, flow cytometry and for microbiological contamination for aerobic and anaerobic microorganisms and *fungi*. A pool from 12 out of 13 of these hUCBP (N = 12) was used for the *in vitro* validation with UC-MSCs and DPSCs viability assessment.

2.4. Donors selection and umbilical cord blood (UCB) collection

Maternal and neonatal pairs were evaluated during the antenatal period in the maternity wards at different hospitals collaborating with the Umbilical Cord Blood Private Bank of Biosskin, Molecular and Cell Therapies, S.A. (Maia, Portugal). Donors signed informed consent before delivery and were clinically evaluated according to Portuguese law (Diário da República, lei N° 12/2009 de 26 de Março de 2009 and lei N° 1/2015 de 8 de Janeiro de 2015). Human UCB was collected from the umbilical vein by gravity into a 150 ml volume simple bag (reference #1385.13, Suru, Portugal) containing 21 ml of citrate-phosphate-dextrose (CPD). The hUCB was stored at 4°C ± 2°C until processing for cryopreservation. Human UCB samples were transported to the Biosskin, Molecular and Cell Therapies S.A. laboratory at refrigerated temperatures ranging between 4°C and 22°C, within 72 hours of collection. The collection method was the same for all the units included in this study.

2.5. Volume reduction with the AXP automated system

The AXP system[®] (Thermogenesis) was used to achieve the separation of a concentrated mononuclear cell (MNC) fraction of uniform volume. During the two-step centrifugation, whole blood is separated into three layers that are delivered into a red blood cells (RBC) bag and a freezing bag. Plasma remains in the processing bag which is also the plasma bag. The programmed final volume in the cryopreservation bag was 21 ml. Samples of the hUCB were taken by sampling pillows integrated within the kit for hematology auto-analyzer and flow cytometry analysis [8, 41–43].

2.6. Haematology auto-analyzer, flow cytometry and microbiological analysis of the UCB

Maternal screening for Cytomegalovirus, Venereal disease/ Syphilis, Hepatitis B and C, HIV-I and II, Toxoplasmosis (and Human T-Cell Lymphotropic Virus Types I and II (HTLV-I/-II), if relevant) was mandatory for sample acceptance for processing. All cryopreserved samples were negative to the screened agents. Microbiological analysis was performed after volume reduction and before cryopreservation (samples are maintained in quarantine until final

results are disclosed) using an automated blood culture system (BacT/ALERT[®], BioMérieux) at 35°C for 14 days, and included microbiologic analysis for aerobic and anaerobic microorganisms and *fungi*. For the UCB units that presented microbial contamination, the microorganism was identified and was performed the antibiogram for identification of the antibiotic sensitivity of the identified microorganisms [8].

Total nucleated cells (TNC) and the number of white blood cells (WBC) were counted with a hematology auto-analyzer (Ac T diff[™], Beckman Coulter, Inc.) before and after the AXP volume reduction procedure. These counts were not corrected for nucleated RBC. The CD34⁺ cell number and viability were quantified by flow cytometry (BD FACSCalibur[™] 3 CA Becton Dickinson, BD Biosciences), the software for acquisition and analysis were BD CellQuest[™] and BDCellQuest Pro Templates, respectively. The clusters of differentiation used to enumerate the total number of CD34⁺ cells and the total number of leucocytes (CD45⁺), the 7-Amino-Actinomycin D (7AAD) nucleic acid dye was used for viability measure (BD Stem Cell Enumeration kit, Becton Dickinson, BD Biosciences), according to the manufacturer's protocol. The BD Stem Cell Enumeration simultaneously enumerates the total viable dual-positive (CD45⁺/CD34⁺) hematopoietic stem cells in absolute counts of CD34⁺ cells per μ L and the percentage (%) of viable leucocytes (CD45⁺) that are CD34⁺ [8].

2.7. FBS commercial samples

The two FBS formulations commercially available as a supplement for hMSCs culture routinely used by our research group for *in vitro* isolation and expansion were tested. The FBS_I was obtained from GIBCO (Gibco[®] Invitrogen, EU Approved FBS, Origin South America, Heat Inactivated, and reference #10500–064). The FBS_II was obtained from BI, Biological Industries (BI LTD, Certified FBS, reference #04-400-1A), and is classified for human MSCs, 0.1 μ m membrane filtered, tested for mycoplasma, and virus screened. This FBS_II is heat inactivated, sterile-filtered, and according to the manufacturer information, presents hemoglobin in a concentration ≤ 25 mg/dL, and ≤ 10 EU/ml endotoxin, and it was used for the *in vitro* validation. For both formulations, 3 different samples obtained from a different lot were tested in the ¹H-NMR experiments (N = 3).

2.8. Part I: Supplements metabolomic characterization

2.8.1. NMR spectroscopy. A 600 μ L aliquot of each sample was transferred into 5 mm NMR tubes and mixed with 50 μ L deuterium oxide (D₂O) containing 0.05mM sodium trimethylsilyl-[2,2,3,3-d₄]-propionate (TSP) as an internal reference. The NMR experiments were recorded at 300K on a Bruker Avance III 600 HD spectrometer, equipped with Cryo-Probe Prodigy. All ¹H NMR spectra were acquired with water suppression using a 1D NOESY (noesygppr1d) and a Carr-Purcell-Meiboom-Gill (CPMG, cpmgpr1d) pulse sequences [44, 45]. The 1D NOESY spectra were collected using 5s relaxation delay and mixing time of 0.01s. The CPMG experiments (cpmgpr1d) were acquired with relaxation delay 4s, during which the water resonance signal was selectively irradiated. The echo time was optimized for each sample and was between 0.3 and 0.8 ms, and a loop for T₂ filter of 20 was used. For all ¹H-NMR spectra 128 or 256 transients of a spectral width of 10000 Hz were collected into 32 K time domain points. The spectra were processed with the Bruker Topspin software package (version 3.2). The time domain data were multiplied by an exponential function with line-broadening factor of 0.3 Hz and zero-filled to 64 k prior to Fourier transformation. Two dimensional (2D) ¹H/¹H COSY and TOCSY spectra were recorded in phase sensitive mode and with water suppression; a relaxation delay of 2s, 16 or 32 scans, a total 2K data points in F2 and 256 or 512 data points in F1 over a spectral width of 10000 Hz. 2D ¹H/¹³C heteronuclear single quantum

coherence (HSQC) experiments were carried out with a spectral width of 10000 Hz for ^1H and 27000 Hz for ^{13}C , relaxation delay 1.5 s, Fourier transform (FT) size 2Kx1K. The quantitative distribution of NMR-detectible metabolites in hUCBP and FBS (FBS_I and FBS_II) analysed samples was determined from the integral intensity of characteristic signals in ^1H -NMR spectra of the samples referenced to the total integral intensity of the signals in the spectra, considering the number of the contributing nuclei for that particular resonance signal.

2.8.2. Multivariate statistical analysis of NMR spectroscopy data. In the present study, the integral intensities over small chemical shift regions (bins) of size 0.01 ppm over the spectral region between 0.50 and 9.00 ppm were used as descriptors for interpretation and statistical analysis of the NMR data. Data was normalized by the total sum of the integral intensities in the spectral region of interest. In all studies the water and citric acid spectral regions were excluded. Additionally, a separate NMR-data set was prepared with the regions containing sugars and lactate resonance signals excluded. The Principal Component Analysis (PCA), an unsupervised pattern recognition approach, was applied to examine the intrinsic variation in the data set and compare the metabolic profiles of the 13 hUCBP samples and 2 commercial formulations of FBS (FBS_I and FBS_II) [46]. PCA determines the independent sources of variance across a set of spectra and ranks these Principal Components (PCs) in order of their contribution to the overall variance. PCA facilitates the identification of the sources of variation relevant to systematic changes in a number of key features in the NMR spectra. Based on the singular values decomposition (SVD) technique, Principal Components (PCs) are extracted from the initial NMR data matrix. Each PC is orthogonal (uncorrelated) with all other PCs. The PCs are ordered based on their information content, with the first few PCs containing the largest part of the variance of the data set. Two dimensional PC maps (PC1 vs. PC2) were used as a convenient way to visualize inherent data clusters and outliers, and make conclusions on the degree of correlation between the hUCBP and both FBS samples. If there are systematic metabolic differences between samples, similar samples may cluster together in the scores plot, and classification of the samples may be possible. Further, PC loading plots (ppm vs. PC1 or PC2) were used to reveal the main metabolites and the associated spectral regions responsible for the NMR data variability. The loading plots define the contribution of variables, i.e. the metabolite content for the divergence or clustering between the samples [34–36].

2.9. Part II: *In vitro* validation

2.9.1. Cell viability assessment. Cell culture and maintenance: UC-MSCs and DPSCs were thawed and *in vitro* expanded using standard protocols previously reported by our research group [4–12, 29, 47–49]. UC-MSCs and DPSCs were maintained at 37°C and 95% humidified atmosphere with 5% CO_2 environment. UC-MSCs were obtained from PromoCell, Cat. C-12971; Lot No. 1112304.2) and DPSCs from AllCells, LLC (Cat. DP0037F, Lot No. DPSC090411-01). MSCs' batches identity was confirmed through flow cytometry analysis. Passage 5–7 cultures were utilized in the presented assays in both cell populations. Both populations were initially maintained in αMEM , with GlutaMAX™, without nucleosides (Gibco, reference #32561029) supplemented with 10% (v/v) FBS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin (Gibco, reference #15140122), 2.05 $\mu\text{g}/\text{ml}$ amphotericin B (Gibco, reference #15290026) and 10 mM HEPES Buffer solution (Gibco, 15630122). For the *in vitro* viability studies, the FBS_II was selected for culture medium supplementation of both hMSCs types under study.

PrestoBlue Cell Viability Protocol: The PrestoBlue (Thermo Fisher Scientific, Molecular Probes) assay is a commercially available, ready-to-use, water-soluble preparation. Cells were seeded in 24 well plate at 4×10^3 cells/well. The cells were left adhering in complete culture

medium, overnight. After this period, culture media was replaced by test media [α MEM with GlutaMAX, without nucleosides supplemented with either 2%, 4%, 6%, or 8% of hUCBP pool or 10% FBS (v/v), 100 IU/mL penicillin, 0.1 mg/ml streptomycin, 2.05 μ g/ml amphotericin B and 10 mM HEPES buffer solution]. Previous experimental set ups revealed jellyfication of the hUCBP supplemented medium, despite of its collection and transportation in CPD containing collection bags. Hence, heparin (Heparin Sodium, 5.000 IU/ml, BBraun®) was added to the hUCBP supplemented medium formulations, at 2 UI/ml. Preliminary assays presented no significant effect of heparin addition on standard culture conditions, using 10% FBS supplemented α MEM (data not shown).

At every time point [24 hours (1 day), 72 hours (3 days), 120 hours (5 days), 168 hours (7 days), and 216 hours (9 days)], the culture media was removed and fresh adequate culture media was added to each well, with 10% (v/v) of 10x PrestoBlue cell viability reagent (Thermo Fisher Scientific, Molecular Probes, Invitrogen, reference #A13262), and UC-MSCs and DPSCs were incubated for 1 hour at 37°C, 5% CO₂. Changes in cell viability were detected by absorbance spectroscopy of cell culture supernatant, and absorbance was read at 570nm and 595 nm in a Thermo Scientific Multiskan FC plate reader. Absorbance readings were normalized and data corrected to unseeded control wells' readings. At each time point, sample wells were harvested using Trypsin-EDTA and triplicate cell counts were performed with Trypan Blue exclusion dye assay (15250061, Gibco), using an automated counter (Countess™ II FL Automated Cell Counter, Invitrogen).

2.9.2. Senescence and apoptosis. To assess for signs of senescence in the cellular populations, β -Galactosidase activity was assayed. Cells were plated in 96-well plates, as described for the growth dynamics assessment, maintaining the assigned hUCBP or FBS supplemented media. At 3, 5 and 7 days [72, 120 and 168 hours], culture media was removed, and cells were washed once with DPBS and incubated with β -Galactosidase Assay Reagent (75705, Thermo Fisher Scientific), for 30 minutes at 37°C. Absorbance was read at 405 nm. UC-MSCs and DPSCs cultured in either hUCBP or FBS supplemented media were further assessed for apoptosis events, through the detection of Annexin V and Propidium Iodine (PI) staining (BMS500FI, eBioscience). For such, cells cultured for 5 days in each condition were harvested using Trypsin-EDTA, counted and resuspended in provided Binding Buffer. Cells were incubated with Annexin V-FITC and PI, as per manufacturer's protocol, and FACS analysis was performed using a Coulter Epics XL Flow Cytometer (Beckman Coulter Inc., Miami, FL, USA). Flow cytometry data was processed using FlowJo Engine X10.4 (v3.05478, LLC).

2.9.3. MSCs' Phenotype identity. The surface marker profiles of UC-MSCs and DPSCs cultured in either hUCBP or FBS supplemented media were assessed through Flow Cytometry. MSCs populations were cultured for 5 days, as described previously and harvested using Accutase™ Cell detachment solution (561527, BD Biosciences), counted and resuspended in Stain Buffer (554676, BD Biosciences). Cells were incubated with anti-positive (CD90, CD105, CD44) and negative marker (CD34, CD11b, CD19, CD45, MHC class II) antibodies and assayed as per manufacturer's instructions (Human MSC Analysis Kit, 562245, BD Biosciences), using a BD FACSCalibur 3 CA Becton Dickinson, BD Biosciences. Flow cytometry data was processed using FlowJo Engine X10.4 (v3.05478, LLC).

2.9.4. Gene expression. Reverse transcriptase Polymerase chain reaction (RT-PCR) and qPCR was performed targeting housekeeping and MSCs' related gene sequences listed in [Table 1](#).

MSCs populations cultured in the described media were harvested using Trypsin-EDTA, as previously described, and pellets of 10⁶ cells of each group were used for total RNA extraction, using the Aurum™ Total RNA Mini kit (732–6820, Bio Rad). Briefly, cell pellets were lysed, DNA was removed with DNase I enzyme and obtained RNA was eluted. Total RNA was

Table 1. Primer sequence information for qRT-PCR custom plate array.

Gene	Length (bp)	Bio Rad Assay ID
<i>MSCs targets</i>		
<i>CD34</i>	99	qHsaCID0007456
<i>CD90/ THY1</i>	120	qHsaCED0036661
<i>CD73/ NT5E</i>	117	qHsaCID0036556
<i>CD105/ ENG</i>	124	qHsaCID0010800
<i>CD166/ ALCAM</i>	173	qHsaCID0037887
<i>CD117/ c-kit</i>	63	qHsaCID0008692
<i>SOX2</i>	98	qHsaCED0036871
<i>OCT3-4/ POU5F1</i>	100	qHsaCED0038334
<i>MHC class I/ HLA-A</i>	115	qHsaCED0037388
<i>MHC class II/ HLA-DRA</i>	119	qHsaCED0037296
<i>Housekeeping genes:</i>		
<i>β-actin</i>	62	qHsaCED0036269
<i>GAPDH</i>	117	qHsaCED0038674

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quantified using a nanophotometer readings at 260 and 280 nm (NanoPhotometer™ Pearl, Implen GmbH). Once RNA concentrations were tuned between samples, cDNA was synthesized from the purified RNA using the iScript Reverse Transcription kit (170–8891, Bio Rad) and T100 Terma Cyclar Thermocycler (Bio Rad), as per manufacturer's instructions. Quantitative PCR (qPCR) was performed in a CFX96 Touch™ (BioRad) apparatus using the iTAQ™ SYBR® Green Supermix (172–5120, BioRad) and custom PCR plates encompassing duplicates of targeted human genes and negative control (Table 1). Recommended PrimePCR cycling protocol was employed: 95°C for 2 minutes (activation), 40 cycles comprising 95°C for 5 seconds (denaturation) - 60°C for 30 seconds (annealing), and 65 to 95°C (0.5°C increments), 5 secs/step (melt curve). The number of cycle threshold for each well was recorded. Data was processed using BioRad CFX® Manager Software 3.1 (Bio Rad Laboratories). Fold differences were calculated using the standard $\Delta\Delta C_q$ method with GAPDH and β -actin as housekeeping genes. The reference gene on the hUCBP supplemented samples was calculated relative to calibrator sample (FBS10%) (fold change between two C_q values) through the equation: Relative quantification (RQ) = $2^{\Delta\Delta C_q}$

2.9.5 Multilineage differentiation. Osteogenic and Adipogenic differentiation protocols—MSCs were seeded onto 24-well plates (8,000 viable cells/cm²), and cultured with either FBS or hUCBP supplemented media until 70–80% confluence was reached (after 3 days). At this point, cells were transitioned to specific differentiation media: Adipogenesis (StemPro Adipogenesis Differentiation kit, A10070-01, Gibco), following manufacturer's instructions, or formulated Osteogenesis medium, composed of expansion media further supplemented with 5nM Dexamethasone (D8893 –Sigma Aldrich®), 250 μ M de ascorbic acid-2- phosphate (AsA2-P, A4403, Sigma Aldrich)) e 10mM β -glycerphosphate (β -GP, G9422, Sigma Aldrich). Xeno-free Osteogenic formulations were prepared using 4%, 6% or 8% hUCBP supplementation [50]. Control wells were maintained in culture media absent osteogenic supplements. Media were changed every 3 days, for 21 days, for Adipogenesis and Osteogenesis, respectively. After 14 days, cells under Adipogenic differentiation were stained with Oil Red O, for lipid droplets detection and semi-quantification. Alizarin Red S Assay was employed to asses for Osteogenic differentiation after 21 days; **Oil Red O (Qualitative and Semi-Quantitative):** Cells were fixated with 4% formaldehyde (3.7–4% buffered to pH7, 252931.1315, Panreac AppliChem) and incubated with Oil Red O (O0625, Sigma-Aldrich) working solution for 10–

20 minutes at room temperature. Oil Red O was discarded and excess dye removed. Differentiated cells were observed under inverted microscope (Axiovert 40 CFL, Zeiss). For semi-quantification, Oil Red O stain was eluted through the addition of 100% isopropanol (59300, Merck Milipore®), and absorbance was read at 570 nm; **Alizarin Red S (Qualitative and Semi-Quantitative)**: ARS Assay was used to qualitatively and semi-quantitatively determine Osteogenic differentiation of hMSCs, as described in [50]. Cells were fixed with 4% formaldehyde and stained with 40 mM ARS (2003999, Milipore) and incubated for 30 min with gentle shaking. Following incubation, unbound dye was removed and wells were washed with diH₂O until supernatant became clear. Red stained mineralized layers produced by differentiated cells were observed macroscopically and under inverted microscope. For the extraction and quantification of the ARS, 10% acetic (ARK2183, Sigma-Aldrich) was added to the wells. Cells and mineral deposits were scraped from the plate and collected, heated at 85°C for 10 minutes and immediately transferred to ice, for 5 minutes. The samples were centrifuged, and individual absorbance values were measured at 405 nm, along with a standard curve for the calculation ARS concentration (μM).

Chondrogenic differentiation protocol: MSCs were suspended at 2×10^4 viable cells/well of 96-well plate, and let to adhere for 48 hours. Chondrogenic culture medium (StemPro Chondrogenesis Differentiation kit, A10071-01, Gibco) was added to each well. Control wells remained in FBS or hUCBP supplemented media. Media was replaced every other day in both groups. After 14 days, wells were stained with Alcian Blue to assess for Proteoglycan's synthesis by differentiated chondrocytes and Sulfated Glycosaminoglycans (GAGs) production was quantified using the Blyscan™ Glycosaminoglycan Assay (Biocolor, UK). **Alcian Blue Staining (Qualitative)**: Cells were fixated with 4% formaldehyde, and stained with Alcian Blue (A9186, Sigma) solution. Alcian Blue was discarded and wells were rinsed with acetic acid 3% (v/v). Acidity was neutralized with distilled water, and left in each well for visualization. Differentiated cells were observed under inverted; **GAGs Production (Quantitative)**: Sulfated GAGs Assay was carried as per manufacturers' instructions. Culture media was removed from each well and reserved. GAGs in the ECM produced by cultured cells was extracted using Papain (P3125 Sigma-Aldrich) and added to the previously collected supernatant, for total GAGs quantification). After centrifugation and removal of cellular debris, Blyscan dye reagent was added and incubated for 30 minutes, and then centrifuged to isolate the precipitated coloured complex. The precipitate was dissolved and individual absorbance values were measured at 656 nm, along with a standard curve for the extrapolation of GAGs concentration (μg/ml).

2.10. Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla California USA). The experiments were performed in triplicates and the results were presented as Mean ± SEM. Significant differences are also presented as percentage difference, in which 100% viability corresponds to the non-treated cells. Dunnett's multiple comparisons test against control group (10% FBS) were performed by one-way ANOVA supplemented with Tukey's HSD post-hoc test. Quantitative data of β-Galactosidase activity, Flow cytometry data and Multilineage differentiation assays were subjected to one-way ANOVA test, followed by pairwise comparisons using Tukey's HSD post hoc test. Statistical significance was established as $P < 0.05$. Differences were considered statistically significant at $P < 0.05$. Significance of the results is indicated according to P values with one, two, three or four symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively.

3. Results

Results obtained are herein presented, and the most relevant findings highlighted.

3.1. Haematology auto-analyser, flow cytometry and microbiological analysis of the umbilical cord blood (UCB) units

S1 Table (found in Supporting information) summarizes the data obtained by haematology auto-analyzer, which includes the initial WBC count, performed before the volume reduction with the AXP automated system (in cells $\times 10^9$ per L) and the final WBC count (in cells $\times 10^9$ per l), performed after the volume reduction procedure of the UCB samples. The obtained mean values were $9.61 \pm 2.96 \times 10^9$ cells/l and $29.42 \pm 14.79 \times 10^9$ cells / l, respectively. The samples presented an average of 66.17 ± 60.19 viable CD34⁺ cells/ μ l, with $93.13 \pm 6.65\%$ viability, and $17.63 \pm 7.27 \times 10^9$ viable CD45⁺/l, with $89.72 \pm 4.96\%$ viability (N = 13), demonstrating individual variability considering the UCB hematopoietic stem cells concentration and CD45⁺ / CD34⁺ cell viability. This individual variability has been previously reported in UCB units processed by AXP automated system [41–43].

Considering the microbiological evaluation of the UCBP, all the samples were negative for aerobic, anaerobic microorganisms and *fungi*, except for the sample hUCBP#5 which was positive for *Actinomyces meyeri* in anaerobiosis. Although contamination was identified in this samples, all of the haematological parameters remained within the average counts (**S1 Table**, hUCBP#5 sample). The bacterial contamination did not interfere with ¹H-NMR analysis and no statistical differences were observed between plasma sample hUCBP#5 and the mean values calculated for the 13 plasma samples analysed (P<0.05) (**S1 Table**). However, hUCBP#5 was excluded from the pool used for the *in vitro* validation, in order to prevent the cell culture contamination, and the hUCBP pool included only 12 out of 13 samples analysed (N = 12).

3.2. Part I: Supplements metabolomic characterization

3.2.1. NMR spectroscopy. ¹H-NMR spectra of all hUCBP and FBS samples were acquired with water suppression using 1D NOESY and CPMG pulse sequences. The implementation of 1D NOESY has led to ¹H-NMR spectra with improved solvent peak (at 4.70 ppm) suppression but complex line shapes and highly overlapped resonances from the low molecular weight metabolites and macromolecular components. To reduce the complexity of the spectra, T2-edited CPMG experiments with appropriate T2 delays were used to attenuate the broad signals from high molecular weight species and facilitate the identification of small molecular components. The average ¹H (CPMG) NMR spectra of hUCBP and FBS samples are shown in **Fig 1A** and **Fig 1B**, respectively. The assignment of the resonance signals in the spectra was based on results obtained from various 1D and 2D (¹H/¹H COSY, ¹H/¹H TOCSY, ¹H/¹³C HSQC) NMR experiments and reference data [51, 52]. Characteristic resonance signals of metabolites identified in ¹H -NMR spectra of hUCBP and FBS samples are labelled in **Fig 1**. The analysis of ¹H-NMR spectra has allowed the identification and quantification of a number of metabolites in hUCBP and FBS listed in **Table 2**.

The presence of essential amino acids useful for protein metabolism and protein synthesis (alanine, arginine, glutamine, glutamate, isoleucine, histidine, leucine, lysine, phenylalanine, proline, tyrosine, and valine), organic acids and derivatives (lactate, β -hidroxibutirate, ethanol, formate, creatine), glucose (α glucose and β glucose), lipids, and nucleotides (adenosine, uridine, guanosine species) were detected in the hUCBP, FBS_I, and FBS_II samples spectra. The relative integral intensity of characteristic signals in ¹H-NMR spectra of hUCBP samples and FBS samples (both FBS_I and FBS_II) was used to quantify the detected metabolites. The

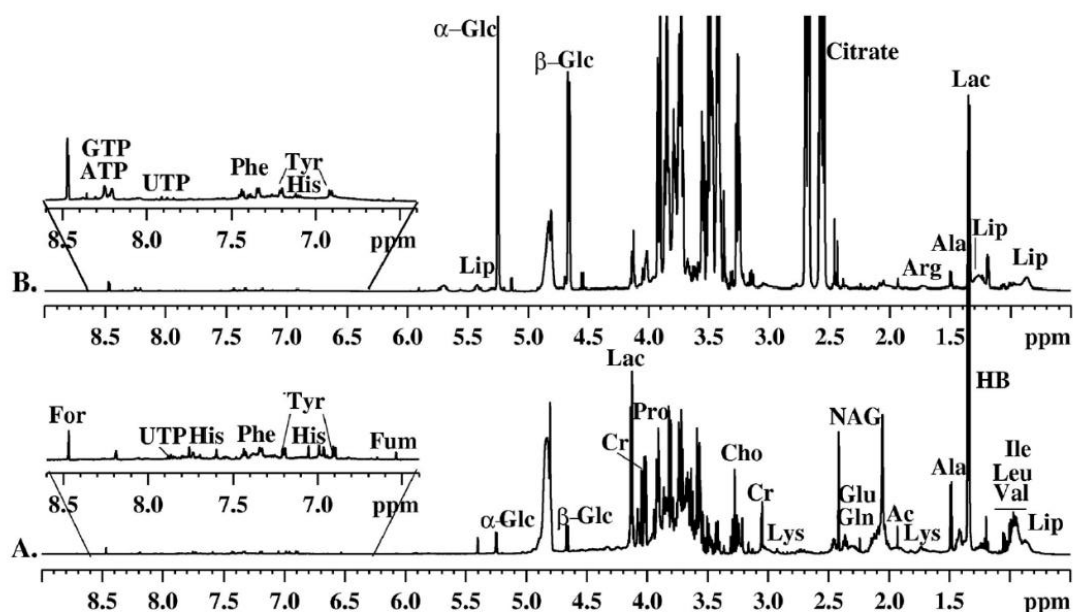


Fig 1. MHz ^1H -NMR spectra of FBS_I and FBS_II and hUCBP. Average 600 MHz ^1H -NMR spectra (A) of FBS_I, and FBS_II and (B) hUCBP samples.

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Table 2. Relative quantitative distribution of metabolites observed in the ^1H -NMR spectra of hUCBP, FBS_I, and FBS_II samples.

Metabolites	Code	Group	Chem. Shifts (ppm)	hUCBP		FBS_I		FBS_II	
1-Methylhistidine	1-Me-His	CH	7,80–7,86	0,47	± 0,23	0,43	± 0,05	0,34	± 0,08
3-Methylhistidine	3-Me-His	CH	7,60–7,68	0,47	± 0,21	0,43	± 0,06	0,31	± 0,09
Acetate	Ace	CH ₃	1,93	0,14	± 0,05	0,18	± 0,01	0,18	± 0,02
Adenosine	ADP/ATP	CH	8,22	0,48	± 0,21	0,41	± 0,06	0,28	± 0,09
Alanine	Ala	CH ₃	1,46	0,35	± 0,12	0,86	± 0,04	0,89	± 0,02
Bile Acids	BA	CH ₃	0,50–0,75	0,37	± 0,16	0,26	± 0,02	0,21	± 0,07
Choline	Cho	NCH ₃	3,24	0,13	± 0,04	0,12	± 0,00	0,11	± 0,01
Citrulline	Citr	CH ₂	3,12	0,25	± 0,07	0,20	± 0,00	0,17	± 0,03
Creatine	Cr	CH ₃	3,04	0,22	± 0,16	0,35	± 0,01	0,38	± 0,01
Ethanol/ Hydroxybutyrate	EtOH/HB	CH ₃	1,21	0,31	± 0,11	0,62	± 0,02	0,22	± 0,01
Formate	For	CH	8,52	0,53	± 0,21	0,41	± 0,06	0,29	± 0,09
Glutamate/ Glutamine	Glu/Gln	CH ₂	2,34	0,85	± 0,30	1,40	± 0,01	1,44	± 0,07
Histidine	His	CH	7,74	0,23	± 0,11	0,20	± 0,03	0,18	± 0,04
Lactate	Lac	CH	4,14	1,01	± 0,25	3,33	± 0,10	3,25	± 0,25
Lipids	Lip	CH ₃	0,8–0,9	0,62	± 0,18	0,59	± 0,00	0,66	± 0,01
Lysine/Arginine	Lys/Arg	CH/CH ₂	1,6–1,8	0,49	± 0,15	0,50	± 0,01	0,47	± 0,06
Phenylalanine	PheAla	CH _{3,5}	7,44	0,16	± 0,07	0,15	± 0,02	0,13	± 0,02
Prolina	Pro	CH	4,2	0,44	± 0,16	0,64	± 0,06	0,80	± 0,15
Threonine	Thr	CH	4,26	0,32	± 0,13	0,51	± 0,05	0,57	± 0,04
Tryptophan	Try	CH ₇	7,56	0,24	± 0,11	0,20	± 0,03	0,15	± 0,04
Tyrosine	Tyr	CH _{2,6}	6,9	0,13	± 0,11	0,12	± 0,03	0,08	± 0,05
Valine/ Leucine/ Isoleucine	Val/Leu/Ile	CH ₃	0,9–1,1	0,30	± 0,09	0,69	± 0,02	0,77	± 0,04
α-Glucose	α-Glu	CH(H ₁)	5,28	2,93	± 0,63	0,47	± 0,02	0,42	± 0,01
β-Glucose	β-Glu	CH(H ₁)	4,68	3,40	± 0,70	0,73	± 0,15	0,69	± 0,05

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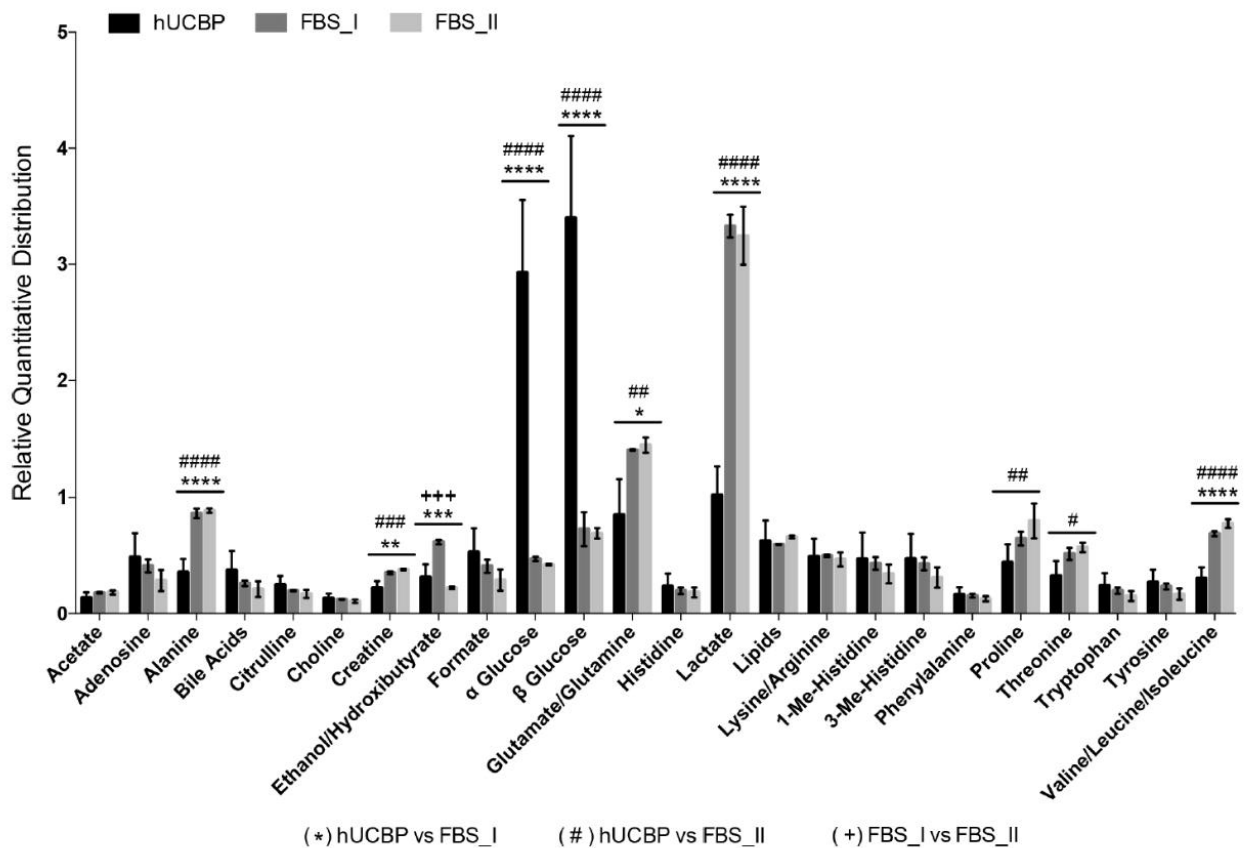


Fig 2. Relative quantitative distribution of the metabolites in hUCBP, FBS_I and FBS_II. Results presented as Mean $\pm$ SEM. Symbols reflect differences between hUCBP and FBS_I (*), hUCBP and FBS_II (#), and FBS_I and FBS_II (+), where the numbers of symbols represents the P value (1 symbol: $0.01 \leq P \leq 0.05$; 2 symbols: $0.001 \leq P < 0.01$; 3 symbols: $0.0001 \leq P < 0.001$; 4 symbols: $P < 0.0001$).

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results allowed us to estimate the relative quantitative distribution of metabolites in all studied samples. The metabolic profiles of the FBS samples (both FBS_I and FBS_II) were found to be very similar but demonstrated some differences when compared to analysed hUCBP samples. The average data for the metabolites distribution in hUCBP, FBS_I and FBS_II samples are graphically presented in Fig 2.

The results have shown that the major difference between the metabolic profiles of hUCBP and FBS samples is due to the different content of glucose, lactate, glutamate, alanine and branched chain amino acids (see Table 2 for detailed relative quantities). Significantly higher levels of glucose (α glucose and β glucose) and lower levels of lactate were found for the hUCBP than for FBS samples. The analysis of ^1H -NMR spectra further revealed an increased level of alanine, glutamate, isoleucine, leucine and valine in both commercial FBS (FBS_I and FBS_II) when compared to hUCBP samples. This seems to be the main difference between the amino acids profiles of hUCBP and FBS. Increased levels of alanine and glutamine can be identified in the commercial FBS sera. Apart from the amino acids discussed above, all hUCBP samples were found to have similar or slightly higher contents of arginine, citrulline, choline, histidine, 1(3)-methyl-histidine, lysine, phenylalanine, tryptophan and tyrosine, as well as of nucleotides ($\delta = 8.4\text{--}7.8$ ppm), lipids ($\delta = 0.5\text{--}0.9$; 1.3; 5.3 ppm), choline and formate when compared to the FBS samples analysed.

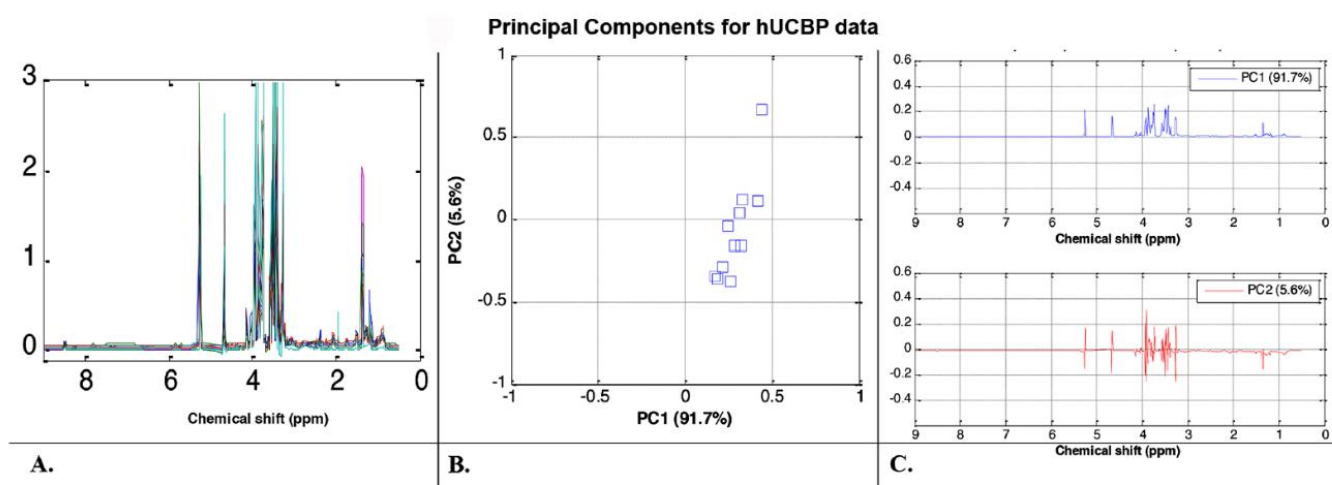


Fig 3. Metabolomic analysis of hUCBP samples. (A) ¹H-NMR spectral trajectories of the hUCBP samples; (B) 2D (PC1 vs. PC2) PCA score plot shows that hUCBP data are well clustered (i.e. there is high similarity between the donor samples) with exception of one donor (considered as an outlier) and (C) corresponding loadings plots illustrate that sugars and lactate content are the main metabolites responsible for the divergence between the hUCBP samples.

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3.2.2. PCA of hUCBP samples. PCA was used to classify the NMR spectral data and revealed factors contributing to the variability between the three groups of data sets. The NMR data in the spectral area between 0.5 and 9.0 ppm was analysed with the water and citric acid spectral regions always excluded prior to PCA analysis and provided as input to the PCA algorithm. The algorithms extracts are the so called Principal Components (PC) that explain data similarity and variability. The hUCBP was first analysed independently of the FBS samples. The ¹H-NMR spectral data of all hUCBP samples (normalized by the total sum of the integral intensities) are plotted in Fig 3A. The observed variations are confirmed by the 2D (PC1 vs. PC2) score plot in Fig 3B, where the hUCBP samples are clearly scattered and one typical outlier sample is also detected. Note that the first two PCs accumulated more than 97% of data variance (PC1 has 91.7% and PC2 has 5.9% amount of variance), therefore they are sufficient features to represent the data. The corresponding loadings plots for PC1 and PC2 in Fig 3C illustrate that sugars and lactate content are the main metabolites responsible for the divergence between the hUCBP samples.

3.2.3. PCA of hUCBP and FBS samples (FBS_I and FBS_II). Statistical analysis was further applied to the ¹H-NMR spectra of all samples in order to identify the source of variability between the metabolic profiles of hUCBP, FBS_I and FBS_II. The ¹H-NMR spectral trajectories of the hUCBP samples (blue colour) and FBS samples (red colour) groups are plotted in Fig 4A. Visual data inspection shows the higher levels of glucose but lower of lactate, ethanol, acetates, and several amino acids (alanine, glutamate, valine, leucine, and isoleucine) in the hUCBP when compared to the FBS samples. A matrix with 19 columns (correspond to the samples) over the spectral area between 0.5 and 9.0 ppm with the water and citric acid spectral regions excluded was provided as input to the PCA algorithm. The 2D (PC1 vs. PC2) PCA score plot is depicted in Fig 4B. The results illustrated the close similarity of FBS_I and FBS_II data sets (a well-defined cluster with overlapping samples) and revealed their separation from the hUCBP data (a scattered cluster) particularly with respect to PC2. PC1 contains 70% and PC2 23% of data variance, thus these two principal components are sufficient to represent the data information content. The corresponding loadings plots (Fig 4C) provide evidence that glucose, lactate and acetates are the dominant metabolites contributing to the dissimilarity

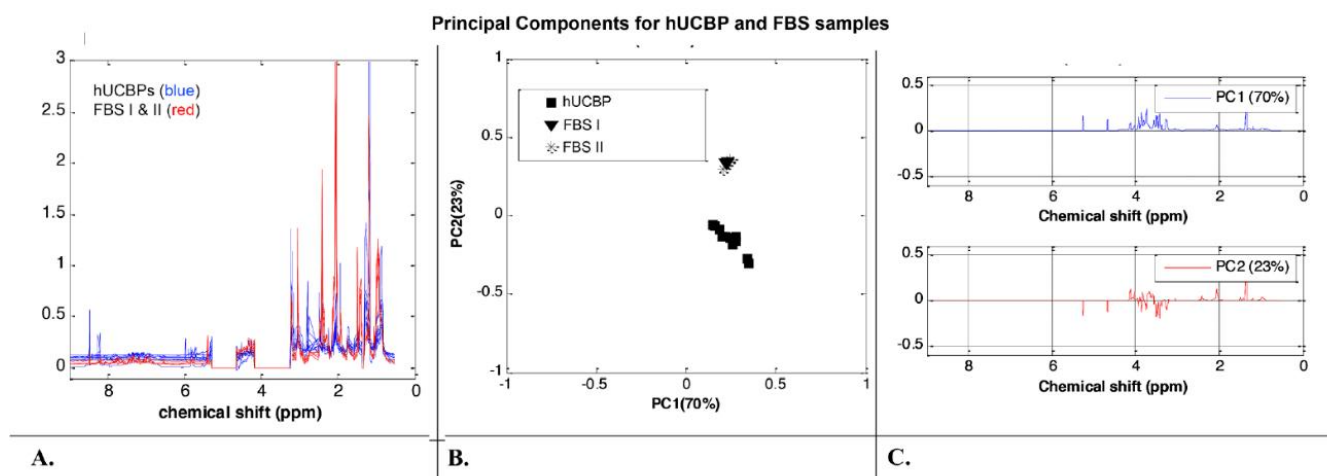


Fig 4. Metabolomic analysis of hUCBP and FBS samples. (A) ¹H-NMR spectral trajectories of the hUCBP samples (blue colour) and FBS (FBS_I and FBS_II samples) (red colour) groups; (B) 2D (PC1 vs. PC2) PCA score plot shows close similarity of FBS_I and FBS_II data (a well-defined cluster with overlapping samples) and a separation from the hUCBP data and (C) corresponding loadings plots illustrate that glucose, lactate and acetates are the main metabolites contributing to the dissimilarity between the human plasma (hUCBP) and bovine sera (FBS_I and FBS_II) samples. Loading plots around zero line suggest close behaviour of the metabolites profiles in the spectral region from 9.0 ppm to 5.3 ppm of the three groups of samples (hUCBP, FBS_I and FBS_II samples).

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between the human plasma (hUCBP) and bovine sera (FBS_I and FBS_II) data sets. Loading plots around zero line suggest close behaviour of the metabolites profiles in the spectral region from 9.0 ppm to 5.3 ppm of the three groups of samples (hUCBP, FBS_I and FBS_II samples). These results coincide with those obtained from the ¹H-NMR spectral analysis of the samples.

3.2.4. PCA of hUCBP samples and FBS samples (FBS_I and FBS_II) excluding glucose and lactate spectral areas. In order to assess intrinsic variations in the content of proteinogenic amino acids, lipids and organic acids and derivatives between hUCBP, FBS_I and FBS_II samples, the same PCA analysis was applied on the ¹H-NMR data after exclusion of glucose (5.28 ppm—4.65 ppm; 4.17 ppm—3.24 ppm) and lactate (1.37 ppm—1.32 ppm) spectral areas. The ¹H-NMR spectral trajectories are plotted in Fig 5A and the PCA score plot is shown in Fig 5B. The hUCBP samples analysis was less scattered, while the FBS_I and FBS_II samples continued to behave as a well-shaped cluster. This observation suggests that glucose and lactate concentrations significantly vary between the hUCBP samples of different donors and their exclusion increased the metabolite profiles proximity. The hUCBP and FBS (FBS_I and FBS_II) samples clusters diverge mainly in the PC2 score (representing only 13% of data variance) and have a very close PC1 score (representing 80% of data variance). The respective loading plots (Fig 5C) confirm these results. They illustrate that the main differences between the hUCBP and FBS data are due to different concentrations of particular amino acids (alanine, glutamate, isoleucine, leucine, and valine) and organic acids and derivatives (N-acetyl residues, 3-hydroxybutyric acid, and creatine) in the spectral region from 3.3 ppm to 0.5 ppm. From the spectral intervals where the loading plots are close to zero, i.e. from 9.0 ppm to 3.3 ppm, with the glucose spectral area excluded, it can be concluded that the metabolites profiles of the three groups (hUCBP, FBS_I and FBS_II samples) are similar.

PCA analysis was performed in three different scenarios based only on the first two Principal Components (PC). Fig 6 illustrates that PC1 and PC2 accumulate more than 90% of data variability and therefore their statistical significance is reliable.

