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In Vitro Models for Cartilage Engineering Using Primary Cells and Biodegradable Scaffolds

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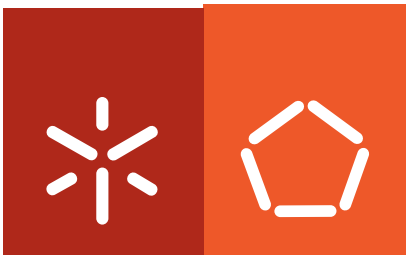

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In Vitro Models for Cartilage Engineering Using Primary Cells and Biodegradable Scaffolds

Tese de Doutoramento em Engenharia de Tecidos, Medicina Regenerativa e Células Estaminais

Trabalho efectuado sob a orientação do
Professor Nuno João Meleiro Alves das Neves
e co-orientação do
Professor Rui Luís Gonçalves dos Reis

Dezembro de 2010

In loving memory of my grandparents

“*It is not knowledge but the act of learning, not possession but the act of getting there, which grants the greatest enjoyment*”

Carl Friedrich Gauss

“Vem por aqui" — dizem-me alguns com os olhos doces
Estendendo-me os braços, e seguros
De que seria bom que eu os ouvisse
Quando me dizem: "vem por aqui!"
Eu olho-os com olhos lassos,
(Há, nos olhos meus, ironias e cansaços)
E cruzo os braços,
E nunca vou por ali...”

In *Cântico Negro* de José Régio

Acknowledgements

When it comes to acknowledgements, I like to cite a Portuguese writer, Virgílio Ferreira, who describes words as rocks ("as palavras são como pedras")…I keep stumbling on them and they do not seem to run as smoothly as I would like. So I would say that the acknowledgements section is one of the most difficult to write!

During the journey of my PhD, several people helped me and made this achievement possible. I would like to thank all the people that direct or indirectly have contributed for the development of this work and that helped me to grow as a researcher and as a person. If I someway forget to acknowledge somebody, my apologies and please consider yourself thanked.

In first place, I would like to acknowledge my supervisor, prof. Nuno Neves, for all the valuable help throughout the five years of work for this thesis. His critical review of the practical work, as well as his guidance in the writing of projects, papers and this thesis contributed for my evolution as a researcher. The multiple brainstorming sessions about tissue engineering and cartilage strategies provided me with skills beyond the simple research.

I would also like to thank my co-supervisor, prof. Rui Reis, for giving me the opportunity to join the 3B´s Research Group. His strong will and perseverance are an example for any researcher, and he passes this strength to his students. I have learn so much in the dynamics of a research group, I acquired skills for writing projects, organizing conferences and so much more, and for that I am sincerely grateful.

To prof. Paul Hatton, my supervisor at the University of Sheffield, I dedicate a very special thank you, for everything. He is one of the kindest human beings that came across my path, and I will be forever thankful for all the strength, sympathy and his restless commitment to simplify my staying at Sheffield. In the personal field, I am deeply thankful for your advices and all the support, especially in my second staying at Sheffield. In the scientific field, in a first place I would like to thank you the opportunity to spend more than one year in your lab. Also, all the sessions of discussion around our work and your criticisms were valuable for the improvement of my journey as a

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Thanks to my family, my parents in law and a special acknowledgment to my grandparents and great-aunt (titi). Although most of my grandparents are not here anymore to see me become a PhD, I know that they are up there cheering for me. A word for my grandmother Cila, that passed away in January…you were my model of personality and behaviour, and I truly hope that I can follow your example to handle life in the bright way that you always did. I miss our talks, but I feel your presence here with me all the time. Thank you for always being one step forward!

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A warm thanks to my parents, for all the support along my life and for allowing me to go along my path. This thesis is for you. I know that this was not the job that you would have imagined for me, but you had the courage to accept my choices, to let me follow my dreams and doing what I always loved to do: science. Sorry Dad, but I am not going to be a medical doctor. But maybe the Nobel Prize… as you always say, "I have to be the number one"!

And last, but always first in my heart, a deeply thank you to Simão. Thank you for being who you are, and for making me a better person. I could write multiple things about all that you mean to me, how you are my rock in a storming sea, but you already know all of them by heart. So, I just wanted to say thank you for shaking me when I was numb with regret, to always make me see the bright side of things, and to be my partner in every single thing in this world, and hopefully, in the next.

IN VITRO MODELS FOR CARTILAGE ENGINEERING USING PRIMARY CELLS AND BIODEGRADABLE SCAFFOLDS

Abstract

Cartilage tissue engineering investigation has been developing strongly in the last years. The main difficulty in cartilage regeneration is its restricted self-repair capacity. Thus, investigation has been focused in promoting the regeneration of functional tissues. There are already products in the market using tissue engineering concepts to promote cartilage regeneration. The results of these treatments, although positive, still need improvements. Current treatments with joint implants are not long lasting and frequently require revision. Tissue engineering thus may provide different solutions based on tissues repair, contrarily to substitution by joint implants.