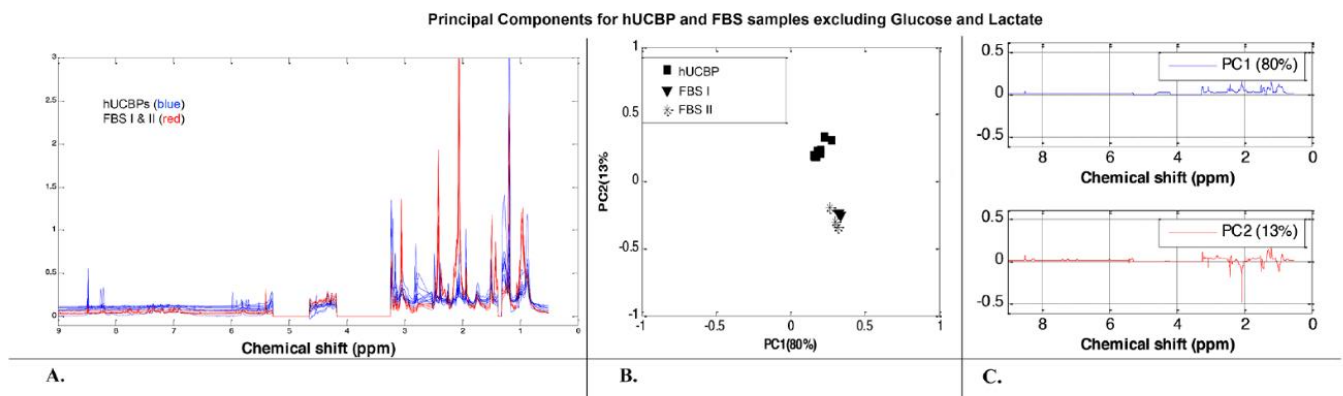


Fig 5. Metabolomic analysis of hUCBP and FBS samples excluding glucose and lactate spectral areas. (A) The ¹H-NMR spectral trajectories; (B) the PCA score plots shows that the hUCBP samples (a well-defined cluster) and the FBS samples (also a well-defined cluster) are less divergent now (they all have a very close PC1 score) and (C) respective loading plots illustrate that the main differences between the hUCBP and FBS data are due to different concentrations of particular amino acids (alanine, glutamate, isoleucine, leucine, and valine) and organic acids and derivatives (N-acetyl residues, 3- hydroxybutyric acid, and creatine) in the spectral region from 3.3 ppm to 0.5 ppm. For spectral area between 9.0 ppm to 3.3 ppm (with excluded glucose) the metabolites profiles of the three groups (hUCBP, FBS_I and FBS_II samples) are very similar.

<https://doi.org/10.1371/journal.pone.0203936.g005>

3.3. Part II: *In vitro* validation

3.3.1. Cell viability assessment. The application of hUCBP as a culture medium supplement for *in vitro* culture and expansion of hMSCs was evaluated using UC-MSCs and DPSCs. Fully characterised cell lots were used for the purpose. PrestoBlue[®] viability test was

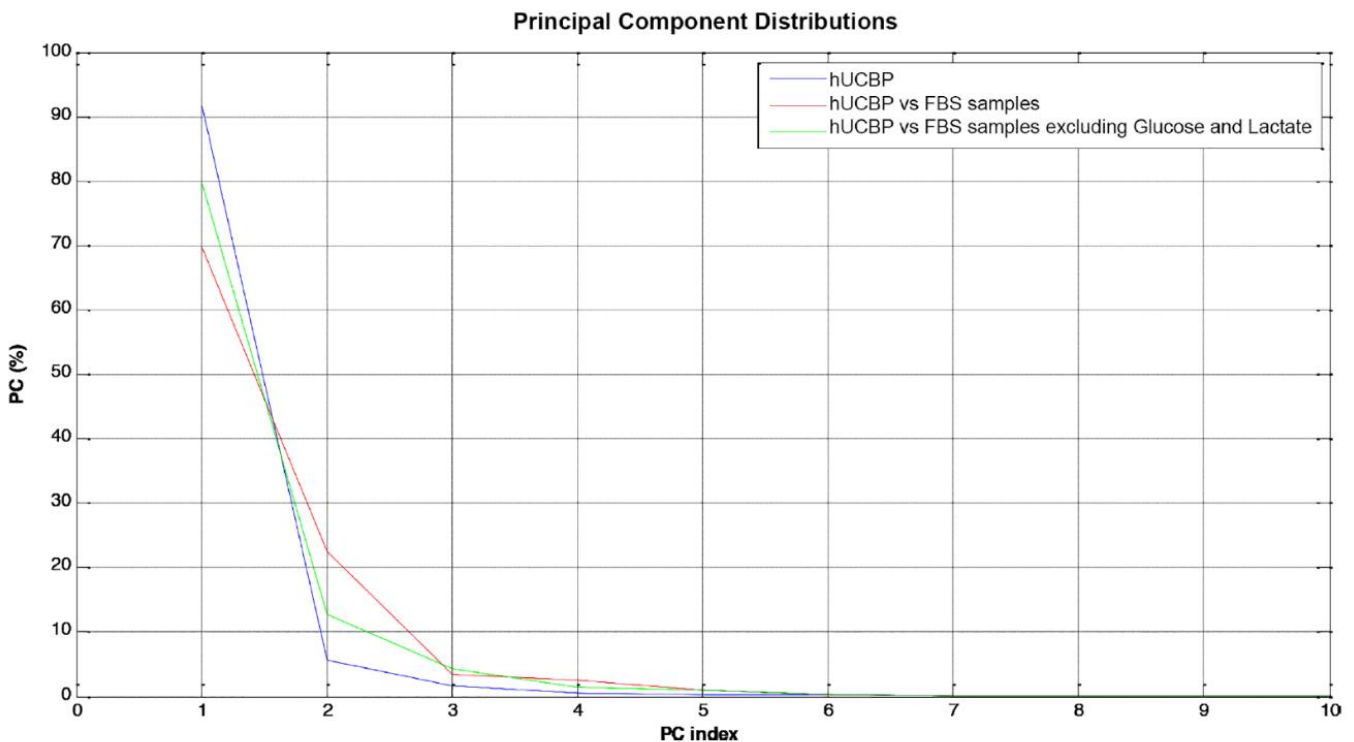


Fig 6. PC contributions for data representation.

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undertaken at determined time points and the supernatant absorbance was measured, normalized and corrected according to manufacturers' instructions. Corrected absorbance values are presented in [S2 Table](#), in Supporting information.

The FBS_II supplemented samples were set as the reference control for the behaviour of hUCBP test samples. Obtained results are graphically presented in [Fig 7](#) and absorbance data is available in [S2 Table](#), in Supporting information.

At 24 hours, UC-MSCs cultures with hUCBP at 4% depicted significantly superior measurements concerning % of cell viability and absorbance, when compared to 10% FBS_II, corresponding to a 200.02% increase over the FBS_II control at that time point. DPSCs presented similar behaviour in most of the tested groups, with no significant differences from the FBS_II control. Here, only the hUCBP 8% group presented inferior measurements (76.28% of cell viability). At 72 hours in culture, UC-MSCs in both hUCBP 2% and hUCBP 4% raised the absorbance to 73.59% and 67.86% over the matched control, respectively. In the DPSCs set, all groups performed superiorly, to the FBS control. In the following time point of 120 hours, no statistical differences were found between the tested groups, for hMSCs from both sources. Maximum measurements were obtained at 168 hours in culture with UC-MSCs at hUCBP 6% and hUCBP 8%, matching 10% FBS_II. On the other hand, hUCBP 4% remained superior to 10% FBS_II control. In the DPSCs, only hUCBP 6% performed comparably to 10% FBS_II, with all other tested groups presenting inferior corrected absorbances.

Both hMSCs types reached the plateau before 216 hours in culture, and hence, metabolic rates (and resazurin reduction) arrested. In all cell culture groups, including the 10% FBS_II, a decrease of the corrected absorbance measurements was observed. Only UC-MSCs cultured in hUCBP 4% supplemented medium sustained activity matching the control.

Absorbance intensity is correlated to the metabolic activity of cells and not directly to their number, but superior number of cells relate to increased viability readings. Retrieved cells numbers and viability (total cell numbers and life cell numbers recovered) by Trypan blue exclusion assay at 24 hours (1 day), 72 hours (3 days), 120 hours (5 days), 168 hours (7 days), and 216 hours (9 days) are presented in [Table 3](#).

3.3.2. Senescence and apoptosis. The assessment of β -Galactosidase activity as an indicator of senescent decline of hMSCs' populations cultures in hUCBP supplemented media revealed no striking differences when compared to FBS supplemented control media, for both assayed populations ([Fig 8A](#) and [S3 Table](#), in Supporting information). Exception was noted for cultured UC-MSCs, at 5 days in culture, where FBS supplemented groups displayed increased β -Galactosidase activity when compared to 4 and 8% hUCBP supplemented groups.

Annexin-V/ PI assay was employed for the detection and definition of apoptosis in cultured cells. Early apoptosis is characterized by plasma membrane reorganization, detected by positive staining for Annexin V, while later stage of apoptosis presents membrane damage, therefore PI can bind to DNA in cytoplasm resulting in positive staining for both Annexin V and PI [53]. Cells cultured under hUCBP or FBS supplementation revealed no significant differences in Annexin-V or PI staining, with exception to hUC-MSCs cultured in hUCBP 8% supplementation (*), which presented increased early apoptosis events, and decreased overall cell viability over the remaining UC-MSCs' groups (% Viable cells (An⁻PI⁻): 99,50 $\pm$ 0,06; 99,40 $\pm$ 0,06; 92,93 $\pm$ 0,52; 99,37 $\pm$ 0,13, and % Early apoptosis (An⁺PI⁺): 0,29 $\pm$ 0,03; 0,40 $\pm$ 0,04; 6,42 $\pm$ 0,53; 0,47 $\pm$ 0,07, for 4%, 6%, 8% hUCBP and 10% FBS supplemented media) ([Fig 8B](#) and [S4 Table](#) in Supporting information).

3.3.3. MSCs' Phenotype identity. The surface marker profiles of UC-MSCs and DPSCs cultured in either hUCBP or FBS supplemented media were assessed through Flow Cytometry. MSCs populations presented characteristic MSCs phenotypical markers in all culturing conditions ([Fig 9](#) and [Table 4](#)), and although some slight differences are noted, populations remain

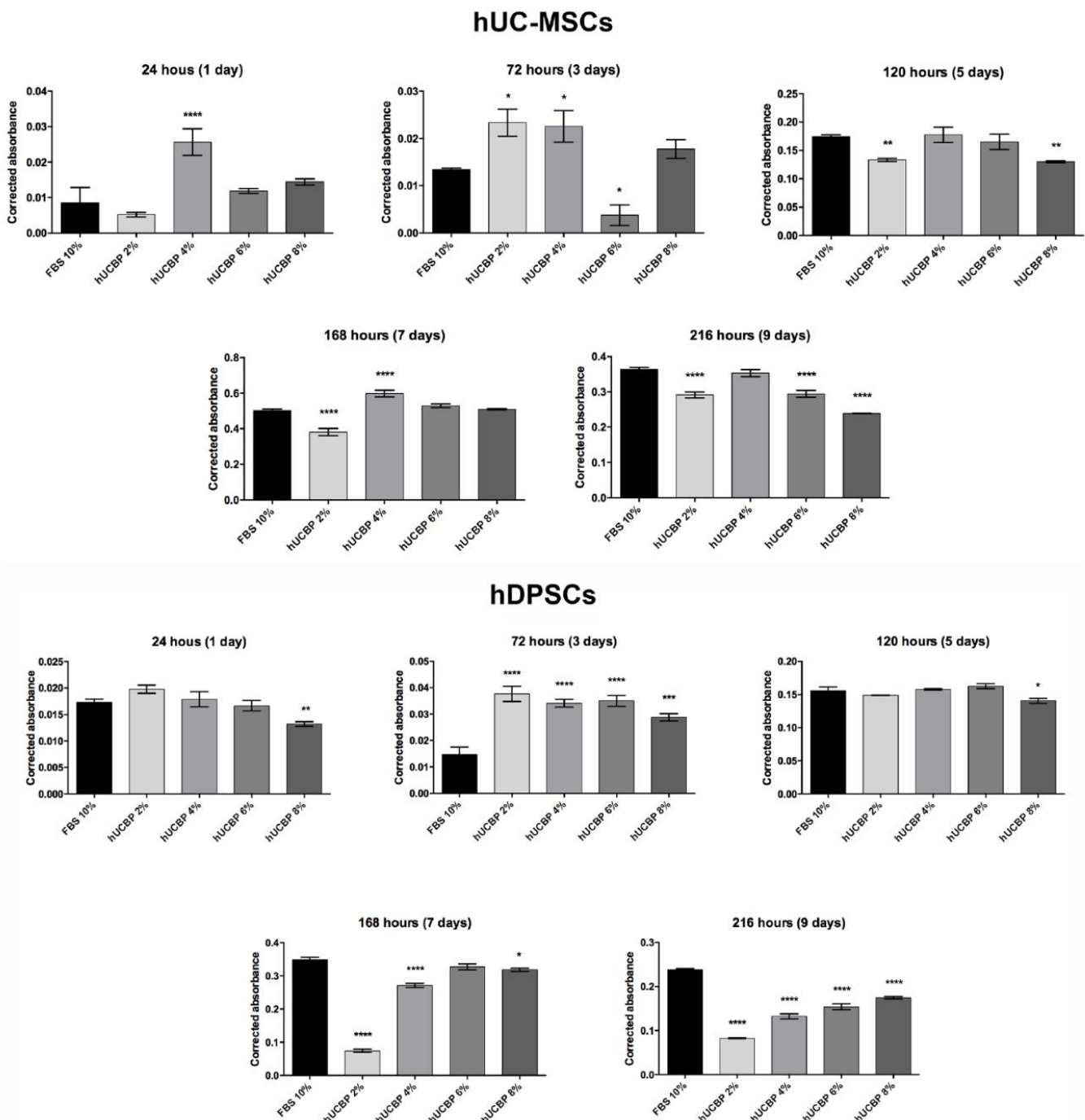


Fig 7. Corrected absorbance readings hUC-MSCs and hDPSCs viability. (A) Corrected absorbance at each assessed time point of the hUC-MSCs cultured in 10% FBS_II, hUCBP 4%, 6% and 8%. (B) Corrected absorbance at each assessed time point of the hDPSCs cultured in 10% FBS_II, hUCBP 4%, 6% and 8%. Values are presented as Mean $\pm$ SEM. Statistical differences were analysed for each hUCBP test samples *versus* control (10% FBS_II supplemented medium) and presented with one to four symbol (*) (*: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$; ***: $0.0001 \leq P < 0.001$; ****: $P < 0.0001$).

<https://doi.org/10.1371/journal.pone.0203936.g007>

Table 3. Retrieved cell numbers ($\times 10^3$) assessed by Trypan Blue exclusion dye assay of hMSCs (UC-MSCs and DPSCs), in the presence of supplemented medium with FBS, II or variable concentrations of hUCBP after 24 hours (1 day), 72 hours (3 days), 120 hours (5 days), 168 hours (7 days), and 216 hours (9 days) incubation. Results presented as Mean $\pm$ SEM.

Retrieved Cells (10^3)	UC-MSCs														
	FBS 10%			hUCBP 2%			hUCBP 4%			hUCBP 6%			hUCBP 8%		
24 h (1 day)	4,23	$\pm$	6,35	4,46	$\pm$	1,64	6,28	$\pm$	2,76	5,97	$\pm$	1,08	5,50	$\pm$	0,97
72 h (3 days)	11,09	$\pm$	0,73	19,94	$\pm$	7,36	20,70	$\pm$	9,17	13,82	$\pm$	24,11	16,24	$\pm$	5,40
120 h (5 days)	94,92	$\pm$	5,40	83,54	$\pm$	5,46	108,48	$\pm$	24,60	118,41	$\pm$	28,99	98,87	$\pm$	3,60
168 h (7 days)	210,17	$\pm$	11,24	173,46	$\pm$	28,12	229,63	$\pm$	23,09	221,25	$\pm$	16,71	174,57	$\pm$	3,11
216 h (9 days)	200,00	$\pm$	14,98	166,00	$\pm$	11,53	216,00	$\pm$	23,69	211,00	$\pm$	12,53	182,00	$\pm$	3,00
	DPSCs														
	FBS 10%			hUCBP 2%			hUCBP 4%			hUCBP 6%			hUCBP 8%		
24 h (1 day)	18,16	$\pm$	1,82	24,82	$\pm$	2,86	25,82	$\pm$	6,14	24,96	$\pm$	4,37	16,95	$\pm$	1,67
72 h (3 days)	39,96	$\pm$	23,12	47,26	$\pm$	10,85	49,23	$\pm$	6,39	52,42	$\pm$	9,14	36,88	$\pm$	5,28
120 h (5 days)	163,13	$\pm$	17,46	112,09	$\pm$	0,96	227,88	$\pm$	3,98	243,48	$\pm$	16,68	180,14	$\pm$	14,66
168 h (7 days)	255,78	$\pm$	16,77	110,72	$\pm$	22,56	215,30	$\pm$	15,45	245,15	$\pm$	20,21	204,28	$\pm$	9,18
216 h (9 days)	250,00	$\pm$	7,23	104,00	$\pm$	10,21	191,00	$\pm$	11,59	231,00	$\pm$	7,51	224,00	$\pm$	10,82

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within the $\geq 92\%$ positive population for CD90, CD105 and CD44, and $\leq 2\%$ negative staining for CD34, CD11b, CD19, CD45 and MHC II. Remarks are to be made regarding the CD90 expression by hDPSCs, which presented only 87–91% positive population, contrasting with 99–100% expressing population of hUC-MSCs.

3.3.4. Gene expression. Gene expression was performed through RT-qPCR analysis. Total RNA was successfully extracted from cultured hMSCs (S5 Table, in Supporting information) and specific genes' expression assessed, after 5 days in specific culturing media conditions. CD34 and Sox2 were undetected in any of the samples from hUCBP or FBS cultured cells, in either population. Strong expression (<29 Cq) of CD105 and CD166, and moderate expression (30–35 Cq) of CD115 were detected in all assayed groups, and no change in expression was observed in differently supplemented UC-MSCs and DPSCs cultures. Particular changes in fold-expression of specific genes was identified in hMSCs from each source (Fig 10 and S6 Table, in Supporting information). In UC-MSCs, CD90 and MHC class I were down-regulated in all hUCBP supplemented groups (although maintaining a strong expression), and moderately expressed OCT-4 was down-regulated in the highest % supplementation groups (6 and 8%). Expression of MHC class II sequence was not detected in 10% FBS supplemented cultures, while it became weakly expressed (>35 Cq) in hUCBP supplemented groups. In DPSCs, strong and moderate expression of CD73 and OCT-4, respectively, was observed in 10% FBS and up-regulated in hUCBP supplementation groups. Contrarily, weak expression of MHC class II was detected in 10% FBS supplemented hDPSCs, that tended to attenuate (down-regulate) in hUCBP supplemented groups.

3.3.5. Multilineage differentiation. Osteogenic differentiation—Macroscopic observation revealed strong Alizarin Red S staining in the FBS supplemented Osteogenic media groups, in both UC-MSCs and DPSCs. All Undifferentiated control groups presented weak ARS staining. Whereas, in all hUCBP supplemented Osteo-differentiation groups, in both cell types, weak ARS staining was observed, not empirically distinguishable from respective control groups. Observation suggest an effective osteo-differentiation and mineral matrix deposition by hMSCs cultures in Osteogenic media containing FBS, while hUCBP supplemented groups did not differentiate towards the intended lineage, after 21 days in specific culture (data not shown). ARS binding quantification confirmed these observations (Fig 11A). Some of the

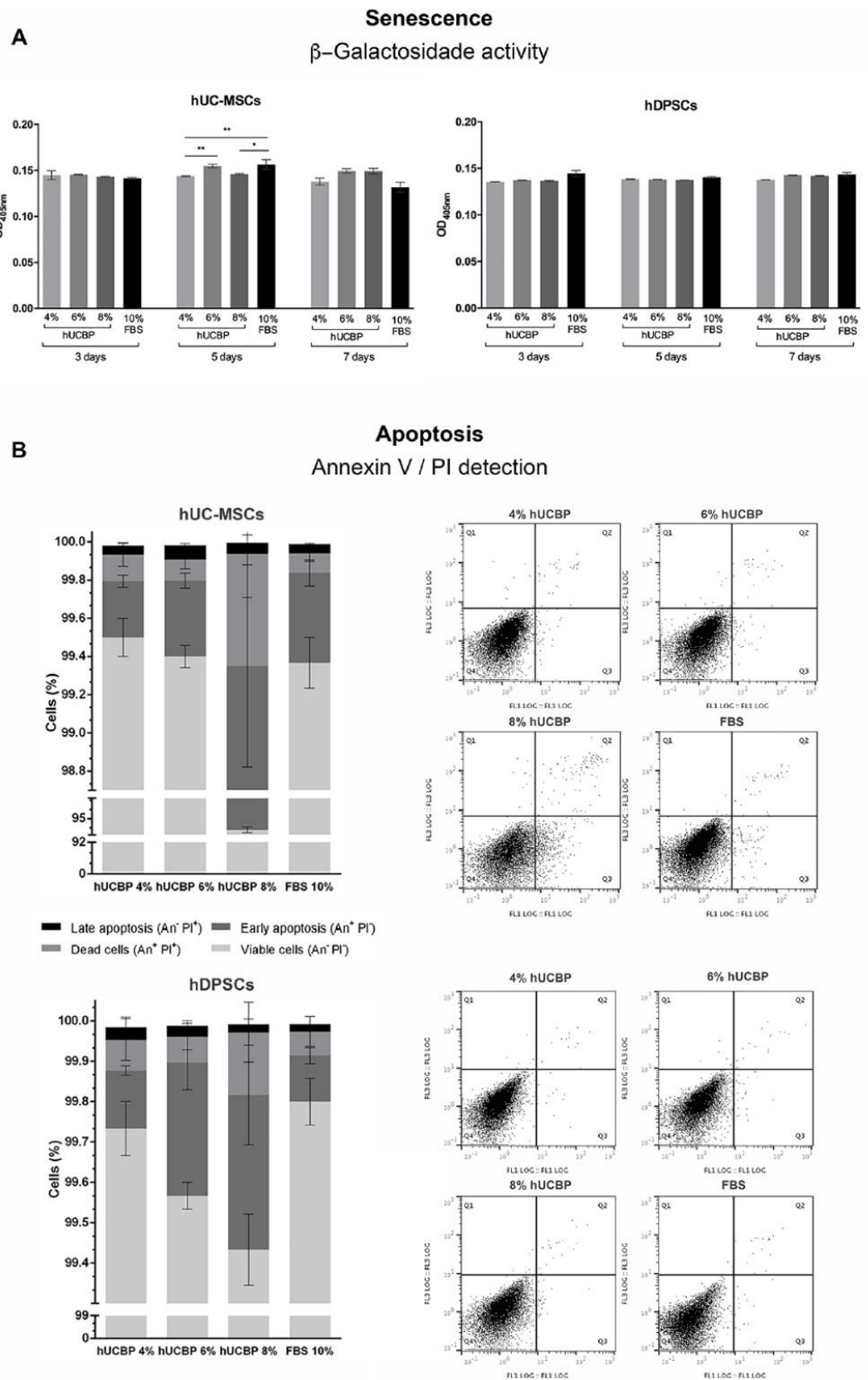


Fig 8. Senescence and Apoptosis: A) Senescence - β -Galactosidase activity (OD_{405nm}) on UC-MSCs and DPSCs at 3, 5 and 7 days. Results Presented as Mean $\pm$ SEM. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; B) Apoptosis: Annexin-V/ PI detection of UC-MSCs (upper panel) and DPSCs (lower panel). Results Presented as Mean $\pm$ SEM. Dot Plots of the correspondent data are presented at the right side of the panels. Top left quadrants match annexin V negative and PI positive cells; Top right quadrants correspond to dead cells that express annexin V and PI positive; Bottom right quadrants pairs with apoptotic cells that express annexin V positive and PI negative; and for last, bottom left quadrants, viable cells that express neither annexin V or PI.

<https://doi.org/10.1371/journal.pone.0203936.g008>

differentiating groups presented no statistical difference towards the respective undifferentiated controls (S7 and S8 Tables, in Supporting information). Furthermore, the semi-quantitative analysis points towards an increased efficiency in the Osteogenic differentiation of DPSCs when compare to UC-MSCs, under the same differentiating conditions ($2299.31 \pm 7.35 \mu M$ and $1449.57 \pm 0.00 \mu M$ of ARS, respectively).

Adipogenic differentiation—Microscopic observation of ORO stained cells evidenced comparable lipid droplets accumulation in the FBS supplemented Adipogenic media groups, in both UC-MSCs and DPSCs. Controls presented occasional lipid droplets accumulation. All hUCBP supplemented Adipo-differentiation groups presented similar differentiation pattern to FBS supplemented groups, amongst all groups of UC-MSCs and DPSCs. Cellular populations maintained in hUCBP presented lipid droplets accumulation in the cytoplasm (data not shown). Spectrophotometric measurement of ORO staining indicated no significant difference between different supplementation levels and/or FBS, within each cell type. However, control groups maintained in hUCBP indicate significantly superior ORO staining over FBS supplemented controls (Fig 11B and S9 and S10 Tables, in Supporting information).

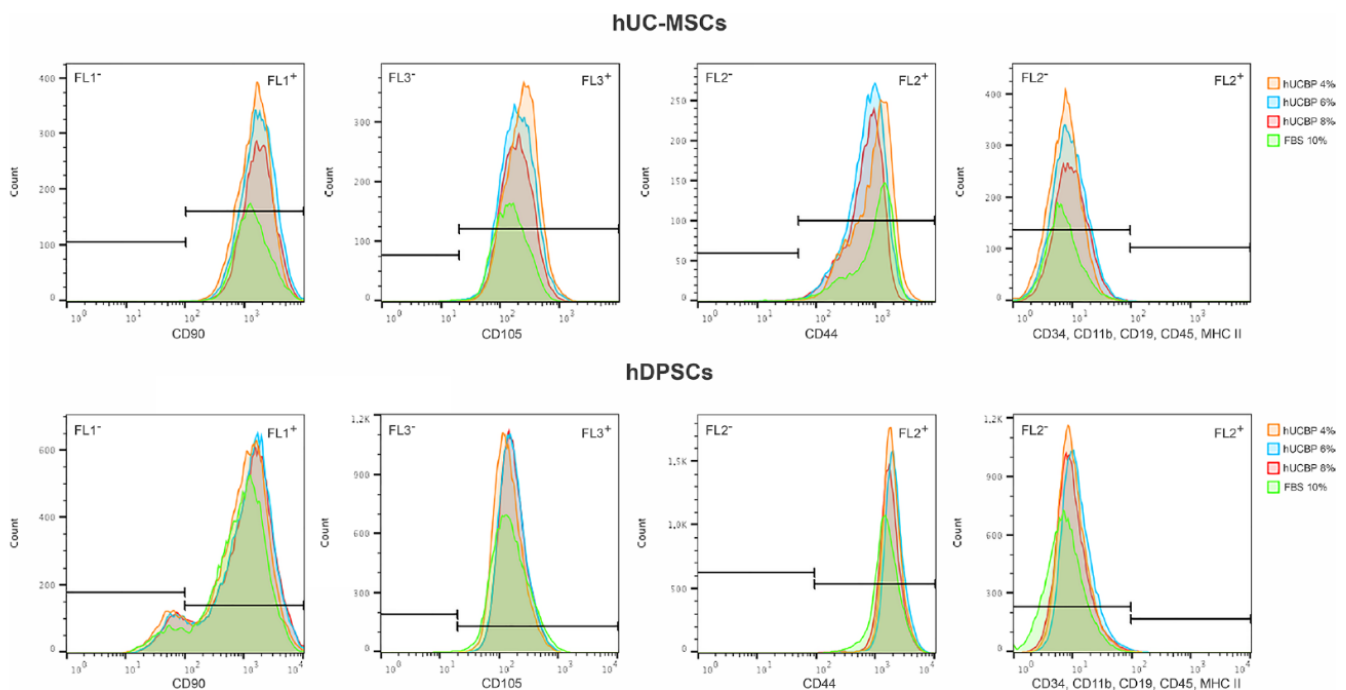


Fig 9. Surface marker expression for MSCs' identity of hUC-MSCs and hDPSCs, assessed by Flow cytometry.

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Table 4. Surface marker profiles of hUC-MSCs and hDPSCs cultured in either hUCBP or FBS supplemented media, assessed through Flow Cytometry. *NEG+ cocktail corresponds to anti-CD34; -CD11b; -CD19; -CD45; and -MHC II antibodies.

Surface Markers	hUC-MSCs											
	hUCBP 4%			hUCBP 6%			hUCBP 8%			FBS 10%		
CD90 ⁺	99,90	±	0,00	99,83	±	0,03	99,87	±	0,03	99,23	±	0,12
CD105 ⁺	99,97	±	0,03	99,77	±	0,03	99,87	±	0,03	99,07	±	0,09
CD44 ⁺	99,47	±	0,09	99,40	±	0,15	99,27	±	0,20	95,70	±	1,65
NEG ⁺⁺	0,05	±	0,01	0,14	±	0,04	0,25	±	0,14	0,13	±	0,08
	hDPSCs											
	hUCBP 4%			hUCBP 6%			hUCBP 8%			FBS 10%		
CD90 ⁺	90,23	±	0,45	90,70	±	0,15	90,67	±	0,07	90,50	±	0,35
CD105 ⁺	99,90	±	0,00	100,00	±	0,00	99,97	±	0,03	99,30	±	0,06
CD44 ⁺	99,97	±	0,03	99,80	±	0,00	99,83	±	0,03	99,80	±	0,00
NEG ⁺⁺	0,37	±	0,03	0,52	±	0,05	0,52	±	0,09	0,13	±	0,04

<https://doi.org/10.1371/journal.pone.0203936.t004>

Chondrogenic differentiation—Groups cultured in Chondrogenic Differentiation media all presented marked light blue staining under macroscopic and microscopic observation, indicating proteoglycan deposition. Control groups from DPSCs in UCBP and FBS remained unstained, indicating no differentiation, as did UC-MSCs in FBS supplemented media. Control groups of UC-MSCs maintained in hUCBP for the assay period presented mild Alcian blue staining and aggregation, suggesting formation of chondrogenic pellets and deposition of proteoglycan rich matrix (data not shown). UC-MSCs and DPSCs maintained in FBS supplemented control media presented no difference in terms of GAGs production, and cells transitioned to differentiating media after 3 days showed increased GAGs production, demonstrating differentiation towards a chondrogenic phenotype, with no significant differences between cell types (Fig 11C and S11 and S12 Tables, in Supporting information). Cells expanded in hUCBP at different concentrations and transitioned to Chondrogenic differentiation media, displayed comparable GAGs production. In the DPSCs, cell maintained in hUCBP for the 14 days of the differentiation period did not differ from the ones maintained in FBS 10%. Undifferentiated controls of UC-MSCs' maintained in the hUCBP supplemented media

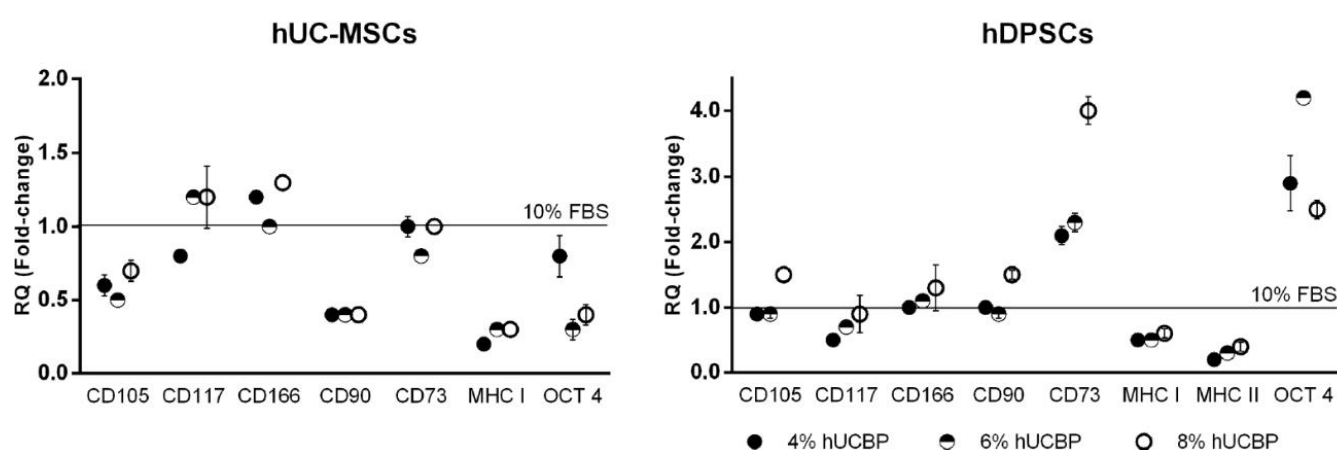


Fig 10. Quantitative RT-PCR of UC-MSCs and DPSCs cultured in hUCBP or FBS supplemented media. RQ: relative quantification (fold change compared to the FBS 10% group), in mean fold change $\pm$ SEM.

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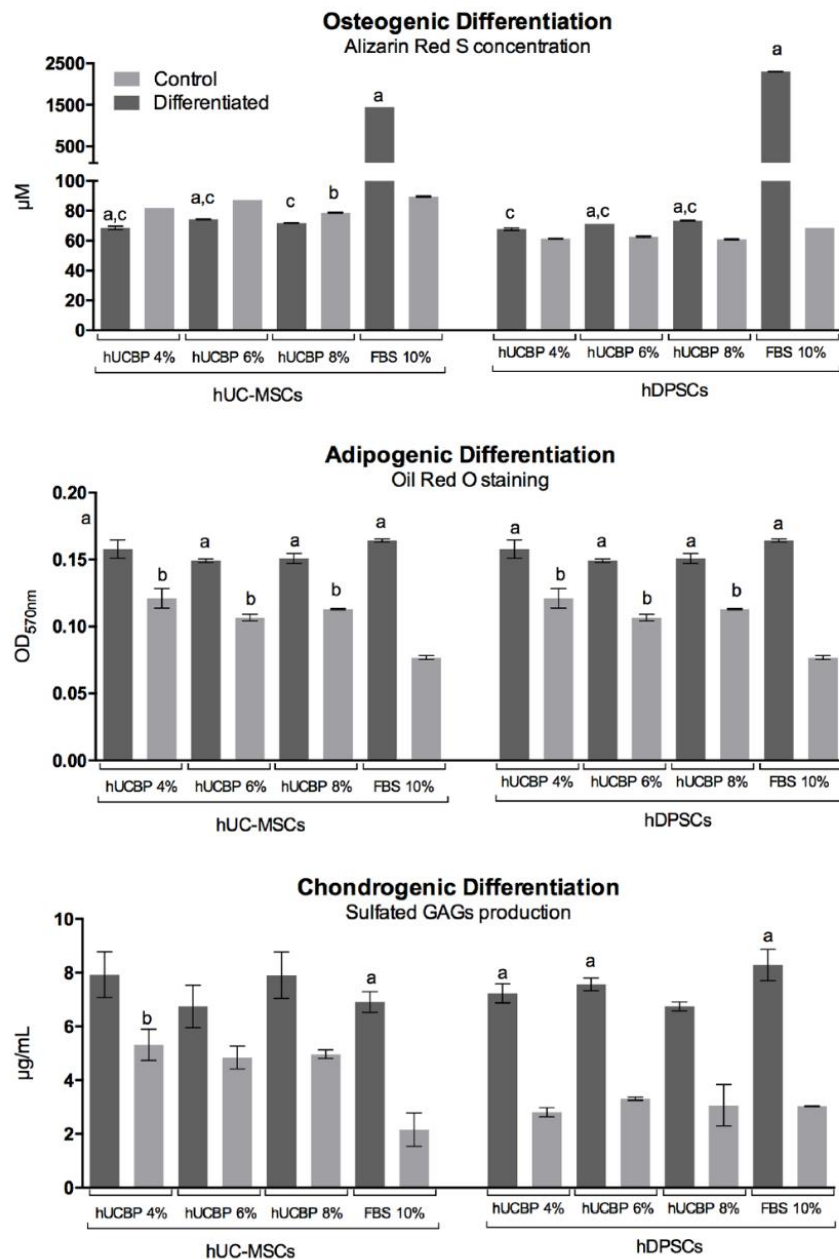


Fig 11. Multilineage differentiation: A) Osteogenic differentiation: Alizarin Red S concentration (µM) after 21 days; B) Adipogenic differentiation: Oil Red O (OD_{570nm}) after 14 days; and C) Chondrogenic differentiation: Sulfated GAGs production (µg/mL) after 14 days, assessed by Blyscan™ Glycosaminoglycan Assay (Biocolor, UK). Control: Undifferentiated control; Results Presented as Mean ± SEM. a: significantly different from undifferentiated group within the same media condition and cellular population; b: undifferentiated control group significantly different from undifferentiated FBS 10% group, within the cellular population; c: differentiated group significantly different from differentiated FBS 10%, within the cellular population.

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demonstrated a tendency to produce increased GAGs, when compared to controls, with mean values approaching differentiated groups, and not being significantly different from these.

4. Discussion and conclusions

In light of the observations from this study, the option of utilizing hUCBP as a potential replacement to FBS supplementation for hMSCs expansion gains further support. The first part of the study focused on the characterization the metabolite composition of hUCBP and its comparison to conventionally utilised FBS, to address its suitability as a culture medium supplement and FBS substituent. In primary samples analysis, individual variations were observed concerning the CD34⁺/CD45⁺ and WBC cells counting, where different donors contribute to a wide range of values. One of the samples was detected as contaminated by anaerobic agent *Actinomyces meyeri* (hUCBP#5), which reinforces the need for thorough quality control of hUCBP samples before usage for cellular therapies preparation. To the authors' knowledge, this is the first extensive comparative study on the metabolomic profile of FBS and hUCBP. Previous published work contributed to a deeper understanding on the growth factor, cytokine and chemokines content of hUCBP, suggesting that it could play a relevant role in advanced therapies development [8].

Within hUCBP samples, glucose and lactate are the major contributors to inter-samples metabolite composition variations. Overall, these observations suggest that, similarly to FBS supplements, the hUCBP are not homogeneous, and present great individual variability, thus requiring to be primarily homogenized before being used in cell-based therapies preparation [41–43]. Hence, the composition of pooled hUCBP batches is well suited for standardization, to which this work aims to contribute with relevant data in terms of individual samples compositions. This will aid in the definition of reference ranges of key components for the approval and release of representative batches, targeting at the reduction of inter-batch variability.

In addition to the detailed definition of individual samples and pooled plasma batch composition as well as the standardization of reference ranges, other concerns must be observed regarding hUCBP quality and safety analysis. Strict control of potential infectious diseases transmission is required at the various steps of the process. One limitation from the necessity of pooling of multiple donor samples is the increased potential risks of infection via donors' hUCBP. To counterbalance this risk, control step reinforcement is advised when aiming at therapeutic applications, with further monitoring of individual samples upon collection and following homogenization/ pooling process.

Comparatively, metabolite profiles of both supplements present distinct features, as demonstrated by NMR spectroscopy and subsequent PCA analysis. The main difference between the metabolic profiles of the two proposed supplements was due to the significantly higher levels of glucose (both α and β glucose) and lower of lactate in the hUCBP. The increased glucose content in hUCBP was attributed to the function of UCB to provide energetic and other metabolic support for the foetus during intrauterine development. The observed variation in the glucose levels throughout hUCBP samples has also been ascribed to the particular diet and health conditions of the different donors, as described by other researchers [41–43]. The presence of anticoagulant citrate phosphate dextrose solution (CPD) [composed of 2.63g of sodium citrate (dehydrate), 0.299 g of citric acid (monohydrate), 2.55g of dextrose (monohydrate) and 0.222g of sodium monobasic bisphosphate (monohydrate)] in the UCB sterile collecting bag might have contributed to the high levels of carbohydrates, as well as of citric acid, detected in ¹H-NMR spectra of hUCBP. Culturing media usually contain varying concentrations of glucose ranging from 1 to 4.5 g/l (5.6 to 25 mM). Glucose is a central source of energy for hMSCs, and recently isolated cells normally benefit from high glucose during the first 48 hours in culture, as we have observed [12, 54, 55]. However, sustained elevated glucose concentrations may impair cellular functions, increase the intracellular calcium concentration ([Ca²⁺]_i) and induce apoptosis [12, 47, 54]. It has also been demonstrated that high glucose

stimulates the glucose-responsive gene thioredoxin-interacting protein (Txnip) in MSCs and induces MSCs to a pro-apoptotic situation, which reduces their regenerative qualities after chronic but not short-term exposure [55]. Nevertheless, high glucose contents must not always be regarded as deleterious for hMSCs populations, as it appears to greatly improve survival in severe hypoxia situations that are often associated with pathological events of ischemia and stroke [56]. The glucose content of the culture medium is therefore of the utmost importance. Increased levels of lactate are detected in FBS samples. In the haemoderived cellular culture supplements, particularly those collected in perinatal stages, as is the case of FBS and hUCBP, observed lactate levels strictly relate labor process and metabolic acidosis of the newborn, and is an important indicator of intrapartum asphyxia [57]. Lactate is a product of anaerobic metabolism of pyruvate (that mostly derives from supplied glucose), and accumulates in cellular culture supernatant during cell culturing, indicating a coexistence of both aerobic and anaerobic bioenergetic pathways. We have recently focused on the characterization of the metabolic profile of these precise cellular populations, and have verified that small amounts (in the μM range) of lactate are provided by basal culture media, and that this metabolite accumulates steadily for up to 48 hours in the culture media (reaching concentration in the 100–200 μM range) (Unpublished data). Most of the research targeting this topic focuses on lactate production by cultured populations. Fewer references focus on the effects of the lactate on these population, and mostly focus on its effects when accumulated in result of bioenergetic production. Lactate is described to impact on cell proliferation, resulting in inhibition over 20mM lactate in the culture media [58], but little is commented on the effects of low concentration of such metabolite. FBS samples assayed are commercially available and have been successfully employed in the hMSCs culture, as represented by the control groups herein utilized. Metabolomic analysis indicated increased increase relative amounts of lactate in these FBS samples, when compared to the hUCBP. FBS and hUCBP are supplemented to culture media in small percentages, ranging from 2–10% (in the presented experimental work) and hence observed variations in the metabolite content are diluted in the final media formulation. Since lower content in lactate is observed in the hUCBP samples, it is not expected to negatively impact cell performance. Although lactate is recognised to affect MSCs proliferative and differentiation features at high concentrations [59], FBS supplementation is not assigned to provide inhibitory levels of lactate. Both FBS groups show similar metabolite profile that differ from hUCBP samples in their high content of lactate, alanine, glutamine and branched chain amino acids and lower of glucose. Similar or slightly different levels of important proteinogenic amino acids, and of nucleotides, lipids, choline and formate were found in both samples. These proteinogenic amino acids are proteins precursors, and thus play a crucial role in the growth, proliferation and differentiation of hMSCs. The decreased content of alanine and glutamine in the hUCBP samples could be theoretically compensated by adding culture media supplements such as GlutaMAXTM (Thermo Fischer Scientific). This animal-origin free supplement (L-alanyl-L-glutamine) is a dipeptide substitute for L-Glutamine and can be used as a direct substitute for L-Glutamine at equimolar concentrations in mammalian and stem cell culture with minimal or no adaptation [8, 60]. At a cellular level, there are many important functions of amino acids such as glutamine, being essential as precursor for peptide and consequently substrate for protein synthesis [61–63]. Choi and colleagues [64] reported on importance of adequate essential and non-essential amino acids in cell culture, as over supplementation of the later was demonstrated to negatively affect MSCs proliferation. On the other hand, increased levels of essential amino acids, with balanced non-essential amino acids intake, were associated with increased cell proliferation. MSCs' surface markers expression did not seem to be affected by differential amino acid contents.

Other minor differences in key proteins' content are observed. Proline, a non-essential amino acid, is overexpressed in FBS_II samples, and has been associated with suppressing reactive oxygen species (ROS), thereby protecting mammalian cells from various stresses [65, 66]. FBS_I showed higher ethanol content in comparison to FBS_II. It has been reported that stem cells are extremely sensitive to alcohol by-products such as acetaldehyde that induces irreversible DNA mutation [67–69], and that it induces adipogenesis, resulting in cell death [70]. In other reports, ethanol exposure interfered with pluripotency marker patterns [68]. Therefore, hUCBP's lower content in ethanol arises as a beneficial point for the maintenance of the regenerative potential of expanding hMSCs. This particular finding converges with our preliminary experience in hMSCs *in vitro* proliferation and expansion, which supported the choice of FBS_II to further study the comparative effect of hUCBP culture medium supplementation [4–12, 29, 47].

The second part of the study aimed at the validation of hUCBP for the expansion of umbilical cord and dental pulp derived mesenchymal stem cells. Proliferation and viability/metabolic assays demonstrated that hMSCs were able to proliferate in 4%, 6% and 8% hUCBP supplemented culture media, and that metabolic rates observed (determined by Presto Blue day reduction assay) were superior to those of DPSCs and UC-MSCs for up to 5 and 7 days, respectively. At this point, cultured cells reached confluency, eliciting inhibitory signaling within hMSCs populations, and hUCBP supplemented cultures increased metabolic rates arrested. Zhang and colleagues reported that high glucose content in the MSCs culture media (which is the most striking difference between hUCBP and FBS) resulted in impaired cellular proliferation [71]. However, this study referred to glucose concentrations ranging from 11 to 20 mM (1.98 to 3.6 g/L, respectively), which exceeds the maximum concentration provided by hUCBP supplemented media [10% FBS: 4,9mM; 4%hUCBP: 6,6mM; 6%hUCBP: 7,5mM; and 8%hUCBP: 8,6mM], and refers to 14 days of culturing, with no mention of passaging or culture confluency. Another study reports no deleterious effect on MSCs' proliferation of 20–30mM of glucose, but this parameter was only assessed at 48 hours of culturing [72]. The arrest in proliferation rates observed in our study is directly linked to the confluent state of the cultured populations, which are described to shift to inhibitory intercellular signaling (reflected by metabolic rate decrease), due to close cell-to-cell contact. The same study [71], reported the induction of cellular senescence in the high glucose groups. Cellular senescence of cultured cellular populations reflect cellular aging *in vivo*, and this process is associated (although not solely bound to) β -Galactosidase enzyme expression and activity [73]. We observed that short term culturing (for up to 7 days) of passage 5 hMSCs in hUCBP supplemented media does not elicit increased cellular senescence events, as reported by Chang and colleagues, for the same time of assessment [74]. Again, contradictory observation may be due to the extreme glucose concentrations and culturing periods reported [71]. Similarly, cultured populations were assessed for apoptotic events, though the detection of Annexin-V and PI. Annexin V identification indicates the externalization of the membrane phospholipid phosphatidylserine (PS), which happens in the early stage of apoptosis. The positive of PI indicates access to intracellular genetic material, due to cell membrane compromise, which occurs either in the end stage of apoptosis or in dead cells. Therefore, viable, early apoptotic, late apoptotic and dead cellular populations can be distinguished [53]. We observed that culturing in hUCBP supplemented media resulted in a dose related tendency for increased early apoptosis events (Annexin V⁺ and PI⁺ cells) in both UC-MSCs and DPSCs. Supplementation with 8% hUCBP presented seemingly inferior viable populations, with increased dead cells. These events were particularly evident in UC-MSCs populations. High glucose contents are reported to induce oxidative stress in exposed population, which drive MSCs towards apoptosis, through the activation of

intrinsic cellular Caspase pathways [75]. These observations align with the apparent dose dependent increase in early apoptosis observed herein [74].

The classification of specific populations as MSCs is related to the expression of a particular immunophenotype. As such, it was agreed by the scientific community [2] that 95% of the candidate population ought to express CD105, CD73 and CD90, as well as to lack expression (<5% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and MHC class II. Other markers, such as CD44 are also identified in hMSCs populations. Cultured hMSCs populations maintained >95% expression of CD105, CD44 and CD73, while remaining <2% positive for CD34, CD11b, CD19, CD45 and MHC class II. Despite the ISCT's guidelines for the classification of MSCs populations, surface marker identity is a controversial topic. The obligatory expression of high levels (>95%) of CD90 in addressed populations is not always confirmed, while all other MSCs characteristics are verified, such as tri-lineage differentiation capacity [64, 76]. We observed maximal expression of CD90 in hUC-MSCs, but lower levels are equally reported [77]. The expression levels of CD90 have been related to MSCs their immunosuppressive capabilities, as Campioni and colleagues have disclosed [78]. Interestingly, qRT-PCR revealed that, although remaining strongly expressed (in accordance to external marker expression revealed by Flow Cytometry), CD90 depicted decreasing mRNA expression in hUCBP cultured groups, which has been associated to decreased immunosuppressive capacities [78]. MSCs are also described to express MHC class I and, to a much smaller extent, MHC class II [79]. DPSCs presented weak expression of MHC class II molecules, which attenuated in xeno-free cultured groups. Contrarily, MHC class II was not detected UC-MSCs in FBS supplemented groups, but became apparent in hUCBP cultured groups. These expression up- and down-regulations are smooth, a did not reflect in the membrane expression of the molecule [80]. MSCs are described to express pluripotency genes in resemblance to embryonic stem cells. We confirmed c-kit and OCT-4 expression but were unable to detect Sox-2 mRNA. Similarly to the character and degree of expression of surface markers, pluripotency genes expression provides conflicting reports [81, 82]. Distinct patterns of up- and down-regulation of specific genes in UC-MSCs and DPSCs, along with the distinct responses verified in terms of apoptosis and multilineage differentiation capacity, reinforce that differently sourced MSCs respond differently to environmental cues, whether they are provided by their specific niches or by *ex-vivo* culturing conditions. The replacement of FBS in the specific Osteo-differentiation media presents a deleterious effect, as only minor staining was observed after incubation with ARS (a Ca²⁺ binding dye). Quantification confirmed the inferior uptake in all hUCBP groups, as compared to the FBS supplemented groups. Standard FBS supplemented differentiation efficiency also support reported superior capacity from DPSCs to produce mineralized matrix when compared to UC-MSCs [83]. Expansion of hMSCs in media supplemented with hUCBP does not preclude their capacity to differentiate towards adipogenic phenotypes in either cell type, when compared to FBS supplemented groups. The maintenance of UC-MSCs and DPSCs in hUCBP supplemented media for 14 days induced lipid droplet deposition, which although statistically inferior to cells maintained in differentiation media, was superior to the respective FBS supplemented control. The presence of AGE (advanced glycation products, associated with high glucose levels) in culture media is associated to reduced MSCs' differentiation capacity [54]. Cramer and colleagues [75], also report impaired osteogenic and chondrogenic potential of MSCs, but observed enhanced adipogenic tendency. Again, maximum concentration provided by hUCBP formulated media remains under the studied ranges reported in literature, but similar effects were already observed in osteogenesis and adipogenesis of UC-MSCs and DPSCs. We do not observe the same inhibition in chondrogenesis. Although high glucose is a marking feature oh hUCBP, other relevant components must be addressed in its composition, such as growth factors, cytokines and chemokines [8].

Preliminary assays demonstrate substantially increased levels of TGF- β 1–3 in hUCBP samples when compared to FBS (7-, 2- and 28-fold increase in TGF- β 1, 2 and 3, respectively, data not shown). TGF- β 1 is a strong modulator of TGF β receptors expression, and thereof of chondrogenesis of MSCs. Although the effects of high glucose are seemingly deleterious to this differentiation pathway, it is associated with increased TGF β RII expression. The combination of both factors (high concentration of glucose and TGF- β s) may attenuate the reported deleterious effect of glucose. We observed that chondro-differentiation efficiency was unaffected by the expansion in hUCBP supplemented media, and that the maintenance of undifferentiated control groups in hUCBP induced UC-MSCs to form chondrogenic-like pellets and produce GAGs rich matrix. The differential response observed in the assessed cell population could relate to the differentiation aptitude of MSCs derived from particular niches [83].

In conclusion, regardless of the discussed differences, FBS and hUCBP present relevant similarities. This study reveals the existence of essential components (including glucose and proteinogenic amino acids) for the growth and proliferation of hMSCs in both supplement options. These observations suggest that hUCBP could be considered as an alternative to FBS for *in vitro* production of hMSCs for cell-based therapies and for clinical applications. Nevertheless, investigation must deepen regarding the applicability of hUCBP through the entire hMSCs production process, and studies are ongoing as for their suitability for the primary isolation of cells from source tissues, as well as for cryopreservation and final formulations of hMSCs derived therapeutic products. Further, we have observed some of the hUCBP composition on the MSCs features and capacities, such as in their differentiation potential, whose impact on the therapeutic properties desired for specific diseases requires further investigation.

Supporting information

S1 Table. UCB samples analysis. Initial WBC count, performed before the volume reduction with the AXP automated system (cells x 10⁹ per liter) and the final WBC count (cells x 10⁹ per liter), performed after the volume reduction procedure of the UCB samples, values obtained by using the hematology auto-analyser (Ac T diff2™, Beckman Coulter, Inc.). Total number of viable leucocytes (CD45⁺) per liter, the percentage (%) of viable leucocytes (CD45⁺), the total number of viable CD34⁺ cells per μ l, and the % of viable CD34⁺, measured by flow cytometry of the UCB samples used for ¹H-NMR analysis. The average (Mean), the maximum (Max) and minimum (Min) values, standard deviation (SD) and the standard error of the mean (SEM) of N = 13 are also presented. *Count using the hematology auto-analyzer. #Extrapolated values considering that the collected hUCBP volume comprises of approximately 1/3 of the volume remaining after the removal of the Buffy coat layer, Microbiological analysis were performed after volume reduction and before cryopreservation and tested for microbiological contamination using an automated blood culture system (BacT/ALERT®, BioMérieux) at 35°C for 14 days.

(DOCX)

S2 Table. Corrected absorbance assessed by PrestoBlue viability assay of hMSCs (UC-MSCs and DPSCs), in the presence of supplemented medium with FBS_II or variable concentrations of hUCBP for up to 9 days. Results presented as Mean $\pm$ SEM.

(DOCX)

S3 Table. β -Galactosidase activity assay (OD_{405nm}) on UC-MSCs and DPSCs at 3, 5 and 7 days. Results Presented as Mean $\pm$ SEM.

(DOCX)

S4 Table. Annexin V/ PI detection on UC-MSCs and DPSCs after 5 days of culture in hUCBP or FBS supplemented media. Results Presented as Mean $\pm$ SEM.
(DOCX)

S5 Table. Total RNA extracted from UC-MSCs and DPSCs cultured in hUCBP or FBS supplemented media, readings at 260 and 280 nm.
(DOCX)

S6 Table. Quantitative PCR of UC-MSCs and DPSCs cultured in hUCBP or FBS supplemented media. Avg Δ Cq: average quantification cycle (differential expression of target and housekeeping genes); $\Delta\Delta$ Cq: differential expression of sample (4%, 6% and 8% hUCBP) and reference sample (FBS 10%) genes; RQ: relative quantification (fold change compared to the FBS 10% group), in mean fold change $\pm$ SEM; nd: not detected; na: not applicable; $\uparrow$: up-regulated over 2-fold; $\downarrow$: down-regulated under 0.5 fold.
(DOCX)

S7 Table. Osteogenic differentiation. Alizarin Red S concentration (μ M) after 21 days. Control: Undifferentiated control; Osteo Diff: Osteogenic Differentiation. Results Presented as Mean $\pm$ SEM.
(DOCX)

S8 Table. Statistical significance in Alizarin Red S concentration (μ M) after 21 days. C: Undifferentiated control; D: Osteogenic Differentiation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.
(DOCX)

S9 Table. Adipogenic differentiation. Oil Red O (OD_{570nm}) after 14 days. Control: Undifferentiated control; Adipo Diff: Adipogenic Differentiation. Results Presented as Mean $\pm$ SEM.
(DOCX)

S10 Table. Statistical significance in Oil Red O (OD_{570nm}) after 14 days. C: Undifferentiated control; D: Adipogenic Differentiation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.
(DOCX)

S11 Table. Chondrogenic differentiation. Sulfated GAGs production (μ g/ml) after 14 days, assessed by Blyscan Glycosaminoglycan Assay (Biocolor, UK). Control: Undifferentiated control; Chondro Diff: Chondrogenic Differentiation. Results Presented as Mean $\pm$ SEM.
(DOCX)

S12 Table. Statistical significance differences in sulfated GAGs production (μ g/ml) after 14 days, assessed by Blyscan Glycosaminoglycan Assay (Biocolor, UK). C: Undifferentiated control; D: Chondrogenic Differentiation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.
(DOCX)

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References

1. Tekkatté C, Gunasingh GP, Cherian KM, Sankaranarayanan K. "Humanized" stem cell culture techniques: the animal serum controversy. *Stem Cells Int*. 2011;2011:504723. <https://doi.org/10.4061/2011/504723> PMID: 21603148; PubMed Central PMCID: PMC3096451.
2. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006; 8(4):315–7. Epub 2006/08/23. <https://doi.org/10.1080/14653240600855905> PMID: 16923606.
3. Buyl K, Vanhaecke T, Desmae T, Lagneaux L, Rogiers V, Najjar M, et al. Evaluation of a new standardized enzymatic isolation protocol for human umbilical cord-derived stem cells. *Toxicol In Vitro*. 2015; 29(6):1254–62. <https://doi.org/10.1016/j.tiv.2014.12.008> PMID: 25541070.
4. Caseiro AR, Pereira T, Ivanova G, Luis AL, Mauricio AC. Neuromuscular Regeneration: Perspective on the Application of Mesenchymal Stem Cells and Their Secretion Products. *Stem Cells Int*. 2016;2016:9756973. <https://doi.org/10.1155/2016/9756973> PMID: 26880998; PubMed Central PMCID: PMC4736584.
5. Gartner A, Pereira T, Simoes MJ, Armada-da-Silva PA, Franca ML, Sousa R, et al. Use of hybrid chitosan membranes and human mesenchymal stem cells from the Wharton jelly of umbilical cord for promoting nerve regeneration in an axonotmesis rat model. *Neural Regen Res*. 2012; 7(29):2247–58. <https://doi.org/10.3969/j.issn.1673-5374.2012.29.002> PMID: 25538746; PubMed Central PMCID: PMC4268725.
6. Pereira T, Armada-da-Silva PA, Amorim I, Rema A, Caseiro AR, Gartner A, et al. Effects of Human Mesenchymal Stem Cells Isolated from Wharton's Jelly of the Umbilical Cord and Conditioned Media on Skeletal Muscle Regeneration Using a Myectomy Model. *Stem Cells Int*. 2014;2014:376918. <https://doi.org/10.1155/2014/376918> PMID: 25379040; PubMed Central PMCID: PMC4212633.
7. Pereira T, Gartner A, Amorim I, Almeida A, Caseiro AR, Armada-da-Silva PA, et al. Promoting nerve regeneration in a neurotmesis rat model using poly(DL-lactide-epsilon-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly: in vitro and in vivo analysis. *Biomed Res Int*. 2014;2014:302659. <https://doi.org/10.1155/2014/302659> PMID: 25121094; PubMed Central PMCID: PMC4119891.
8. Pereira T, Ivanova G, Caseiro AR, Barbosa P, Bartolo PJ, Santos JD, et al. MSCs Conditioned Media and Umbilical Cord Blood Plasma Metabolomics and Composition. *PloS one*. 2014; 9(11):e113769. <https://doi.org/10.1371/journal.pone.0113769> PMID: 25423186.
9. Ribeiro J, Gartner A, Pereira T, Gomes R, Lopes MA, Goncalves C, et al. Perspectives of employing mesenchymal stem cells from the Wharton's jelly of the umbilical cord for peripheral nerve repair. *Int Rev Neurobiol*. 2013; 108:79–120. <https://doi.org/10.1016/B978-0-12-410499-0.00004-6> PMID: 24083432.
10. Ribeiro J, Pereira T, Amorim I, Caseiro AR, Lopes MA, Lima J, et al. Cell therapy with human MSCs isolated from the umbilical cord Wharton jelly associated to a PVA membrane in the treatment of chronic skin wounds. *Int J Med Sci*. 2014; 11(10):979–87. <https://doi.org/10.7150/ijms.9139> PMID: 25076843; PubMed Central PMCID: PMC4115236.
11. Ribeiro J, Pereira T, Caseiro AR, Armada-da-Silva P, Pires I, Prada J, et al. Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells. *World journal of stem cells*. 2015; 7(6):956–75. <https://doi.org/10.4252/wjsc.v7.i6.956> PMID: 26240682; PubMed Central PMCID: PMC4515438.
12. Silva DM, Caseiro AR, Amorim I, Pereira I, Faria F, Pereira T, et al. Inflammatory response to dextrin-based hydrogel associated with human mesenchymal stem cells, urinary bladder matrix and Bonelike (R) granules in rat subcutaneous implants. *Biomed Mater*. 2016; 11(6):065004. <https://doi.org/10.1088/1748-6041/11/6/065004> PMID: 27786165.
13. Fekete N, Rojewski MT, Furst D, Kreja L, Ignatius A, Dausend J, et al. GMP-compliant isolation and large-scale expansion of bone marrow-derived MSC. *PloS one*. 2012; 7(8):e43255. <https://doi.org/10.1371/journal.pone.0043255> PMID: 22905242; PubMed Central PMCID: PMC3419200.
14. Pham PV, Vu NB, Pham VM, Truong NH, Pham TL, Dang LT, et al. Good manufacturing practice-compliant isolation and culture of human umbilical cord blood-derived mesenchymal stem cells. *Journal of translational medicine*. 2014; 12:56. <https://doi.org/10.1186/1479-5876-12-56> PMID: 24565047; PubMed Central PMCID: PMC4393935.
15. Bieback K, Hecker A, Kocaomer A, Lannert H, Schallmoser K, Strunk D, et al. Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. *Stem cells*. 2009; 27(9):2331–41. <https://doi.org/10.1002/stem.139> PMID: 19544413.
16. Selvaggi TA, Walker RE, Fleisher TA. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. *Blood*. 1997; 89(3):776–9. Epub 1997/02/01. PMID: 9028307.

17. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. *Mol Ther*. 2004; 9(5):747–56. <https://doi.org/10.1016/j.ymthe.2004.02.012> PMID: 15120336.
18. Phinney DG. Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. *Cell Cycle*. 2007; 6(23):2884–9. <https://doi.org/10.4161/cc.6.23.5095> PMID: 18000405.
19. Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views. *Stem cells*. 2007; 25(11):2896–902. <https://doi.org/10.1634/stemcells.2007-0637> PMID: 17901396.
20. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet*. 1970; 3(4):393–403. PMID: 5523063.
21. Simonetti AB, Englert GE, Campos K, Mergener M, De David C, De Oliveira AP, et al. Nanobacteria-like particles: A threat to cell cultures. *Brazilian Journal of Microbiology*. 2007; 38(1):153–8.
22. Halme DG, Kessler DA. FDA regulation of stem-cell-based therapies. *The New England journal of medicine*. 2006; 355(16):1730–5. <https://doi.org/10.1056/NEJMp063086> PMID: 17050899.
23. Halme DG, Khodor J, Mitchell R, Walker GC. A small-scale concept-based laboratory component: the best of both worlds. *CBE Life Sci Educ*. 2006; 5(1):41–51. <https://doi.org/10.1187/cbe.05-02-0065> PMID: 17012190; PubMed Central PMCID: PMC1635131.
24. Schallmoser K, Bartmann C, Rohde E, Reinisch A, Kashofer K, Stadelmeyer E, et al. Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. *Transfusion*. 2007; 47(8):1436–46. <https://doi.org/10.1111/j.1537-2995.2007.01220.x> PMID: 17655588.
25. Bernardo ME, Avanzini MA, Perotti C, Cometa AM, Moretta A, Lenta E, et al. Optimization of in vitro expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: further insights in the search for a fetal calf serum substitute. *Journal of cellular physiology*. 2007; 211(1):121–30. <https://doi.org/10.1002/jcp.20911> PMID: 17187344.
26. Huang L, Critser PJ, Grimes BR, Yoder MC. Human umbilical cord blood plasma can replace fetal bovine serum for in vitro expansion of functional human endothelial colony-forming cells. *Cytotherapy*. 2011; 13(6):712–21. <https://doi.org/10.3109/14653249.2010.548380> PMID: 21250867; PubMed Central PMCID: PMC163387926.
27. Hong J, Jin H, Han J, Hu H, Liu J, Li L, et al. Infusion of human umbilical cord-derived mesenchymal stem cells effectively relieves liver cirrhosis in DEN-induced rats. *Mol Med Rep*. 2014; 9(4):1103–11. <https://doi.org/10.3892/mmr.2014.1927> PMID: 24481983.
28. Pineault N, Abu-Khader A. Advances in umbilical cord blood stem cell expansion and clinical translation. *Exp Hematol*. 2015; 43(7):498–513. <https://doi.org/10.1016/j.exphem.2015.04.011> PMID: 25970610.
29. Gartner A, Pereira T, Alves MG, Armada-da-Silva PA, Amorim I, Gomes R, et al. Use of poly(DL-lactide-epsilon-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. *Differentiation*. 2012; 84(5):355–65. <https://doi.org/10.1016/j.diff.2012.10.001> PMID: 23142731.
30. Simoes IN, Boura JS, dos Santos F, Andrade PZ, Cardoso CM, Gimble JM, et al. Human mesenchymal stem cells from the umbilical cord matrix: successful isolation and ex vivo expansion using serum-/xeno-free culture media. *Biotechnology journal*. 2013; 8(4):448–58. <https://doi.org/10.1002/biot.201200340> PMID: 23420807.
31. Jung J, Moon N, Ahn JY, Oh EJ, Kim M, Cho CS, et al. Mesenchymal stromal cells expanded in human allogenic cord blood serum display higher self-renewal and enhanced osteogenic potential. *Stem Cells Dev*. 2009; 18(4):559–71. <https://doi.org/10.1089/scd.2008.0105> PMID: 18754716.
32. Ding DC, Chang YH, Shyu WC, Lin SZ. Human umbilical cord mesenchymal stem cells: a new era for stem cell therapy. *Cell Transplant*. 2015; 24(3):339–47. <https://doi.org/10.3727/096368915X686841> PMID: 25622293.
33. Shetty P, Bharucha K, Tanavde V. Human umbilical cord blood serum can replace fetal bovine serum in the culture of mesenchymal stem cells. *Cell Biol Int*. 2007; 31(3):293–8. <https://doi.org/10.1016/j.cellbi.2006.11.010> PMID: 17208468.
34. Dona AC, Jimenez B, Schafer H, Humpfer E, Spraul M, Lewis MR, et al. Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping. *Anal Chem*. 2014; 86(19):9887–94. <https://doi.org/10.1021/ac5025039> PMID: 25180432.
35. Beckonert O, Keun HC, Ebbels TMD, Bundy JG, Holmes E, Lindon JC, et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature Protocols*. 2007; 2(11):2692–703. <https://doi.org/10.1038/nprot.2007.376> PubMed PMID: WOS:000253140000005. PMID: 18007604

36. Zhang S, Nagana Gowda GA, Ye T, Raftery D. Advances in NMR-based biofluid analysis and metabolite profiling. *Analyst*. 2010; 135(7):1490–8. <https://doi.org/10.1039/c000091d> PMID: [20379603](#); PubMed Central PMCID: PMC4720135.
37. Gartland KP, Beddell CR, Lindon JC, Nicholson JK. Application of pattern recognition methods to the analysis and classification of toxicological data derived from proton nuclear magnetic resonance spectroscopy of urine. *Mol Pharmacol*. 1991; 39(5):629–42. PMID: [2034235](#).
38. Spraul M, Neidig P, Klauck U, Kessler P, Holmes E, Nicholson JK, et al. Automatic reduction of NMR spectroscopic data for statistical and pattern recognition classification of samples. *J Pharm Biomed Anal*. 1994; 12(10):1215–25. PMID: [7841215](#).
39. Corcoran O, Lindon JC, Hall R, Ismail IM, Nicholson JK. The potential of 19F NMR spectroscopy for rapid screening of cell cultures for models of mammalian drug metabolism. *Analyst*. 2001; 126(12):2103–6. PMID: [11814184](#).
40. Powers R. Advances in Nuclear Magnetic Resonance for Drug Discovery. *Expert Opin Drug Discov*. 2009; 4(10):1077–98. <https://doi.org/10.1517/17460440903232623> PMID: [20333269](#); PubMed Central PMCID: PMC4720135.
41. Perales A, Solves P, Perales-Puchalt A, Moraga R, Lopez M, Diago V. Obstetric Factors influencing the hematopoietic content of cord blood units. *Advances in Perinatal Medicine*. 2010:695–8. PubMed PMID: WOS:000281970400134.
42. Solves P, Mirabet V, Blanquer A, Delgado F, Larrea L, Torres A, et al. Apx in Routine Cord Blood Banking: Critical Analysis of Different Volume Reduction Methodologies. *Haematol-Hematol J*. 2009; 94:301–. PubMed PMID: WOS:000266931900740.
43. Solves P, Planelles D, Mirabet V, Blanquer A, Carbonell-Uberos F. Qualitative and quantitative cell recovery in umbilical cord blood processed by two automated devices in routine cord blood banking: a comparative study. *Blood Transfus-Italy*. 2013; 11(3):405–11. <https://doi.org/10.2450/2012.0037-12> PubMed PMID: WOS:000322288600016.
44. Jeener J, Meier BH, Bachmann P, Ernst RR. Investigation of Exchange Processes by 2-Dimensional Nmr-Spectroscopy. *J Chem Phys*. 1979; 71(11):4546–53. <https://doi.org/10.1063/1.438208> PubMed PMID: WOS:A1979HW09500041.
45. Meiboom S, Gill D. Modified Spin-Echo Method for Measuring Nuclear Relaxation Times. *Review of Scientific Instruments*. 1958; 29(8):688–91. <https://doi.org/10.1063/1.1716296> PubMed PMID: WOS: A1958WH51500003.
46. Mevik BH, Wehrens R. The pls package: Principal component and partial least squares regression in R. *J Stat Softw*. 2007; 18(2):1–23. PubMed PMID: WOS:000244067100001.
47. Alexandre N, Amorim I, Caseiro AR, Pereira T, Alvites R, Rema A, et al. Long term performance evaluation of small-diameter vascular grafts based on polyvinyl alcohol hydrogel and dextran and MSCs-based therapies using the ovine pre-clinical animal model. *Int J Pharm*. 2017. <https://doi.org/10.1016/j.ijpharm.2017.02.043> PMID: [28283218](#).
48. Alexandre N, Costa E, Coimbra S, Silva A, Lopes A, Rodrigues M, et al. In vitro and in vivo evaluation of blood coagulation activation of polyvinyl alcohol hydrogel plus dextran-based vascular grafts. *Journal of biomedical materials research Part A*. 2015; 103(4):1366–79. <https://doi.org/10.1002/jbm.a.35275> PMID: [25044790](#).
49. Alexandre N, Ribeiro J, Gartner A, Pereira T, Amorim I, Fragoso J, et al. Biocompatibility and hemocompatibility of polyvinyl alcohol hydrogel used for vascular grafting—In vitro and in vivo studies. *Journal of biomedical materials research Part A*. 2014; 102(12):4262–75. <https://doi.org/10.1002/jbm.a.35098> PMID: [24488670](#).
50. Kyllönen L, Haimi S, Mannerström B, Huhtala H, Rajala KM, Skottman H, et al. Effects of different serum conditions on osteogenic differentiation of human adipose stem cells in vitro. *Stem cell research & therapy*. 2013; 4(1):17. <https://doi.org/10.1186/sct165> PMID: [23415114](#)
51. Foxall PJD, Spraul M, Farrant RD, Lindon LC, Neild GH, Nicholson JK. 750 Mhz 1h-Nmr Spectroscopy of Human Blood-Plasma. *Journal of Pharmaceutical and Biomedical Analysis*. 1993; 11(4–5):267–76. [https://doi.org/10.1016/0731-7085\(93\)80017-U](https://doi.org/10.1016/0731-7085(93)80017-U) PubMed PMID: WOS:A1993LJ29400001. PMID: [8357863](#)
52. Nicholson JK, Foxall PJD, Spraul M, Farrant RD, Lindon JC. 750-Mhz H-1 and H-1-C-13 Nmr-Spectroscopy of Human Blood-Plasma. *Analytical Chemistry*. 1995; 67(5):793–811. <https://doi.org/10.1021/ac00101a004> PubMed PMID: WOS:A1995QK14400012. PMID: [7762816](#)
53. Pedrosa SS, Pereira P, Correia A, Moreira S, Rocha H, Gama FM. Biocompatibility of a Self-Assembled Crosslinkable Hyaluronic Acid Nanogel. *Macromolecular bioscience*. 2016; 16(11):1610–20.
54. Stolzing A, Coleman N, Scutt A. Glucose-induced replicative senescence in mesenchymal stem cells. *Rejuvenation Res*. 2006; 9(1):31–5. <https://doi.org/10.1089/rej.2006.9.31> PMID: [16608393](#).

55. Li YM, Schilling T, Benisch P, Zeck S, Meissner-Weigl J, Schneider D, et al. Effects of high glucose on mesenchymal stem cell proliferation and differentiation. *Biochemical and biophysical research communications*. 2007; 363(1):209–15. <https://doi.org/10.1016/j.bbrc.2007.08.161> PubMed PMID: WOS:000250060200035. PMID: [17868648](#)
56. Deschepper M, Oudina K, David B, Myrttil V, Collet C, Bensidhoum M, et al. Survival and function of mesenchymal stem cells (MSCs) depend on glucose to overcome exposure to long-term, severe and continuous hypoxia. *Journal of cellular and molecular medicine*. 2011; 15(7):1505–14. <https://doi.org/10.1111/j.1582-4934.2010.01138.x> PMID: [20716129](#); PubMed Central PMCID: PMC3823195.
57. Gjerris AC, Staer-Jensen J, Jørgensen JS, Bergholt T, Nickelsen C. Umbilical cord blood lactate: A valuable tool in the assessment of fetal metabolic acidosis. *European Journal of Obstetrics and Gynecology and Reproductive Biology*. 2008; 139(1):16–20. <https://doi.org/10.1016/j.ejogrb.2007.10.004> PMID: [18063469](#)
58. Schop D, Janssen FW, van Rijn LD, Fernandes H, Bloem RM, de Bruijn JD, et al. Growth, metabolism, and growth inhibitors of mesenchymal stem cells. *Tissue Engineering Part A*. 2009; 15(8):1877–86. <https://doi.org/10.1089/ten.tea.2008.0345> PMID: [19196147](#)
59. Chen T, Zhou Y, Tan W-S. Influence of lactic acid on the proliferation, metabolism, and differentiation of rabbit mesenchymal stem cells. *Cell biology and toxicology*. 2009; 25(6):573.
60. Fresney R. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*. In: Wiley-Blackwell, editor. New York 2010.
61. Newsholme P, Lima MMR, Porcopio J, Pithon-Curi TC, Doi SQ, Bazotte RB, et al. Glutamine and glutamate as vital metabolites. *Brazilian Journal of Medical and Biological Research*. 2003; 36(2):153–63. <https://doi.org/10.1590/S0100-879x2003000200002> PubMed PMID: WOS:000181135700002.
62. Newsholme P, Procopio J, Lima MMR, Pithon-Curi TC, Curi R. Glutamine and glutamate—their central role in cell metabolism and function. *Cell Biochem Funct*. 2003; 21(1):1–9. <https://doi.org/10.1002/cbf.1003> PubMed PMID: WOS:000181097100001. PMID: [12579515](#)
63. MacIntyre DA, Melguizo Sanchis D, Jimenez B, Moreno R, Stojkovic M, Pineda-Lucena A. Characterisation of human embryonic stem cells conditioning media by ¹H-nuclear magnetic resonance spectroscopy. *PloS one*. 2011; 6(2):e16732. <https://doi.org/10.1371/journal.pone.0016732> PMID: [21347425](#); PubMed Central PMCID: PMC3036660.
64. Choi K-M, Yoon H-H, Seo Y-K, Song K-Y, Kwon S-Y, Lee H-S, et al. Effect of essential and nonessential amino acid compositions on the in vitro behavior of human mesenchymal stem cells. *Korean Journal of Chemical Engineering*. 2007; 24(6):1058–63.
65. Maddocks ODK, Berkers CR, Mason SM, Zheng L, Blyth K, Gottlieb E, et al. Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. *Nature*. 2013; 493(7433):542–+. <https://doi.org/10.1038/nature11743> PubMed PMID: WOS:000313871400040. PMID: [23242140](#)
66. Sahu N, Dela Cruz D, Gao M, Sandoval W, Haverty PM, Liu JF, et al. Proline Starvation Induces Unresolved ER Stress and Hinders mTORC1-Dependent Tumorigenesis. *Cell metabolism*. 2016; 24(5):753–61. <https://doi.org/10.1016/j.cmet.2016.08.008> PubMed PMID: WOS:000389508000015. PMID: [27618686](#)
67. Worley SL, Vaughn BJ, Terry AI, Gardiner CS, DeKrey GK. Time- and dose-dependent effects of ethanol on mouse embryonic stem cells. *Reprod Toxicol*. 2015; 57:157–64. <https://doi.org/10.1016/j.reprotox.2015.06.044> PubMed PMID: WOS:000360322300018. PMID: [26073001](#)
68. Arzumayan A, Anni H, Rubin R, Rubin E. Effects of ethanol on mouse embryonic stem cells. *Alcoholism-Clinical and Experimental Research*. 2011; 35(4):773–. <https://doi.org/10.1111/j.1530-0277.2010.01377.x> PubMed PMID: WOS:000288867100022.
69. Chen JR, Lazarenko OP, Shankar K, Blackburn ML, Badger TM, Ronis MJ. A Role for Ethanol-Induced Oxidative Stress in Controlling Lineage Commitment of Mesenchymal Stromal Cells Through Inhibition of Wnt/beta-Catenin Signaling. *Journal of Bone and Mineral Research*. 2010; 25(5):1117–27. <https://doi.org/10.1002/jbmr.7> PubMed PMID: WOS:000278292100022. PMID: [20200986](#)
70. Yeh CH, Chang JK, Wang YH, Ho ML, Wang GJ. Ethanol may suppress Wnt/beta-catenin signaling on human bone marrow stroma cells. *Clinical orthopaedics and related research*. 2008; 466(5):1047–53. <https://doi.org/10.1007/s11999-008-0171-1> PubMed PMID: WOS:000254772200006. PMID: [18288545](#)
71. Zhang D, Lu H, Chen Z, Wang Y, Lin J, Xu S, et al. High glucose induces the aging of mesenchymal stem cells via Akt/mTOR signaling. *Molecular medicine reports*. 2017; 16(2):1685–90.
72. Weil BR, Abarbanell AM, Herrmann JL, Wang Y, Meldrum DR. High glucose concentration in cell culture medium does not acutely affect human mesenchymal stem cell growth factor production or proliferation. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 2009; 296(6):R1735–R43. <https://doi.org/10.1152/ajpregu.90876.2008> PMID: [19386985](#)

73. Lee BY, Han JA, Im JS, Morrone A, Johung K, Goodwin EC, et al. Senescence-associated β -galactosidase is lysosomal β -galactosidase. *Aging cell*. 2006; 5(2):187–95.
74. Chang T-C, Hsu M-F, Wu KK. High glucose induces bone marrow-derived mesenchymal stem cell senescence by upregulating autophagy. *PLoS one*. 2015; 10(5):e0126537. <https://doi.org/10.1371/journal.pone.0126537> PMID: 25961745
75. Cramer C, Freisinger E, Jones RK, Slakey DP, Dupin CL, Newsome ER, et al. Persistent high glucose concentrations alter the regenerative potential of mesenchymal stem cells. *Stem cells and development*. 2010; 19(12):1875–84. <https://doi.org/10.1089/scd.2010.0009> PMID: 20380516
76. Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem cells*. 2006; 24(5):1294–301. <https://doi.org/10.1634/stemcells.2005-0342> PMID: 16410387
77. Maleki M, Ghanbarvand F, Behvarz MR, Ejtemaei M, Ghadirkhomi E. Comparison of mesenchymal stem cell markers in multiple human adult stem cells. *International journal of stem cells*. 2014; 7(2):118. <https://doi.org/10.15283/ijsc.2014.7.2.118> PMID: 25473449
78. Campioni D, Rizzo R, Stignani M, Melchiorri L, Ferrari L, Moretti S, et al. A decreased positivity for CD90 on human mesenchymal stromal cells (MSCs) is associated with a loss of immunosuppressive activity by MSCs. *Cytometry Part B: Clinical Cytometry*. 2009; 76(3):225–30.
79. Machado CdV, Telles PDdS, Nascimento ILO. Immunological characteristics of mesenchymal stem cells. *Revista brasileira de hematologia e hemoterapia*. 2013; 35(1):62–7. <https://doi.org/10.5581/1516-8484.20130017> PMID: 23580887
80. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringdén O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Experimental Hematology*. 2003; 31(10):890–6. [https://doi.org/10.1016/S0301-472X\(03\)00110-3](https://doi.org/10.1016/S0301-472X(03)00110-3) PMID: 14550804
81. Pierantozzi E, Gava B, Manini I, Roviello F, Marotta G, Chiavarelli M, et al. Pluripotency regulators in human mesenchymal stem cells: expression of NANOG but not of OCT-4 and SOX-2. *Stem cells and development*. 2010; 20(5):915–23. <https://doi.org/10.1089/scd.2010.0353> PMID: 20879854
82. Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M, Jankovskis G, Muiznieks I, et al. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. *Stem Cell Reviews and Reports*. 2009; 5(4):378–86. <https://doi.org/10.1007/s12015-009-9094-9> PMID: 20058201
83. Caseiro RA AR, Pedrosa SS, Campos JM, Reis IL, Santos JD, Mendonça C, Atayde LM and Maurício AC. The Potential Clinical Application of Mesenchymal Stem Cells from the Dental Pulp (DPSCs) for Bone Regeneration. In: Anjum A-u-RaS, editor. *Frontiers in Stem Cell and Regenerative Medicine Research*. Bentham Science Publishers; 2017.

Supporting information:

S1 Table: UCB Samples Analysis. Initial WBC count, performed before the volume reduction with the AXP automated system (cells $\times 10^9$ per liter) and the final WBC count (cells $\times 10^9$ per liter), performed after the volume reduction procedure of the UCB samples, values obtained by using the hematology auto-analyzer (Ac T diff2™, Beckman Coulter, Inc.). Total number of viable leucocytes (CD45⁺) per liter, the percentage (%) of viable leucocytes (CD45⁺), the total number of viable CD34⁺ cells per μ L, and the % of viable CD34⁺, measured by flow cytometry of the UCB samples used for ¹H-NMR analysis. The average (Mean), the maximum (Max) and minimum (Min) values, standard deviation (SD) and the standard error of the mean (SEM) of N=13 are also presented. *Count using the hematology auto-analyzer. #Extrapolated values considering that the collected hUCBP volume comprises of approximately 1/3 of the volume remaining after the removal of the Buffy coat layer, Microbiological analysis were performed after volume reduction and before cryopreservation and tested for microbiological contamination using an automated blood culture system (BacT/ALERT®, BioMérieux) at 35°C for 14 days.

<i>hUCBP sample</i>	<i>Initial WBC (cells $\times 10^9$/L)*</i>	<i>Final WBC (cells $\times 10^9$/L)*</i>	<i>Total viable CD45⁺ (cells $\times 10^9$/L)</i>	<i>Cell viability (CD45⁺)</i>	<i>Total viable CD34⁺ (cells $\times 10^9$/L)</i>	<i>Cell viability (CD34⁺)</i>	<i>Processed hUCBP volume (mL)</i>	<i>Collected hUCBP volume (mL)#</i>	<i>Microbiological analysis (aerobic, anaerobic and fungi)</i>
<i>hUCBP#1</i>	8.15	12.45	10.00	95.39	40.00	95.68	51.06	10.15	negative
<i>hUCBP#2</i>	7.00	17.30	14.30	97.99	43.33	94.08	70.10	16.47	negative
<i>hUCBP#3</i>	8.50	17.80	10.80	85.19	66.34	95.18	59.24	12.81	negative
<i>hUCBP#4</i>	15.70	52.60	27.50	89.32	158.49	95.05	78.92	19.34	negative
<i>hUCBP#5</i>	8.00	25.80	15.80	97.87	44.93	97.26	84.09	21.13	<i>Actinomyces meyer</i>
<i>hUCBP#6</i>	15.30	54.80	29.90	84.80	17.66	96.86	98.27	25.79	negative
<i>hUCBP#7</i>	7.00	37.60	19.90	90.98	27.29	98.56	124.10	34.37	negative
<i>hUCBP#8</i>	11.10	43.90	27.90	85.41	168.05	98.85	125.23	34.81	negative
<i>hUCBP#9</i>	6.80	12.60	9.10	91.91	29.34	80.90	47.30	8.87	negative
<i>hUCBP#10</i>	8.30	34.65	19.60	87.22	40.57	95.83	105.53	28.24	negative
<i>hUCBP#11</i>	8.70	15.60	10.90	90.65	17.02	86.67	51.44	10.21	negative
<i>hUCBP#12</i>	7.80	16.40	9.90	88.46	15.72	77.56	60.54	13.21	negative
<i>hUCBP#13</i>	12.60	40.95	24.40	81.12	191.45	98.25	87.61	22.27	negative
Mean	9.61	29.42	17.63	89.72	66.17	93.13	80.26	19.82	
SD	2.96	14.79	7.27	4.96	60.19	6.65	26.90	8.95	
SEM	2.50	13.54	6.56	4.08	49.18	5.27	7.46	2.48	
Max	15.70	54.80	29.09	97.99	191.45	98.85	34.81	34.81	
Min	6.80	12.45	9.10	81.12	15.72	77.56	8.87	8.87	
n	13	13	13	13	13	13	13	13	

S2 Table: Corrected absorbance assessed by PrestoBlue® viability assay of hMSCs (UC-MSCs and DPSCs), in the presence of supplemented medium with FBS_II or variable concentrations of hUCBP for up to 9 days. Results presented as Mean $\pm$ SEM.

<i>PrestoBlue® Cell Viability Assay</i>	<i>hUC-MSCs</i>				
	<i>FBS 10%</i>	<i>hUCBP 2%</i>	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>
24 hours (1 day)	0.0086 $\pm$ 0.0043	0.0052 $\pm$ 0.0006	0.0257 $\pm$ 0.0038	0.0119 $\pm$ 0.0007	0.0144 $\pm$ 0.0009
72 hours (3 days)	0.0134 $\pm$ 0.0003	0.0233 $\pm$ 0.0029	0.0226 $\pm$ 0.0033	0.0038 $\pm$ 0.0022	0.0178 $\pm$ 0.0020
120 hours (5 days)	0.1743 $\pm$ 0.0033	0.1333 $\pm$ 0.0029	0.1773 $\pm$ 0.0134	0.1651 $\pm$ 0.0135	0.1299 $\pm$ 0.0016
168 hours (7 days)	0.5022 $\pm$ 0.0084	0.3807 $\pm$ 0.0206	0.5982 $\pm$ 0.0190	0.5288 $\pm$ 0.0104	0.5097 $\pm$ 0.0030
216 hours (9 days)	0.3637 $\pm$ 0.0055	0.2914 $\pm$ 0.0084	0.3531 $\pm$ 0.0101	0.2942 $\pm$ 0.0098	0.2391 $\pm$ 0.0007

	<i>hDPSCs</i>				
	<i>FBS 10%</i>	<i>hUCBP 2%</i>	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>
24 hours (1 day)	0.0173 $\pm$ 0.0006	0.0198 $\pm$ 0.0008	0.0179 $\pm$ 0.0014	0.0167 $\pm$ 0.0010	0.0132 $\pm$ 0.0004
72 hours (3 days)	0.0147 $\pm$ 0.0028	0.0377 $\pm$ 0.0029	0.0341 $\pm$ 0.0015	0.0350 $\pm$ 0.0020	0.0288 $\pm$ 0.0014
120 hours (5 days)	0.1557 $\pm$ 0.0056	0.1489 $\pm$ 0.0004	0.1579 $\pm$ 0.0009	0.1626 $\pm$ 0.0037	0.1406 $\pm$ 0.0038
168 hours (7 days)	0.3487 $\pm$ 0.0076	0.0741 $\pm$ 0.0048	0.2712 $\pm$ 0.0065	0.3273 $\pm$ 0.0090	0.3188 $\pm$ 0.0048
216 hours (9 days)	0.2386 $\pm$ 0.0021	0.0829 $\pm$ 0.0009	0.1323 $\pm$ 0.0054	0.1542 $\pm$ 0.0065	0.1748 $\pm$ 0.0028

S3 Table: β -Galactosidase activity assay (OD_{405nm}) on hUC-MSCs and hDPSCs at 3, 5 and 7 days. Results presented as Mean $\pm$ SEM.

<i>β-Galactosidase (OD_{405nm})</i>	<i>hUC-MSCs</i>			
	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>	<i>FBS 10%</i>
3 days	0.145 $\pm$ 0.005	0.146 $\pm$ 0.000	0.143 $\pm$ 0.001	0.141 $\pm$ 0.001
5 days	0.144 $\pm$ 0.001	0.155 $\pm$ 0.002	0.146 $\pm$ 0.001	0.156 $\pm$ 0.005
7 days	0.138 $\pm$ 0.004	0.149 $\pm$ 0.002	0.149 $\pm$ 0.003	0.132 $\pm$ 0.005

	<i>hDPSCs</i>			
	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>	<i>FBS 10%</i>
3 days	0.136 $\pm$ 0.000	0.137 $\pm$ 0.000	0.137 $\pm$ 0.001	0.145 $\pm$ 0.003
5 days	0.138 $\pm$ 0.000	0.138 $\pm$ 0.000	0.137 $\pm$ 0.000	0.140 $\pm$ 0.001
7 days	0.138 $\pm$ 0.000	0.143 $\pm$ 0.000	0.142 $\pm$ 0.001	0.144 $\pm$ 0.002

S4 Table: Annexin V/ PI detection on hUC-MSCs and hDPSCs after 5 days of culture in hUCBP or FBS supplemented media. Results presented as Mean $\pm$ SEM.

<i>Annexin V/ PI detection (% cells)</i>	<i>hUC-MSCs</i>			
	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>	<i>FBS 10%</i>
<i>Late apoptosis (An-PI+)</i>	0.05 $\pm$ 0.02	0.07 $\pm$ 0.01	0.06 $\pm$ 0.04	0.05 $\pm$ 0.00
<i>Dead cells (An+PI+)</i>	0.14 $\pm$ 0.06	0.11 $\pm$ 0.05	0.59 $\pm$ 0.23	0.10 $\pm$ 0.04
<i>Early apoptosis (An+PI-)</i>	0.29 $\pm$ 0.03	0.40 $\pm$ 0.04	6.42 $\pm$ 0.53	0.47 $\pm$ 0.07
<i>Viable cells (An- PI-)</i>	99.50 $\pm$ 0.10	99.40 $\pm$ 0.06	92.93 $\pm$ 0.52	99.37 $\pm$ 0.13

	<i>hDPSCs</i>			
	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>	<i>FBS 10%</i>
<i>Late apoptosis (An-PI+)</i>	0.03 $\pm$ 0.02	0.03 $\pm$ 0.01	0.02 $\pm$ 0.01	0.02 $\pm$ 0.02
<i>Dead cells (An+PI+)</i>	0.08 $\pm$ 0.05	0.06 $\pm$ 0.03	0.16 $\pm$ 0.07	0.06 $\pm$ 0.04
<i>Early apoptosis (An+PI-)</i>	0.14 $\pm$ 0.01	0.33 $\pm$ 0.07	0.38 $\pm$ 0.12	0.11 $\pm$ 0.02
<i>Viable cells (An- PI-)</i>	99.73 $\pm$ 0.07	99.57 $\pm$ 0.03	99.43 $\pm$ 0.09	99.80 $\pm$ 0.06

S5 Table: Total RNA extracted from hUC-MSCs and hDPSCs cultured in hUCBP or FBS supplemented media, readings at 260 and 280 nm.

<i>Total extracted RNA</i>		<i>Concentration (ng/μg)</i>	<i>Absorbance ratio (260/280 nm)</i>
<i>hUC-MSCs</i>	<i>hUCBP 4%</i>	400	2.04
	<i>hUCBP 6%</i>	386	2.05
	<i>hUCBP 8%</i>	372	2.44
	<i>FBS 10%</i>	338	2.04
<i>hDPSCs</i>	<i>hUCBP 4%</i>	196	2.09
	<i>hUCBP 6%</i>	210	2.02
	<i>hUCBP 8%</i>	202	2.10
	<i>FBS 10%</i>	190	2.16

S6 Table: Quantitative PCR of hUC-MSCs and hDPSCs cultured in hUCBP or FBS supplemented media. Avg ΔCq : average quantification cycle (differential expression of target and housekeeping genes); $\Delta\Delta Cq$: differential expression of sample (4%, 6% and 8% hUCBP) and reference sample (FBS 10%) genes; RQ: relative quantification (fold change compared to the FBS 10% group), in mean fold change $\pm$ SEM; nd: not detected; na: not applicable; $\uparrow$: up-regulated over 2-fold; $\downarrow$: down-regulated under 0.5 fold.

hUC-MSCs

Target gene	FBS 10%	hUCBP 4%			hUCBP 6%			hUCBP 8%		
	Avg ΔCq	Avg ΔCq	$\Delta\Delta Cq$	RQ	Avg ΔCq	$\Delta\Delta Cq$	RQ	Avg ΔCq	$\Delta\Delta Cq$	RQ
CD34	nd	nd	na	na	nd	na	na	nd	na	na
CD105	6.8	6.0	-0.7	0.6 ± 0.1	5.8	-0.9	0.5 ± 0.0	6.2	-0.6	0.7 ± 0.1
CD117	12.1	11.8	-0.4	0.8 ± 0.0	12.4	0.2	1.2 ± 0.0	12.4	0.2	1.2 ± 0.2
Sox2	nd	nd	na	na	nd	na	na	nd	na	na
CD166	6.0	6.3	0.3	1.2 ± 0.0	6.0	0.0	1.0 ± 0.0	6.4	0.4	1.3 ± 0.0
CD90	4.2	2.9	-1.4	$0.4 \pm 0.0 \downarrow$	2.8	-1.4	$0.4 \pm 0.0 \downarrow$	3.0	-1.3	$0.4 \pm 0.0 \downarrow$
CD73	6.8	6.8	0.0	1.0 ± 0.1	6.6	-0.3	0.8 ± 0.0	6.9	0.1	1.0 ± 0.0
MHC I	5.7	3.7	-2.0	$0.2 \pm 0.0 \downarrow$	3.9	-1.8	$0.3 \pm 0.0 \downarrow$	3.9	-1.8	$0.3 \pm 0.0 \downarrow$
MHC II	nd	18.4	na	na $\uparrow$	19.3	na	na $\uparrow$	15.9	na	na $\uparrow$
OCT 4	15.6	15.3	-0.3	0.8 ± 0.2	14.0	-1.6	$0.3 \pm 0.1 \downarrow$	14.2	-1.4	$0.4 \pm 0.1 \downarrow$

hDPSCs

Target gene	FBS 10%	hUCBP 4%			hUCBP 6%			hUCBP 8%		
	Avg ΔCq	Avg ΔCq	$\Delta\Delta Cq$	RQ	Avg ΔCq	$\Delta\Delta Cq$	RQ	Avg ΔCq	$\Delta\Delta Cq$	RQ
CD34	nd	nd	na	na	nd	na	na	nd	na	na
CD105	7.1	6.9	-0.2	0.9 ± 0.0	6.9	-0.2	0.9 ± 0.1	7.7	0.6	1.5 ± 0.0
CD117	11.3	10.2	-1.1	0.5 ± 0.0	10.8	-0.5	0.7 ± 0.0	11.1	-0.2	0.9 ± 0.3
Sox2	nd	nd	na	na	nd	na	na	nd	na	na
CD166	4.6	4.5	-0.1	1.0 ± 0.0	4.7	0.2	1.1 ± 0.0	4.9	0.3	1.3 ± 0.3
CD90	5.9	5.9	-0.1	1.0 ± 0.0	5.8	-0.1	0.9 ± 0.1	6.5	0.6	1.5 ± 0.0
CD73	4.6	5.7	1.1	$2.1 \pm 0.2 \uparrow$	5.9	1.2	$2.3 \pm 0.1 \uparrow$	6.6	2.0	$4.0 \pm 0.2 \uparrow$
MHC I	4.3	3.3	-1.0	0.5 ± 0.0	3.2	-1.1	0.5 ± 0.0	3.6	-0.7	0.6 ± 0.1
MHC II	11.2	8.9	-2.3	$0.2 \pm 0.0 \downarrow$	9.4	-1.8	$0.3 \pm 0.0 \downarrow$	9.9	-1.4	$0.4 \pm 0.1 \downarrow$
OCT 4	14.2	15.8	1.5	$2.9 \pm 0.4 \uparrow$	16.3	2.1	$4.2 \pm 0.0 \uparrow$	15.5	1.3	$2.5 \pm 1.5 \uparrow$

S7 Table: Osteogenic differentiation. Alizarin Red S concentration (μM) after 21 days. Control: Undifferentiated control; Osteo Diff: Osteogenic Differentiation. Results presented as Mean $\pm$ SEM.

Alizarin Red S (μM)	hUC-MSCs			
	hUCBP 4%	hUCBP 6%	hUCBP 8%	FBS 10%
Osteo Diff	68.45 $\pm$ 1.20	74.48 $\pm$ 0.32	72.14 $\pm$ 0.27	1449.57 $\pm$ 0.00
Control	81.85 $\pm$ 0.00	87.21 $\pm$ 0.00	78.72 $\pm$ 0.45	89.44 $\pm$ 0.45

	hDPSCs			
	hUCBP 4%	hUCBP 6%	hUCBP 8%	FBS 10%
Osteo Diff	68.79 $\pm$ 0.82	71.13 $\pm$ 0.00	73.81 $\pm$ 0.00	2299.31 $\pm$ 7.35
Control	61.31 $\pm$ 0.45	62.65 $\pm$ 0.45	60.86 $\pm$ 0.45	68.45 $\pm$ 0.00

S8 Table: Statistical Significance in Alizarin Red S concentration (μM) after 21 days. C: Undifferentiated control; D: Osteogenic Differentiation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.

ARS Assay	Statistical significance	hUC-MSCs								hDPSCs							
		hUCBP 4%		hUCBP 6%		hUCBP 8%		FBS 10%		hUCBP 4%		hUCBP 6%		hUCBP 8%		FBS 10%	
		D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
hUC-MSCs	hUCBP 4% D		****	ns	****	ns	***	****	****	ns	ns	ns	ns	ns	*	***	ns
	hUCBP 4% C			*	ns	***	ns	****	ns	****	****	***	****	**	****	****	****
	hUCBP 6% D				****	ns	ns	****	****	*	****	ns	****	ns	****	****	ns
	hUCBP 6% C					****	*	****	ns	****	****	****	****	****	****	****	****
	hUCBP 8% D						ns	****	****	ns	***	ns	**	ns	***	****	ns
	hUCBP 8% C							****	**	***	****	*	****	ns	****	****	**
hDPSCs	FBS 10% D							****	****	****	****	****	****	****	****	****	****
	FBS 10% C									****	****	****	****	****	****	****	****
	hUCBP 4% D									ns	ns	ns	ns	ns	****	ns	
	hUCBP 4% C										**	ns	****	ns	****	ns	
	hUCBP 6% D											**	ns	***	****	ns	
	hUCBP 6% C												***	ns	****	ns	
	hUCBP 8% D													****	****	ns	
	hUCBP 8% C														****	ns	
	FBS 10% D															****	
	FBS 10% C																

S9 Table: Adipogenic differentiation. Oil Red O (OD_{570nm}) after 14 days. Control: Undifferentiated control; Adipo Diff: Adipogenic Differentiation. Results presented as Mean ± SEM.

<i>Oil Red O</i> (OD _{570 nm})	<i>hUC-MSCs</i>			
	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>	<i>FBS 10%</i>
Adipo Diff	0.158 ± 0.007	0.149 ± 0.001	0.151 ± 0.004	0.164 ± 0.001
Control	0.121 ± 0.007	0.107 ± 0.003	0.113 ± 0.001	0.077 ± 0.001

	<i>hDPSCs</i>			
	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>	<i>FBS 10%</i>
Adipo Diff	0.158 ± 0.007	0.149 ± 0.001	0.151 ± 0.004	0.164 ± 0.001
Control	0.121 ± 0.007	0.107 ± 0.003	0.113 ± 0.001	0.077 ± 0.001

S10 Table: Statistical Significance in Oil Red O (OD_{570nm}) after 14 days. C: Undifferentiated control; D: Adipogenic Differentiation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to 0.01 ≤ P < 0.05; 0.001 ≤ P < 0.01; 0.0001 ≤ P < 0.001 and P < 0.0001, respectively; ns, not significant.

<i>ORO Assay</i>		<i>hUC-MSCs</i>								<i>hDPSCs</i>							
<i>Statistical significance</i>		<i>hUCBP 4%</i>		<i>hUCBP 6%</i>		<i>hUCBP 8%</i>		<i>FBS 10%</i>		<i>hUCBP 4%</i>		<i>hUCBP 6%</i>		<i>hUCBP 8%</i>		<i>FBS 10%</i>	
		D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
<i>hUC-MSCs</i>	<i>hUCBP 4%</i>	D	****	ns	****	ns	****	ns	****	ns	****	ns	****	ns	****	ns	****
		C		**	ns	****	ns	****	****	****	ns	**	ns	ns	***	****	****
	<i>hUCBP 6%</i>	D			****	ns	****	ns	****	ns	**	ns	****	ns	****	ns	****
		C				****	ns	****	**	****	ns	****	ns	****	ns	****	**
	<i>hUCBP 8%</i>	D					****	ns	****	ns	***	ns	****	ns	****	ns	****
		C						****	***	****	ns	****	ns	****	ns	****	***
	<i>FBS 10%</i>	D							****	ns	****	ns	****	ns	****	ns	****
		C								****	****	****	**	****	***	****	ms
<i>hDPSCs</i>	<i>hUCBP 4%</i>	D								****	ns	****	ns	****	ns	****	****
		C									**	ns	***	ns	****	****	****
	<i>hUCBP 6%</i>	D										****	ns	****	ns	****	****
		C											****	ns	****	****	**
	<i>hUCBP 8%</i>	D													****	ns	****
		C														****	***
	<i>FBS 10%</i>	D															****
		C															

S11 Table: Chondrogenic differentiation. Sulfated GAGs production ($\mu\text{g/mL}$) after 14 days, assessed by Blyscan™ Glycosaminoglycan Assay (Biocolor, UK). Control: Undifferentiated control; Chondro Diff: Chondrogenic Differentiation. Results presented as Mean $\pm$ SEM.

GAGs content ($\mu\text{g/mL}$)	<i>hUC-MSCs</i>			
	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>	<i>FBS 10%</i>
Chondro Diff	7.92 $\pm$ 0.86	6.74 $\pm$ 0.79	7.91 $\pm$ 0.87	6.91 $\pm$ 0.39
Control	5.32 $\pm$ 0.58	4.84 $\pm$ 0.43	4.97 $\pm$ 0.16	2.15 $\pm$ 0.62

<i>hDPSCs</i>				
	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>	<i>FBS 10%</i>
Chondro Diff	7.23 $\pm$ 0.36	7.56 $\pm$ 0.23	6.74 $\pm$ 0.16	8.29 $\pm$ 0.58
Control	2.80 $\pm$ 0.17	3.31 $\pm$ 0.06	3.07 $\pm$ 0.77	3.03 $\pm$ 0.02

S12 Table: Statistical Significance Differences in Sulfated GAGs production ($\mu\text{g/mL}$) after 14 days, assessed by Blyscan™ Glycosaminoglycan Assay (Biocolor, UK). C: Undifferentiated control; D: Chondrogenic Differentiation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.

GAGs Assay Statistical significance		<i>hUC-MSCs</i>								<i>hDPSCs</i>							
		<i>hUCBP 4%</i>		<i>hUCBP 6%</i>		<i>hUCBP 8%</i>		<i>FBS 10%</i>		<i>hUCBP 4%</i>		<i>hUCBP 6%</i>		<i>hUCBP 8%</i>		<i>FBS 10%</i>	
		D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C
<i>hUC-MSCs</i>	<i>hUCBP 4%</i>	D	ns	ns	*	ns	*	ns	****	ns	***	ns	**	ns	**	ns	**
		C		ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
	<i>hUCBP 6%</i>	D			ns	ns	ns	ns	ns	ns	*	ns	ns	ns	*	ns	*
		C				*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	<i>hUCBP 8%</i>	D					ns	ns	ns	ns	***	ns	**	ns	**	ns	**
		C						ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	<i>FBS 10%</i>	D							**	ns	*	ns	ns	ns	*	ns	*
		C								**	ns	***	ns	**	ns	****	ns
<i>hDPSCs</i>	<i>hUCBP 4%</i>	D								*	ns	ns	ns	ns	ns	ns	*
		C									*	ns	ns	ns	**	ns	ns
	<i>hUCBP 6%</i>	D										*	ns	*	ns	*	*
		C											ns	ns	**	ns	ns
	<i>hUCBP 8%</i>	D													ns	ns	ns
		C													**	ns	ns
	<i>FBS 10%</i>	D															**
		C															

3.2. Bioactive factors content of human Umbilical Cord Blood Plasma and Foetal Bovine Serum: a preliminary comparative characterisation

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1. Introduction

Pairwise with the investigation of the metabolite mixture provided by the media supplements to the culturing cells, the knowledge on other bioactive factors supplied presents great relevance to the comprehension of cellular dynamics.

The detailed composition of FBS remains virtually unknown (Van der Valk et al., 2004), turning the mission of finding an adequate replacement an even greater quest.

Bieback and colleagues (Bieback et al., 2009) recognised the relevance of the specific media composition for the cellular populations and performed an interesting study, where they attempted a comparative analysis of human-derived culture supplements and FBS, in search for their growth factors profiles. They assessed media formulated at a 10% supplementation of FBS, human serum (HS), thrombin-activated platelet releasate in plasma (tPRP) and human platelet lysate (pHPL). From a panel of 174 growth factors, cytokines and chemokines they detected signals for 48 of these compounds, including bFGF, EGF, MCP-1, PDGFs, RANTES and VEGF. These and other factors are recognised regulators of MSCs activity (Rodrigues et al., 2010), and their presence and concentration in culture influence MSCs *in vitro* behaviour (Bieback et al., 2009).

More recently, the hUCBP was proposed as an additional potential substitute for the FBS, and it was preliminarily determined if its metabolomic and bioactive factors content would suit the purpose (Pereira et al., 2014). Fair amounts of TGF- β 1-3, GRO, MDC, PDGF-AA and -BB, sCD40L, MCP-1 and RANTES were detected and compared its profile to that of Conditioned Media produced by hUC-MSCs. Ding and colleagues (Ding et al., 2013) also detected relative levels of a panel of growth factors in hUCBP (including FGFs, HGF, SCF, IGF-1, IGFBPs, and PDGFs), and further tested it as FBS substituent in hUC-MSCs culturing and differentiation. Ehrhart and colleagues (Ehrhart et al., 2018) compared the bioactive factors content of the hUCBP with that of peripheral blood serum or plasma, reporting the first to present low levels of pro-inflammatory cytokines (IL-2, IL-6, IFN- γ , and TNF- α) while bearing more expressive concentrations of IL-8, VEGF, G-CSF, EGF and bFGF.

Therefore, the bioactive factors content of the cell culture supplements was further explored, to complement on the metabolomic characterisation of the standard FBS supplement and proposed substitute (hUCBP) and aid the interpretation of the differential effects observed on the hUC-MSCs and hDPSCs populations.

2. Materials and Methods

Samples of FBS and hUCBP were prepared alongside the samples analysed through NMR, as thoroughly detailed in the previous section (Caseiro et al., 2018). Supplement samples were centrifuged 1800xg for 10 min, to remove debris and preserved at -80°C. Samples were transported in dry ice and maintained frozen until analysis.

2.1. Bioactive Factors Detection and Quantification

For the detection and quantification of the bioactive factors, Multiplexing LASER Bead Analysis was performed by Eve Technologies (Calgary, Alberta, Canada) using the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA)(Pereira et al., 2014). A multispecies panel of bioactive factor was selected [Discovery Assay® TGFβ 3-Plex Cytokine Array (Eve Technologies Corp)] targeting the detection of Transforming growth factor-beta 1, 2 and 3 (TGF-β 1, -2, and -3).

An additional panel was performed, to widen the number of factors investigated - Human Primary Cytokine Array/Chemokine Array 41-Plex Panel, which included cytokines, chemokines and growth factors: epidermal growth factor (EGF), eotaxin-1, fibroblast growth factor 2 (FGF-2), fms-related tyrosine kinase 3 ligand (Flt-3L), fractalkine, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), GRO(pan), interferon- alpha 2 (IFNα 2), interferon-gama (IFNγ), several interleukins (IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A), interferon gama-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), monocyte chemotactic protein-3 (MCP-3), macrophage-derived chemokine (MDC), macrophage inflammatory protein- 1 alpha (MIP-1α), macrophage inflammatory protein-1 beta (MIP-1β), platelet-derived growth factor – AA (PDGF-AA), platelet-derived growth factor – AB/BB (PDGF-AB/BB), chemokine (C-C motif) ligand 5 (RANTES or CCL5), soluble CD40 ligand (sCD40L), transforming growth factor alpha (TGFα), tumor necrosis factor alpha (TNFα), tumor necrosis factor beta (TNFβ), vascular endothelial growth factor A (VEGF-A).

For data quantification purpose, and subsequent interpretation and statistical analysis, measurements inferior to the assay's detection limits (evidenced by n.d.) were corresponded to 0 pg/mL.

2.2. Statistical Analysis

Quantitative data was statistically analysed through one-way ANOVA, followed by pairwise comparison Tukey's HSD post hoc test. Statistical analysis was performed

using the GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla California USA), and significance defined for $P < 0.05$, represented using respectively one-four (*) referring to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$.

3. Results

A multispecies panel was selected considering the species of origin of the supplements under analysis. Significantly higher quantity of TGF- β 1 is highlighted in hUCBP when compared to FBS samples, and to TGF- β 2 and - β 3 isoforms (**Table 1 and Figure 1**).

Table 1: TGF- β isoforms detection and quantification (pg/mL) calculated from Multiplexing LASER Bead Analysis of hUCBP and FBS. Data presented in Mean $\pm$ SEM.

(pg/mL)	<i>hUCBP</i>	<i>FBS</i>
TGF-β1	5680.66 $\pm$ 824.93	790.29 $\pm$ 41.06
TGF-β2	581.27 $\pm$ 138.09	299.80 $\pm$ 4.99
TGF-β3	131.75 $\pm$ 79.14	4.61 $\pm$ 0.63

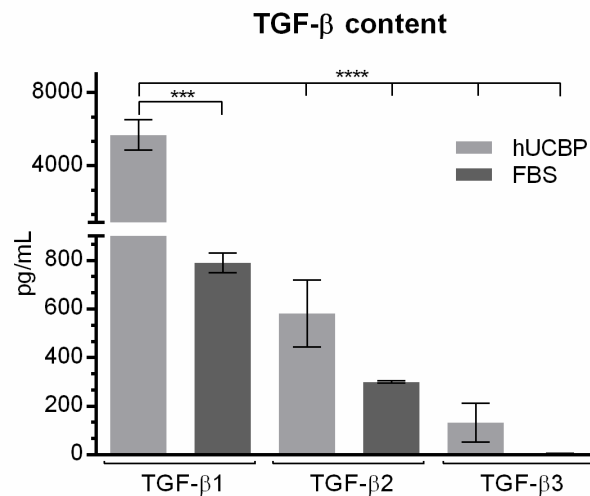


Figure 1: TGF- β isoforms detection and quantification (pg/mL) calculated from Multiplexing LASER Bead Analysis of hUCBP and FBS. Data presented in Mean $\pm$ SEM. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively.

Considering that hUCBP and FBS containing media are prepared using distinct supplementation percentages, the growth factor content in each formulation was extrapolated (**Table 2**).

Table 2: TGF- β isoforms concentration (pg/mL) in formulated media, extrapolated from Multiplexing LASER Bead Analysis of hUCBP and FBS. Data presented in Mean $\pm$ SEM.

(pg/mL)	<i>hUCBP 4%</i>	<i>hUCBP 6%</i>	<i>hUCBP 8%</i>	<i>10% FBS</i>
TGF-β1	227.23 $\pm$ 33.00	340.84 $\pm$ 49.50	454.45 $\pm$ 65.99	79.03 $\pm$ 4.11
TGF-β2	23.25 $\pm$ 5.52	34.88 $\pm$ 8.29	46.50 $\pm$ 11.05	29.98 $\pm$ 0.50
TGF-β3	5.27 $\pm$ 3.17	7.90 $\pm$ 4.75	10.54 $\pm$ 6.33	0.46 $\pm$ 0.06

Regarding the Human Primary Cytokine Array/Chemokine Array 41-Plex Panel, hUCBP demonstrated to bare supplementation of a wide variety of bioactive factors. Eight of the addressed molecules were not detected in >50% of the thirteen (13) samples analysed (Flt-3L, IL13, IL15, IL1 β , IL-2, IL-4, MCP-3 and TNF- β) and other three (3) were found in very residual amounts (IL-5, IL-7, IL-9). The most expressive factors, presenting >400 pg/mL in the hUCBP were GROpan, MCP-1, MDC, PDGF-AA, PDGF-BB, RANTES and sCD40L. The rather large SD of the observed Mean concentrations suggest great variability, which is confirmed through individual sample data analysis (**Figure 2** and **Table 3**).

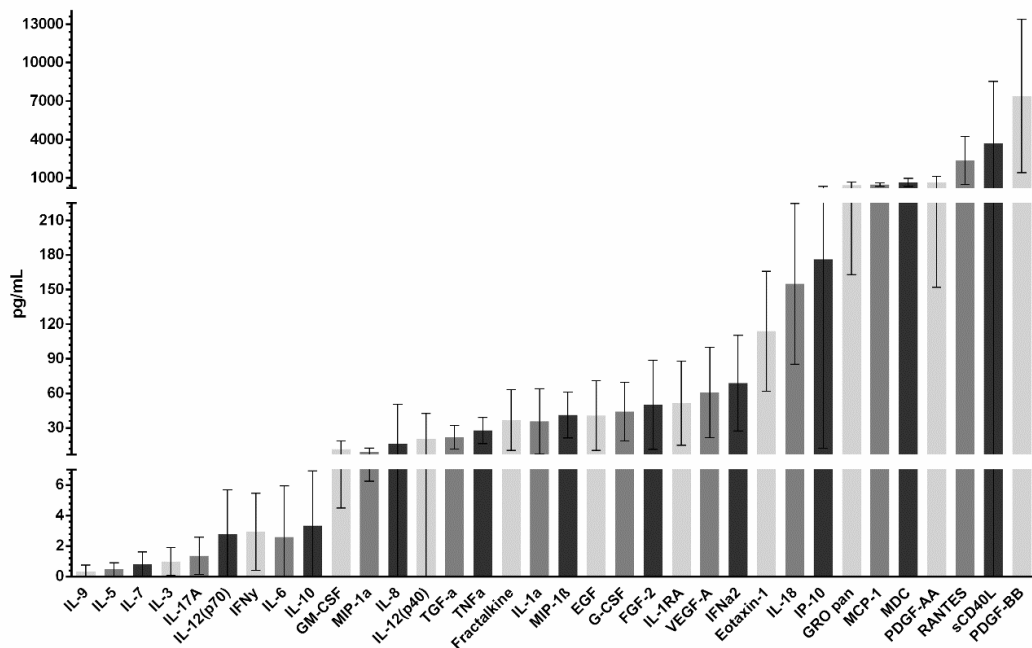


Figure 2: Bioactive factors detection and quantification (pg/mL) in hUCBP, calculated from Multiplexing LASER Bead Analysis. Data presented in Mean $\pm$ SEM.

Table 3: Bioactive factors detection and quantification (pg/mL) in hUCBP and FBS, calculated from Multiplexing LASER Bead Analysis.

	hUCBP													FBS								
	individual sample data (#13)													individual sample data (#3)								
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM							
EGF	27.79	41.94	21.99	16.74	22.38	16.77	112.51	34.23	88.68	69.67	23.3	27.64	27.46	40.85	30.26	8.39	2.34	1.5	3.69	2.51	1.10	0.64
Eotaxin-1	123.01	76.51	223.09	56.53	135.03	89.89	50.96	72.87	59.74	113.31	161.53	161.31	156.25	113.85	52.13	14.46	n.d.	n.d.	n.d.	25.53	27.13	15.66
FGF-2	n.d.	n.d.	25.66	n.d.	25.66	68.16	90.75	55.05	45.47	69.33	81.22	124.75	65.74	50.14	38.63	10.71	22.56	n.d.	54.02	0.89	0.96	0.55
Flt-3L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.38	n.d.	n.d.	n.d.	-	n.d.	1.9	0.78	n.d.	6.65	11.51	6.65
Fractalkine	24.84	38.57	18.95	16.98	16.98	40.46	9.79	77.68	34.48	34.48	16.00	99.21	51.98	36.95	26.19	7.26	n.d.	19.94	n.d.	1.08	0.96	0.55
G-CSF	21.59	42.06	10.42	1.76	22.45	42.67	64.45	44.04	70.17	56.46	71.9	39.66	86.87	44.197	25.46	7.06	n.d.	n.d.	n.d.	-	-	-
GM-CSF	18.06	21.76	8.96	9.67	18.74	3.73	14.81	12.58	1.76	1.31	7.23	22.49	10.79	11.68	7.19	1.99	n.d.	1.82	1.43	0.38	0.40	0.23
GRO pan	840.65	451.33	312.57	145.59	212.43	160.61	306.08	251.68	1013.95	543.12	620.29	448.15	267.85	428.79	265.74	73.70	n.d.	n.d.	n.d.	0.62	0.55	0.32
IFN α 2	65.28	36.32	22.57	32.73	44.72	64.86	63.38	51.66	100.32	180.87	51.66	78.47	103.51	68.95	41.40	11.48	0.78	1.07	n.d.	0.33	0.40	0.23
IFN γ	1.45	2.12	1.89	1.75	3.6	9.42	1.00	7.15	1.71	1.53	1.35	2.06	3.11	2.93	2.52	0.70	0.8	0.8	0.33	0.48	0.31	0.18
IL-10	n.d.	n.d.	n.d.	n.d.	n.d.	8.44	0.35	6.91	1.49	7.56	5.18	8.04	5.35	3.33	3.58	0.99	0.15	0.51	0.77	3.07	3.53	2.04
IL-12(p40)	n.d.	n.d.	n.d.	n.d.	n.d.	34.7	n.d.	18.67	32.03	62.11	27.58	47.12	43.58	20.45	22.14	6.14	1.9	7.04	0.28	0.57	0.51	0.29
IL-12(p70)	n.d.	2.75	n.d.	n.d.	n.d.	6.31	2.16	6.31	1.39	8.91	1.65	1.65	4.75	2.76	2.92	0.81	0.75	0.97	n.d.	0.20	0.23	0.14
IL-13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.54	n.d.	1.61	-	-	-	n.d.	0.18	n.d.	-	-	-
IL15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.89	n.d.	0.72	n.d.	n.d.	1.35	1.22	0.34	0.15	n.d.	n.d.	0.77	0.34	0.20
IL-17A	1.28	1.73	n.d.	0.51	n.d.	1.89	n.d.	1.09	2.43	1.98	3.79	0.1	2.79	35.76	28.22	7.83	0.01	n.d.	n.d.	0.25	0.19	0.11
IL-1a	3.81	117.3	23.1	23.1	4.07	28.84	32.51	33.4	34.28	42.57	56.12	29.78	36.00	-	-	-	n.d.	n.d.	n.d.	18.53	0.44	0.26
IL-1b	n.d.	n.d.	n.d.	n.d.	n.d.	1.62	2.29	4.04	0.42	1.95	n.d.	n.d.	n.d.	51.58	36.48	10.12	0.51	1.15	0.64	0.22	0.13	0.08
IL-1RA	57.49	63.97	13.81	9.42	n.d.	81.56	23.75	52.05	50.68	141.44	55.87	60.09	60.44	-	-	-	n.d.	n.d.	n.d.	0.77	0.34	0.20
IL-2	n.d.	n.d.	n.d.	n.d.	n.d.	2.9	2.13	4.32	2.01	n.d.	0.32	0.67	2.46	1.00	0.90	0.25	0.47	0.1	0.18	0.25	0.19	0.11
IL-3	0.39	0.58	0.43	0.23	0.43	1.53	0.25	0.35	3.06	0.54	1.4	1.4	2.36	1.00	0.90	0.25	n.d.	n.d.	n.d.	18.53	0.44	0.26
IL-4	0.54	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.28	0.82	n.d.	1.72	0.28	n.d.	0.47	0.44	0.12	0.23	0.08	19.03	0.22	0.13	0.08
IL-5	0.38	0.28	1.81	0.34	0.31	0.16	0.3	0.62	0.32	0.28	0.82	0.32	0.23	2.60	3.34	0.93	0.23	n.d.	n.d.	0.77	0.34	0.20
IL-6	0.66	n.d.	1.59	n.d.	n.d.	3.47	10.88	0.74	0.46	7.59	4.71	1.11	2.53	0.82	0.80	0.22	n.d.	n.d.	n.d.	0.25	0.19	0.11
IL-7	0.67	0.34	n.d.	n.d.	n.d.	2.55	1.64	1.22	0.91	1.02	1.69	n.d.	0.63	16.37	34.18	9.48	n.d.	n.d.	n.d.	0.13	0.12	0.07
IL-8	11.03	5.08	5.74	5.8	128.99	2.18	1.17	0.9	11.41	6.99	7.59	18.86	7.09	0.33	0.42	0.12	0.22	0.2	0.2	0.13	0.12	0.07
IL-9	n.d.	n.d.	n.d.	n.d.	n.d.	0.33	0.35	0.53	0.51	0.29	0.42	0.32	1.55	0.33	0.42	0.12	0.22	0.2	n.d.	0.13	0.12	0.07
IP-10	31.83	66.48	79.37	73.44	96.83	649.45	203.49	158.47	106.84	165.68	92.41	246.9	320.48	176.28	163.78	45.43	2.01	3.62	n.d.	1.88	1.81	1.05
MCP-1	444.68	555.33	225.22	281.08	481.59	619.52	398.68	419.6	426.51	603.6	597.93	633.84	637.69	486.56	135.75	37.65	1.54	n.d.	2.36	1.30	1.20	0.69
MCP-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	486.56	135.75	37.65	8.52	9.09	7.92	8.51	0.59	0.34
MDC	319.57	351.59	228.75	518.79	247.66	814.73	778.7	683.25	938.09	1070.3	541.98	1275.13	772.4	657.00	326.09	90.44	4.32	3.79	4.32	4.14	0.31	0.18
MIP-1a	6.49	7.16	5.25	5.31	6.77	14.6	7.99	13.76	10.04	11.55	12.39	9.41	12.39	9.47	3.23	0.90	4.32	n.d.	n.d.	-	-	-
MIP-1b	37.79	39.05	26.3	13.36	21.92	22.86	53.5	35.8	55.98	32.86	43.09	81.26	73.12	41.30	20.04	5.56	n.d.	4.58	n.d.	-	-	-
PDGFAA	1866.38	679.35	191.57	257.42	224.62	434.49	741.41	614.46	854.57	1160.59	820.41	56.1	389.12	637.73	485.81	134.74	0.14	0.11	0.1	0.12	0.02	0.01
PDGFB	n.d.	3909.87	2169.72	1689.35	3624.64	7731.69	4207.25	6225.24	17117.4	13314.92	14643.49	16772.57	4665.82	7390.15	5986.42	1660.34	20.3	21.43	23.22	21.65	1.47	0.85
RANTES	2554.36	3050.49	3526.37	3973.04	2330.72	2027.23	760.21	904.28	7247.08	2000.53	n.d.	1341.04	1107.54	2370.99	1858.64	515.49	5.5	3.74	2.62	3.95	1.45	0.84
SCD4L	17171.00	10460.07	4432.81	2428.29	3350.15	479.76	1523.39	985.02	2603.65	1895.39	864.59	1324.75	627.32	3703.55	4826.17	1338.54	n.d.	0.13	0.25	0.13	0.13	0.07
TGF-a	18.06	16.2	8.66	17.01	8.84	38.24	10.53	19.15	20.74	35.5	32.72	26.61	33.32	21.97	10.31	2.86	2.82	3.04	2.89	2.92	0.11	0.06
TNFa	17.33	25.36	22.71	14.95	14.65	53.57	23.88	28.98	35.52	33.91	35.46	38.99	17.05	27.87	11.39	3.16	n.d.	n.d.	n.d.	0.56	0.66	0.38
TNFb	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.87	n.d.	n.d.	n.d.	n.d.	n.d.	60.77	39.02	10.82	1.29	0.39	n.d.	9.57	3.15	1.82
VEGF-A	85.6	90.81	5.22	31.83	34.05	80.00	49.89	83.93	80.00	39.9	33.36	151.29	24.19	60.77	39.02	10.82	13.01	8.88	6.83	-	-	-

Concerning the FBS samples, a very sensible approach to data interpretation must be made, since the employed array is specific for human molecules detection and, to date, no parallel bovine panel or cross-reactivity studies are available to validate the obtained data (based on literature research and information kindly provided by Eve Technologies, Canada). Therefore, in the data withdrawn from this panel, no statistical comparison between the human and non-human derived samples was performed.

Nevertheless, the analysis of the FBS samples in light of the human-specific array, it is suggested that FGF-2, IL-4, MCP-3, MDC, PDGF-BB and VEGF-A are the dominant bioactive molecules encountered. The suggested concentrations are far inferior to those present in the hUCBP, not exceeding 25 pg/mL. Fourteen (14) of the assayed molecules were not detected in >50% of the samples.

4. Discussion and Conclusions

The in-depth analysis of the bioactive factors content of the FBS and hUCBP provided relevant information on the formulation they vehiculate to the culturing MSCs, and on the effects they may be exerting on these.

The fact that the supplements under comparison derive from distinct species poses an additional challenge and limit the available assays that are simultaneously for human and non-human proteins. This evidence also poses a limitation to future studies on the individual and comparative studies on the composition of veterinary species-derived products, such as the equine or canine derived UCBP.

A multispecies array is available for the detection and quantification of TGF- β family members. This data was preliminarily discussed in the previous section of this Chapter (Caseiro et al., 2018). The comparative analysis of the hUCBP indicates superior levels of isoforms 1 to 3 of TGF- β , respectively presenting 7-, 2- and 28-fold increased concentration when compared to the FBS supplement. Bieback and colleagues (Bieback et al., 2009) also reported the detection of TGF- β s in FBS samples. However, they only discussed on signal intensities and did not titrate to the observed concentrations. They observed increased TGF- β 1 signal in the human-derived samples (serum, thrombin-activated platelet releasate in plasma and platelet lysate) over the FBS, which coincides with the concentration profile we observed in the hUCBP. Regarding TGF- β 2, all of the supplements are suggested to be similarly detected, and to a greater extent than TGF- β 1, contrarily to the present assessment, where TGF- β 1 was present in significantly higher amounts than TGF- β 2. As for TGF- β 3, they interestingly reported negligible signal in all the samples, while a concentration in the range of 100 pg/mL on hUCBP samples was herein observed.

As discussed previously, the expressive concentration of TGF- β 1 in the hUCBP supplemented media may counterpart the inhibitory effects of the high glucose levels towards the chondrogenic differentiation of hMSCs. The rich TGF- β content may also relate to the apparent spontaneous differentiation observed in the hUC-MSCs maintained in hUCBP supplemented media.

The Human Primary Cytokine Array/Chemokine Array provided additional detail on the hUCBP constitution. Bieback and colleagues (Bieback et al., 2009) faced the same interpretation dilemma due to the species specificity of the array panel and comparably decided on a conscious approach to the FBS data analysis/ interpretation. They observed that PDGF-AA, PDGF-AB/BB and RANTES were the most prominently detected signals/ concentrations in the hUCBP samples. In fact, a similar signal magnitude was observed in FBS samples for PDGF-AB/BB. The same compound was observed in the FBS, but to a much smaller magnitude than the one found in hUCBP. They also report IL-6 and IL-8 expression in the human-derived supplement options. Basic FGF was also identified in both sample types, and EGF was mostly observed in human-derived options. They do not report very expressive detection of GRO, which was one of the dominant factors in this assessment. MCP-1 and MDC displayed similar expression, both more relevant in FBS than in hUCBP samples.

The individual bioactive factors quantification on the hUCBP samples clearly reflects the extensively reported inter-donor variability, as earlier discussed in the Metabolomic study section, regarding the umbilical cord blood haematology and metabolite profile.

Ehrhart and colleagues (Ehrhart et al., 2018) also observed low pro-inflammatory cytokine content in the UCBP samples (IL1- β , IL-2, IL-6, and IFN- γ). Regarding TNF- α , the present study observed far superior cytokine concentration ($\approx 22x$), as occurred to most of the growth factors concurrently assayed (bFGF, EGF, GM-CSF; ≈ 3 to $8x$). Both studies identified moderate IL-8 presence (ranging from 10-20 pg/mL). Interestingly, VEGF was identified in relatively low concentration (≈ 7 pg/mL) in the study, contrasting to the mean value of 60,77 pg/mL (VEGF-A – approximately $8x$) herein quantified. However, the targeted VEGF isoform is not detailed in the study, precluding further discussion.

Many of the bioactive factors herein identified are well recognised as regulators of MSCs survival, proliferation (Rodrigues et al., 2010) and differentiation (Lee et al., 2011). Some factors, like PDGF, RANTES, are potent mitogens to MSCs (Ponte et al., 2007, Kroeze et al., 2009) and are present in very significant amounts in the hUCBP, which may relate to the observation that the hUCBP supplementation is required in much smaller concentrations than FBS, granting similar or improved proliferative rates in the cultured hUC-MSCs and DPSCs. Additionally, this detailed characterisation provides additional

information for the comprehension of the deleterious impact on osteodifferentiation observed in the hUCBP supplemented cultures, reported in the previous section (Caseiro et al., 2018). The osteodifferentiation capacity impairment could also potentially result from the presence of substantial concentrations of PDGF in the media (Tamama et al., 2006).

Other molecules presenting greater concentration in human-derived components, like members of the EGF family, have been demonstrated to promote mitogenesis of MSCs and prevent their differentiation, maintaining the population in self-renewal rather than differentiative pathways (Krampera et al., 2005). BasicFGF is another well described mitogen for MSCs expansion. It is however reported to result in the induction of chondrogenic differentiation in long term exposure (Tsutsumi et al., 2001). An apparent spontaneous induction of chondrogenesis was observed in the hUCBP cultured hUC-MSCs, but it is not suggested to derive from the action of bFGF since its extrapolated concentrations would be similar to those observed in the FBS group, which did not differentiate when not induced. Instead, this observation is expected to relate to the TGF- β presence.

In brief, the Multiplexing LASER Bead Analysis approach was proved useful in the screening of growth factors, cytokines and chemokines found in the proposed hUCBP supplement. Although several species-specific panels are available, the challenge of interpreting data from different species remains.

This detailed analysis enabled the profiling of 44 bioactive factors in the raw supplement options prior to media formulation. It substantiated the richness that was empirically attributed to this haemoderived by-product. Further, a comparative approach with FBS composition can be preliminarily introduced. The hUCBP is herein suggested to provide far superior amounts of a variety of bioactive molecules described to influence MSCs dynamics.

Complementing the metabolomic characterisation previously endured, the bioactive factors profile analysis assisted on the comprehension of the *in vitro* behaviour of hUC-MSCs and hDPSCs systems observed in the presence of hUCBP as a substitute of the classical FBS supplement.

3.3. References

- Bartholomew, S., S. D. Owens, G. Ferraro, D. Carrade, D. Lara, F. Librach, D. L. Borjesson and L. D. Galuppo (2009). Collection of equine cord blood and placental tissues in 40 thoroughbred mares. Equine veterinary journal **41**(8): 724-728.
- Bieback, K., A. Hecker, A. Kocaomer, H. Lannert, K. Schallmoser, D. Strunk and H. Kluter (2009). Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells **27**(9): 2331-2341.
- Caseiro, A., G. Ivanova, S. Pedrosa, M. Branquinho, P. Georgieva, P. Barbosa, J. Santos, R. Magalhães, P. Teixeira and T. Pereira (2018). Human umbilical cord blood plasma as an alternative to animal sera for mesenchymal stromal cells in vitro expansion—A multicomponent metabolomic analysis. PloS one **13**(10): e0203936.
- Ding, Y., H. Yang, J. B. Feng, Y. Qiu, D. S. Li and Y. Zeng (2013). Human umbilical cord-derived MSC culture: the replacement of animal sera with human cord blood plasma. In Vitro Cellular & Developmental Biology-Animal **49**(10): 771-777.
- Ehrhart, J., P. R. Sanberg and S. Garbuzova-Davis (2018). Plasma derived from human umbilical cord blood: Potential cell-additive or cell-substitute therapeutic for neurodegenerative diseases. Journal of cellular and molecular medicine **22**(12): 6157-6166.
- Fekete, N., M. T. Rojewski, D. Furst, L. Kreja, A. Ignatius, J. Dausend and H. Schrezenmeier (2012). GMP-compliant isolation and large-scale expansion of bone marrow-derived MSC. PLoS One **7**(8): e43255.
- Friedenstein, A. J., R. K. Chailakhjan and K. S. Lalykina (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet **3**(4): 393-403.
- Hong, J., H. Jin, J. Han, H. Hu, J. Liu, L. Li, Y. Huang, D. Wang, M. Wu, L. Qiu and Q. Qian (2014). Infusion of human umbilical cord-derived mesenchymal stem cells effectively relieves liver cirrhosis in DEN-induced rats. Mol Med Rep **9**(4): 1103-1111.
- Huang, L., P. J. Critser, B. R. Grimes and M. C. Yoder (2011). Human umbilical cord blood plasma can replace fetal bovine serum for in vitro expansion of functional human endothelial colony-forming cells. Cytotherapy **13**(6): 712-721.
- Krampera, M., A. Pasini, A. Rigo, M. T. Scupoli, C. Tecchio, G. Malpeli, A. Scarpa, F. Dazzi, G. Pizzolo and F. Vinante (2005). HB-EGF/HER-1 signaling in bone marrow mesenchymal stem cells: inducing cell expansion and reversibly preventing multilineage differentiation. Blood **106**(1): 59-66.

- Kroeze, K. L., W. J. Jurgens, B. Z. Doulabi, F. J. Van Milligen, R. J. Scheper and S. Gibbs (2009). Chemokine-mediated migration of skin-derived stem cells: predominant role for CCL5/RANTES. Journal of Investigative Dermatology **129**(6): 1569-1581.
- Lee, J. Y., H. Nam, Y. J. Park, S. J. Lee, C. P. Chung, S. B. Han and G. Lee (2011). The effects of platelet-rich plasma derived from human umbilical cord blood on the osteogenic differentiation of human dental stem cells. In Vitro Cell Dev Biol Anim **47**(2): 157-164.
- Pereira, T., A. Gartner, I. Amorim, A. Almeida, A. R. Caseiro, P. A. Armada-da-Silva, S. Amado, F. Fregnan, A. S. Varejao, J. D. Santos, P. J. Bartolo, S. Geuna, A. L. Luis and A. C. Mauricio (2014). Promoting nerve regeneration in a neurotmesis rat model using poly(DL-lactide-epsilon-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly: in vitro and in vivo analysis. Biomed Res Int **2014**: 302659.
- Pereira, T., G. Ivanova, A. R. Caseiro, P. Barbosa, P. J. Bartolo, J. D. Santos, A. L. Luis and A. C. Mauricio (2014). MSCs Conditioned Media and Umbilical Cord Blood Plasma Metabolomics and Composition. PLoS One **9**(11): e113769.
- Pham, P. V., N. B. Vu, V. M. Pham, N. H. Truong, T. L. Pham, L. T. Dang, T. T. Nguyen, A. N. Bui and N. K. Phan (2014). Good manufacturing practice-compliant isolation and culture of human umbilical cord blood-derived mesenchymal stem cells. J Transl Med **12**: 56.
- Phinney, D. G. (2007). Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. Cell Cycle **6**(23): 2884-2889.
- Phinney, D. G. and D. J. Prockop (2007). Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. Stem Cells **25**(11): 2896-2902.
- Ponte, A. L., E. Marais, N. Gallay, A. Langonné, B. Delorme, O. Hérault, P. Charbord and J. Domenech (2007). The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. Stem cells **25**(7): 1737-1745.
- Roberts, E. L., T. Dang, S. I. Lepage, A. H. Alizadeh, T. Walsh, T. G. Koch and M. S. Kallos (2019). Improved expansion of equine cord blood derived mesenchymal stromal cells by using microcarriers in stirred suspension bioreactors. Journal of Biological Engineering **13**(1): 25.
- Rodrigues, M., L. G. Griffith and A. Wells (2010). Growth factor regulation of proliferation and survival of multipotential stromal cells. Stem cell research & therapy **1**(4): 32.

- Simonetti, A. B., G. E. Englert, K. Campos, M. Mergener, C. De David, A. P. De Oliveira and P. M. Roehe (2007). Nanobacteria-like particles: A threat to cell cultures. Brazilian Journal of Microbiology **38**(1): 153-158.
- Sultana, T., S. Lee, H.-Y. Yoon and J. I. Lee (2018). Current Status of Canine Umbilical Cord Blood-Derived Mesenchymal Stem Cells in Veterinary Medicine. Stem cells international **2018**.
- Tamama, K., V. H. Fan, L. G. Griffith, H. C. Blair and A. Wells (2006). Epidermal growth factor as a candidate for ex vivo expansion of bone marrow–derived mesenchymal stem cells. Stem cells **24**(3): 686-695.
- Tekkatte, C., G. P. Gunasingh, K. M. Cherian and K. Sankaranarayanan (2011). "Humanized" stem cell culture techniques: the animal serum controversy. Stem Cells Int **2011**: 504723.
- Tsutsumi, S., A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi and Y. Kato (2001). Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochemical and biophysical research communications **288**(2): 413-419.
- Van der Valk, J., D. Brunner, K. De Smet, Å. F. Svenningsen, P. Honegger, L. E. Knudsen, T. Lindl, J. Noraberg, A. Price and M. Scarino (2010). Optimization of chemically defined cell culture media–replacing fetal bovine serum in mammalian in vitro methods. Toxicology in vitro **24**(4): 1053-1063.
- Van der Valk, J., D. Mellor, R. Brands, R. Fischer, F. Gruber, G. Gstraunthaler, L. Hellebrekers, J. Hyllner, F. Jonker and P. Prieto (2004). The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. Toxicology in vitro **18**(1): 1-12.

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Chapter IV

The paracrine pathway of Mesenchymal Stem/
Stromal Cells regenerative effect

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Introductory note

The role of MSCs in regenerative events is globally assigned to two major mechanisms of action: the lineage-specific differentiation, substituting the damaged cells in their functions, and the secretion of paracrine factors, that modulate the intrinsic tissue responses.

The lineage-specific differentiation pathway of MSCs has been well established for hard tissue regeneration (da Silva Meirelles et al., 2009, Doorn et al., 2012). In bone tissue applications, implanted MSCs have been demonstrated capable of long-term engraftment, osteogenic differentiation and specific tissue matrix production (Graziano et al., 2008, Seo et al., 2008).

In tissues of a softer nature, this pathway does not present as dominant in the regenerative process. As previously detailed, MSCs systems applied to skeletal muscle tissue undergoing regeneration display limited engraftment efficiency and are rarely observed as individually differentiated entities or hybrid muscular fibres (Natsu et al., 2004, de la Garza-Rodea et al., 2011, Grabowska et al., 2013). Rather, they are assigned to contribute to the satellite cells' pool (native skeletal muscle regenerative populations) and to remain in close proximity to the lesion, in an undifferentiated state (Rossi et al., 2011). In peripheral nerve, implanted MSCs are also observed in the vicinity of the regenerating site, not generally penetrating the recovering nervous structure itself (Pan et al., 2007, Han et al., 2016).

Therefore, evidence strongly supports that the regenerative mechanisms of MSCs on neuromuscular tissue primarily rely on their capacity to secrete relevant paracrine factors (Natsu et al., 2004, Pan et al., 2007, de la Garza-Rodea et al., 2012, Pereira et al., 2014), leading to the necessity of addressing the specific composition of the secreted arrays, to substantiate on their interactions with the host environment (and *vice-versa*).

In both components of the neuromuscular complex, literature reports are convergent in the beneficial effects observed: modulation of inflammatory environment and degenerative events, acceleration of tissue formation and improvement of structural organisation, optimised re-vascularisation of the lesion site and reduction of the fibrotic scarring.

The re-vascularisation events are ubiquitous for successful tissue regeneration (few exceptions to be noted, such as cartilage regeneration (Santos et al., 2013)), irrespective of the tissue source from which MSCs systems were obtained.

Extensive research has elaborated on the effective composition of the MSCs secretion (Chen et al., 2008, Lai et al., 2010, Timmers et al., 2011, Pereira et al., 2014), aiming at the identification of tissue-specific modulators (myogenic or neurogenic bioactive factors) or transversal actions regulators, such as fibrosis and angiogenesis.

Previous research demonstrated UC-MSCs to secrete a broad range of proliferative/ anti-apoptotic factors and chemokines known to impact on neuromuscular regenerative events (Pereira et al., 2014).

The present Chapter presents a comparative analysis of the metabolomic and bioactive factors components of the secretome of the previously explored UC-MSCs and the newly implemented DPSCs. This study intends to characterise the metabolite profile of these populations and contribute to the comprehension of the metabolic pathways presiding to the conditioning process. The composition of the secretome in terms of bioactive factors was also addressed, investigating the presence and concentration of a panel of regulatory growth factors, cytokines and chemokines. Finally, in light of the reported impact of MSCs implantation on local vascularisation, the effects of these MSCs secretion products were analysed *in vitro* and *in vivo* through angiogenic/ vasculogenic assays.

4.1. Metabolomic and bioactive factors secretory profiles of Mesenchymal Stem/ Stromal Cells populations

**Mesenchymal Stem/ Stromal Cells metabolomic and bioactive factors profiles:
a comparative analysis on the Umbilical Cord and Dental Pulp derived
Stem/ Stromal Cells secretome**

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RESEARCH ARTICLE

Mesenchymal Stem/ Stromal Cells metabolomic and bioactive factors profiles: A comparative analysis on the umbilical cord and dental pulp derived Stem/ Stromal Cells secretome

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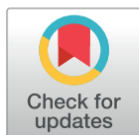
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Abstract

Mesenchymal Stem/ Stromal Cells assume a supporting role to the intrinsic mechanisms of tissue regeneration, a feature mostly assigned to the contents of their secretome. A comparative study on the metabolomic and bioactive molecules/factors content of the secretome of Mesenchymal Stem/ Stromal Cells derived from two expanding sources: the umbilical cord stroma and the dental pulp is presented and discussed. The metabolic profile (Nuclear Magnetic Resonance Spectroscopy) evidenced some differences in the metabolite dynamics through the conditioning period, particularly on the glucose metabolism. Despite, overall similar profiles are suggested. More prominent differences are highlighted for the bioactive factors (Multiplexing Laser Beam Analysis), in which Follistatin, Growth Regulates Protein, Hepatocyte Growth Factor, Interleukin-8 and Monocyte Chemotactic Protein-1 dominate in Umbilical Cord Mesenchymal Stem/ Stromal Cells secretion, while in Dental Pulp Stem/ Stromal Cells the Vascular Endothelial Growth Factor-A and Follistatin are more evident. The distinct secretory cocktail did not result in significantly different effects on endothelial cell populations dynamics including proliferation, migration, tube formation capacity and *in vivo* angiogenesis, or in chemotaxis for both Mesenchymal Stem/ Stromal Cells populations.



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1. Introduction

Mesenchymal Stem/ Stromal Cells (MSCs) are at the forefront of research for the development of cell-based therapies, due to their capacity to self-renew and differentiate into several cell types, to secrete soluble factors with paracrine actions, as well as due to their immunosuppressive and immunomodulatory properties [1–6].

MSCs have been described to reside on nearly every body tissue [7–9] since Friedenstein and colleagues firstly described the bone marrow derived population [10, 11].

Currently, the umbilical cord stroma (Wharton jelly) and the dental pulp may come to gain ground as sources for MSCs-based therapies, due to the non/ minimally invasive and ethically accepted collection procedures (umbilical cords and extracted healthy teeth were previously considered medical waste), as well as for the increasingly available private and public banking options worldwide [12].

The first evidence of the MSCs contribution to the healing processes was assigned to their specific differentiation skills, replacing the damaged native cells in their functions [13, 14]. However, current trends demonstrate that in some instances MSCs remain undifferentiated at the lesion site or in its vicinity, for limited periods of time, or even that only minimal percentages of the MSCs would effectively differentiate and integrate host tissues [15]. Regardless of their differentiation into tissue specific phenotypes, MSCs are often correlated to improved regenerative outcomes [16].

These observations were then attributed to the secretion products of those MSCs [17–19] and, in recent years, research has focused on deepening the knowledge on the effective composition of the MSCs secretion, in the form of soluble molecules or extracellular vesicles [6, 20–23].

In most tissues, the key for regenerative efficiency is the re-vascularization of the lesion site and MSCs have been associated with improved angiogenesis in a number of models of disease [24, 25]. As such, MSCs assume a supporting role to the intrinsic mechanisms of tissue regeneration, promoting the re-vascularization processes, providing adequate perfusion to active healing sites, as well as urging resident regenerative populations to home towards these locations [26].

Further, some groups investigated the extent to which the presence of the cells themselves was absolutely essential to the observation of beneficial effects, since regenerative benefit can be observed by the application of MSCs secretion products alone, conventionally designated as the secretome [8, 17–19, 27].

The secretome comprises a range of bioactive molecules/factors secreted to the extracellular space. Its composition is particular to individual cells and tissues, and is modulated in response to physiological and/or pathological stimuli [24]. The application of these cell-based products may bring several advantages to the advanced therapies field, namely the decreased cell number requirements and allocated cell storage necessities, ease of tailoring, quality control and dosing, reduced risk of rejection and malignancy, as well as the ready availability for administration in acute scenarios [28].

Therefore, studies on the composition of the MSCs secretome through metabolic analysis are a valuable tool to the comprehension of the underlying mechanisms to MSCs dynamics and therapeutic effects [29–32].

Metabolomic profiling techniques [33–37] yield information on targeted metabolites’ structure and quantitative distribution [33, 34], and despite the significant progress made in the field of structural biology and bio-chemistry, the development and application of these techniques towards the MSCs secretome are still sparse [8].

We recently demonstrated the application of proton NMR spectroscopy and implementation of appropriate one (1D) and two (2D) dimensional NMR techniques to the analysis of the metabolic composition of Umbilical Cord Stem/ Stromal Cells (UC-MSCs) conditioned media and changes in the metabolic profile of the culture media in the process of conditioning [21].

Alongside the metabolite content, a wide range of growth factors, cytokines, chemokines and extracellular matrix components have already been identified in the CM obtained from differently sourced MSCs [38, 39], and many of them are known to impact on most tissues structure, function and regeneration [25, 40, 41]. Beyond modulating their surrounding environment MSCs are sensitive themselves to signaling factors, altering their secretory profile in response to, as an example, the presence of inflammatory cytokines. This evidence may contribute to the understanding of the eventual difference in the tissue response to cells or to their secretome alone [42], and to the development of strategies to manipulate secretion profiles towards specific needs.

In a recent study, we determined that UC-MSCs CM becomes rich in a range of proliferative and anti-apoptotic factors, particularly in transforming growth factor beta 1 (TGF- β 1), epidermal growth factor (EGF), granulocyte and granulocyte-macrophage colony-stimulating factor (G-CSF and GM-CSF), platelet derived growth factor (PDGF-AA) and vascular endothelial growth factor (VEGF). Also, several other chemokines such as monocyte chemoattractant protein-1 e 3 (MCP-1 and MCP-3), chemokine (C-C motif) ligand 5 (CCL5 or RANTES), GRO, and interleukin 8 (IL-8) were observed in increasing levels [21]. This previous analysis enabled the primary definition of UC-MSCs CM composition [21], which we herein expand and compare to Dental Pulp Stem/ Stromal Cells (DPSCs) CM profile.

The first part of the present study focuses on a comparative analysis of the metabolomic and bioactive factors secretion profile of UC-MSCs and DPSCs secretome, through the conditioning process. This aimed to characterize the metabolite profile of the collected CM and to enlighten on the metabolic pathways presiding to the process. Further, the bioactive factors content of the CM was analyzed, focusing on a series of growth factors, cytokines and chemokines, aiming at the comparison of the regenerative potential of the two MSCs population. Finally, the effects of the characterized CMs were addressed on *in vitro* and *in vivo* models of angiogenesis.

2. Materials and methods

2.1. Human Mesenchymal Stem/ Stromal Cells characterization and conditioning protocol

Umbilical Cord -MSCs [PromoCell™ (C-12971; Lot No. 1112304.2)] and DPSCs [AllCells, LLC (DP0037F, Lot No. DPSC090411-01)] were cultured with MEM- α , GlutaMAX™ (32561–029, Gibco®) supplemented with MSCs certified FBS (04-400-1A, Biological Industries Israel Beit-Haemek Ltd)(10% v/v), penicillin (100 U/mL)-streptomycin (100 μ g/mL) (151140–122, Gibco®), and amphotericin B (2.5 μ g/mL)(15290–026, Gibco®). Both cell types were maintained at 37 °C in a 5% CO₂ humidified atmosphere. Passage 4 cells were seeded in 75 cm³ T-flasks until confluence was reached. Detachment of confluent cells was achieved by a 5 minutes incubation in 0,05% Trypsin-EDTA (Trypsin-EDTA 0.25%, 25200–072, Gibco®). Cells were re-suspended and counted using 0.4% Trypan Blue (T8154-20ML, Sigma-Aldrich®) and the Countess II FL Automated Cell Counter (Invitrogen™).

2.1.1. Mesenchymal Stem/ Stromal Cells phenotype identity. The surface marker profiles of UC-MSCs and DPSCs were confirmed through Flow Cytometry, with anti-positive (CD90, CD105, CD44) and negative marker (CD34, CD11b, CD19, CD45, MHC class II) antibodies (Human MSC Analysis Kit, 562245, BD Biosciences), as described in detail in [43].

Data was acquired using BD FACSCalibur™ 3 CA Becton Dickinson, BD Biosciences, and data was processed using FlowJo Engine X10.4 (v3.05478, LLC).

2.1.2. Reverse transcriptase polymerase chain reaction. Reverse transcriptase Polymerase chain reaction (RT-PCR) and qPCR targeting specific genes expressed by pluripotent stem cells was performed. Gene DNA sequences were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank) and aligned using the Clustal Omega bioinformatic tool from EMBL-EBI (<http://www.ebi.ac.uk/Tools/msa/clustalo>). The primers sequences are listed in [Table 1](#).

Cultures were harvested and pelleted for total RNA extraction (High Pure RNA Isolation kit (Roche™)). DNA traces were eliminated with DNase I, and RNA quantity and quality assessed by Nanodrop ND-1000 Spectrophotometer, reading from 220 nm to 350 nm, and stored at -80°C. After, cDNA was synthesized from the purified RNA (kit Ready-To-Go You-Prime First-Strand Beads (GE Healthcare®)). The synthesized cDNA, corresponding to the mRNA present in the sample, was run for the expression of six genes: two housekeeping genes (β -actin and GAPDH) and four genes used as pluripotent stem cells markers (c-kit, Oct-4, Nanog and ALP). Quantitative PCR (qPCR) was performed in a CFX96™ (BioRad®) apparatus using the iQ™ SYBR® Green Supermix (BioRad®). Each pair of primers targeting a gene was used to analyze its expression in the UC-MSCs and DPSCs cDNA, in duplicate, along with a negative control, through defined temperature cycles [95°C for 4 minutes, 35 cycles comprising 95°C for 20 seconds, 55°C for 20 seconds and 72°C for 30 seconds ending with Real-Time acquisition, and final extension of 72°C for 7 minutes], and the number of cycle threshold for each well were recorded. The plate containing the amplified genes or qPCR products was kept in ice and observed in a 2% agarose gel (NuSieve® 3:1 Agarose (Lonza)) to check and reinforce the identity of the amplicons, in horizontal electrophoresis apparatus. Under 120 V potential difference for 40 minutes to separate the amplicons. Gel was then observed under UV light and pictures recorded using the GelDoc® 2000 (BioRad®) and Quantity One® software (BioRad®).

2.1.3. Multilineage differentiation. Multilineage differentiation of the UC-MSCs and DPSCs was induced towards Osteogenic, Adipogenic and Chondrogenic phenotypes using specific differentiation media. Differentiation efficiency was assessed by Alizarin Red S, Oil Red O and Alcian Blue/ Sulfated Glycosaminoglycans (GAGs) quantification, as detailed in [\[43\]](#).

2.2. Metabolomic and bioactive factors secretion profiles

2.2.1. Conditioned medium collection and analysis. For the production of CM, both cellular populations were plated at 6000 cells/ cm², in triplicates, and cultured in standard culture medium (α MEM 10% FBS) until approximately 80% confluence was reached. Then, the cultures were washed to remove any trace of FBS and other supplements and placed in plain DMEM/F12 GlutaMAX™ (10565018, Gibco®) for 24 and 48 hours. Conditioned media samples were collected at both time-points, centrifuged 1800 xg for 10 min to remove any cellular debris and preserved at -80°C until analysis.

2.2.2 Metabolomic analysis. Proton NMR spectroscopy was used to identify and quantify metabolites in plain/unconditioned and conditioned media collected at two time-points (24 and 48 hours) of UC-MSCs and DPSCs in proliferation. A total 600 μ L aliquot of each sample was placed into 5 mm NMR tubes and 50 μ L deuterium oxide (D₂O) containing 0.05 mM sodium trimethylsilyl-[2,2,3,3-d₄]-propionate (TSP) was added as a chemical shift and quantitation standard. All NMR spectra were recorded at 300K on a Bruker Avance III 600 HD spectrometer, equipped with CryoProbe Prodigy. ¹H NMR spectra with water suppression using a 1D NOESY (noesygppr1d) and a Carr-Purcell-Meiboom-Gill (CPMG, cpmgpr1d) pulse

Table 1. List of primers used, target gene and size of the PCR product.

Primer		GenBank target gene	PCR product
Pluripotent Stem Cells Markers genes			
ALP human	Fwd: 5' -CCTAAAAGGGCAGAAGAAGGAC-3'	NM_001632.4	444 bp
	Rev: 5' -TCCACCTAGGATCACGTCAATG-3'		
c-Kit human	Fwd: 5' -AACGCTCGACTACCTGTGAA-3'	NM_000222	401 bp
	Rev: 5' -GACAGAATTGATCCGCACAG-3'		
Nanog human	Fwd: 5' -CTTCCTCCATGGATCTGCTTATTC-3'	XM_011520851.1	265 bp
	Rev: 5' -AGGTCTTCACCTGTTTGTAGCTGAG-3'		
Oct4 human	Fwd: 5' -GAAGCTGGAGAAGGAGAAGCT-3'	NM_002701.5	243 bp
	Rev: 5' -CAAGGGCCGCAGCTTACACAT-3'		
Housekeeping genes			
β-actin human	Fwd: 5' -GGCACCCAGCACAATGAAGA-3'	NM_001101.3	100 bp
	Rev: 5' -CTGGAAGGTGGACAGCGAGGC-3'		
GAPDH human	Fwd: 5' -AGCCGCATCTTCTTTTGCCTC-3'	NM_002046.5	815 bp
	Rev: 5' -TCATATTGGCAGGTTTTTCT-3'		

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sequences [44, 45] were acquired with spectral width of 10000 Hz, 32 K data points and 32 scans. The 1D NOESY spectra were collected using 5 s relaxation delay and mixing time of 100 ms. The CPMG experiments (cpmgrp1d) were acquired with relaxation delay 4 s, spin-echo delay between 0.4 and 0.6 ms, and a loop for T2 filter of 20 was used. All free induction decays (FIDs) were processed by 0.3 Hz line broadening and zero filling to 64 K, manually phased, and baseline corrected. Two dimensional 1H/1H COSY and TOCSY spectra were recorded in phase sensitive mode and with water suppression; a relaxation delay of 2 s, 16 or 32 scans, a total 2K data points in F2 and 256 or 512 data points in F1 over a spectral width of 10000 Hz. Two dimensional 1H/13C heteronuclear single quantum coherence (HSQC) experiments were carried out with a spectral width of 10000 Hz for 1H and 27000 Hz for 13C, relaxation delay 1.5 s, Fourier transform (FT) size 2K × 1K. The quantitative distribution of NMR-detectable metabolites in the samples was determined from the integral intensity of characteristic signals in ¹H NMR spectra of the samples referenced to the integral intensity of TSP signal, considering the number of the contributing nuclei for that particular resonance signal [46].

2.2.3. Bioactive factors detection and quantification. Multiplexing LASER Bead Analysis was performed by Eve Technologies (Calgary, Alberta, Canada) using the Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), similarly to previously described in [47]. The multiplexing technology is based on color-coded polystyrene beads with unique color / fluorophore signatures, and a dual-laser system and a flow-cytometry system. One laser activates the fluorescent dye within the beads which identifies the specific analyte, the second laser excites the fluorescent conjugate (streptavidin-phycoerythrin), and the amount of the conjugate detected by the analyzer is in direct proportion to the amount of the target analyte. The results are quantified according to a standard curve.

A broad panel of 57 bioactive factor was assayed [Discovery Assay[®] TGFβ 3-Plex Cytokine Array (Eve Technologies Corp); Milliplex Human Angiogenesis / Growth Factor kit Discovery Assay[®] and the Human Cytokine Array / Chemokine Array (Millipore, St. Charles, MO, USA)], including angiopoietin-2 (Ang), bone morphogenetic protein 9 (BMP-9), EGF, endoglin (ENG), endothelin-1 (EDN1), eotaxin-1, fibroblast growth factor 1 and 2 (FGF-1 and -2), fms-related tyrosine kinase 3 ligand (Flt-3L), follistatin (Fst), fractalkine, G-CSF, GM-CSF, GRO(pan), heparin-binding-EGF (HB-EGF), hepatocyte growth factor (HGF), interferon-alpha 2 (IFNα2), interferon-gama (IFNγ), several interleukins (IL-1α, IL-1β, IL-1ra, IL-2, IL-3,

IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-18), interferon gamma-induced protein 10 (IP-10), leptin, MCP-1, MCP-3, macrophage-derived chemokine (MDC), macrophage inflammatory protein-1 alpha (MIP-1 α), macrophage inflammatory protein-1 beta (MIP-1 β), platelet-derived growth factor-AA (PDGF-AA) and -AB/BB (PDGF-AB/BB), placental growth factor (PLGF), RANTES/ CCL5, soluble CD40 ligand (sCD40L), transforming growth factor alpha (TGF α), transforming growth beta 1, 2 and 3 (TGF- β 1, -2, and -3), tumor necrosis factor alpha (TNF α), tumor necrosis factor beta (TNF β), VEGF-A, -C, and -D.

2.3. Effects of MSCs CM on angiogenesis

2.3.1. Conditioned medium concentration protocol. Conditioned Medium obtained from the UC-MSCs and DPSCs was concentrated 5 times (5x), using Pierce™ Protein Concentrator, 3k MWCO, 5–20 mL (88525, Thermo Scientific). Tubes were sterilized *as per* manufacturer's instructions, through immersion in 70% (v/v) ethanol for 30 minutes, followed by 2 cycles of 70% ethanol and DPBS (14190144, Gibco®) centrifugations at 3000 xg, for 10 minutes each). Tubes were air dried for 10 minutes and CM placed at the top compartment. Samples were centrifuged until 5x concentration of sample volume in the upper compartment was achieved.

2.3.2. Human umbilical vein endothelial cells expansion and maintenance. Human Umbilical Vein Endothelial Cells (UVECs) were obtained from Sigma® (200P-05N; Lot No. 3257, Cell Applications, Inc) and expanded using specific expansion medium (211–500, Cell Applications, Inc). Cells were maintained at 37°C and 95% humidified atmosphere with 5% CO₂ environment. Passage 4–5 cultures were utilized in the presented assays.

2.3.3. Cell viability assay. Cells were seeded in 24 well plate at 6000 cells/cm². The cells were allowed to adhere in UVECs expansion medium, overnight. After this period, culture media was replaced by CM supplemented culture media [composed of 80% UVECs expansion medium and 20% 5x concentrated UC-MSCs' CM or DPSCs' CM; control medium was composed of 80% UVECs expansion media and 20% DPBS].

At every time point (0, 24, 48, 72 and 96 hours), the culture media was removed and fresh adequate culture media was added to each well, with 10% (v/v) of 10x PrestoBlue® cell viability reagent (A13262, Thermo Fisher Scientific, Molecular Probes, Invitrogen), and UVECs were incubated for 1 hour at 37°C, 5% CO₂. The absorbance of the cell culture supernatant was read at 570 nm and 595 nm in a Thermo Scientific Multiskan FC plate reader, to assess for changes in cell viability. Absorbance readings were normalized, and data corrected to unseeded control wells' readings.

2.3.4. Senescence and apoptosis assays. To assess for signs of senescence in the cellular populations, β -Galactosidase activity was assayed. Cells were plated in 96-well plates, similarly as described for the cell viability assessment. After 48 hours in CM-supplemented and control media, culture media was removed, and cells were washed once with DPBS and incubated with β -Galactosidase Assay Reagent (75705, Thermo Fisher Scientific), for 30 minutes at 37°C. Absorbance was read at 405 nm.

Cultured UVECs were further assessed for apoptosis events, through the detection of Annexin V and Propidium Iodine (PI) staining (BMS500FI, eBioscience). For such, cells cultured for 2 days in each condition were harvested using Trypsin-EDTA, counted and resuspended in provided Binding Buffer. Cells were incubated with Annexin V-FITC and PI, *as per* manufacturer's protocol, and FACS analysis was performed using a Coulter Epics XL Flow Cytometer (Beckman Coulter Inc., Miami, FL, USA). Flow cytometry data was processed using FlowJo Engine X10.4 (v3.05478, LLC).

2.3.5. Migration assays. Human UVECs, UC-MSCs and DPSCs were plated at 10000 cells/cm² in a 12 well-plate. Cells were further allowed to expand up to 90% confluency and the centre of the well scratched using a P200 sterile pipette tip.

Wells were individually photographed, and standard culture medium was replaced by defined experimental conditions and appropriate controls [UVECs assay—CM media 80% UVECs expansion medium and 20% 5x concentrated UC-MSCs' CM or DPSCs' CM; Control Medium: 80% UVECs expansion media and 20% DPBS; Complete Medium: 100% UVECs expansion media; MSCs assay—CM media 80% α MEM 10% FBS and 20% 5x concentrated UC-MSCs' CM or DPSCs' CM; Control Medium: 80% α MEM 10% FBS and 20% DPBS; Complete Medium: 100% α MEM 10% FBS].

Cells were cultured in the conditions above described and observed for migration and proliferation into the scratched area. Photographs were taken at 0, 10, 14 and 16 hours (for UVECs) and 0, 6 and 24 hours (for MSCs) after experimental media addition and media was changed after each procedure. Photographs were obtained from marked areas along each scratch line, allowing for the monitoring of cell response in multiple areas (Axiovert 40 CFL, Zeiss[®]).

Photographs were then analysed using the ImageJ Software (ImageJ 1.51k, NIH, USA) and the scratched area was measured in time sequenced images, using the 'MRI Wound Healing Tool' (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool). Six measurements per condition were considered and the results were presented as 'percentage decrease in scratched area'.

2.3.6. *In vitro* endothelial tube formation assay. For the evaluation of the effect of the CMs on the *in vitro* endothelial tube formation capacity of the UVECs, cells were plated on Matrigel[®] (Growth Factor Reduced Matrigel[®], Cat. 354230, Corning[®]) coated 96-well culture plates. A final density of 16000 cells *per* well were plated and specific media added to each group. Endothelial tubes formation was accompanied for up to 12 hours, and photographic record at 40x magnification was obtained at this timepoint (Axiovert 40 CFL, Zeiss[®]). Recorded images were then analysed using the ImageJ Software (ImageJ 1.51k, NIH, USA).

Recorded measurements concerned covered area (%), branching points (#), total tubes (#) and total tube length (px), total loops (#), total loop area (px²) and total loop perimeter (px).

2.3.7. *In vivo* vascularization assay. For the *in vivo* assessment, adult male Sasco Sprague Dawley rats [300-350g body weight (b.w.)] were selected. Experimental animals were housed in environmentally controlled facilities, under 12h light-dark cycles. Standard rodent chow and water were provided *ad libitum*. Normal cage activities were allowed, under standard laboratory conditions. All experimental procedures were approved by the Organism Responsible for Animal Welfare (ORBEA) of the Abel Salazar Institute for Biomedical Sciences (ICBAS), University of Porto (UP) (project 165/2016) and by the Veterinary Authorities of Portugal (DGAV) (project DGAV: 2018-07-11 014510), complying with Directive 2010/63/EU of the European Parliament and the European Communities Council Directive 86/609/EEC. Humane Endpoints were considered as recommended by the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000). Before surgical procedure, animals were maintained for two weeks under normal routine, for acclimation.

Anaesthesia protocol consisted on intraperitoneal (IP) injection of xylazine-ketamine mixture (Rompun[®] 20 mg/mL, Bayer[®], 12 mg/kg b.w., and Imalgene 1000[®], Merial[®], 100 mg/kg b.w.). The dorsum of each animal was clipped, and the skin asepsis performed with iodopovidone 10% solution (Betadine[®]).

Matrigel[®] was thoroughly mixed with the Control, Complete and Conditioned Media (80% Matrigel[®] and 20% of the respective medium). The formulation was maintained in ice,

to prevent Matrigel[®] gelation. The dorsum was virtually divided into four quadrants (each assigned to an experimental group) and a final volume of 500 μ L was injected subcutaneously. Upon injection, the formation of a protruding plug was confirmed. Animals were recovered from anaesthesia. After 7 days, animals were placed again under general anaesthesia and sacrificed by lethal intraperitoneal injection of 5% sodium pentobarbital (Eutasil[®] 200 mg/mL, Ceva).

Injection sites were confirmed and dorsal subcutaneous tissue enclosing the vascularised plug were collected and preserved in paraformaldehyde (3.7–4% buffered to pH 7, Panreac AppliChem[®]).

Collected tissues were processed for routine histopathologic analysis. Sequential sections (3 μ m) were prepared and stained with haematoxylin-eosin (H&E) and assessed for the presence and pattern of endotheliocyte/ capillary penetration into the matrix plug. For the immunohistochemical study, sections were deparaffinized in xylene and rehydrated in sequential graded alcohols. Antigen retrieval was performed in EDTA, pH 8.0 for 30 minutes in water bath 100° C. The Novolink[™] Max-Polymer detection system (Novocastra, Newcastle, UK) was used for visualization, according to the manufacturer's instructions. Slides were then incubated with anti-VEGFR2 antibody (clone 55B11; Cell Signaling Technology, Boston, USA), diluted 1:300, overnight at 4°C. Colour was developed with 3,3'-diamino-benzidine (DAB; Sigma, St. Louis, MO, USA) and sections were then counterstained with haematoxylin, dehydrated and mounted. Sections of normal rat skin were used as positive control and negative controls were performed by replacing the primary antibody with another of the same immunoglobulin isotype.

The tissue areas containing Matrigel[®] plugs and the surrounding tissue were microscopically evaluated in order to identify the regions of highest vascular density. Vessels which showed unequivocal brown VEGFR2 immunostaining were counted manually at higher magnification ($\times 40$) in at least 10 different regions of each sample.

2.4. Statistical analysis

Data was plotted and treated using GraphPad Prism version 6.00 (GraphPad Software, La Jolla California USA). Data was further analyzed for significant differences. Multiple comparison tests were performed by one-way ANOVA supplemented with Tukey's HSD post-hoc test. Differences were considered statistically significant at $P < 0.05$. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively.

3. Results

3.1. Mesenchymal Stem/ Stromal Cells characterization and conditioning protocol

Mesenchymal Stem/ Stromal Cells populations depicted characteristic phenotypic markers (Fig 1), presenting $\geq 92\%$ positive population for CD90, CD105 and CD44, and $\leq 2\%$ negative marking for CD34, CD11b, CD19, CD45 and MHC II. Exception was noted for DPSCs population with regard to CD90, which marked positive for only 87–91% of assessed population (Positive population: UC-MSCs—CD90⁺: 99.23 ± 0.12 ; CD105⁺: 99.07 ± 0.09 ; CD44⁺: 95.70 ± 1.65 ; NEG⁺: 0.13 ± 0.08 ; DPSCs: CD90⁺: 90.50 ± 0.35 ; CD105⁺: 99.30 ± 0.06 ; CD44⁺: 99.80 ± 0.00 ; NEG⁺: 0.13 ± 0.04 ; *NEG+ cocktail corresponds to anti-CD34; -CD11b; -CD19; -CD45; and -MHC II antibodies).

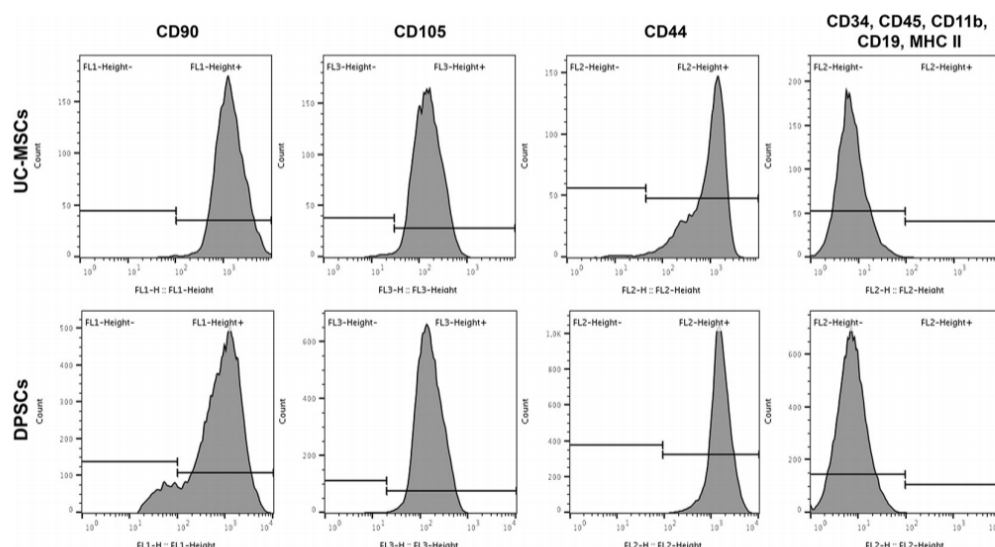


Fig 1. Surface marker expression for MSCs' identity of UC-MSCs and DPSCs. Positivity for CD90, CD105 and CD44, negativity for a cocktail including CD34, CD11b, CD19, CD45 and MHC II, assessed by Flow cytometry.

<https://doi.org/10.1371/journal.pone.0221378.g001>

RNA was purified from both UC-MSCs and DPSCs and converted to cDNA using adequate procedures. Primers targeting two housekeeping genes (β -actin and GAPDH) and four typical pluripotency markers (c-kit, Oct-4, Nanog and ALP) were used to support its identity. [Table 2](#) details on the average of Threshold cycle (Ct) values. Total RNA was successfully purified from UC-MSCs and DPSCs using the High Pure RNA Isolation kit (Roche), obtaining a concentration of 883.2 ng/ μ l from UC-MSCs and 158.7 ng/ μ l from DPSCs. Volumes were adjusted to use only 1.5 μ g of total RNA to synthesize corresponding cDNA. This cDNA was used as template in the qPCR reaction. The Ct values show a reasonable amplification of the target genes, in both cell types, resulting in an active expression of these genes in both UC-MSCs and DPSCs. The agarose gel confirms the identity of the genes from the observed molecular weight.

The strongest expression is observed in the housekeeping genes, while the lowest expression was observed for the Oct-4 sequence (as evidenced by the lower and higher Δ Ct values determined for the amplification cycles, respectively) ([Table 2](#)).

The differentiation capacity of MSCs towards three mesodermal lineages was confirmed ([Fig 2](#)). Macroscopic observation revealed calcified matrix formation, evidenced by ARS staining in both UC-MSCs and DPSCs under osteodifferentiation conditions. Semi-quantitative

Table 2. Average of Delta Threshold cycle (Ct) values. Of the amplification of the selected genes from UC-MSCs and DPSCs.

Gene	Δ Ct value					
	UC-MSCs			DPSCs		
Pluripotent Stem Cells Markers genes						
ALP	6.03	±	0.03	6.36	±	0.01
c-kit	1.96	±	0.01	1.49	±	0.02
Nanog	6.41	±	0.06	6.15	±	0.02
Oct-4	7.95	±	0.00	7.26	±	0.01

<https://doi.org/10.1371/journal.pone.0221378.t002>

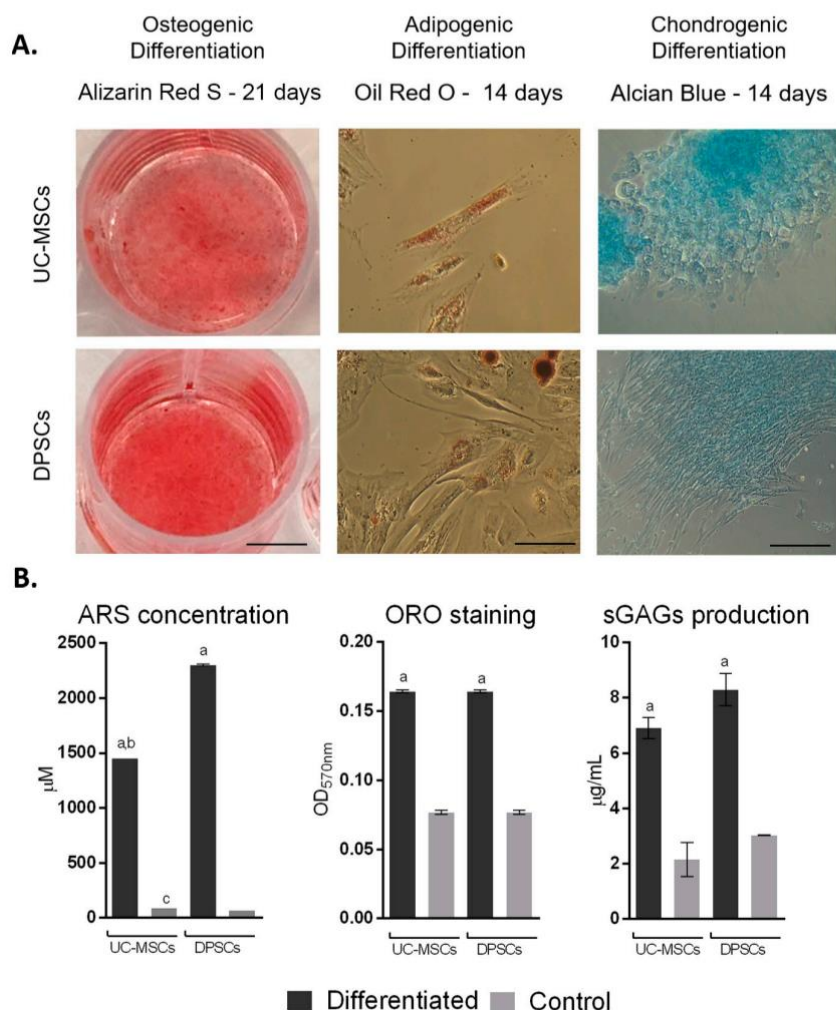


Fig 2. Multilineage differentiation. A) Qualitative evaluation—Osteogenic differentiation: Alizarin Red S (ARS) staining after 21 days (scale bar = 6000 μm); Adipogenic differentiation: Oil Red O (ORO) staining after 14 days (scale bar = 100 μm); Chondrogenic differentiation: Alcian blue staining after 14 days (scale bar = 400 μm). B) Semi-Quantitative evaluation—Osteogenic differentiation: ARS concentration (μM) after 21 days; Adipogenic differentiation: Oil Red O (OD_{570nm}) after 14 days; and C) Chondrogenic differentiation: Sulfated GAGs production (μg/ml) after 14 days, assessed by Blyscan™ Glycosaminoglycan Assay (Biocolor, UK). Control: Undifferentiated control; Results presented as Mean ± SEM. a: significantly different from undifferentiated group; b: UC-MSCs differentiated group significantly different from DPSCs differentiated group; c: UC-MSCs undifferentiated group significantly different from DPSCs undifferentiated group ($P < 0.05$).

<https://doi.org/10.1371/journal.pone.0221378.g002>

analysis confirmed observed differentiation, further indicating an increased efficiency and osteogenic propensity of DPSCs when compared to UC-MSCs (2299.31 ± 7.35 μM and 1449.57 ± 0.00 μM of ARS, respectively). In adipogenic differentiation, microscopic observation of ORO stained cells evidenced comparable lipid droplets accumulation in differentiated UC-MSCs and DPSCs. Spectrophotometric quantification of ORO staining confirmed observed differentiation and indicate no significant difference between UC-MSCs and DPSCs efficiency. Chondrogenic differentiation groups presented marked light blue staining under macroscopic and microscopic observation, indicating proteoglycan deposition by Alcian Blue staining, confirmed by sGAGs production quantification. UC-MSCs and DPSCs presented no

difference in terms of sGAGs production within the differentiated and the undifferentiated wells.

3.2. Metabolomic and bioactive factors secretion profiles

3.2.1. Conditioned medium collection and analysis

Populations from both tissue sources were successfully seeded and reached the desired density within 3 to 4 days. For conditioning time, cells remained well attached and preserved their characteristic spindled shape, although a decrease in the proliferation rate could be empirically observed.

3.2.2. Metabolomic analysis

Proton NMR spectroscopy was used to define the metabolic profiles of UC-MSCs and DPSCs through the analysis of produced CMs. To identify metabolite changes induced by DPSCs and UC-MSCs conditioning, various 1D and 2D NMR experiments were performed, and ^1H NMR spectra acquired using appropriate NMR techniques for suppression of the solvent and other interfering signals. The same experimental conditions (temperature, sample/solvent volume, standard (TSP) concentration, NMR acquisition and processing parameters) were used in order to avoid external interferences and to identify particular metabolite variations. The assignment of the proton resonances in NMR spectra and the assessment of the metabolite composition of the samples were achieved by high resolution ^1H NMR spectra analysis, considering specific NMR parameters which reflect structural characteristics of the respective species. The results were verified by the implementation of appropriate 2D NMR ($^1\text{H}/^1\text{H}$ COSY, $^1\text{H}/^1\text{H}$ TOCSY, $^1\text{H}/^{13}\text{C}$ HSQC) spectroscopic techniques and compared to available data in the literature [33, 34]. The assignment of the resonance signals of metabolites in ^1H NMR spectra of the samples studied is presented in Table 3. Characteristic resonance signals of metabolites identified in ^1H NMR spectra of Plain (unconditioned), UC-MSCs and DPSCs CM are labelled in Fig 3.

The visual inspection of the ^1H NMR spectra (Fig 3) suggests similar profile shapes but different concentration distribution of the metabolites. To assess characteristic metabolic changes

Table 3. ^1H NMR chemical shifts and multiplicity. Of the main metabolites observed in the spectra of Plain, UC-MSCs and DPSCs CM.

Metabolites	Abbrev.	Chemical Shifts (ppm) multiplicity
Acetate	Ace	1.92(s, CH_3)
Alanine	Ala	1.48(d, bCH_3), 3.78(q, aCH)
Choline	Cho	3.19 (s, CH_3), 3.51(dd, CH_2), 4.06(dd, CH_2),
Ethanol	Et	1.19 (t, CH_3), 3.57 (q, CH_2)
Formate	For	8.46 (CH)
Glutamate	Glu	2.36(t, gCH_2), 2.08(m, bCH_2), 3.74(dd, aCH)
Glutamine	Gln	2.44(t, gCH_2), 2.51/2.04(m, bCH_2), 3.76(dd, aCH)
Lactate	Lac	1.34(d, bCH_3), 4.13(q, aCH)
Nicotinamide	NA	7.58 (dd, CH), 8.24 (dd, CH), 8.70 (dd, CH), 8.94 (s, CH)
Pyruvate	CH_3	2.39(s)
Tyrosine	Tyr	3.05/3.18 (bCH_2), 3.93 (aCH), 7.19(d, H2,6), 6.90(d, H3,5)
α -Glucose	$\alpha\text{-}\Gamma\lambda\upsilon$	5.24(d, H1), 3.54(dd, H2), 3.72(t, H3), 3.41(t, H4), 3.47(ddd, H5), 3.91/3.72 (dd, H6)
β -Glucose	$\beta\text{-}\Gamma\lambda\upsilon$	4.65(d, H1), 3.24(t, H2), 3.49(t, H3), 3.42(t, H4), 3.84(m, H5,6), 3.78(dd, H6)

<https://doi.org/10.1371/journal.pone.0221378.t003>

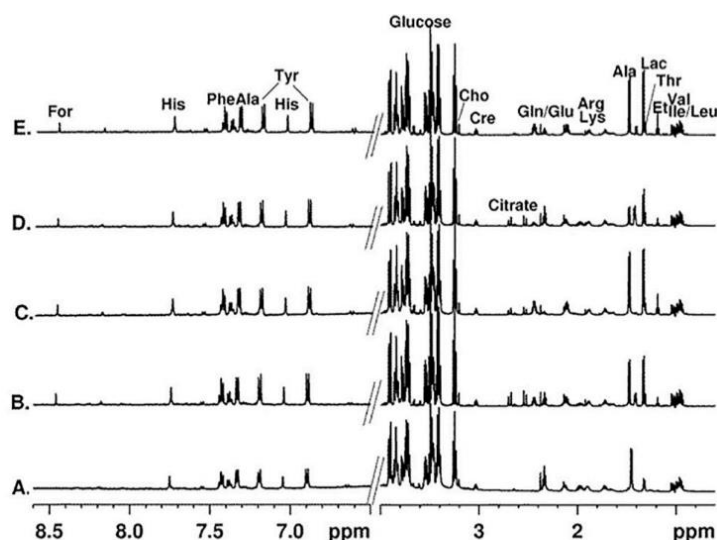


Fig 3. Average 600 MHz ^1H NMR spectra. (in H_2O with 10% D_2O) of Plain Medium (A), UC-MSCs CM collected after 24 (B) and 48 hours (C), and of DPSCs CM collected after 24 (D) and 48 hours (E). The intensity in the aromatic spectral area (6.5–8.6 ppm) incremented by 10x; the water signal is excluded from the spectra.

<https://doi.org/10.1371/journal.pone.0221378.g003>

occurring during hMSCs conditioning, comparative quantitative analysis using ^1H NMR spectra of the samples from the three groups was performed.

The metabolites concentration was determined from the integral intensity of characteristic signals in ^1H NMR spectra of the samples referenced to the integral intensity of TSP signal used as an internal standard and considering the number of the contributing nuclei for that particular resonance signal. The concentration of the metabolites identified in the ^1H NMR

Table 4. Metabolite concentration (μM). Calculated from ^1H -NMR spectra of unconditioned (Plain Medium), and UC-MSCs and DPSCs conditioned media, after 24 and 48 hours. Data presented in Mean $\pm$ SD.

Metabolite	Group	Chem. Shift (ppm)	Concentration (μM)				
			Plain Medium	UC-MSCs 24h	UC-MSCs 48h	DPSCs 24h	DPSCs 48h
Acetate	CH_3	1.93	2.91 $\pm$ 0.02	6.16 $\pm$ 0.81	5.15 $\pm$ 0.34	2.03 $\pm$ 1.17	2.00 $\pm$ 0.06
Alanine	CH_3	1.48	16.34 $\pm$ 0.52	120.65 $\pm$ 1.76	165.76 $\pm$ 2.75	46.42 $\pm$ 0.89	141.99 $\pm$ 6.17
Choline	$\text{N}(\text{CH}_3)_3$	3.21	2.53 $\pm$ 0.09	4.60 $\pm$ 0.89	3.87 $\pm$ 0.17	3.93 $\pm$ 0.14	4.20 $\pm$ 0.52
Ethanol	CH_3	1.19	0.84 $\pm$ 0.13	8.22 $\pm$ 0.18	16.13 $\pm$ 0.07	8.47 $\pm$ 0.56	21.00 $\pm$ 0.30
Formate	CH	8.46	0.39 $\pm$ 0.19	1.58 $\pm$ 0.27	1.92 $\pm$ 0.77	1.07 $\pm$ 0.09	1.48 $\pm$ 0.26
GlutaMAX I (L-Ala)	CH_3	1.42	94.64 $\pm$ 1.24	77.09 $\pm$ 0.18	4.97 $\pm$ 1.94	88.83 $\pm$ 1.77	25.39 $\pm$ 9.74
GlutaMAX II (L-Glu)	CH_2	2.34	153.38 $\pm$ 4.29	160.99 $\pm$ 4.52	29.72 $\pm$ 0.36	160.17 $\pm$ 5.22	62.61 $\pm$ 8.03
Glutamine	CH_2	2.45	6.62 $\pm$ 0.44	134.98 $\pm$ 4.32	175.09 $\pm$ 2.54	37.85 $\pm$ 0.60	157.38 $\pm$ 15.51
Lactate	CH	4.12	11.36 $\pm$ 0.44	111.44 $\pm$ 8.04	131.75 $\pm$ 6.58	87.20 $\pm$ 1.45	137.52 $\pm$ 11.95
Nicotinamide	CH	8.94	0.44 $\pm$ 0.37	2.55 $\pm$ 0.72	2.04 $\pm$ 1.77	1.14 $\pm$ 0.13	1.16 $\pm$ 0.13
Pyruvate	CH_3	2.37	14.40 $\pm$ 0.15	15.67 $\pm$ 1.07	7.53 $\pm$ 0.37	12.49 $\pm$ 4.69	6.97 $\pm$ 0.22
Tyrosine	2CH	7.19	21.59 $\pm$ 0.26	35.59 $\pm$ 1.07	27.51 $\pm$ 0.44	26.61 $\pm$ 2.11	27.36 $\pm$ 1.39
α -Glucose	CH	5.24	773.63 $\pm$ 1.52	1 112.15 $\pm$ 58.57	813.21 $\pm$ 16.77	805.17 $\pm$ 25.38	860.69 $\pm$ 15.68
β -Glucose	CH	4.65	945.19 $\pm$ 14.00	1 307.92 $\pm$ 9.58	1 038.61 $\pm$ 63.04	913.16 $\pm$ 2.84	998.30 $\pm$ 19.05

<https://doi.org/10.1371/journal.pone.0221378.t004>

spectra of the samples was calculated using the equation [48]:

$$W_x = \frac{I_x \times N_{std} \times M_x \times m_{std}}{I_{std} \times N_x \times M_{std}}$$

Where:

- W_x represent the mass of the metabolite;
- M_x and M_{std} are the molar masses of the metabolite and the standard (TSP was used), respectively;
- I_x and I_{std} are the integrated signal area of the metabolite and the standard, respectively;
- N_x and N_{std} are the number of protons in the integrated signal area of the metabolite and the standard;
- m_{std} represent the mass of the standard.

The concentration of the metabolites (μM) identified in each group, as well as the chemical shift and assignment of the signal used for quantification are presented in [Table 4](#). Statistical significance of encountered differences in Metabolite concentration (μM) is presented in [S1 Table](#).

All ^1H NMR spectra are dominated by the proton resonance signals of glucose but several low molecular weight compounds such as amino acids (e.g. alanine, tyrosine), organic acids (e.g. lactate, acetate, citrate, formate) and choline are identified ([Fig 3](#)).

Additionally, in the spectra of the Plain Medium, resonance signals characteristic for L-alanyl-L-glutamine (GlutaMAXTM I (L-Ala) at 1.42 and GlutaMAXTM II (L-Glu) at 2.37 ppm) dipeptide from GlutaMAXTM are clearly observed. The intensity of these resonances significantly decreases through the cell conditioning process, which is especially evident after 48 hours of culture conditioning. Glutamine and alanine, the resulting compound from GlutaMAXTM and glutamate's metabolism, expectedly rise through culturing times. Remarkably, UC-MSCs' CM increase in these metabolites' content occurs at 24 hours, before significant GlutaMAXTM consumption can be observed. For the DPSCs population, this process appears to occur at a slower rate ([Fig 4A](#) and [Fig 4B](#)).

Glucose is the most prominent metabolite identified in the spectra and therefore in greatest concentration. In the media collected after 24 hours of conditioning of UC-MSCs, a significant increase of α and β -Glucose content is observed, when compared to the Plain Medium (which was supplied to the culture at the start of the conditioning period), as well as when compared to the DPSCs at the same conditioning time. This initial spike in glucose content decreases in the following hours, reaching back to values not significantly different from the Plain Medium. Human DPSCs present no significant variation in glucose content throughout the study period.

Pyruvate is an energetic compound that is catabolised through aerobic or anaerobic pathways. The analysis of the Plan Media confirms it is one of the supplemented metabolites. Both cell types appear to present a tendency for pyruvate consumption in the 48 hours of conditioning ([Fig 4C](#) and [Fig 4D](#)), but significance in such reduction is only found for UC-MSCs. Catabolites resulting from its metabolism are also detected in the spectra, such as acetate, lactate and ethanol. Acetate accumulated in UC-MSCs cultures, particularly in the first 24h ([Fig 4C](#)). In DPSCs CM, Acetate concentrations remained stable though the evaluation ([Fig 4D](#)). Lactate, on the other hand, accumulated strongly within the first 24 hours in both cases, with little increase afterwards, reaching maximum concentration at 48 hours of incubation. Ethanol

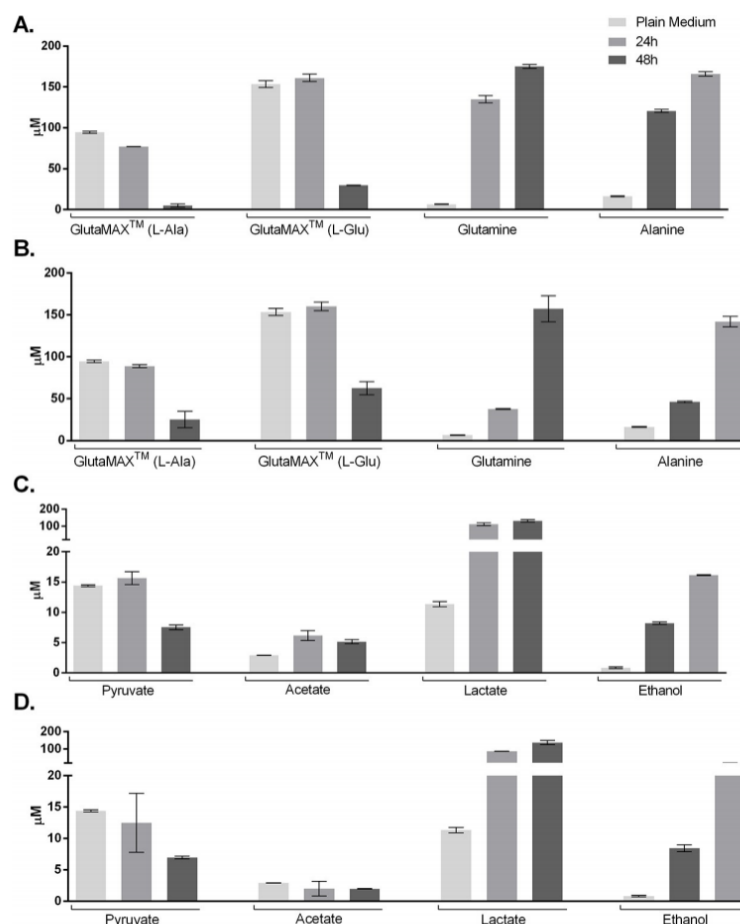


Fig 4. Metabolite dynamics. Dynamics of GlutaMAX™ breakdown to glutamine and alanine of UC-MSCs (A) and DPSCs (B) populations; and Dynamics of Pyruvate catabolism into acetate, lactate and ethanol of UC-MSCs (C) and DPSCs (D) populations. Statistical significance of observed differences available in [S1 Table](#), as Supporting Information.

<https://doi.org/10.1371/journal.pone.0221378.g004>

presented a steady rate of accumulation in the culture supernatant for both UC-MSCs and DPSCs.

Other compounds such as choline, formate, tyrosine and nicotinamide are also detected at low levels in both Plain and Conditioned media.

3.2.3. Bioactive factors detection and quantification. Multiplexing LASER Bead Analysis was performed for the detection of a total of 57 Bioactive factors in the CM obtained from UC-MSCs and DPSCs, after 24 and 48 hours of conditioning. Obtained measurements were normalized to the Unconditioned/ Plain medium (naturally devoid of artificial bioactive factors), run as blank sample. Fluorescent readings were plotted against a standard curve and calculated concentrations are presented in [Table 5](#). Statistical significance of encountered differences in the detected Bioactive factors (pg/mL) is presented in [S2 Table](#).

After 24 hours of conditioning most factors presented no or little expression. Some of the factors gained expression after 48 hours, becoming detectable and, occasionally, in significant concentrations. Overall, none to minimal signal intensity was detected in 36 of the 57 assayed factors, combining the two timepoints.

Table 5. Bioactive factors detection and quantification (pg/mL). Calculated from Multiplexing LASER Bead Analysis of UC-MSCs and DPSCs CM after 24 and 48 hours of conditioning. Data presented in Mean $\pm$ SEM. Bioactive factors presenting no to minimal signal intensity in grey. ND, not detected.

Bioactive Factor	Concentration (pg/mL)										
	UC-MSCs 24h			UC-MSCs 48h			DPSCs 24h			DPSCs 48h	
Angiop-2	ND			10.66	$\pm$	0.45	ND			1.86	$\pm$ 1.00
BMP-9	ND			0.22	$\pm$	0.02	ND			0.00	$\pm$ 0.00
EGF	0.23	$\pm$	0.13	0.07	$\pm$	0.04	0.28	$\pm$	0.02	0.68	$\pm$ 0.10
Endoglin	ND			47.74	$\pm$	3.25	0.93	$\pm$	0.93	2.56	$\pm$ 0.39
Endothelin-1	ND			1.21	$\pm$	0.07	ND			0.03	$\pm$ 0.03
Eotaxin-1	ND			1.50	$\pm$	0.49	4.37	$\pm$	2.08	3.09	$\pm$ 1.54
FGF-1	ND			1.97	$\pm$	0.63	ND			0.26	$\pm$ 0.22
FGF-2	ND			212.27	$\pm$	12.39	ND			7.06	$\pm$ 7.06
Flt-3L	ND			1.37	$\pm$	0.69	ND			0.76	$\pm$ 0.22
Follistatin	143.45	$\pm$	10.69	1101.53	$\pm$	293.83	202.04	$\pm$	29.56	1309.40	$\pm$ 110.60
Fractalkine	3.37	$\pm$	0.95	23.76	$\pm$	13.03	ND			ND	
G-CSF	ND			1041.30	$\pm$	113.67	ND			0.49	$\pm$ 0.25
GM-CSF	1.24	$\pm$	0.34	2.45	$\pm$	1.07	ND			1.04	$\pm$ 0.25
GRO pan	17.80	$\pm$	7.49	966.84	$\pm$	177.44	ND			12.19	$\pm$ 1.45
HB-EGF	0.02	$\pm$	0.00	0.97	$\pm$	0.11	0.04	$\pm$	0.04	0.10	$\pm$ 0.03
HGF	113.99	$\pm$	7.25	4436.31	$\pm$	516.74	0.37	$\pm$	0.37	69.86	$\pm$ 18.50
IFN α 2	3.19	$\pm$	0.99	3.04	$\pm$	0.41	10.95	$\pm$	5.08	2.28	$\pm$ 1.11
IFN γ	0.27	$\pm$	0.05	1.22	$\pm$	0.11	0.40	$\pm$	0.16	ND	
IL-10	ND			0.29	$\pm$	0.05	ND			ND	
IL-12(p40)	2.80	$\pm$	0.89	3.49	$\pm$	0.63	0.64	$\pm$	0.11	ND	
IL-12(p70)	0.32	$\pm$	0.17	0.40	$\pm$	0.04	0.38	$\pm$	0.03	0.41	$\pm$ 0.14
IL-13	1.02	$\pm$	0.06	0.76	$\pm$	0.12	0.27	$\pm$	0.04	1.15	$\pm$ 0.13
IL-15	0.65	$\pm$	0.02	0.80	$\pm$	0.19	0.37	$\pm$	0.04	0.49	$\pm$ 0.03
IL-17A	ND			ND			ND			ND	
IL-18	ND			ND			ND			ND	
IL-1B	0.56	$\pm$	0.08	0.35	$\pm$	0.05	0.50	$\pm$	0.26	0.43	$\pm$ 0.17
IL-1RA	0.54	$\pm$	0.14	0.78	$\pm$	0.27	ND			ND	
IL-1 α	4.45	$\pm$	0.31	7.10	$\pm$	0.73	ND			ND	
IL-2	ND			ND			ND			ND	
IL-3	0.38	$\pm$	0.21	ND			ND			ND	
IL-4	0.81	$\pm$	0.35	ND			ND			ND	
IL-5	ND			ND			0.01	$\pm$	0.00	0.05	$\pm$ 0.03
IL-6	31.24	$\pm$	2.47	470.56	$\pm$	33.61	ND			ND	
IL-7	0.12	$\pm$	0.05	0.65	$\pm$	0.10	ND			ND	
IL-8	48.18	$\pm$	2.89	1863.05	$\pm$	10.77	0.02	$\pm$	0.01	1.23	$\pm$ 0.14
IL-9	ND			0.30	$\pm$	0.06	ND			0.38	$\pm$ 0.29
IP-10	1.90	$\pm$	0.10	ND			ND			1.23	$\pm$ 0.10
Leptin	1.76	$\pm$	0.00	216.56	$\pm$	17.66	39.62	$\pm$	24.47	12.09	$\pm$ 12.09
MCP-1	972.36	$\pm$	83.42	2626.08	$\pm$	236.18	N.D.			0.44	$\pm$ 0.33
MCP-3	6.84	$\pm$	3.45	53.53	$\pm$	6.48	2.43	$\pm$	0.89	7.23	$\pm$ 2.77
MDC	ND			32.13	$\pm$	6.16	ND			ND	
MIP-1 α	ND			ND			ND			ND	
MIP-1 β	ND			ND			ND			ND	
PDGF-AA	0.14	$\pm$	0.01	0.56	$\pm$	0.04	ND			0.02	$\pm$ 0.01
PDGF-BB	2.06	$\pm$	0.56	3.15	$\pm$	1.35	2.24	$\pm$	0.67	4.29	$\pm$ 1.97

(Continued)

Table 5. (Continued)

Bioactive Factor	Concentration (pg/mL)											
	UC-MSCs 24h			UC-MSCs 48h			DPSCs 24h			DPSCs 48h		
PLGF	0.17	±	0.04	0.11	±	0.03	0.13	±	0.06	4.35	±	0.30
RANTES	12.35	±	2.32	73.61	±	5.56	4.52	±	1.58	1.53	±	1.12
sCD4L	ND			ND			ND			ND		
TGF- α	ND			ND			0.26	±	0.08	ND		
TGF- β 1	11.96	±	2.18	582.49	±	56.37	4.08	±	2.08	53.01	±	23.04
TGF- β 2	0.69	±	0.15	174.20	±	17.52	0.13	±	0.13	29.33	±	5.09
TGF- β 3	0.09	±	0.09	1.77	±	1.25	ND			0.20	±	0.16
TNF α	0.05	±	0.01	0.24	±	0.07	ND			0.08	±	0.00
TNF β	ND			0.86	±	0.17	ND			1.38	±	0.27
VEGF-A	17.25	±	3.48	1.34	±	0.28	14.31	±	8.75	2041.55	±	45.66
VEGF-C	1.90	±	0.13	486.48	±	23.48	ND			30.71	±	1.67
VEGF-D	0.57	±	0.22	1.90	±	0.48	0.52	±	0.17	ND		

<https://doi.org/10.1371/journal.pone.0221378.t005>

A similar pattern of superior secretion by UC-MSCs is observed throughout, highlighting HGF, IL-8, and MCP-1 expressive quantities. Exception is appointed to follistatin and, most significantly, to VEGF-A, where DPSCs depict increased levels when compared to UC-MSCs.

3.3. Effects of MSCs CM on angiogenesis

3.3.1. Cell viability, senescence and apoptosis assays. The CM obtained from MSCs cultures was employed as culture medium supplement for *in vitro* culture of UVECs, to assess for its ability to sustain the activity and proliferation of the endothelial populations. PrestoBlue[®] viability was used as a metabolic indicator. Obtained corrected absorbance values are

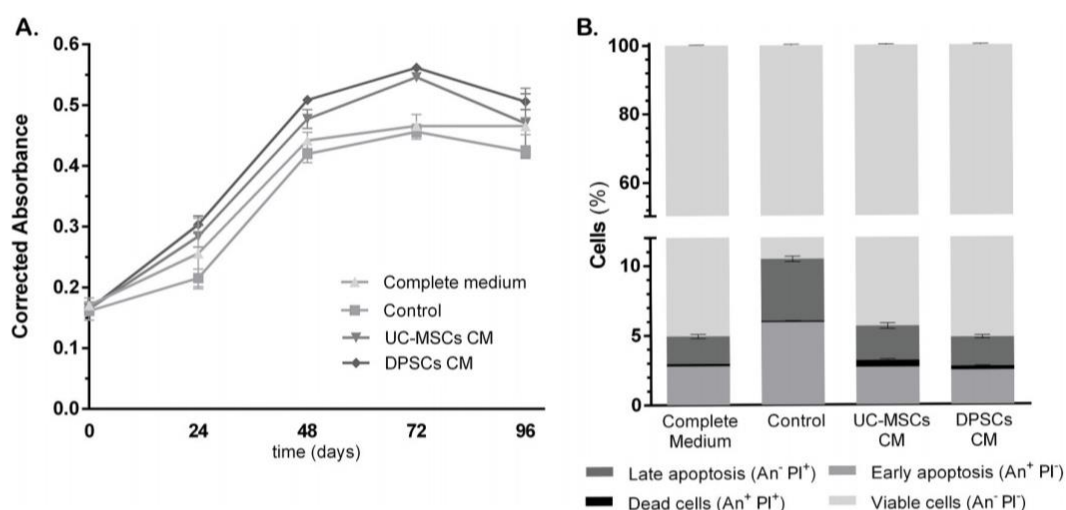


Fig 5. Corrected absorbance readings of Presto Blue[®] Assay of UVECs. At 0, 24, 48, 72 and 96 hours of expansion in control or CMs supplemented media. Values are presented as Mean $\pm$ SD (A). Statistical significance of observed differences available in [S3 Table](#), as Supporting Information; Apoptosis (Annexin-V/ PI) assay of UVECs, after 48 hours of expansion in Complete, Control or CMs supplemented media. Results presented as percentage (%) of cells of Viable, Early Apoptotic, Late Apoptotic and Death cells, as Mean $\pm$ SD (B). Statistical significance of observed differences available in [S4 Table](#), as Supporting Information.

<https://doi.org/10.1371/journal.pone.0221378.g005>

Table 6. Corrected absorbance readings of Presto Blue[®] Assay of UVECs. At 0, 24, 48, 72 and 96 hours of expansion in control or CMs supplemented media. Values are presented as Mean $\pm$ SD.

Corrected Absorbance	Complete Medium			Control			UC-MSCs CM			DPSCs CM		
0 h	0.171	$\pm$	0.012	0.161	$\pm$	0.015	0.165	$\pm$	0.011	0.165	$\pm$	0.008
24 h	0.254	$\pm$	0.056	0.215	$\pm$	0.014	0.284	$\pm$	0.018	0.304	$\pm$	0.014
48 h	0.442	$\pm$	0.014	0.420	$\pm$	0.015	0.477	$\pm$	0.015	0.509	$\pm$	0.007
72 h	0.464	$\pm$	0.015	0.456	$\pm$	0.012	0.546	$\pm$	0.005	0.562	$\pm$	0.003
96 h	0.465	$\pm$	0.014	0.424	$\pm$	0.010	0.459	$\pm$	0.040	0.502	$\pm$	0.019

<https://doi.org/10.1371/journal.pone.0221378.t006>

presented in Fig 5 and Table 6 (statistical differences on observed measurements detailed in S3 Table).

At 24 hours, UVECs cultured in medium supplemented with DPSCs CM depict slightly increased activity, with no other noteworthy differences. As time progresses (48 and 72 hours), both DPSCs and UC-MSCs CM supplemented groups sustain superior cellular activity than Complete and Control media. At about 72 hours of culture, the UVECs cultured in CM supplemented media reached confluence and entered the plateau stage. The activity of Control medium group also decreased from that point onwards, although cellular confluence was unmatched to the CM supplemented groups. At 96 hours, only the Complete medium continued to present an increase in cellular metabolism but remained slightly inferior to that provided by DPSCs CM.

Senescent events on the cultured populations were assessed through the β -galactosidase activity. Control medium presented increased enzyme activity when compared to the CM supplemented groups (Corrected absorbance, as mean $\pm$ SD—Control: 0.0025 ± 0.0030 ; UC-MSCs CM 0.0003 ± 0.0004 ; DPSCs CM: 0.0000 ± 0.0000 ; $0.001 \leq P < 0.01$). CM supplemented groups presented no significant difference from the UVECs maintained in their standard Complete medium (Corrected absorbance, as mean $\pm$ SD: 0.0009 ± 0.0007).

As for apoptosis events in the cultured population, none of the CM supplemented groups revealed noteworthy impact in cellular viability (Fig 5B, Table 7 and S4 Table). The most striking observation is that the Control group presented increased events of early and late stage apoptosis, significantly decreasing the percentage of viable cells. As for the groups supplemented with DPSCs or UC-MSCs CM, the first presented increased cellular viability, as opposed to the early apoptotic and cell death events identified in the UC-MSCs CM supplemented group. The CM supplemented groups matched the events of each stage recorded for UVECs cultured in their standard Complete media, except for the early apoptosis, where DPSCs CM provided an inferior percentage.

Table 7. Apoptosis (Annexin-V/ PI) assay of UVECs. After 48 hours of expansion in Complete, Control or CMs supplemented media. Results presented as percentage (%) of cells of Viable, Early Apoptotic, Late Apoptotic and Dead cells, as Mean $\pm$ SD.

Apoptosis (Annexin V/ PI)	Complete Medium			Control			UC-MSCs CM			DPSCs CM		
Viable Cells	95.07	$\pm$	0.25	89.47	$\pm$	0.41	94.33	$\pm$	0.41	95.17	$\pm$	0.29
Early Apoptosis	2.79	$\pm$	0.06	5.93	$\pm$	0.09	2.71	$\pm$	0.06	2.47	$\pm$	0.05
Late Apoptosis	1.97	$\pm$	0.21	4.43	$\pm$	0.28	2.71	$\pm$	0.28	2.09	$\pm$	0.16
Dead Cells	0.17	$\pm$	0.01	0.11	$\pm$	0.02	0.48	$\pm$	0.10	0.27	$\pm$	0.07

<https://doi.org/10.1371/journal.pone.0221378.t007>

3.3.2. Migration assays

After wound induction on the cellular monolayers with the pipette tip, the area of the produced defect did not differ between groups/wells, deeming them adequate for comparison through the healing time.

In one instance, we investigated the chemotactic effect of the MSCs CMs on endothelial populations, represented by HUVECs. No significant differences in recovered area of the wound were identified until 14 hours into the assay, when DPSCs CM supplemented groups depicted increased percentage of covered wound area, when compared to the Complete and Control media ($0.0001 \leq P < 0.001$) (Fig 6 and Table 8). At the final assessment time (16 hours), both CM supplemented groups presented over 90% area coverage, while Complete and Control media groups remained under 85% coverage of the originally induced 'scratch' ($0.0001 \leq P < 0.001$ to $P < 0.0001$).

Further, we assessed the effect of the CMs on the chemotaxis of the two MSCs populations under study. After 6 hours of 'scratching' and media replacement of the MSCs monolayers, no

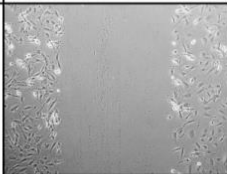
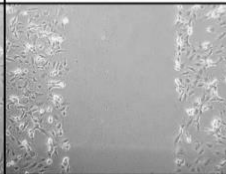
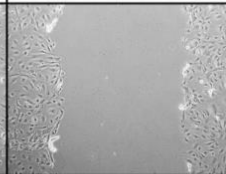
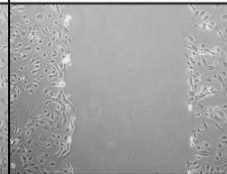
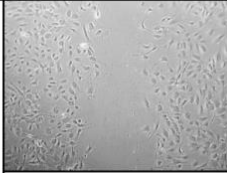
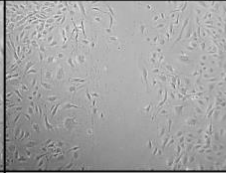
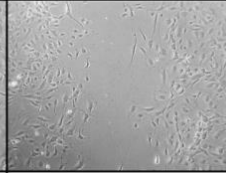
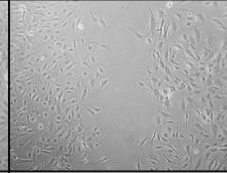
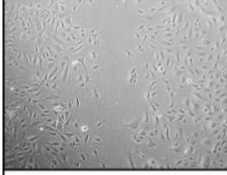
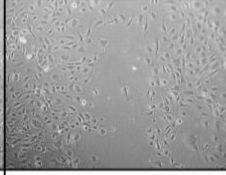
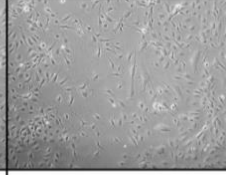
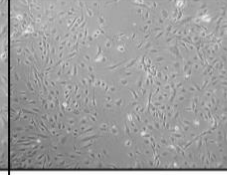
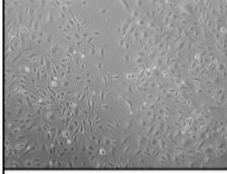
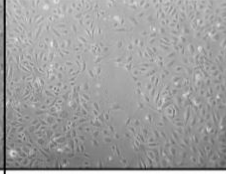
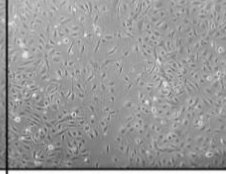
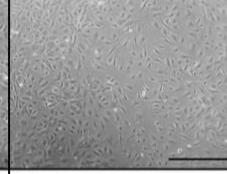
UVECs Migration Assay				
	Complete Medium	Control	UC-MSCs CM	DPSCs CM
0h				
	0%			
10h				
	59,9 %	53,8 %	59,7 %	62,9 %
14h				
	67,8 %	68,6 %	77,2 %	87,0 %
16h				
	84,0 %	84,6 %	90,1 %	90,3 %

Fig 6. Migration assay of UVECs. At 0, 10, 14 and 16 hours in control or CMs supplemented media. Values are presented as percentage (%) of wound area coverage from the 0 hours baseline (scale bar = 200 μ m).

<https://doi.org/10.1371/journal.pone.0221378.g006>

Table 8. Migration assay of UVECs. At 0, 10, 14 and 16 hours in control or CMs supplemented media, wounded area in 10^5 pixels. Values are presented as Mean $\pm$ SD.

Area (10^5 pixels)	Complete Medium			Control			UC-MSCs CM			DPSCs CM		
0 h	33.207	$\pm$	2.198	33.378	$\pm$	1.573	35.089	$\pm$	0.718	33.486	$\pm$	2.322
10 h	13.332	$\pm$	1.547	15.436	$\pm$	2.372	14.132	$\pm$	2.694	12.431	$\pm$	3.408
14 h	10.703	$\pm$	3.403	10.490	$\pm$	2.028	7.993	$\pm$	3.641	4.366	$\pm$	2.235
16 h	5.298	$\pm$	1.202	5.132	$\pm$	0.915	3.470	$\pm$	0.295	3.244	$\pm$	0.779

<https://doi.org/10.1371/journal.pone.0221378.t008>

differences were observed in cellular migration in Control and Complete Medium groups between, and within the two MSCs sources (Fig 7 and Table 9). The CM supplemented groups depicted superior area, with exception for CMs supplemented DPSCs, which did not differ significantly from the same population cultured in α MEM 10% FBS. No differences were patent in UC-MSCs and DPSCs supplemented with either CM ($0.0001 \leq P < 0.001$ to $P < 0.0001$).

At the final assessment of 24 hours, UC-MSCs in CM supplemented media presented increased migration into the area than the un-supplemented Control and α MEM 10% FBS groups. They also presented increased motility than the DPSCs populations ($0.0001 \leq P < 0.001$ to $P < 0.0001$). No difference in response to the two CMs was observed to within each MSCs type, but UC-MSCs responded with superior migration when paired to the DPSCs ($0.0001 \leq P < 0.001$ to $P < 0.0001$).

3.3.3. *In vitro* endothelial tube formation assay. The *in vitro* angiogenic potential of the CMs was evaluated through the tube formation capacity of UVECs, cultured in Complete, Control, UC-MSCs and DPSCs CM supplemented media. Control group presented the least effective tube formation capacity, while CM supplemented groups provided enhanced *in vitro* angiogenic potential (Fig 8).

It was noticeable that UVECs exposed to UC-MSCs and DPSCs CM supplemented media formed consistently well-defined tubular networks, with evenly distributed and regularly shaped loops in the Matrigel[®] matrix. Complete medium supplementation resulted in defined tubes, although loop distribution appears less homogeneous than in the CMs groups. Occasional events of incomplete tube connection from the branching points were observed. In the Control group, the dominant observation is the incomplete bridging attempts and short unconnected tubes, resulting in large and hill shaped loops.

Regarding the number of branching points, no differences were encountered between groups. In terms of total loops (formed from the connection of the branched tubes) and total tube length, Complete medium and CM supplemented groups showed significative superior performance, when compared to Control medium. Although both CMs provided increased results over the Complete medium group, statistical significance was only noted for the DPSCs CM supplemented group. No significant difference was found between the two CMs. The surface area covered by UVECs reflected the tube formation efficiency observed (Fig 8 and Table 10).

3.3.4. *In vivo* vascularization assay. After 7 days of subcutaneous implantation, the Matrigel[®] plugs were evidenced and collected. Discrete vascular penetration was observed macroscopically in all groups.

Microscopically, the Complete Medium and the UC-MSCs CM groups presented apparent increased capillary density than the Control group (Fig 9). In the CM groups, the capillary penetration reached deeper into the matrix. These observations were further confirmed through VEGFR2 staining of the microvessels within the subcutaneous tissue at the penetrating interface with the Matrigel[®] plug (Fig 9 and Table 11).

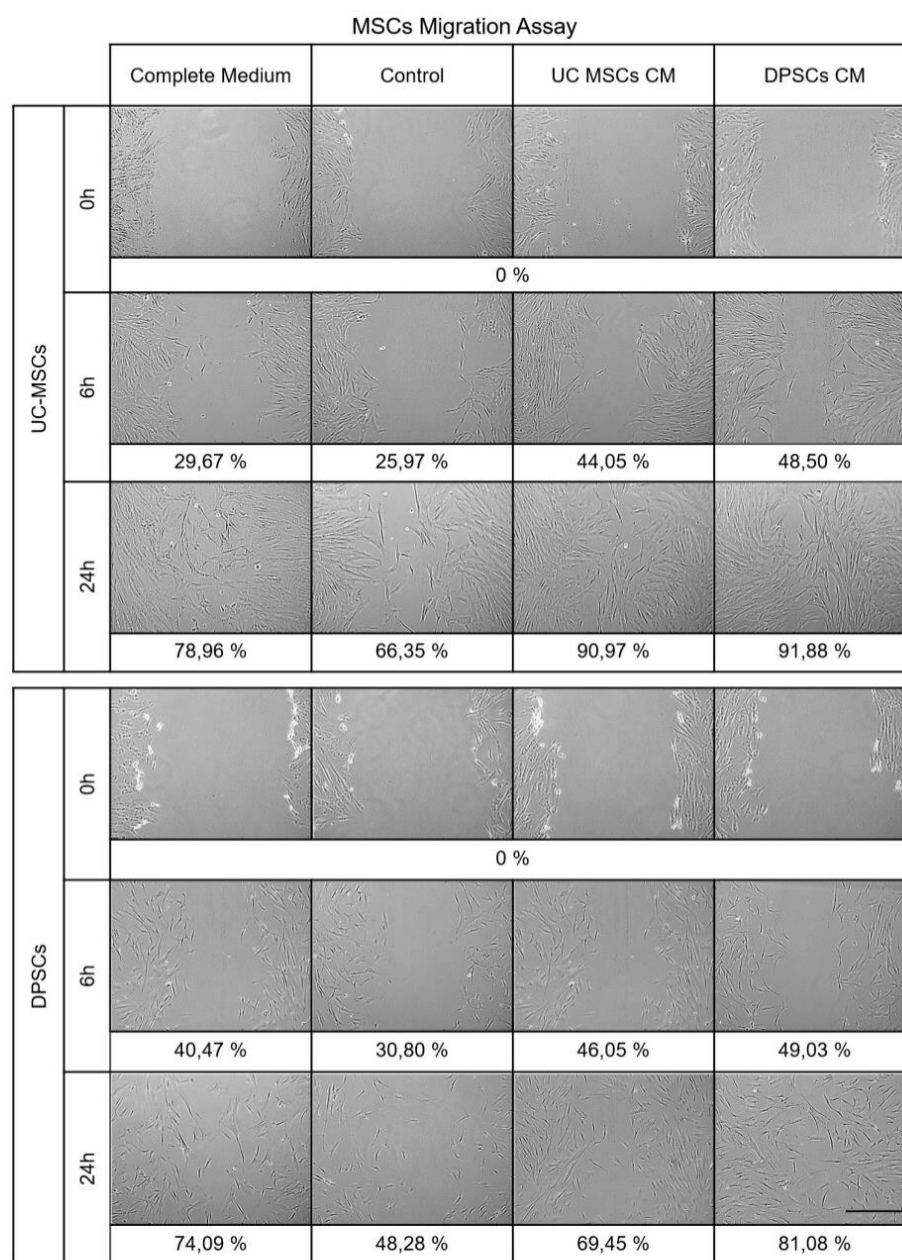


Fig 7. Migration assay of hMSCs. At 0, 6 and 24 hours of expansion in control or CMs supplemented media. Values are presented as percentage (%) of wound area coverage from the 0 hours baseline (scale bar = 200 μ m).

<https://doi.org/10.1371/journal.pone.0221378.g007>

4. Discussion and conclusions

The disclosure of the composition of MSCs populations' secretome is key to the comprehension of the underlying mechanisms of their therapeutic action. Therefore, it is key to understand the basal profile of secretion of these MSCs populations, to grant basis for the selection of the MSCs systems most suitable for each intended therapeutic application. For such, we

Table 9. Migration assay of hMSCs. At 0, 6 and 24 hours of expansion in control or CMs supplemented media, wounded area in 10^5 pixels. Values are presented as Mean $\pm$ SD.

Area (10^5 pixels)		Complete Medium			Control			UC-MSCs CM			DPSCs CM		
UC-MSCs	0 h	41.42	$\pm$	2.61	37.55	$\pm$	4.54	35.06	$\pm$	3.98	36.56	$\pm$	5.14
	6 h	29.13	$\pm$	3.82	27.80	$\pm$	2.73	19.62	$\pm$	2.98	18.83	$\pm$	2.11
	24 h	8.72	$\pm$	3.45	12.64	$\pm$	2.43	3.17	$\pm$	1.08	2.97	$\pm$	1.97
DPSCs	0 h	42.51	$\pm$	5.87	41.26	$\pm$	3.27	41.42	$\pm$	2.87	42.74	$\pm$	2.05
	6 h	25.30	$\pm$	3.71	28.55	$\pm$	3.53	22.34	$\pm$	2.28	21.79	$\pm$	2.75
	24 h	11.01	$\pm$	3.65	21.34	$\pm$	5.73	12.65	$\pm$	2.54	8.09	$\pm$	2.66

<https://doi.org/10.1371/journal.pone.0221378.t009>

conducted an analytical study focusing on the investigation of the metabolomic and bioactive factors composition of the secretome of hMSCs originated from two of the most promising sources for medical applications: the UC-MSCs and the DPSCs. These sources may come to gain ground for MSCs-based therapies due to the non-/ minimally invasive and ethically accepted collection procedures, as well as for the increasingly available private and public banking options worldwide.

The international scientific community has long debated the criteria for MSCs character assignment and issued a conciliatory guiding line of features for the definition of populations classified as MSCs [2], considering their phenotypic and differentiation capacities, which was confirmed on the hMSCs herein explored. We observed an increased capacity for DPSCs to differentiate towards mineralising populations, as reported by [49], but did not confirm their decreased capacity for adipogenesis since both UC-MSCs and DPSCs displayed comparable Oil Red O uptake. Further, a panel of specific genes has been reported to correlate with the proliferative capacity of undifferentiated MSCs and pluripotency/ multilineage differentiation capacity. Both UC-MSCs and DPSCs presented comparable expression of ALP, c-kit and Nanog. ALP has been described to relate to the proliferative stage of undifferentiated stem cells of embryonic and mesenchymal origin, while Nanog expression in MSCs is associated with the transition from *in vivo* quiescence to adaptation to *in vitro* growth condition. Both genes are described to downregulate as lineage commitment occurs, except for the osteoblastic phenotype conversion, where it is characteristically upregulated [50, 51]. C-kit is the gene coding for the receptor for the stem cell factor, also observed highly proliferative populations and related to differential lineage differentiation capacity [52]. Oct-4 was expressed to a lower extent in both cellular populations. Oct-4, along with Nanog, is associated to MSCs populations plasticity [53]. Other authors report that Oct-4 was not identified in cultured human adult MSCs [51].

These populations were therefore expanded in culture and elicited to secrete bioactive molecules to the surrounding media, through the conditioning process. The conditioning process was handled under serum-free conditions, so that the only factors detected in the CM were those produced by the UC-MSCs and DPSCs and not those bared to the system by exogenous supplementation.

Addressing the obtained CM from a metabolomic perspective (through ^1H -NMR spectroscopy), we identified glucose as the dominant metabolite in the spectra. Nuschke et al. recently investigated the metabolism of glucose in MSCs populations (bone marrow) and determined this to be a major limiting factor for MSCs survival. They describe a steady consumption rate for glucose in semi- and confluent cultures [54], a tendency that we could not observe in the cultured UC-MSCs and DPSCs. Indeed, UC-MSCs present an increase in both α - and β -glucose content, suggesting a possible engagement to gluconeogenesis and the utilization of other

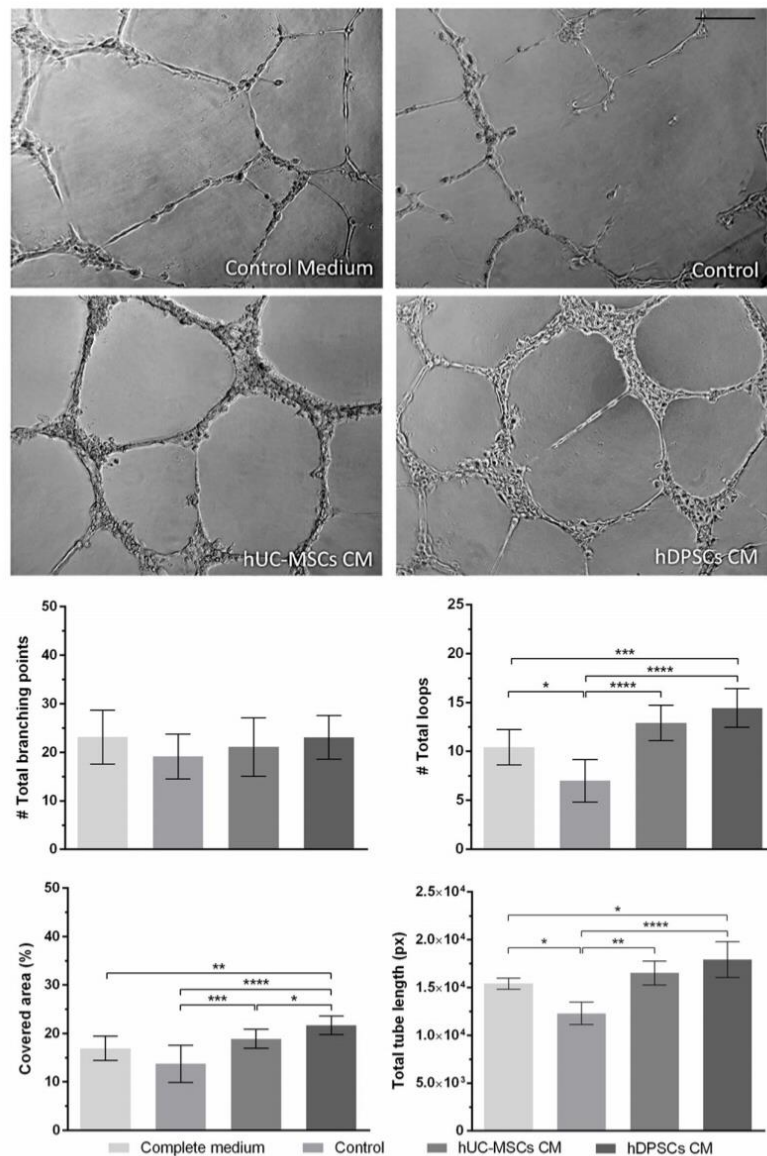


Fig 8. Tube formation assay. After 12 hours of UVECs exposure to Complete, Control, UC-MSCs or DPSCs supplemented media (upper panel). Graphical representation of total branching points, total loops, covered area and total tube length observed in each group (lower panel) (scale bar = 200 μ m). Values are presented as Mean $\pm$ SD. Significant differences indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively.

<https://doi.org/10.1371/journal.pone.0221378.g008>

bioenergetic substrates for energy production. The observed profiles suggest that, although significant amounts of glucose are provided by plain media, conditioning cells do not solely rely on glycolytic pathways for ATP and NADP production.

Pyruvate is therefore suggested as one of the crucial energetic metabolites that enters a variety of metabolic pathways, for the aerobic production of ATP [through its conversion into acetate/acetylCoA and entrance into the citric acid cycle]. Pyruvate is detected at relatively low

Table 10. Tube formation assay. After 12 hours of UVECs exposure to Complete, Control, UC-MSCs or DPSCs supplemented media: total branching points, total loops, covered area and total tube length observed in each group. Values are presented as Mean $\pm$ SD.

Tube Formation Assay	Complete medium			Control			UC-MSCs CM			DPSCs CM		
Covered area (%)	16.93	$\pm$	2.50	13.75	$\pm$	3.82	18.89	$\pm$	1.98	21.69	$\pm$	1.93
Branching points (#)	23.14	$\pm$	5.52	19.17	$\pm$	4.62	21.09	$\pm$	6.04	23.10	$\pm$	4.51
Total tubes (#)	47.29	$\pm$	12.57	44.67	$\pm$	12.61	43.27	$\pm$	13.91	46.60	$\pm$	9.00
Total tube length (px)	15415.00	$\pm$	582.38	12292.00	$\pm$	1169.33	16522.86	$\pm$	1239.79	17933.00	$\pm$	1858.48
Total loops (#)	10.43	$\pm$	1.81	7.00	$\pm$	2.19	12.91	$\pm$	1.81	14.45	$\pm$	1.97
Total loop area (px ²)	503384	$\pm$	476226	374678	$\pm$	331769	335432	$\pm$	229543	279728	$\pm$	170898
Total loop perimeter (px)	3087.11	$\pm$	2047.40	3738.14	$\pm$	2591.60	2833.46	$\pm$	1241.24	2459.92	$\pm$	1054.61

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concentrations, with a small tendency for consumption as conditioning time progresses. Pyruvate is initially provided by the media, but the maintenance of its concentration in the media suggest intrinsic production by the MSCs, through non-glucose dependent alternative synthesis pathways. Acetate levels, an intermediate product for the aerobic pathway of pyruvate catabolism, also remain fairly constant, as it is not expected to accumulate, but to continue its path through the citric acid cycle towards energy production. The build-up of choline and formate (related to the production of acetate) converge to this assumption.

Evidence of anaerobic metabolism is also observed throughout the conditioning period, providing accumulation of ethanol and lactate, both of which derived mainly from fermentation processes of the same energetic precursor pyruvate.

The observed accumulation of metabolites related to both bioenergetic processes supports the combination of both aerobic and anaerobic pathways for energy production by conditioning MSCs populations.

Other substrates dynamics are worth highlighting. GlutaMAXTM is a formulation whose hydrolysis results in the release of alanine and, ultimately, of L-glutamine, which is an essential nutrient in cell cultures for energy production as well as protein and nucleic acid synthesis [55]. This profile of consumption of GlutaMAXTM and build-up of its by-products is observable in both cellular populations. Little consumption is detected at 24 hours, but the process becomes very significant after 48 hours in culture. Additionally, UC-MSCs seem to entail on this metabolic pathway faster than DPSCs. The metabolic dynamics observed supports the survival and biosynthetic pathway endured by MSCs that, amongst other compounds, results in the production and/or release of bioactive molecules into their surroundings.

Through the evaluation of the bioactive factors content of the UC-MSCs and DPSCs secretome, the paracrine function of MSCs in therapeutic setups is substantiated. This topic has been granted greater attention in research when compared to the metabolomic composition of the MSCs secretome, and various cell sources have been researched besides the bone marrow standard [56–64], such as the adipose tissue [65–68], the dental pulp and other dental tissues [62, 69–72], the umbilical cord tissue [64, 73], cord blood [74], and other placental/ foetal tissues [64, 75]. These works report the identification of most of the angiogenic factors herein investigated, such as Ang-2 [64], Endothelin-1 [66, 71], FGF-2 [58–60, 64, 65, 70, 74], G-CSF [68], HB-EGF [74], HGF [60, 65, 66, 73, 74], IL-8 [58, 64, 69, 73, 75], PLGF [59] [64], VEGF [56–63, 65, 66, 69–71, 74], and TGF- β [61, 65] [64].

We identified a series of pro-proliferative/ anti-apoptotic (TGF β s, FGF-2, G-CSF, HGF and VEGFs) and chemotactic factors (RANTES, GRO, MCP and MDC), as well as relevant cytokines (IL-6 and IL-8). Some other factors are described in the literature, which we were not able to detect in significant amounts, such as GM-CSF [65]. PDGF is another angiogenic factor

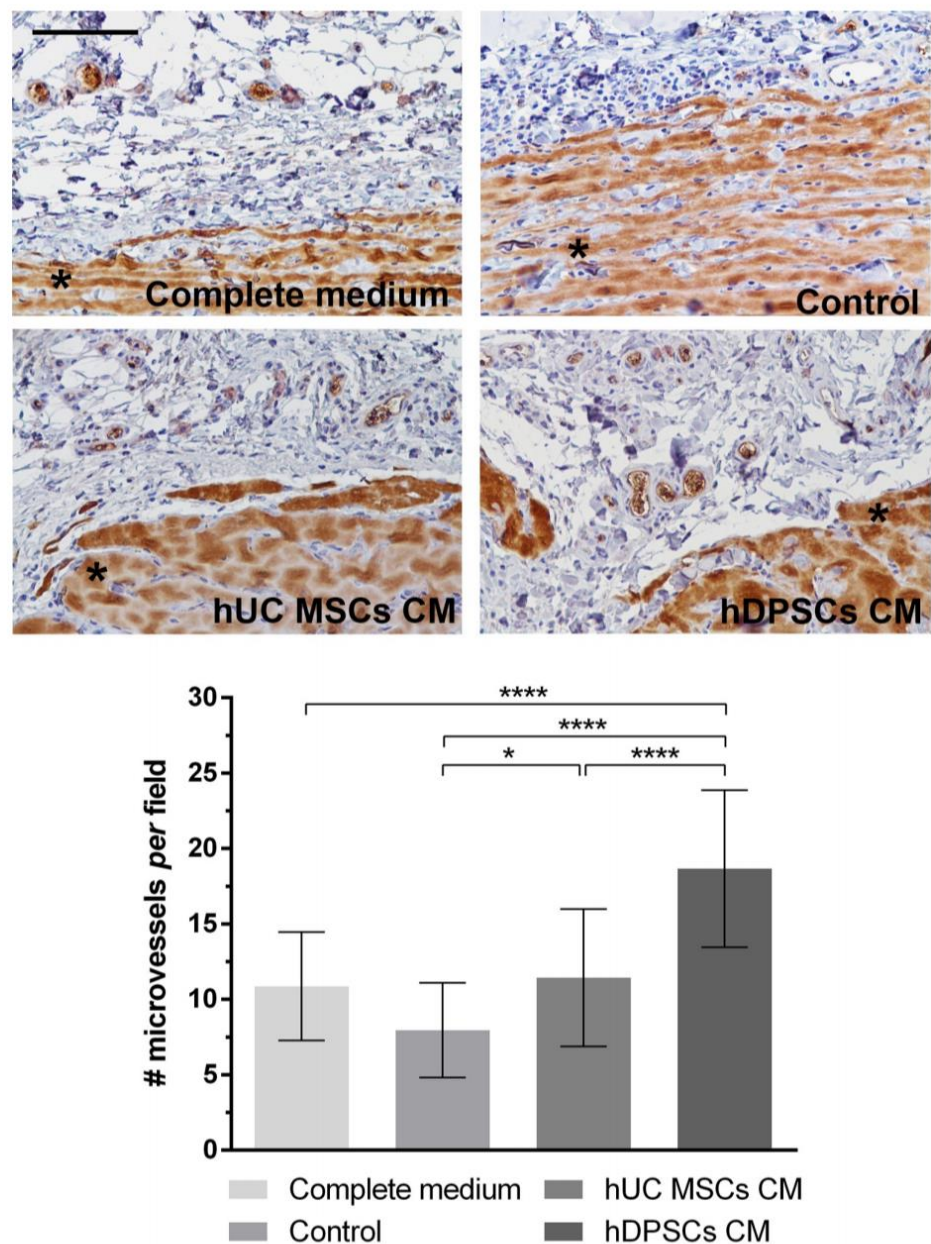


Fig 9. *In vivo* re-vascularization (Matrigel® Plug) assay. After 7 days of implantation of Complete, Control, UC-MSCs or DPSCs supplemented media groups. VEGFR2 immunohistochemical staining (upper panel); *: background uptake of DAB chromogen by the Matrigel®. Graphical representation of VEGFR2 immunopositive microvessels *per field* (#) observed in each group (lower panel) (scale bar = 100 μm). Values are presented as Mean ± SD. Significant differences indicated according to P values with one, two, three or four of the symbols (*) corresponding to 0.01 ≤ P < 0.05; 0.001 ≤ P < 0.01; 0.0001 ≤ P < 0.001 and P < 0.0001, respectively.

<https://doi.org/10.1371/journal.pone.0221378.g009>

previously reported to be produced by DPSCs [70] that we were unable to detect in the conditioned media.

Table 11. *In vivo* re-vascularization (Matrigel® Plug) assay. After 7 days of implantation of Complete, Control, UC-MSCs or DPSCs supplemented media groups. Microvessels *per field* at 200x magnification, following VEGFR2 immunohistochemical staining. Values are presented as Mean $\pm$ SD.

<i>In vitro</i> re-vascularization after 7 days	Complete medium			Control			UCMSCs CM			DPSCs CM		
Microvessels <i>per field</i> (#)	10,87	$\pm$	3,60	7,96	$\pm$	3,14	11,43	$\pm$	4,56	18,66	$\pm$	5,21

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Exploring into the specific functions of the identified factors, the VEGF family members are primary factors in the MSCs pro-angiogenic character [76, 77]. Interestingly, most authors do not take into account the existence of the different isoforms of VEGF, and rather quantify its presence in bulk. We herein demonstrate that the differential detection of VEGF isoforms is of great importance, since the assayed cellular sources demonstrated clear differences in the isoforms profile content. Human DPSCs were here demonstrated to predominantly secrete VEGF-A (that connects to endogenous re-vascularization response [78]), while UC-MSCs were more prone to VEGF-C secretion (prone to neurogenesis induction, without exerting angiogenic effects [79]), which may impact on their potential for tissue regeneration therapies. Janebodin and colleagues [62] also report source dependent differential isoform secretion, and demonstrated that DPSCs secreted superior levels of VEGF-A than BM-MSCs. They also report the secretion of VEGF-D, and to significantly greater extents than BM-MSCs, while we were unable to detect significant VEGF-D signal, especially after 48 hours of secretion. VEGFs (and Ang) directly stimulate endothelial cells populations to proliferate and migrate, and to organise in tube like vascular networks of increased stability and maturation [56]. Other mitogens are described for endothelial populations [80], such as FGF-2 and PLGF, which we failed to observe in relevant amounts [81], in line with the recent reports for UC-MSCs [64] and for DPSCs [63, 69]. HGF, the dominant bioactive factor identified in the UC-MSCs secretome, is recognised as anti-apoptotic and pro-mitogenic in various tissues [82], and was previously identified in UC-MSCs [77] and adipose derived-MSCs [65]. The strong expression of TGF- β s (particularly TGF- β 1) by UC-MSCs could raise flags to their potential effect on the stimulation of fibroblastic population at a lesion site, but the combined secretion with antagonistic molecules, such as HGF and Follistatin may be the key to the antifibrotic character of MSCs populations [83]. IL-8 similarly displays pro-angiogenic features [65], promoting endothelial proliferation, migration and *in vitro* tube network formation [73].

These observations lead to the conclusion that both cell sources successfully supplied endothelial populations with stimulatory factors for their *in vitro* proliferation, migration and tube formation capacity, and forestalled senescence and apoptosis factors deprived endothelial cells. Indeed, the CM efficiently outweighed the complete endothelial cell expansion media utilised as positive control.

IL-8 and G-CSF are potent chemoattractant molecules [84], with recognised role in the homing of endogenous and delivered MSCs to lesion sites [85]. We observed great amounts of MCP-1 secretion by UC-MSCs, but not from DPSCs, colliding with Bronckaers' observations [69]. Potapova [58] reports BM-MSCs to also secrete MCP-1, in inferior proportion than IL-8 and IL-6. We observed an inverse pattern, since UC-MSCs secreted much expressive concentrations of MCP-1 instead of the ILs. Other entities such as eotaxin, fractalkine, GRO, and MDC and RANTES have been demonstrated to be chemoattract to MSCs [26, 86, 87]. The UC-MSCs maintain increased secretion of the molecules and, similarly to what was observed in the endothelial populations, the action of the CMs in the migration of the MSCs populations did not display equivalent differences. Conditioned media from both UC-MSCs and DPSCs promoted overall increased migration of the MSCs, seldom comparable to the stimuli provided by standard expansion media. The UC-derived populations appear to be more

responsive to the CM vehiculated stimuli. Additionally, the UC-MSCs also appear to respond to the bioactive factors produced by themselves during the migration challenge, as the un-supplemented controls display some degree of in-defect proliferation, exceeding that of DPSCs. The distinct response profile may relate to the expression of chemokine receptors by MSCs, that varies though species, culturing time and, suggestively, though tissue source for isolation [26].

Our report on the composition of the CMs is therefore consistent with the properties attributed to MSCs, suggesting that UC-MSCs provide a wider variety and greater concentration of relevant growth factors and cytokines. The proposed difference in the secretory potential of UC-MSCs and DPSCs was explored *in vitro* and *in vivo* focusing on their effects on endothelial populations, as well as on the MSCs themselves. The observed effects on the endothelial populations discarded the initial assumption that UC-MSCs presented increased potential than DPSCs, since both CM presented comparable stimulatory and protective effects on this population. Significant differences between the two media performances were seldom observed and, whenever present, indicated superior performance to DPSCs-CM supplemented groups. The variety of growth factors and observed effects emphasises on the complementarity of functions between the known and unknown factors that compose MSCs secretion cocktail [64]. This complementarity is also well patent in some works, where the abolishment of one factor only partially attenuate the effects displayed [59].

Amid the direct effects of the MSC's secretome on the angiogenic populations and, hence, on the revascularization of lesion sites, many of the bioactive factors herein identified exert additional effects on other cellular populations, which contribute to their its significantly broad therapeutic potential in multiple applications. The MSCs secretome has been described to positively interact with gastric mucosa epithelial cells [88], dermal fibroblasts [89, 90] and keratinocytes [90], pulmonary alveolar and small airway epithelial cells [91], retinal cells [92], hepatocytes [93], central and peripheral neuronal cells [94, 95], and osteoblasts [96], as well as on interacting inflammatory populations [96].

To the authors' knowledge, this is the first comparative approach to the umbilical cord and dental pulp derived stem/ stromal cells populations' metabolomic and bioactive secretion profiles. In summary, the present work provided insight on the metabolic profile of UC-MSCs and DPSCs in culture during the serum-free conditioning process through which MSCs conditioned media was obtained. Some differences were evidenced in the metabolite dynamics on the CM from MSCs derived from the umbilical cord stroma and from the dental pulp through the conditioning period (particularly on glucose metabolism), but similar global metabolic profiles are suggested. Limited literature is available to compare on the metabolic profiles of MSCs.

More prominent differences are highlighted for the bioactive factors content of these CMs, where FST, HGF, G-CSF, IL-8 and MCP-1 dominate in the UC-MSCs secretion, while VEGF-A and FST are the most prominently produced by DPSCs.

Finally, the distinct secretory cocktail did not result in significantly different effects on endothelial cell populations (proliferation, migration and tube formation capacity). The apparent decreased chemotactic factors content of DPSCs was additionally refuted by the comparable capacity to induce MSCs migration *in vitro*.

Supporting information

S1 Table. Statistically significant differences in Metabolites concentration (μM). Calculated from 1H-NMR spectra of unconditioned (Plain Medium), and UC-MSCs and DPSCs conditioned media, after 24 and 48 hours. Significance of the results is indicated according to P

values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant. (DOCX)

S2 Table. Statistically significant differences on detected Bioactive factors (pg/mL). Calculated from Multiplexing LASER Bead Analysis of UC-MSCs and DPSCs Conditioned Media after 24 and 48 hours of conditioning. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant. (DOCX)

S3 Table. Significance of the results of the Presto Blue[®] Assay of UVECs. At 24, 48, 72 and 96 hours of expansion in control or CMs supplemented media, according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant. (DOCX)

S4 Table. Significance of the results of the Apoptosis (Annexin-V/ PI) assay of UVECs. After 48 hours of expansion in Complete, Control or CMs supplemented media, according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant. (DOCX)

S1 Dataset.
(XLSX)

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References

1. Bianco P, Robey PG, Simmons PJ. Mesenchymal Stem Cells: Revisiting History, Concepts, and Assays. *Cell Stem Cell*. 2008; 2(4):313–9. <http://dx.doi.org/10.1016/j.stem.2008.03.002>.
2. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006; 8(4):315–7. Epub 2006/08/23. <https://doi.org/10.1080/14653240600855905> PMID: 16923606.
3. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol*. 2004; 36(4):568–84. <https://doi.org/10.1016/j.biocel.2003.11.001> PMID: 15010324.
4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science (New York, NY)*. 1999; 284(5411):143–7. Epub 1999/04/02. <https://doi.org/10.1126/science.284.5411.143> PMID: 10102814.
5. Mendicino M, Bailey AM, Wonnacott K, Puri RK, Bauer SR. MSC-based product characterization for clinical trials: an FDA perspective. *Cell Stem Cell*. 2014; 14(2):141–5. <https://doi.org/10.1016/j.stem.2014.01.013> PMID: 24506881.
6. Silachev DN, Goryunov KV, Shpilyuk MA, Beznoschenko OS, Morozova NY, Kraevaya EE, et al. Effect of MSCs and MSC-Derived Extracellular Vesicles on Human Blood Coagulation. *Cells*. 2019; 8(3):258.
7. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *Journal of Cell Science*. 2006; 119(11):2204–13.
8. Carvalho MM, Teixeira FG, Reis RL, Sousa N, Salgado AJ. Mesenchymal Stem Cells in the Umbilical Cord: Phenotypic Characterization, Secretome and Applications in Central Nervous System Regenerative Medicine. *Curr Stem Cell Res T*. 2011; 6(3):221–8. WOS:000301639800005.
9. Patel AN, Vargas V, Revello P, Bull DA. Mesenchymal Stem Cell Population Isolated From the Subepithelial Layer of Umbilical Cord Tissue. *Cell Transplant*. 2013; 22(3):513–9. <https://doi.org/10.3727/096368912x655064> WOS:000317257000013. PMID: 23057960
10. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation*. 1968; 6(2):230–47. Epub 1968/03/01. PMID: 5654088.
11. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keilissb. Iv. Stromal Cells Responsible for Transferring Microenvironment of Hematopoietic Tissues—Cloning In Vitro and Replantation In Vivo. *Transplantation*. 1974; 17(4):331–40. <https://doi.org/10.1097/00007890-197404000-00001> WOS:A1974S668500001. PMID: 4150881
12. Arutyunyan I, Fatkhudinov T, Sukhikh G. Umbilical cord tissue cryopreservation: a short review. *Stem cell research & therapy*. 2018; 9(1):236.
13. Doorn J, Moll G, Le Blanc K, van Blitterswijk C, de Boer J. Therapeutic applications of mesenchymal stromal cells: paracrine effects and potential improvements. *Tissue Engineering Part B: Reviews*. 2012; 18(2):101–15.
14. da Silva Meirelles L, Fontes AM, Covas DT, Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine & Growth Factor Reviews*. 2009; 20(5–6):419–27. <http://dx.doi.org/10.1016/j.cytogfr.2009.10.002>.
15. Natsu K, Ochi M, Mochizuki Y, Hachisuka H, Yanada S, Yasunaga Y. Allogeneic bone marrow-derived mesenchymal stromal cells promote the regeneration of injured skeletal muscle without differentiation into myofibers. *Tissue engineering*. 2004; 10(7–8):1093–112. Epub 2004/09/15. <https://doi.org/10.1089/ten.2004.10.1093> PMID: 15363167.
16. Merritt EK, Cannon MV, Hammers DW, Le LN, Gokhale R, Sarathy A, et al. Repair of traumatic skeletal muscle injury with bone-marrow-derived mesenchymal stem cells seeded on extracellular matrix. *Tissue Eng Part A*. 2010; 16(9):2871–81. Epub 2010/04/24. <https://doi.org/10.1089/ten.TEA.2009.0826> PMID: 20412030.
17. Haynesworth SE, Baber MA, Caplan AI. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: Effects of dexamethasone and IL-1 α . *Journal of cellular physiology*. 1996; 166(3):585–92.

18. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. *Journal of Cellular Biochemistry*. 2006; 98(5):1076–84. <https://doi.org/10.1002/jcb.20886> PMID: 16619257
19. Zimmerlin L, Park TS, Zambidis ET, Donnenberg VS, Donnenberg AD. Mesenchymal stem cell secretome and regenerative therapy after cancer. *Biochimie*. 2013; 95(12):2235–45. <https://doi.org/10.1016/j.biochi.2013.05.010> WOS:000327805400006. PMID: 23747841
20. Chen L, Tredget EE, Wu PYG, Wu Y. Paracrine Factors of Mesenchymal Stem Cells Recruit Macrophages and Endothelial Lineage Cells and Enhance Wound Healing. *PloS one*. 2008; 3(4):e1886. <https://doi.org/10.1371/journal.pone.0001886> PMID: 18382669
21. Pereira T, Ivanova G, Caseiro AR, Barbosa P, Bartolo PJ, Santos JD, et al. MSCs Conditioned Media and Umbilical Cord Blood Plasma Metabolomics and Composition. *PloS one*. 2014; 9(11):e113769. <https://doi.org/10.1371/journal.pone.0113769> PMID: 25423186.
22. Lai RC, Arslan F, Tan SS, Tan B, Choo A, Lee MM, et al. Derivation and characterization of human fetal MSCs: An alternative cell source for large-scale production of cardioprotective microparticles. *Journal of molecular and cellular cardiology*. 2010; 48(6):1215–24. <https://doi.org/10.1016/j.yjmcc.2009.12.021> WOS:000277944700025. PMID: 20064522
23. Timmers L, Lim SK, Hoefer IE, Arslan F, Lai RC, van Oorschot AAM, et al. Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. *Stem cell research*. 2011; 6(3):206–14. <https://doi.org/10.1016/j.scr.2011.01.001> WOS:000291295800002. PMID: 21419744
24. Vizoso F, Eiro N, Cid S, Schneider J, Perez-Fernandez R. Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. *International journal of molecular sciences*. 2017; 18(9):1852. <https://doi.org/10.3390/ijms18091852> PMID: 28841158
25. Bronckaers A, Hilken P, Martens W, Gervois P, Ratajczak J, Struys T, et al. Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. *Pharmacology & therapeutics*. 2014; 143(2):181–96.
26. Hocking AM. The role of chemokines in mesenchymal stem cell homing to wounds. *Advances in wound care*. 2015; 4(11):623–30. <https://doi.org/10.1089/wound.2014.0579> PMID: 26543676
27. Pereira T, Armada-da Silva P, Amorim I, Rêma A, Caseiro A, Gartner A, et al. Effects of Human Mesenchymal Stem Cells isolated from the Wharton's jelly of the umbilical cord and conditioned media on skeletal muscle regeneration using a myectomy model. *Cells, tissues, organs*. 2014;(in revision).
28. Volarevic V, Markovic BS, Gazdic M, Volarevic A, Jovicic N, Arsenijevic N, et al. Ethical and safety issues of stem cell-based therapy. *International journal of medical sciences*. 2018; 15(1):36.
29. Skalnikova HK. Proteomic techniques for characterisation of mesenchymal stem cell secretome. *Biochimie*. 2013; 95(12):2196–211. <https://doi.org/10.1016/j.biochi.2013.07.015> WOS:000327805400002. PMID: 23880644
30. Panfoli I, Calzia D, Santucci L, Ravera S, Bruschi M, Candiano G. A blue dive: from 'blue fingers' to 'blue silver'. A comparative overview of staining methods for in-gel proteomics. *Expert Rev Proteomic*. 2012; 9(6):627–34. <https://doi.org/10.1586/Epr.12.63> WOS:000312604700013. PMID: 23256673
31. Malmstrom J, Lee H, Aebersold R. Advances in proteomic workflows for systems biology. *Curr Opin Biotech*. 2007; 18(4):378–84. <https://doi.org/10.1016/j.copbio.2007.07.005> WOS:000249980400015. PMID: 17698335
32. Brown KJ, Formolo CA, Seol H, Marathi RL, Duguez S, An E, et al. Advances in the proteomic investigation of the cell secretome. *Expert Rev Proteomic*. 2012; 9(3):337–45. <https://doi.org/10.1586/Epr.12.21> WOS:000306542300015. PMID: 22809211
33. Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature protocols*. 2007; 2(11):2692–703. <https://doi.org/10.1038/nprot.2007.376> PMID: 18007604
34. Zhang S, Nagana Gowda GA, Ye T, Raftery D. Advances in NMR-based biofluid analysis and metabolite profiling. *Analyst*. 2010; 135(7):1490–8. <https://doi.org/10.1039/c000091d> PMID: 20379603
35. Madhu B, Narita M, Jauhainen A, Menon S, Stubbs M, Tavare S, et al. Metabolomic changes during cellular transformation monitored by metabolite-metabolite correlation analysis and correlated with gene expression. *Metabolomics*. 2015; 11(6):1848–63. <https://doi.org/10.1007/s11306-015-0838-z> WOS:000363040600032. PMID: 26491426
36. Beger RD. A review of applications of metabolomics in cancer. *Metabolites*. 2013; 3(3):552–74. <https://doi.org/10.3390/metabo3030552> PMID: 24958139
37. MacIntyre DA, Melguizo Sanchis D, Jiménez B, Moreno R, Stojkovic M, Pineda-Lucena A. Characterisation of Human Embryonic Stem Cells Conditioning Media by 1H-Nuclear Magnetic Resonance Spectroscopy. *PloS one*. 2011; 6(2):e16732. <https://doi.org/10.1371/journal.pone.0016732> PMID: 21347425

38. Baraniak PR, McDevitt TC. Stem cell paracrine actions and tissue regeneration. *Regenerative medicine*. 2010; 5(1):121–43. <https://doi.org/10.2217/rme.09.74> PMID: 20017699
39. Skalnikova H, Motlik J, Gadher SJ, Kovarova H. Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines. *Proteomics*. 2011; 11(4):691–708. <https://doi.org/10.1002/pmic.201000402> WOS:000287941800013. PMID: 21241017
40. Caseiro AR, Pereira T, Bártolo PJ, Santos JD, Luís AL, Maurício AC. Chapter—Trends in Mesenchymal Stem Cells Applications for Skeletal Muscle Repair and Regeneration, in *PROGRESS IN STEM CELL TRANSPLANTATION*: T Demirel 2015.
41. Caseiro AR, Pereira T, Ivanova G, Lu, #xed, s AL, et al. Neuromuscular Regeneration: Perspective on the Application of Mesenchymal Stem Cells and Their Secretion Products. *Stem Cells International*. 2016; 2016:16. <https://doi.org/10.1155/2016/9756973> PMID: 26880998
42. Pereira T, Armada-da Silva P, Amorim I, Rêma A, Caseiro A, Gärtner A, et al. Effects of Human Mesenchymal Stem Cells Isolated from Wharton's Jelly of the Umbilical Cord and Conditioned Media on Skeletal Muscle Regeneration Using a Myectomy Model. *Stem Cells International*. 2014; 2014.
43. Caseiro A, Ivanova G, Pedrosa S, Branquinho M, Georgieva P, Barbosa P, et al. Human umbilical cord blood plasma as an alternative to animal sera for mesenchymal stromal cells in vitro expansion—A multi-component metabolomic analysis. *PloS one*. 2018; 13(10):e0203936. <https://doi.org/10.1371/journal.pone.0203936> PMID: 30304014
44. Jeener J, Meier BH, Bachmann P, Ernst RR. Investigation of Exchange Processes by 2-Dimensional Nmr-Spectroscopy. *J Chem Phys*. 1979; 71(11):4546–53. <https://doi.org/10.1063/1.438208> WOS: A1979HW09500041.
45. Meiboom S, Gill D. Modified Spin-Echo Method for Measuring Nuclear Relaxation Times. *Review of Scientific Instruments*. 1958; 29(8):688–91. <https://doi.org/10.1063/1.1716296> WOS:A1958WH51500003.
46. Liang X, Du L, Su F, Parekh HS, Su W. The application of quantitative NMR for the facile, rapid and reliable determination of clindamycin phosphate in a conventional tablet formulation. *Magnetic Resonance in Chemistry*. 2014.
47. Pereira T, Ivanova G, Caseiro AR, Barbosa P, Bártolo PJ, Santos JD, et al. MSCs conditioned media and umbilical cord blood plasma metabolomics and composition. *PloS one*. 2014; 9(11):e113769. <https://doi.org/10.1371/journal.pone.0113769> PMID: 25423186
48. Liang XR, Du LP, Su F, Parekh HS, Su WK. The application of quantitative NMR for the facile, rapid and reliable determination of clindamycin phosphate in a conventional tablet formulation. *Magnetic Resonance in Chemistry*. 2014; 52(4):178–82. <https://doi.org/10.1002/mrc.4048> WOS:000332972700005. PMID: 24464591
49. Struys T, Moreels M, Martens W, Donders R, Wolfs E, Lambrechts I. Ultrastructural and immunocytochemical analysis of multilineage differentiated human dental pulp- and umbilical cord-derived mesenchymal stem cells. *Cells Tissues Organs*. 2011; 193(6):366–78. <https://doi.org/10.1159/000321400> PMID: 21124001.
50. Stefkova K, Prochazkova J, Pachernik J. Alkaline Phosphatase in Stem Cells. *Stem cells international*. 2015. Artn 628368 <https://doi.org/10.1155/2015/628368> WOS:000350163700001. PMID: 25767512
51. Pierantozzi E, Gava B, Manini I, Roviello F, Marotta G, Chiavarelli M, et al. Pluripotency Regulators in Human Mesenchymal Stem Cells: Expression of NANOG But Not of OCT-4 and SOX-2. *Stem Cells and Development*. 2011; 20(5):915–23. <https://doi.org/10.1089/scd.2010.0353> WOS:000290255300015. PMID: 20879854
52. Blazquez-Martinez A, Chiesa M, Arnalich F, Fernandez-Delgado J, Nistal M, De Miguel MP. c-Kit identifies a subpopulation of mesenchymal stem cells in adipose tissue with higher telomerase expression and differentiation potential. *Differentiation*. 2014; 87(3–4):147–60. <https://doi.org/10.1016/j.diff.2014.02.007> WOS:000339959000006. PMID: 24713343
53. Tsai C-C, Su P-F, Huang Y-F, Yew T-L, Hung S-C. Oct4 and Nanog Directly Regulate Dnmt1 to Maintain Self-Renewal and Undifferentiated State in Mesenchymal Stem Cells. *Molecular Cell*. 2012; 47(2):169–82. <https://doi.org/10.1016/j.molcel.2012.06.020> PMID: 22795133
54. Nuschke A, Rodrigues M, Wells AW, Sylakowski K, Wells A. Mesenchymal stem cells/multipotent stromal cells (MSCs) are glycolytic and thus glucose is a limiting factor of in vitro models of MSC starvation. *Stem Cell Res Ther*. 2016; 7(1):179. <https://doi.org/10.1186/s13287-016-0436-7> PMID: 27906055; PubMed Central PMCID: PMC5134064.
55. MacIntyre DA, Melguizo Sanchis D, Jimenez B, Moreno R, Stojkovic M, Pineda-Lucena A. Characterisation of human embryonic stem cells conditioning media by 1H-nuclear magnetic resonance spectroscopy. *PloS one*. 2011; 6(2):e16732. <https://doi.org/10.1371/journal.pone.0016732> PMID: 21347425; PubMed Central PMCID: PMC3036660.

56. Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem cells*. 2007; 25(10):2648–59. <https://doi.org/10.1634/stemcells.2007-0226> PMID: 17615264
57. Gruber R, Kandler B, Holzmann P, Vögele-Kadletz M, Losert U, Fischer MB, et al. Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells. *Tissue engineering*. 2005; 11(5–6):896–903. <https://doi.org/10.1089/ten.2005.11.896> PMID: 15998229
58. Potapova IA, Gaudette GR, Brink PR, Robinson RB, Rosen MR, Cohen IS, et al. Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro. *Stem cells*. 2007; 25(7):1761–8. <https://doi.org/10.1634/stemcells.2007-0022> PMID: 17395769
59. Kinnaird T, Stabile E, Burnett M, Lee C, Barr S, Fuchs S, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circulation research*. 2004; 94(5):678–85.
60. Chang CP, Chio CC, Cheong CU, Chao CM, Cheng BC, Lin MT. Hypoxic preconditioning enhances the therapeutic potential of the secretome from cultured human mesenchymal stem cells in experimental traumatic brain injury. *Clin Sci*. 2013; 124(3–4):165–76. <https://doi.org/10.1042/Cs20120226> WOS:000314083000004. PMID: 22876972
61. Burlacu A, Grigorescu G, Rosca A-M, Preda MB, Simionescu M. Factors secreted by mesenchymal stem cells and endothelial progenitor cells have complementary effects on angiogenesis in vitro. *Stem cells and development*. 2012; 22(4):643–53. <https://doi.org/10.1089/scd.2012.0273> PMID: 22947186
62. Janebodin K, Zeng Y, Buranaphatthana W, Ieronimakis N, Reyes M. VEGFR2-dependent angiogenic capacity of pericyte-like dental pulp stem cells. *Journal of dental research*. 2013; 92(6):524–31.
63. Ribot J, Caliaferroum G, Paquet J, Boisson-vidal C, Petite H, Anagnostou F. Type 2 diabetes alters mesenchymal stem cell secretome composition and angiogenic properties. *J Cell Mol Med*. 2017; 21(2):349–63.
64. Kamprom W, Kheolamai P, U-Pratya Y, Supokaweij A, Wattanapanitch M, Laowtammathron C, et al. Effects of mesenchymal stem cell-derived cytokines on the functional properties of endothelial progenitor cells. *European Journal of Cell Biology*. 2016; 95(3):153–63. <https://doi.org/10.1016/j.ejcb.2016.02.001>.
65. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation*. 2004; 109(10):1292–8.
66. Waters R, Alam P, Pacelli S, Chakravarti AR, Ahmed RPH, Paul A. Stem cell-inspired secretome-rich injectable hydrogel to repair injured cardiac tissue. *Acta Biomaterialia*. 2018; 69:95–106. <https://doi.org/10.1016/j.actbio.2017.12.025>.
67. Rubina K, Kalinina N, Efimenko A, Lopatina T, Melikhova V, Tsokolaeva Z, et al. Adipose stromal cells stimulate angiogenesis via promoting progenitor cell differentiation, secretion of angiogenic factors, and enhancing vessel maturation. *Tissue Engineering Part A*. 2009; 15(8):2039–50. <https://doi.org/10.1089/ten.tea.2008.0359> PMID: 19368510
68. Kilroy GE, Foster SJ, Wu X, Ruiz J, Sherwood S, Heifetz A, et al. Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. *Journal of cellular physiology*. 2007; 212(3):702–9. <https://doi.org/10.1002/jcp.21068> PMID: 17477371.
69. Bronckaers A, Hilkens P, Fanton Y, Struys T, Gervois P, Politis C, et al. Angiogenic properties of human dental pulp stem cells. *PloS one*. 2013; 8(8):e71104. <https://doi.org/10.1371/journal.pone.0071104> PMID: 23951091
70. Tran-Hung L, Laurent P, Camps J, About I. Quantification of angiogenic growth factors released by human dental cells after injury. *Archives of oral biology*. 2008; 53(1):9–13.
71. Hilkens P, Fanton Y, Martens W, Gervois P, Struys T, Politis C, et al. Pro-angiogenic impact of dental stem cells in vitro and in vivo. *Stem Cell Research*. 2014; 12(3):778–90. <https://doi.org/10.1016/j.scr.2014.03.008> PMID: 24747218
72. Yalvaç ME, Yarat A, Mercan D, Rizvanov AA, Palotás A, Şahin F. Characterization of the secretome of human tooth germ stem cells (hTGSCs) reveals neuro-protection by fine-tuning micro-environment. *Brain, Behavior, and Immunity*. 2013; 32:122–30. <https://doi.org/10.1016/j.bbi.2013.03.007>.
73. Choi M, Lee H-S, Naidansaren P, Kim H-K, Eunju O, Cha J-H, et al. Proangiogenic features of Wharton's jelly-derived mesenchymal stromal/stem cells and their ability to form functional vessels. *Int J Biochem Cell Biol*. 2013; 45(3):560–70. <https://doi.org/10.1016/j.biocel.2012.12.001> PMID: 23246593
74. Montemurro T, Viganò M, Ragni E, Barilani M, Parazzi V, Boldrin V, et al. Angiogenic and anti-inflammatory properties of mesenchymal stem cells from cord blood: soluble factors and extracellular vesicles for cell regeneration. *European Journal of Cell Biology*. 2016; 95(6):228–38. <https://doi.org/10.1016/j.ejcb.2016.04.003>.

75. Kim S-W, Zhang H-Z, Kim C-E, Kim J-M, Kim MH. Amniotic mesenchymal stem cells with robust chemotactic properties are effective in the treatment of a myocardial infarction model. *International journal of cardiology*. 2013; 168(2):1062–9. <https://doi.org/10.1016/j.ijcard.2012.11.003> PMID: 23218573
76. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003; 9(6):669–76. <https://doi.org/10.1038/nm0603-669> PMID: 12778165.
77. Di GH, Liu Y, Lu Y, Liu J, Wu CT, Duan HF. IL-6 Secreted from Senescent Mesenchymal Stem Cells Promotes Proliferation and Migration of Breast Cancer Cells. *PloS one*. 2014; 9(11). ARTN e113572 <https://doi.org/10.1371/journal.pone.0113572> WOS:000346766900049. PMID: 25419563
78. Mecollari V, Nieuwenhuis B, Verhaagen J. A perspective on the role of class III semaphorin signaling in central nervous system trauma. *Frontiers in Cellular Neuroscience*. 2014; 8(328). <https://doi.org/10.3389/fncel.2014.00328> PMID: 25386118
79. Shin YJ, Choi JS, Choi JY, Hou Y, Cha JH, Chun MH, et al. Induction of vascular endothelial growth factor receptor-3 mRNA in glial cells following focal cerebral ischemia in rats. *J Neuroimmunol*. 2010; 229(1–2):81–90. <https://doi.org/10.1016/j.jneuroim.2010.07.008> PMID: 20692049.
80. Cao R, Bråkenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, et al. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nature Medicine*. 2003; 9:604. <https://doi.org/10.1038/nm848> <https://www.nature.com/articles/nm848#supplementary-information>. PMID: 12669032
81. Kinnaird T, Stabile E, Burnett M, Shou M, Lee C, Barr S, et al. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation*. 2004; 109(12):1543–9.
82. Nakamura T, Mizuno S. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. *Proc Jpn Acad Ser B Phys Biol Sci*. 2010; 86(6):588–610. <https://doi.org/10.2183/pjab.86.588> PMID: 20551596; PubMed Central PMCID: PMC3081175.
83. Genovese JA, Spadaccio C, Rivello HG, Toyoda Y, Patel AN. Electrostimulated bone marrow human mesenchymal stem cells produce follistatin. *Cytotherapy*. 2009; 11(4):448–56. Epub 2009/06/17. <https://doi.org/10.1080/14653240902960445> PMID: 19530028.
84. Sumanasinghe RD, Pfeiler TW, Monteiro-Riviere NA, Lobo EG. Expression of proinflammatory cytokines by human mesenchymal stem cells in response to cyclic tensile strain. *Journal of cellular physiology*. 2009; 219(1):77–83.
85. Tang Y, Chen Y, Wang X, Song G, Li Y, Shi L. Combinatorial Intervention with Mesenchymal Stem Cells and Granulocyte Colony-Stimulating Factor in a Rat Model of Ulcerative Colitis. *Digestive diseases and sciences*. 2015; 60(7):1948–57. <https://doi.org/10.1007/s10620-015-3655-3> PMID: 25894931
86. Smith H, Whittall C, Weksler B, Middleton J. Chemokines stimulate bidirectional migration of human mesenchymal stem cells across bone marrow endothelial cells. *Stem cells and development*. 2011; 21(3):476–86. <https://doi.org/10.1089/scd.2011.0025> PMID: 21513440
87. Kroeze KL, Jurgens WJ, Doulabi BZ, Van Milligen FJ, Scheper RJ, Gibbs S. Chemokine-mediated migration of skin-derived stem cells: predominant role for CCL5/RANTES. *Journal of Investigative Dermatology*. 2009; 129(6):1569–81. <https://doi.org/10.1038/jid.2008.405> PMID: 19122644
88. Xia X, Chiu PWY, Lam PK, Chin WC, Ng EKW, Lau JYW. Secretome from hypoxia-conditioned adipose-derived mesenchymal stem cells promotes the healing of gastric mucosal injury in a rodent model. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*. 2018; 1864(1):178–88.
89. Yoon BS, Moon J-H, Jun EK, Kim J, Maeng I, Kim JS, et al. Secretory profiles and wound healing effects of human amniotic fluid-derived mesenchymal stem cells. *Stem cells and development*. 2009; 19(6):887–902. <https://doi.org/10.1089/scd.2009.0138> PMID: 19686050
90. Park S-R, Kim J-W, Jun H-S, Roh JY, Lee H-Y, Hong I-S. Stem cell secretome and its effect on cellular mechanisms relevant to wound healing. *Molecular Therapy*. 2018; 26(2):606–17. <https://doi.org/10.1016/j.ymthe.2017.09.023> PMID: 29066165
91. Akram KM, Samad S, Spiteri MA, Forsyth NR. Mesenchymal stem cells promote alveolar epithelial cell wound repair in vitro through distinct migratory and paracrine mechanisms. *Respiratory Research*. 2013; 14(1):9. <https://doi.org/10.1186/1465-9921-14-9> PMID: 23350749
92. Johnson TV, DeKorver NW, Levasseur VA, Osborne A, Tassoni A, Lorber B, et al. Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. *Brain*. 2013; 137(2):503–19.
93. Azhdari ZT, Mahmoodi M, Hajizadeh MR, Ezzatizadeh V, Baharvand H, Vosough M, et al. Conditioned Media Derived from Human Adipose Tissue Mesenchymal Stromal Cells Improves Primary Hepatocyte Maintenance. *Cell journal*. 2018; 20(3):377–87.
94. Teixeira FG, Panchalingam KM, Assunção-Silva R, Serra SC, Mendes-Pinheiro B, Patrício P, et al. Modulation of the Mesenchymal Stem Cell Secretome Using Computer-Controlled Bioreactors: Impact

- on Neuronal Cell Proliferation, Survival and Differentiation. *Scientific Reports*. 2016; 6:27791. <https://doi.org/10.1038/srep27791> PMID: 27301770
95. Assunção-Silva RC, Mendes-Pinheiro B, Patrício P, Behie LA, Teixeira FG, Pinto L, et al. Exploiting the impact of the secretome of MSCs isolated from different tissue sources on neuronal differentiation and axonal growth. *Biochimie*. 2018; 155:83–91. <https://doi.org/10.1016/j.biochi.2018.07.026>.
 96. Li C, Li G, Liu M, Zhou T, Zhou H. Paracrine effect of inflammatory cytokine-activated bone marrow mesenchymal stem cells and its role in osteoblast function. *Journal of Bioscience and Bioengineering*. 2016; 121(2):213–9. <https://doi.org/10.1016/j.jbiosc.2015.05.017>.

Supporting information:

S1 Table: Statistically significant differences in Metabolites concentration (μM) calculated from $^1\text{H-NMR}$ spectra of unconditioned (Plain Medium), and UC-MSCs and DPSCs conditioned media, after 24 and 48 hours. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.

Metabolite	Group	Chem. Shift (ppm)	Statistically Significant Differences in Metabolite Concentration (μM)							
			Plain Medium		UC-MSCs 24h		UC-MSCs 48h		DPSCs 24h	
			vs UC 24h	vs UC 48h	vs DP 24h	vs DP 48h	vs UC 48h	vs DP 24h	vs DP 48h	vs DP 48h
Acetate	CH ₃	1.93	**	*	ns	ns	***	**	**	ns
Alanine	CH ₃	1.48	****	****	****	****	****	****	****	****
Choline	N(CH ₃) ₃	3.21	**	*	*	ns	ns	ns	ns	ns
Ethanol	CH ₃	1.19	****	****	****	****	****	****	****	****
Formate	CH	8.46	ns	*	ns	ns	ns	ns	ns	ns
GlutaMAX I (L-Ala)	CH ₃	1.42	*	****	ns	****	****	****	****	****
GlutaMAX II (L-Glu)	CH ₂	2.34	ns	****	ns	****	****	****	****	****
Glutamine	CH ₂	2.45	****	****	*	****	**	****	****	****
Lactate	CH	4.12	****	****	****	****	ns	*	***	***
Nicotinamide	CH	8.94	ns	ns	ns	ns	ns	ns	ns	ns
Pyruvate	CH ₃	2.37	ns	ns	ns	*	ns	ns	ns	ns
Tyrosine	2CH	7.19	****	**	*	**	***	***	ns	ns
α -Glucose	CH	5.24	****	ns	ns	****	****	****	ns	ns
β -Glucose	CH	4.65	****	ns	ns	***	****	****	*	ns

S2 Table: Statistically significant differences on detected Bioactive factors (pg/mL) calculated from Multiplexing LASER Bead Analysis of UC-MSCs and DPSCs Conditioned Media after 24 and 48 hours of conditioning. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.

<i>Bioactive Factor</i>	<i>Statistical significance</i>			
	<i>UC 24h vs</i>	<i>DP 24h vs</i>	<i>UC 24h vs</i>	<i>UC 48h vs</i>
	<i>UC 48h</i>	<i>DP 48h</i>	<i>DP 24h</i>	<i>DP 48h</i>
Eotaxin-1	****	ns	ns	****
FGF-2	****	ns	ns	****
Follistatin	-	ns	ns	-
Fractalkine	****	ns	ns	****
GRO pan	*	**	ns	ns
HGF	ns	-	-	-
IFNα2	****	ns	ns	****
IL-1α	***	-	-	***
IL-6	****	ns	ns	****
IL-8	ns	ns	ns	ns
MCP-1	****	-	-	-
MCP-3	****	ns	**	****
PDGF-BB	****	ns	ns	****
RANTES	****	-	-	****
TGF-β1	***	ns	ns	***
TGF-β2	-	-	-	-
VEGF-A	****	ns	ns	****
VEGF-C	****	ns	ns	****

S3 Table: Significance of the results of the Presto Blue® Assay of UVECs, at 24, 48, 72 and 96 hours of expansion in control or CMs supplemented media, according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.

24 hours	Complete medium	Control	UC-MSCs CM	DPSCs CM
Complete medium		ns	ns	ns
Control			ns	*
UC-MSCs CM				ns
DPSCs CM				

48 hours	Complete medium	Control	UC-MSCs CM	DPSCs CM
Complete medium		ns	*	**
Control			**	***
UC-MSCs CM				ns
DPSCs CM				

72 hours	Complete medium	Control	UC-MSCs CM	DPSCs CM
Complete medium		ns	****	****
Control			****	****
UC-MSCs CM				ns
DPSCs CM				

96 hours	Complete medium	Control	UC-MSCs CM	DPSCs CM
Complete medium		ns	ns	ns
Control			ns	*
UC-MSCs CM				ns
DPSCs CM				

S4 Table: Significance of the results of the Apoptosis (Annexin-V/ PI) assay of UVECs, after 48 hours of expansion in Complete, Control or CMs supplemented media, according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.

Viable Cells	Complete medium	Control	UC-MSCs CM	DPSCs CM
Complete media		****	ns	ns
Control			****	****
UC-MSCs CM				*
DPSCs CM				

Early Apoptosis	Complete medium	Control	UC-MSCs CM	DPSCs CM
Complete media		****	*	ns
Control			****	****
UC-MSCs CM				*
DPSCs CM				

Late Apoptosis	Complete medium	Control	UC-MSCs CM	DPSCs CM
Complete media		****	ns	ns
Control			**	**
UC-MSCs CM				ns
DPSCs CM				

Dead Cells	Complete medium	Control	UC-MSCs CM	DPSCs CM
Complete media		ns	ns	ns
Control			ns	ns
UC-MSCs CM				*
DPSCs CM				

4.2. References

- Chen, L., E. E. Tredget, P. Y. G. Wu and Y. Wu (2008). Paracrine Factors of Mesenchymal Stem Cells Recruit Macrophages and Endothelial Lineage Cells and Enhance Wound Healing. PLoS One **3**(4): e1886.
- da Silva Meirelles, L., A. M. Fontes, D. T. Covas and A. I. Caplan (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. Cytokine & Growth Factor Reviews **20**(5–6): 419-427.
- de la Garza-Rodea, A. S., I. van der Velde-van Dijke, H. Boersma, M. A. Gonçalves, D. W. van Bekkum, A. A. de Vries and S. Knaän-Shanzer (2012). Myogenic properties of human mesenchymal stem cells derived from three different sources. Cell Transplantation **21**(1): 153-173.
- de la Garza-Rodea, A. S., I. van der Velde, H. Boersma, M. A. Gonçalves, D. W. van Bekkum, A. A. de Vries and S. Knaän-Shanzer (2011). Long-term contribution of human bone marrow mesenchymal stromal cells to skeletal muscle regeneration in mice. Cell Transplantation **20**(2): 217-231.
- Doorn, J., G. Moll, K. Le Blanc, C. van Blitterswijk and J. de Boer (2012). Therapeutic applications of mesenchymal stromal cells: paracrine effects and potential improvements. Tissue Engineering Part B: Reviews **18**(2): 101-115.
- Grabowska, I., W. Streminska, K. Janczyk-Ilach, E. K. Machaj, Z. Pojda, G. Hoser, J. Kawiak, J. Moraczewski, M. A. Ciemerych and E. Brzoska (2013). Myogenic potential of mesenchymal stem cells - the case of adhesive fraction of human umbilical cord blood cells. Current Stem Cell Research & Therapy **8**(1): 82-90.
- Graziano, A., R. d'Aquino, G. Laino, A. Proto, M. T. Giuliano, G. Pirozzi, A. De Rosa, D. Di Napoli and G. Papaccio (2008). Human CD34+ stem cells produce bone nodules in vivo. Cell Prolif **41**(1): 1-11.
- Han, J. W., D. Choi, M. Y. Lee, Y. H. Huh and Y.-s. Yoon (2016). Bone marrow-derived mesenchymal stem cells improve diabetic neuropathy by direct modulation of both angiogenesis and myelination in peripheral nerves. Cell transplantation **25**(2): 313-326.
- Lai, R. C., F. Arslan, S. S. Tan, B. Tan, A. Choo, M. M. Lee, T. S. Chen, B. J. Teh, J. K. L. Eng, H. Sidik, V. Tanavde, W. S. Hwang, C. N. Lee, R. M. El Oakley, G. Pasterkamp, D. P. V. de Kleijn, K. H. Tan and S. K. Lim (2010). Derivation and characterization of human fetal MSCs: An alternative cell source for large-scale production of cardioprotective microparticles. Journal of Molecular and Cellular Cardiology **48**(6): 1215-1224.

- Natsu, K., M. Ochi, Y. Mochizuki, H. Hachisuka, S. Yanada and Y. Yasunaga (2004). Allogeneic bone marrow-derived mesenchymal stromal cells promote the regeneration of injured skeletal muscle without differentiation into myofibers. Tissue Engineering **10**(7-8): 1093-1112.
- Pan, H. C., F. C. Cheng, C. J. Chen, S. Z. Lai, C. W. Lee, D. Y. Yang, M. H. Chang and S. P. Ho (2007). Post-injury regeneration in rat sciatic nerve facilitated by neurotrophic factors secreted by amniotic fluid mesenchymal stem cells. J Clin Neurosci **14**(11): 1089-1098.
- Pereira, T., A. Gartner, I. Amorim, A. Almeida, A. R. Caseiro, P. A. Armada-da-Silva, S. Amado, F. Fregnan, A. S. Varejao, J. D. Santos, P. J. Bartolo, S. Geuna, A. L. Luis and A. C. Mauricio (2014). Promoting nerve regeneration in a neurotmesis rat model using poly(DL-lactide-epsilon-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly: in vitro and in vivo analysis. Biomed Res Int **2014**: 302659.
- Pereira, T., G. Ivanova, A. R. Caseiro, P. Barbosa, P. J. Bartolo, J. D. Santos, A. L. Luis and A. C. Mauricio (2014). MSCs Conditioned Media and Umbilical Cord Blood Plasma Metabolomics and Composition. PLoS One **9**(11): e113769.
- Rossi, C. A., M. Flaibani, B. Blaauw, M. Pozzobon, E. Figallo, C. Reggiani, L. Vitiello, N. Elvassore and P. De Coppi (2011). In vivo tissue engineering of functional skeletal muscle by freshly isolated satellite cells embedded in a photopolymerizable hydrogel. The FASEB Journal **25**(7): 2296-2304.
- Santos, A. R. C., H. A. Almeida and P. J. Bártolo (2013). Additive manufacturing techniques for scaffold-based cartilage tissue engineering: a review on various additive manufacturing technologies in generating scaffolds for cartilage tissue engineering. Virtual and Physical Prototyping **8**(3): 175-186.
- Seo, B., W. Sonoyama, T. Yamaza, C. Coppe, T. Kikuri, K. Akiyama, J. Lee and S. Shi (2008). SHED repair critical-size calvarial defects in mice. Oral diseases **14**(5): 428-434.
- Timmers, L., S. K. Lim, I. E. Hoefer, F. Arslan, R. C. Lai, A. A. M. van Oorschot, M. J. Goumans, C. Strijder, S. K. Sze, A. Choo, J. J. Piek, P. A. Doevendans, G. Pasterkamp and D. P. V. de Kleijn (2011). Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. Stem Cell Research **6**(3): 206-214.

Chapter V

General Discussion, Conclusions and Future perspectives

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5.1. General Discussion and Conclusions

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Biological systems are inherently programmed to face injury, but the extent or recidivism of the aggression events may challenge the efficiency of the healing mechanism. This finite capacity for tissue repair and regeneration may ultimately lead to organ and/or system failure, posing a risk for the health and well-being of individuals.

Technical and scientific developments through the years have addressed this issue, and therapeutic methodologies have been proposed and implemented, such as tissue and organ transplantation. However, impending limitations present, such as the shortage of healthy organs for donation, the high biological risk associated to potential disease transmission, and significant social and ethical concerns, that preclude these from being portrayed as a definitive solution.

The Tissue Engineering and Regenerative Medicine field of research arose from the lacunae found on such strategies, focusing on the comprehension and replication of the endogenous healing mechanisms or the substitution of damaged tissues, with the ultimate goal of complete function restoration. This integrated approach relies on the combination of three major “pillars” or research fronts, that combine biomaterials, cellular systems and bioactive cues for the development of therapeutic innovations (Berthiaume et al., 2011).

From the earliest forays into Tissue Engineering and Regenerative Medicine, the importance of the ‘living’ entities of the regenerative systems has been well recognised, and the development of cellular systems has been granted fair attention through the years (Murphy et al., 2013).

The current project framed itself within this scope, aiming to contribute to scientific advancements towards the development of innovative cellular systems for therapeutic application, targeting the regeneration of challenging soft tissues, such as the neuromuscular tissue.

For such, the mechanisms presiding to the tissue regeneration processes were investigated, and the ‘state of the art’ on regenerative approaches was defined for each tissue target.

Then, research focused on the selection of suitable cellular sources considering the technical and biological requisites imposed for the effective therapeutic application of the developed systems. A new tissue source was therefore introduced within the research group, derived from the dental pulp tissue, and isolation and culturing methods were proposed and validated.

Further, this project targeted the development of a cellular system compliant with the regulatory requisites that dictate its potential approval for final clinical application and considered the utilisation of xeno-free protocols.

Complementarily, this project investigated the mechanisms of action of the cellular systems in the regenerative scenarios. Literature suggested that the major effects of MSCs systems reside on their capacity to produce bioactive molecules that modulate local tissue responses. As such, bioactive molecules produced by these populations were profiled and their effects assessed *in vitro* and *in vivo*.

The most relevant observations and results of each Chapter are hereafter briefly discussed.

The therapeutic potential of Mesenchymal Stem/ Stromal Cells in Regenerative Medicine

Profound knowledge of the intrinsic healing mechanisms is essential to understand why suboptimal outcomes are so often observed and to determine strategic directions to develop therapeutic approaches to address the issue. In the particular situation of skeletal muscle and peripheral neural tissue, herein addressed as prospective therapeutic targets that drove the presented research, the healing/ regenerative mechanisms take place in sequentially interconnected stages. The first stage entails the insulted tissue earliest response in which haemorrhage, damaged tissues degeneration/ destruction occurs, and inflammatory cells invade the site. In the second phase, the wounded area is invaded by matrix producing fibroblastic populations, and vascular networks are restored, to supply the final remodelling stage. In this last stage, the non-specific matrix is progressively replaced by tissue that closely tries to mimic the native structure and function.

As extensively revised in ***Chapter I – Section 1.1 - Mesenchymal Stem/ Stromal Cells application for skeletal muscle repair and regeneration*** and ***Section 1.2 - Mesenchymal Stem/ Stromal Cells application for peripheral nerve repair and regeneration***, the application of native and non-native tissue cellular systems has been addressed by diverse research groups. Gathered knowledge indicates that populations of mesenchymal stem/ stromal nature bring advantage to the regenerative outcomes by specific effects impacting on each of the repair/ regeneration stages in skeletal muscle and peripheral nervous systems. The research group within which this project was developed has long focused on the application of these cellular populations on both therapeutic perspectives, in several publications.

In the earliest works targeting the peripheral nerve tissue, it was observed that neural derived populations employed as the therapeutic system did not significantly benefit regenerative process in neurotmesis injuries (Amado et al., 2008, Luis et al., 2008, Luis

et al., 2008, Amado et al., 2010). Adding to the challenges of harvesting and culturing of such populations, the search for alternative methods was stimulated.

Hence, alternative cellular populations were hypothesised considering the harvesting and preparation constraints and the potential beneficial effects on the biological systems. This search resulted in the selection of the post-natal mesenchymal stem/ stromal cells derived from the umbilical cord tissue. One of the strongest points cementing the selection of this population was the ease of collection of the source tissue, which did not require specific intervention, as this is a natural result of childbirth. Ethically, it bared advantages to the collection of other tissue sources for the isolation of the cellular fractions (e.g. the classical bone-marrow) that imply specific interventions, adding pain and other risks to the donation process. Furthermore, the timing was paired with the emergence and dissemination of umbilical cord blood and tissue banking options, turning tissue available for cellular isolation throughout an extended period of an individual's life. So, MSCs derived from the umbilical cord tissue were proposed and investigated, aiming at the regeneration of peripheral nerve injuries.

The methods for isolations and culturing were optimized (Gärtner et al., 2013) and the resulting cellular systems were applied in the regeneration of nervous lesions of different severity (from crush - axonotmesis, to complete nerve section - neurotmesis), in combination to diverse biomaterials (Gärtner et al., 2012, Gärtner et al., 2012, Gärtner et al., 2014, Caseiro et al., 2015, Ribeiro et al., 2015), to ensure structural reconstruction of the tissue and guide functional regeneration.

These combined approaches provided interesting observations on the specific effects of the MSCs in the regeneration scenario (Gärtner et al., 2012, Gärtner et al., 2012, Gärtner et al., 2014, Caseiro et al., 2015, Ribeiro et al., 2015). In acute scenarios, the presence of MSCs in the regenerative setup was related to the moderation of the Wallerian degeneration events, reducing initial tissue destruction in response to injury, and promoting improved newly forming fibres' organisation and alignment. It also resulted in diminished fibrotic events, that contribute down the line for facilitated matrix remodelling. As healing time progressed, the chronic effects associated with the MSCs application included increased numbers of regenerating fibres penetrating distally through the nerve lesion, accompanied by increased myelin sheaths thickness, indication supporting neuroglial cells recovery. These structural features were often accompanied by sensory and motor function indicators recovery over the study periods, as well as on the regain of gait normality (through kinematics analysis), suggesting the recuperation of complex integrated peripheral neural functions.

The observation of such indications of regenerative advantage in the peripheral nerve tissue led to the investigation of the applicability of the same UC-MSCs system targeting other complex soft tissue: the skeletal muscle.

In this approach, the UC-MSCs and their products of secretion (obtained through conditioning protocol) were combined with biomaterial vehicles to address severe skeletal muscle lesions. The hybrid cellular-biomaterial approaches lead to interesting morphologic (histologic) and functional outcomes, particularly through less evident scar tissue formation. The most striking observation was the improved histological scores presented by the cell-free system (containing secretion products alone) when compared to the UC-MSCs containing groups (Pereira et al., 2014). These observations suggested that the conditioned media containing MSCs secretion products could be considered as an alternative to the *in vivo* transplantation of the cells themselves. To substantiate this hypothesis, the content of the secretion of UC-MSCs was explored, and a variety of bioactive factors with relevant roles in tissue regeneration were identified (Pereira et al., 2014).

More recently, a *nouvel* source for the isolation of MSCs was proposed, that posed comparable positive ethical boundaries to the UC-MSCs: the stem/ stromal cells derived from the dental pulp. This population entailed an additional advantage: the collection of the source tissue was not limited to a very specific event in life (birth, in the case of the UC-MSCs), enabling their retrieval from individuals of an extended age interval.

These alternative MSCs were therefore hypothesised to present comparable features to those of the UC-MSCs, and that they could similarly suit the purpose of being a base for the development of advanced therapeutic systems for regenerative medicine.

Dental Pulp Stem/ Stromal cells as an alternative system for regenerative therapies development

Extensive literature research on dental pulp stem/ stromal cells (DPSCs) revealed that this cellular population shared inherent features with other MSCs populations, as discussed in detail in ***Chapter II - Section 2.1 - Dental Pulp Stem/ Stromal Cells as an innovative source for therapies development***. Indeed, strong evidence has been reported regarding their positive impact on tissue regeneration, with particular emphasis to hard tissue applications, such as bone and dental tissue regeneration.

However, limited attention had been granted to the application of DPSCs in the regeneration of tissues of a softer nature, such as the skeletal muscle and the peripheral nerve. This work aimed to bring new insight into the herein targeted application, reinforcing the pertinence of the research.

The first step to embody the application and knowledge of this innovative tissue source as a *nouvel* regenerative tool was to define and validate suitable cell isolation and proliferation protocols. These protocols were prospected to distinct from the ones previously defined for the isolation of populations from the umbilical cord stroma due to the different anatomy and histology of the native tissues. Optimisation of dental samples transportation conditions in addition to the isolation protocols were detailed and discussed in **Chapter II - Section 2.2 - Dental Pulp Stem/ Stromal Cells Protocol selection and validation**.

Although *ex vivo* isolation and culture of adherent populations can be achieved through three methods (mechanic destruction, enzymatic digestion and explant/ outgrowth culturing method) the sparse amount of tissue (dental pulp) obtainable from each donor is considered a major limitation, and therefore explant/ outgrowth method was selected accordingly. This method implicates lower manipulation levels of the tissue/cells and the usage of fewer reagents, which have been described as possible factors affecting MSCs features and capabilities. Isolated cell populations were characterised for their replication dynamics and chromosomal stability, and demonstrated to be comparable to the data reported in the literature. Also, adherent populations were further characterised as to their phenotypical and functional nature, to verify their fitness to be termed as an MSCs population. They demonstrated to exhibit the classical CD115, CD73, CD90 markers of the MSCs identity. On the other hand, the isolates lacked representation of phenotypical markers assigned to hematopoietic / non-mesenchymal populations (CD45, 34, 14/11b, 79a/19) and MHC-II. In particular, MHC-II represents the main marker/antigen of one's' identity, being the principal responsible for the donor-recipient histocompatibility match requirements for interindividual (allogeneic) organ/ tissue/ cell transplantation. This feature is one of the hallmarks of MSCs as cellular therapeutic systems, facilitating donor-recipient paring and providing that those therapies can be applied not only in an autologous perspective but also in an allogeneic approach. This provides that DPSCs can not only be obtained from a broader spectrum of donors through a more extensive time frame in life, but that can also benefit recipients other than the donor. Finally, these cells were confirmed to present multilineage differentiation capacity towards the standard mesenchymal lineages to form osseous, adipose and chondral tissues.

Once determined that the populations isolated from the dental pulp tissues were of an MSCs nature (as the previously explored UC-MSCs), research focused on two essential fronts, to follow the requirements that would impose envisioning the ultimate goal of clinical application of the developed cellular system, and to understand the regenerative mechanisms of this cell population through their differentiation ability, metabolic and secretory profiles.

Looking towards the prospective clinical application of these cellular populations, the utilisation of xeno-free culture supplementation and how it would sustain and impact on cellular expansion and features were investigated.

Simultaneously, understanding the regenerative mechanisms of these cellular populations is of key importance. As classically established, the UC-MSCs were selected as a comparative model to DPSCs to comprehend the MSCs secretome content in *ex vivo* culturing settings, and to compare on the potential of the two cell types for *in vivo* regeneration of the soft tissues under study. Also, the definition of the secretome profiles of populations cultured in standard conditions would contribute with a baseline to understand the effect of the proposed xeno-free media formulations.

Xeno-free cellular systems for Clinical application in Regenerative Medicine

The third Chapter (***Chapter III - Xeno-free cellular systems for Clinical application in Regenerative Medicine***) describes the experimental work developed targeting the formulation of a suitable xeno-free medium for the expansion of MSCs.

The vast majority of scientific research in cellular systems development (irrespective of the species, tissue source, cellular type or therapeutic target) rely on the usage of FBS as a xenogeneic supplement to the cell culture media. Most scientific breakthroughs in the field are based on these culturing methods, precluding their direct translation to clinical application since the reported cellular systems are not fully compliant with the regulatory requirements for approval.

The first step was to investigate supplementation alternatives obtained from the same species (in the case: human) or from a fully synthetic nature. General unawareness of FBS detailed composition turns difficult to mimic its formulation or its effects through chemically defined formulations. Human-derived biological alternatives, such as

peripheral blood serum or plasma-derived formulations, have been investigated as prospected alternatives, but conclusive results are still sparse.

As previously discussed, the ethical framework in which biological products are obtained is of considerable significance, which lead to the procurement of a supplement option that would fit this perspective - the umbilical cord blood plasma. As for the cord tissue from which the stromal cells are isolated, the umbilical cord blood is collected at birth. After that, cellular and plasma fractions are separated and cryopreserved. Previously to the implementation of collection and preservation of these tissues (for research or banking purposes), the umbilical cord tissue and blood were conventionally discarded as medical waste.

Aiming at the implementation of FBS substitution in MSCs culturing, it was first investigated the metabolomic and biological factors constitution of the UCBP and how they compare to the FBS. The metabolomic composition and bioactive factor content of the human UCBP had been previously addressed (Pereira et al., 2014) but had not approached the direct comparison with FBS, to substantiate it as a truly suitable alternative substitute.

Nuclear magnetic resonance techniques were employed to detect and determine the relative quantities of 24 metabolites, as presented and discussed in detail in **Chapter III – Section 3.1. - Metabolomic profile of human Umbilical Cord Blood Plasma and Foetal Bovine Serum, and their effects on human Mesenchymal Stem/ Stromal Cells populations, Part I**. Overall, the FBS and UCBP presented comparable metabolite profiles, providing similar relative quantities of relevant compounds, such as several essential and non-essential aminoacids and lipids. Glucose (α and β isoforms) was determined through PCA analysis as the principal component to distinguish the two supplements, and its relative quantity was found to be about 5x higher in the hUCBP samples. Lactate was found in UCBP in inferior relative quantities. FBS was identified as containing superior levels of threonine, valine/leucine/isoleucine (essential aminoacids), alanine, glutamate/ glutamine and proline (non-essential aminoacids).

To further compare the bioactive factors content of FBS and UCBP, multiplexing analysis resources were employed. For this analysis, a primary challenge has presented as the available panels are species-specific, and the serum/plasma samples derived from different species.

The limited availability of cross-species reactive kits precludes a broad comparative analysis of the two supplements, but interesting observations can be highlighted, as

disclosed in **Chapter III – Section 3.2 - Bioactive factors content of human Umbilical Cord Blood Plasma and Foetal Bovine Serum: a preliminary comparative characterisation.**

The TGF- β (1,2 and 3 isoforms) is preserved through mammal species, and the presence and content of the 3 isoforms were assessed in both supplements. The UCBP was demonstrated to include superior concentrations of TGF- β 1 (7x the amount identified in FBS). The remaining isoforms also presented apparent superior amounts. A panel of human-specific factors was also performed on both sample types. The human-derived supplement was demonstrated to provide very significant concentrations of (>400 pg/mL) of GROpan, MCP-1, MDC, PDGF-AA, PDGF-BB and RANTES. The FBS samples assayed using the same panels expressed seemingly inferior bioactive factors concentration. This preliminary comparative assay may suggest that UCBP contains a wider variety of bioactive factors and in greater concentrations than FBS. Unfortunately, technique limitations preclude a more conclusive analysis.

Overall, this methodological approach indicated that UCBP displayed a comparable metabolic profile to that observed in the conventional supplement FBS (with exception to the glucose content). The bioactive factors quantification also ensured that UCBP was able to provide relevant bioactive stimuli to the cultured cells.

The UCBP was then tested as the culturing supplement to develop regulation-compliant cell culturing protocols. At this stage the UC-MSCs cellular system previously developed within the research group was recovered and assessed in parallel to the newly validated DPSCs, as presented in **Chapter III – Section 3.1 - Metabolomic profile of human Umbilical Cord Blood Plasma and Foetal Bovine Serum, and their effects on human Mesenchymal Stem/ Stromal Cells populations, Part II.**

In summary, it was observed that UC-MSCs and DPSCs were both amenable of expansion in culture media containing 4-8% of hUCBP in substitution to the classical 10% FBS and that the new media formulation promoted increased proliferation rates. Senescence and apoptosis events were also investigated in response to the culturing media formulation. Senescence indicator (β -galactosidase) expression and activity was not elicited by the UCBP supplemented cells. On the other hand, early apoptosis events presented a correlated increase in respect to the increasing media content on UCBP. Indeed, MSCs populations (particularly UC-MSCs) cultured in 8% supplemented UCBP medium displayed increased cellular death.

Both populations demonstrated to maintain their phenotypical markers expression pattern, although qPCR suggests internal modifications in mRNA expression in response to the different media formulations (including slight up and downregulation of surface markers gene expression). Other pluripotency related genes demonstrated different up and downregulation patterns, indicating distinct responses induced by the supplement in each population.

Finally, the effects of FBS substitution in multilineage differentiation capacity were addressed. Osteogenic differentiation was demonstrated to be hampered by the UCBP presence. Obstruction to adipogenesis was not herein observed, but rather an exacerbated lipid droplet disposition to that of the FBS was observed. Lastly, cells expanded in UCBP and induced towards chondrogenesis successfully produced tissue-specific matrix. In the UC-MSCs populations, it was further observed that the micromass cultured in UCBP supplemented media (undifferentiated controls) presented increased sGAG content as compared to FBS supplemented cells. The DPSCs maintained in undifferentiating conditions did not respond differently to the undifferentiated controls media, and chondral matrix production remained comparable within all groups.

The interpretation and discussion of these observations proved itself challenging given the sparse literature available on the topic and the high number of variables (i.e. metabolites and bioactive factors) to address. Focusing on the major differences in the supplements' composition, glucose was hinted as a possible factor contributing to the observed responses. Glucose is in fact reported to be a critical factor in cell functions, and deleterious effects on MSCs have been reported under extreme high glucose culturing conditions (Stolzing et al., 2006, Cramer et al., 2010, Chang et al., 2015, Zhang et al., 2017). Additionally, Ding *et al.* (Ding et al., 2013) reported to have successfully expanded and differentiated UC-MSCs in 10-15% UCBP supplemented media. Although no specific data on the metabolic profile of those UCBP samples, one detail on the blood samples processing may shed light on the differential results observed: the anticoagulant present in the UCB collection system. While Ding *et al.* collected the blood samples into heparinised systems, the UCB samples analysed herein were collected in Citrate Phosphate Dextrose (CPD) containing systems. Therefore, the high levels of glucose (synonym of dextrose) observed report directly to the collection methods employed. This information adds to the discussed role of glucose in the deleterious effects observed in the UC-MSCs and DPSCs populations exposed to comparably lower concentrations of UCBP. Similarly, massive TGF- β 1 content in the UCBP can also influence cellular behaviour and differentiation (Tsutsumi et al., 2001, Tamama et al., 2006).

The presented research relied on the investigation of human-derived cells and supplements since the available techniques are mostly developed towards human (and laboratory rodents) research and diagnostics applications, with few specific assays targeting other mammals species. Nevertheless, the finding herein presented and discussed stand as the basis for the investigation of these innovative approaches for advanced therapeutic approaches in Veterinary Medicine.

The collection of UCB has been described in clinically relevant veterinary species. The collection of UCB upon birth has been described in equine (Bartholomew et al., 2009), but aiming only at the isolation of UCB-derived MSCs (Roberts et al., 2019). No literature on the composition or application of the UCBP or UCBS was encountered by the author. Canine UCB collection has also been described for the same purpose and, although the procedure is technically more challenging than in equine individuals (due to the dimension of the umbilical vessels and the volume of UCB), it is increasingly feasible, due to the increasing number of cesarean sections performed in veterinary practice (Sultana et al., 2018).

The paracrine pathway of Mesenchymal Stem/ Stromal Cells regenerative effect

While dedicating to the validation of the *nouvel* culturing protocols, a deeper characterisation of the UC-MSCs and DPSCs on their current formulations was endured, questioning whether the cells from the two tissues would perform similarly in regenerative scenarios. Also, this approach aimed to define the baseline to understand the effects of the new xeno-free system on their regenerative potential.

Considering the target tissues that encouraged this project (skeletal muscle and peripheral nerve tissue), evidence indicates that therapeutic cellular systems interact with the host tissue not through specific lineage differentiation but rather through active secretion of a variety of bioactive cues. This evidence has even driven to the utilisation of the products of MSCs secretion as the therapeutic approach, alone.

As such, work was first focused on the secretory capacity of the UC-MSCs and DPSCs, from both the metabolomic and bioactive factors content perspectives, in **Chapter IV – Section 4.1: Metabolomic and Bioactive Factors Secretory profile of human Mesenchymal Stem/ Stromal Cells populations**. This aimed at the comprehension of the similarities and differences in the general features and content of the CM produced by both cell populations, and how those ought to impact on its regenerative potential.

Regarding the differentiation capacity of these populations, a distinct aptitude towards osteogenic lineages was observed, with DPSCs outperforming UC-MSCs in mineralised tissue formation capacity. On the other hand, adipogenesis and chondrogenesis propensity did not differ significantly.

Further, both UC-MSCs and DPSCs presented comparable expression of specific genes (ALP, c-kit and Nanog) related to the proliferative capacity of undifferentiated MSCs and pluripotency/ multilineage differentiation capacity (Pierantozzi et al., 2011, Blazquez-Martinez et al., 2014, Stefkova et al., 2015). Oct-4 was expressed to a lower extent in both cellular populations.

Addressing the secretion products obtained through the conditioning period, NMR spectroscopy techniques lead to the identification of glucose as the dominant metabolite in both spectra. Significant glucose consumption was not observed, and UC-MSCs presented an increase in both α - and β -glucose content, suggesting that conditioning cells do not rely exclusively on glycolytic pathways for energy production. Pyruvate was also identified and maintained its concentration in the media, which suggest intrinsic production by the MSCs, as does the presence of acetate, and accumulation of choline and formate, indicating the engagement of energy production through the citric acid cycle. Ethanol and lactate are also suggestive of pyruvate metabolism, but rather through anaerobic fermentation pathways. GlutaMAX™, a glutamate analogous supplied in commercial culture media formulations, is subtly consumed in the first 24 hours in culture but boosts after 48 hours. Additionally, UC-MSCs seem to entail on this metabolic pathway faster than DPSCs.

Additional evaluation of bioactive factors content of the UC-MSCs and DPSCs secretome (through multiplexing techniques) enabled the identification of a series of pro-proliferative/ anti-apoptotic factors (TGF β s, FGF-2, G-CSF, HGF and VEGFs), chemokines (RANTES, GRO, MCP and MDC) and cytokines (IL-6 and IL-8). Other factors previously described in the literature were not herein identified (such as GM-CSF and PDGF)(Rehman et al., 2004, Tran-Hung et al., 2008).

The specific functions of the identified factors are extensively discussed in **Chapter IV – Section 4.1: Metabolomic and Bioactive Factors Secretory profile of human Mesenchymal Stem/ Stromal Cells populations**, but most of the identified factors present reported pro-angiogenic (Ferrara et al., 2003, Di et al., 2014), anti-apoptotic, pro-mitogenic (Nakamura and Mizuno, 2010), antifibrotic (Genovese et al., 2009), and chemoattractant (for endogenous or delivered MSCs)(Kroeze et al., 2009,

Sumanasinghe et al., 2009, Smith et al., 2011, Hocking, 2015, Tang et al., 2015) features, that contribute to the overall regenerative milieu.

Particularly, differential detection of VEGF isoforms was evident in this study, and DPSCs demonstrated to predominantly secrete VEGF-A (that connects to endogenous re-vascularization response (Mecollari et al., 2014)), while UC-MSCs depicted stronger VEGF-C secretion (prone to neurogenesis induction, without exerting angiogenic effects (Shin et al., 2010)). These observations will contribute to the interpretation of further data on the effective impact of each cellular system in specific tissue regeneration applications. Another significant observation was the strong expression of TGF- β s (particularly TGF- β 1) by UC-MSCs that bare direct stimulatory features of fibroblastic populations, while major antifibrotic effects are assigned to this particular MSCs population. This final effect probably results from the combined secretion with antagonistic molecules, such as HGF and Follistatin, also expressively produced by these cells (Genovese et al., 2009).

When comparing the secretion power of both cellular populations, the major differences are found in the levels of FST, HGF, G-CSF, IL-8 and MCP-1, which are dominant in the UC-MSCs secretion, while VEGF-A and FST are prominently produced by DPSCs.

Considering the specific regenerative path of neuromuscular tissues, *in vitro* validation of the regenerative potential of the MSCs CM was directed towards the pro-angiogenic and chemotactic potential (also suggested by the secretory content), aiming to understand the impact of the distinct secretory profiles on targeted cells behaviour.

It was concluded that the distinct secretory cocktail did not result in significantly different effects on endothelial cell populations behaviour (*in vitro* proliferation, migration and tube formation capacity) and that both CMs forestalled senescence and apoptosis. Additionally, both CMs provided migratory stimuli of the MSCs themselves. This assay also indicated that UC-MSCs responded not only to the CM supplied stimuli but also to the bioactive factors produced by themselves during the migration assay.

To the authors knowledge, this represents the first comparative approach to these MSCs populations metabolomic and bioactive secretion profiles, that sets a baseline for further research on the contents of the MSCs secretome, as well as on the effects of system optimization approaches (such as the FBS substitution by UCBP), on the potential and performance in the regeneration of the targeted neuromuscular tissues.

The reported findings also set a starting point for an inter-species comparative approach, aiming at the translation of human targeted scientific and technical developments towards other clinically relevant veterinary species, such as equine and canine, that present pathologies comparable to those of human patients.

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5.2. Future Perspectives

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Rather than providing definitive answers to the original questions, most research works perpetuate through the emergence of additional questions, stimulating continuous knowledge development.

Concerning the validated isolation methodologies developed in *Chapter II*, the comparative analysis of cellular yield, proliferative rate and the intrinsic cellular features of cellular populations isolated through the outgrowth method (herein presented) and the enzymatic digestion approach are worth addressing. Other variables should be considered, namely the application of the UCBP (or other non-xenogeneic supplement) in the complete streamline for the cellular systems preparation, quality and safety (from collection to patient application).

The therapeutic advancements herein proposed are amenable of translation into not only towards human clinical application but also for the purpose of presenting innovative therapeutic options in the Veterinary Medicine field. This opportunity turns the preparation of Veterinary targeting systems into a hot topic to pursue. Equine and Canine-derived cellular systems are currently being optimised, focusing on the sourcing of both UC-MSCs and DPSCs. Preliminary isolations (using a reduced number of donated samples) indicate that MSC-like populations can be isolated from both source tissues (umbilical cord stroma and the dental pulp) in both species, utilising the same explant method described in *Chapter II*. Additionally, adherent cellular populations were also obtained from the equine umbilical cord tissue following enzymatic digestion procedures (Collagenase and Dispase cocktail). These populations present good proliferative capacity and have been successfully differentiated towards the standard mesenchymal lineages (osteogenic, adipogenic and chondrogenic). Karyotype analysis on the isolated samples is also being addressed, indicating adequate chromosomal profiles in the earliest passages (equine $2n=64$ chromosomes and canine $2n=78$ chromosomes), but that tend to present alterations in later passages (assayed until Passage 9, to date). Immunohistochemical staining has already demonstrated these populations as being of a mesenchymal/ stromal origin (staining positive for Vimentin, while absent of AE1/AE3, an epithelial marker). The equine and canine derived populations are also required to be characterised regarding the phenotype identity, but the selection of the reference panels for the validation of veterinary MSCs is itself a challenge. A consensual profile of surface markers expression has not yet been defined for each species (unlike for human-derived MSCs (Dominici et al., 2006)), and conflicting findings are often reported (Devireddy et al., 2017).

Another significant line of work would be the investigation of the effect of isolations and culturing protocols on the paracrine factors production by the MSCs, which is recognised to vary in response to exogenous stimuli. This question would continue the work developed in *Chapter IV*, in which the baseline of secretion for UC-MSCs and DPSCs was determined under standard conditions.

The assessment of the secretory capacity of equine and canine-derived MSCs is also up for investigation, to better understand their therapeutic potential. Preliminary quantifications suggest de secretion of IL-6 and IL-8 by MSCs isolated from the umbilical cord and, to a smaller extent, from the dental pulp. However, a significant obstacle has precluded a more in-depth secretome characterisation, since very limited panels are available targeting equine and canine-specific growth factors, cytokines and chemokines (as discussed for the samples analysed in *Chapter III*).

The research presented in *Chapter III* indicates that UCBP is capable of sustaining the expansion of MSCs populations as xeno-free systems. However, it was also demonstrated that UCBP supplementation affects MSCs population in multiple extents. Therefore, further research is imperative and should focus on the impact of the observed cellular responses on safety and regenerative efficacy. Particularly, it would be of great relevance to address possible immunophenotype modulation and its effect of the cell-host interactions (as well as on the engraftment capacity). Since the UCB collection procedures also seem to assume a critical role in the supplement performance, the innovation and validation of more suitable methodologies ought to also be observed, in strict collaboration with the presiding regulatory entities.

Ongoing work is addressing the applicability of the UCBP as adjuvant in the cryopreservation process, replacing FBS or FBS containing media formulations or synthetic derivates currently utilised. Preliminary results on MSCs derived from synovia and dental tissues from veterinary species (canine and equine) indicate that human UCBP grants improved cellular viability of the cryopreserved cells upon thawing (versus autologous serum). Additionally, it allowed the conservation of fully prepared formulation under refrigeration temperatures ($\pm 4^{\circ}\text{C}$) for extended periods of time (up to 72 hours), maintaining adequate cellular viability. This study was performed by DVM Cláudia Costa in her **MSc Thesis “Cryopreservation and Thawing Protocol of Mesenchymal Stem Cells for use in Veterinary Medicine Cell Therapy”**, supervised by Professora Doutora Ana Colette Pereira de Castro Osório Maurício (Lead Supervisor) and the PhD candidate Ana Rita Caseiro Santos (Co-Supervisor). Further work will investigate the applicability

of these methods in species matched populations (equine MSCs with equine UCBP, as an example), and on the performance of the thawed systems.

The DPSCs were not herein investigated on their capacity to differentiate towards myogenic or neural/ neuroglial phenotypes which, although not being reported as an essential requisite for regenerative effects *in situ*, still presents an interesting direction of research, especially in the understanding of the effects of lineage-specific differentiation on the immunophenotype and paracrine secretion capacity and content. It would also be relevant to explore the reciprocal effect of induced modulation of secretive patterns and active lesion sites environment (mimicking the clinical lesion scenarios), which often differ from experimental setups.

The UC-MSCs and DPSCs and their respective CMs are currently being addressed for the regeneration of skeletal muscle lesions, utilising *in vivo* rodent models of laceration and volumetric muscle lesions, and their combination with different biomaterial matrices. Preliminary data converge to the findings previously published (Pereira et al., 2014), cementing that the application of the MSCs CM results in blunted tissular reaction to the implanted biomaterials (Tisseel®, Matrigel® and ‘in-house’ developed Chitosan hydrogel), and in decreased lesion site fibrosis. The UC-MSCs are suggested to present inferior scores to those provided by DPSCs or their CM, proposing a superior inflammatory and fibrosis modulation capacity. Further analysis is being carried to validate these observations and to address the vascularization efficiency of the lesion sites, the skeletal muscle regenerative indexes (myotube formation, Myogenin, Myo-D and/or Myf-5 expression) and , as well as on the interaction of the delivered systems with the native muscular satellite cells (Pax-7⁺) populations.

The DPSCs are currently being explored within the research group, as therapeutic options to the regeneration of severe peripheral nerve injury (neurotmesis). Early observations indicate that the addition of the DPSCs to the biomaterial positively influences the recovery of functional parameters of biomaterial enveloped nerves, in complement to the end-to-end suturing. Preliminary measurements on the effect on the skeletal muscle neurogenic atrophy, however, indicate no significant effect additive benefit bared by the DPSCs, whose results compare to the nerves enveloped by the biomaterial alone (although both present superior to the sutured groups alone). Remaining data on the structural/ stereological analysis of the regenerated nerve structures and gait analysis (kinematics) will substantiate the observed effects.

The DPSCs system has already been successfully employed for *in vivo* bone regeneration in combination to Bonelike® (as osteogenic granular matrix). The combined approach granted overall superior new bone formation in an ovine model of non-critical bone defects at 30, 60 and 120 days post-implantation, as recently published in **Campos JM, Sousa AC, Caseiro AR, Pedrosa SS, Pinto PO, Branquinho MV, Amorim I, Santos JD, Pereira T, Mendonça CM, Afonso A. *Dental pulp stem cells and Bonelike® for bone regeneration in ovine model*. Regenerative biomaterials. 2018 Dec 22;6(1):49-59.**

The absolute end goal of the presented research is the clinical application of the cellular systems developed, in which ‘real’ human and veterinary patients will benefit from the new advanced systems. For this purpose, and since no ideal *in vitro* models are available to date, research must scale-up the validated *in vivo* models of disease. This would approximate scientific data to lesion scenarios closer to the medical reality in which therapies aim to be applied. Here, animal models would not only serve as translational models to human application but would also comprise relevant therapy targets directly benefiting from the tested therapies, in the fast-evolving Veterinary Medicine field (specifically considering companion and sports animals).

5.3. References

- Amado, S., J. M. Rodrigues, A. L. Luis, P. A. Armada-da-Silva, M. Vieira, A. Gartner, M. J. Simoes, A. P. Veloso, M. Fornaro, S. Raimondo, A. S. Varejao, S. Geuna and A. C. Mauricio (2010). Effects of collagen membranes enriched with in vitro-differentiated N1E-115 cells on rat sciatic nerve regeneration after end-to-end repair. Journal of NeuroEngineering and Rehabilitation **7**: 7.
- Amado, S., M. J. Simoes, P. A. Armada da Silva, A. L. Luis, Y. Shirosaki, M. A. Lopes, J. D. Santos, F. Fregnan, G. Gambarotta, S. Raimondo, M. Fornaro, A. P. Veloso, A. S. Varejao, A. C. Mauricio and S. Geuna (2008). Use of hybrid chitosan membranes and N1E-115 cells for promoting nerve regeneration in an axonotmesis rat model. Biomaterials **29**(33): 4409-4419.
- Bartholomew, S., S. D. Owens, G. Ferraro, D. Carrade, D. Lara, F. Librach, D. L. Borjesson and L. D. Galuppo (2009). Collection of equine cord blood and placental tissues in 40 thoroughbred mares. Equine veterinary journal **41**(8): 724-728.
- Berthiaume, F., T. J. Maguire and M. L. Yarmush (2011). Tissue engineering and regenerative medicine: history, progress, and challenges. Annual review of chemical and biomolecular engineering **2**: 403-430.
- Blazquez-Martinez, A., M. Chiesa, F. Arnalich, J. Fernandez-Delgado, M. Nistal and M. P. De Miguel (2014). c-Kit identifies a subpopulation of mesenchymal stem cells in adipose tissue with higher telomerase expression and differentiation potential. Differentiation **87**(3-4): 147-160.
- Caseiro, A. R., T. Pereira, J. Ribeiro, I. Amorim, F. Faria, P. J. Bártolo, P. Armada, A. L. Luís and A. C. Maurício (2015). Neuro-muscular regeneration using scaffolds with mesenchymal stem cells (MSCs) isolated from human umbilical cord Wharton's jelly: functional and morphological analysis using rat sciatic nerve neurotmesis injury model. Procedia Engineering **110**: 106-113.
- Chang, T.-C., M.-F. Hsu and K. K. Wu (2015). High glucose induces bone marrow-derived mesenchymal stem cell senescence by upregulating autophagy. PloS one **10**(5): e0126537.
- Cramer, C., E. Freisinger, R. K. Jones, D. P. Slakey, C. L. Dupin, E. R. Newsome, E. U. Alt and R. Izadpanah (2010). Persistent high glucose concentrations alter the regenerative potential of mesenchymal stem cells. Stem cells and development **19**(12): 1875-1884.
- Devireddy, L. R., L. Boxer, M. J. Myers, M. Skasko and R. Screven (2017). Questions and challenges in the development of mesenchymal stromal/stem cell-based

- therapies in veterinary medicine. Tissue Engineering Part B: Reviews **23**(5): 462-470.
- Di, G. H., Y. Liu, Y. Lu, J. Liu, C. T. Wu and H. F. Duan (2014). IL-6 Secreted from Senescent Mesenchymal Stem Cells Promotes Proliferation and Migration of Breast Cancer Cells. Plos One **9**(11).
- Ding, Y., H. Yang, J. B. Feng, Y. Qiu, D. S. Li and Y. Zeng (2013). Human umbilical cord-derived MSC culture: the replacement of animal sera with human cord blood plasma. In Vitro Cellular & Developmental Biology-Animal **49**(10): 771-777.
- Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop and E. Horwitz (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy **8**(4): 315-317.
- Ferrara, N., H. P. Gerber and J. LeCouter (2003). The biology of VEGF and its receptors. Nat Med **9**(6): 669-676.
- Gärtner, A., T. Pereira, P. Armada-da-Silva, S. Amado, A. Veloso, I. Amorim, J. Ribeiro, R. Bárcia, P. Cruz, H. Cruz., A. Luís, J. Santos, S. Geuna and A. Maurício (2014). Effects of umbilical cord tissue mesenchymal stem cells (UCX[®]) on rat sciatic nerve regeneration after neurotmesis injuries. Journal of Stem Cells & Regenerative Medicine **Vol.X**(1): JSRM Code: 010010300001EPA010010010214.
- Gärtner, A., T. Pereira, P. A. Armada-da-Silva, I. Amorim, R. Gomes, J. Ribeiro, M. L. Franca, C. Lopes, B. Porto, R. Sousa, A. Bombaci, G. Ronchi, F. Fregnan, A. S. Varejao, A. L. Luis, S. Geuna and A. C. Mauricio (2012). 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. Differentiation **84**(5): 355-365.
- Gärtner, A., T. Pereira, R. Gomes, A. L. Luís, M. França, S. Geuna, P. A. S. Armada-da-Silva and A. C. Maurício (2013). Mesenchymal Stem Cells from Extra-Embryonic Tissues for Tissue Engineering – Regeneration of the Peripheral Nerve. Advances in Biomaterials Science and Biomedical Applications. R. Pignatello, InTech.
- Gärtner, A., T. Pereira, M. J. Simões, P. A. Armada-da-Silva, M. L. França, R. Sousa, S. Bompasso, S. Raimondo, Y. Shirosaki and Y. Nakamura (2012). Use of hybrid chitosan membranes and human mesenchymal stem cells from the Wharton jelly of umbilical cord for promoting nerve regeneration in an axonotmesis rat model. Neural regeneration research **7**(29): 2247.
- Genovese, J. A., C. Spadaccio, H. G. Rivello, Y. Toyoda and A. N. Patel (2009). Electrostimulated bone marrow human mesenchymal stem cells produce follistatin. Cytotherapy **11**(4): 448-456.

- Hocking, A. M. (2015). The role of chemokines in mesenchymal stem cell homing to wounds. Advances in wound care **4**(11): 623-630.
- Kroeze, K. L., W. J. Jurgens, B. Z. Doulabi, F. J. Van Milligen, R. J. Scheper and S. Gibbs (2009). Chemokine-mediated migration of skin-derived stem cells: predominant role for CCL5/RANTES. Journal of Investigative Dermatology **129**(6): 1569-1581.
- Luis, A. L., J. M. Rodrigues, S. Geuna, S. Amado, Y. Shirosaki, J. M. Lee, F. Fregnan, M. A. Lopes, A. P. Veloso, A. J. Ferreira, J. D. Santos, P. A. Armada-Da-silva, A. S. Varejao and A. C. Mauricio (2008). Use of PLGA 90:10 scaffolds enriched with in vitro-differentiated neural cells for repairing rat sciatic nerve defects. Tissue Eng Part A **14**(6): 979-993.
- Luis, A. L., J. M. Rodrigues, S. Geuna, S. Amado, M. J. Simoes, F. Fregnan, A. J. Ferreira, A. P. Veloso, P. A. Armada-da-Silva, A. S. Varejao and A. C. Mauricio (2008). Neural cell transplantation effects on sciatic nerve regeneration after a standardized crush injury in the rat. Microsurgery **28**(6): 458-470.
- Mecollari, V., B. Nieuwenhuis and J. Verhaagen (2014). A perspective on the role of class III semaphorin signaling in central nervous system trauma. Frontiers in Cellular Neuroscience **8**(328).
- Murphy, M. B., K. Moncivais and A. I. Caplan (2013). Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. Experimental & molecular medicine **45**(11): e54.
- Nakamura, T. and S. Mizuno (2010). The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. Proc Jpn Acad Ser B Phys Biol Sci **86**(6): 588-610.
- Pereira, T., P. Armada-da Silva, I. Amorim, A. Rêma, A. Caseiro, A. Gärtner, M. Rodrigues, M. Lopes, P. Bártolo and J. Santos (2014). Effects of Human Mesenchymal Stem Cells Isolated from Wharton's Jelly of the Umbilical Cord and Conditioned Media on Skeletal Muscle Regeneration Using a Myectomy Model. Stem Cells International **2014**.
- Pereira, T., G. Ivanova, A. R. Caseiro, P. Barbosa, P. J. Bartolo, J. D. Santos, A. L. Luis and A. C. Mauricio (2014). MSCs Conditioned Media and Umbilical Cord Blood Plasma Metabolomics and Composition. PLoS One **9**(11): e113769.
- Pierantozzi, E., B. Gava, I. Manini, F. Roviello, G. Marotta, M. Chiavarelli and V. Sorrentino (2011). Pluripotency Regulators in Human Mesenchymal Stem Cells: Expression of NANOG But Not of OCT-4 and SOX-2. Stem Cells and Development **20**(5): 915-923.

- Rehman, J., D. Traktuev, J. Li, S. Merfeld-Clauss, C. J. Temm-Grove, J. E. Bovenkerk, C. L. Pell, B. H. Johnstone, R. V. Considine and K. L. March (2004). Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation **109**(10): 1292-1298.
- Ribeiro, J., T. Pereira, A. R. Caseiro, P. Armada-da-Silva, I. Pires, J. Prada, I. Amorim, S. Amado, M. França and C. Gonçalves (2015). Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells. World journal of stem cells **7**(6): 956.
- Roberts, E. L., T. Dang, S. I. Lepage, A. H. Alizadeh, T. Walsh, T. G. Koch and M. S. Kallos (2019). Improved expansion of equine cord blood derived mesenchymal stromal cells by using microcarriers in stirred suspension bioreactors. Journal of Biological Engineering **13**(1): 25.
- Shin, Y. J., J. S. Choi, J. Y. Choi, Y. Hou, J. H. Cha, M. H. Chun and M. Y. Lee (2010). Induction of vascular endothelial growth factor receptor-3 mRNA in glial cells following focal cerebral ischemia in rats. J Neuroimmunol **229**(1-2): 81-90.
- Smith, H., C. Whittall, B. Weksler and J. Middleton (2011). Chemokines stimulate bidirectional migration of human mesenchymal stem cells across bone marrow endothelial cells. Stem cells and development **21**(3): 476-486.
- Stefkova, K., J. Prochazkova and J. Pachernik (2015). Alkaline Phosphatase in Stem Cells. Stem Cells International.
- Stolzing, A., N. Coleman and A. Scutt (2006). Glucose-induced replicative senescence in mesenchymal stem cells. Rejuvenation research **9**(1): 31-35.
- Sultana, T., S. Lee, H.-Y. Yoon and J. I. Lee (2018). Current Status of Canine Umbilical Cord Blood-Derived Mesenchymal Stem Cells in Veterinary Medicine. Stem cells international **2018**.
- Sumanasinghe, R. D., T. W. Pfeiler, N. A. Monteiro-Riviere and E. G. Lobo (2009). Expression of proinflammatory cytokines by human mesenchymal stem cells in response to cyclic tensile strain. Journal of cellular physiology **219**(1): 77-83.
- Tamama, K., V. H. Fan, L. G. Griffith, H. C. Blair and A. Wells (2006). Epidermal growth factor as a candidate for ex vivo expansion of bone marrow-derived mesenchymal stem cells. Stem cells **24**(3): 686-695.
- Tang, Y., Y. Chen, X. Wang, G. Song, Y. Li and L. Shi (2015). Combinatorial Intervention with Mesenchymal Stem Cells and Granulocyte Colony-Stimulating Factor in a Rat Model of Ulcerative Colitis. Digestive Diseases and Sciences **60**(7): 1948-1957.
- Tran-Hung, L., P. Laurent, J. Camps and I. About (2008). Quantification of angiogenic growth factors released by human dental cells after injury. Archives of Oral Biology **53**(1): 9-13.

- Tsutsumi, S., A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi and Y. Kato (2001). Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochemical and biophysical research communications **288**(2): 413-419.
- Zhang, D., H. Lu, Z. Chen, Y. Wang, J. Lin, S. Xu, C. Zhang, B. Wang, Z. Yuan and X. Feng (2017). High glucose induces the aging of mesenchymal stem cells via Akt/mTOR signaling. Molecular medicine reports **16**(2): 1685-1690.

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