This thesis comprises the investigation of three in vitro models aiming to produce cartilage extracellular matrix (ECM), using primary cultures and scaffolds as supports for cell growth and ECM deposition. The scaffold is one of the key points in a tissue engineering strategy, thus several morphologies and formulations based on biodegradable scaffolds were explored herein. Moreover, different culture conditions were also investigated, either by using dynamic cultures (stirred and flow perfusion) or static cultures. Therefore, the thesis is divided in 3 sections concerning each of the in vitro models tested and cells used: bovine articular chondrocytes (BAC), human bone marrow derived mesenchymal stem cells (hBMSCs), and co-cultures of human primary culture of articular chondrocytes (hACs) and MSCs.

The first studies of this thesis were developed with BACs using two types of electrospun scaffolds: polycaprolactone (PCL) and starch compounded with PCL (SPCL) nanofiber meshes and microporous scaffolds. Those were composed by a blend of chitosan-poly(butylene succinate) (CPBS) with two different pore sizes and morphologies. Overall, we concluded that BAC model allowed the production of cartilage ECM on all tested scaffolds. For electrospun nanofiber meshes, no significant differences were found between PCL and SPCL as supports for the cells in terms of ECM deposition. However, results were positive in terms of the matrix deposition in both substrates. Concerning CPBS scaffolds, 80 CPBS formulation presenting larger pores with random distribution proved to have a stronger performance when compared

to 60 CPBS formulation. Those results showed the importance of larger pores for cells to colonize the scaffolds structure and deposit ECM.

In our second model we studied the chondrogenic differentiation of hBMSCs when cultured onto nanofibers (PCL) or chitosan-based microfiber meshes, using an in house developed flow perfusion bioreactor. Human MSCs were able to grow and differentiate when cultured either seeded in electrospun nanofiber meshes or in microfiber meshes. The use of dynamic culture with nanofiber meshes did not provide solid evidences of an enhancement of hBMSCs chondrogenic differentiation. Conversely, microfiber meshes indeed showed being adequate for this type of culture, as chondrogenic differentiation was enhanced when hBMSCs were seeded and cultured on those scaffolds in the bioreactor, compared to the static control. The results may even be further enhanced by the optimization of the flow rate used in those experiments.

Finally, co-cultures using hACs and hMSCs seeded onto chitosan-based microfiber meshes were established. We selected two different sources of hMSCs (hBMSCs or human Wharton´s jelly mesenchymal stem cells - hWJSCs) and compared their chondrogenic potential when co-cultured in direct contact with hACs, or indirectly co-cultured with conditioned medium derived from hACs cultures. Results showed that indirect co-cultures using conditioned medium promoted more cartilage ECM formation, both with hBMSCs or with hWJSCs. Additionally, hWJSCs demonstrated a higher chondrogenic potential than hBMSCs that produced an ECM containing higher expression levels of collagen type I. This result is very interesting because it is believed that has a higher potential to be used in clinic to treat patients, since unrelated chondrocytes may be used to induce the chondrogenic differentiation of autologous adult stem cells.

Overall, the work on this thesis presents some valid concepts for cartilage tissue engineering. We were able to obtain cartilage like tissue using primary cultures, either differentiated or undifferentiated. Deposition of cartilage ECM was observed in all the tested 3D biodegradable scaffolds either in static or in dynamic culture conditions. The advantages of co-culturing differentiated and undifferentiated cells for cartilage engineering were also demonstrated.

MODELOS IN VITRO PARA ENGENHARIA DE CARTILAGEM UTILIZANDO CÉLULAS PRIMÁRIAS E "SCAFFOLDS" BIODEGRADÁVEIS

Resumo

A investigação em engenharia de cartilagem teve um desenvolvimento muito intenso nos últimos anos. Uma das maiores dificuldades em regenerar cartilagem prende-se com o facto de este tecido ter uma capacidade de auto-regeneração muito limitada. Actualmente, já existem no mercado alguns produtos para a regeneração de tecido cartilagíneo, baseados em conceitos de engenharia de tecidos. Apesar de trazerem resultados positivos, estes tratamentos ainda necessitam de ser muito optimizados. A aplicação actual de implantes para recuperar a funcionalidade das articulações não é duradoura, e frequentement os implantes necessitam de revisão após alguns anos. A engenharia de tecidos pode desenvolver soluções mais duradouras, baseadas na regeneração de tecidos e não na substituição da área afectada por implantes articulares.

Esta tese engloba o estudo de três modelos in vitro desenvolvidos com o objectivo de produzir matriz extracelular de cartilagem (ECM), utilizando culturas celulares primárias e "scaffolds" como suportes para o crescimento das células e deposição da referida matriz. O "scaffold" é um dos aspectos críticos numa estratégia de engenharia de tecidos, por isso vários "scaffolds" biodegradáveis com morfologias e formulações diferentes foram avaliados. Várias condições de cultura foram também investigadas, usando culturas dinâmicas (agitação ou fluxo de perfusão) ou estáticas. Consequentemente, a tese está dividida em três secções correspondentes a cada um dos modelos in vitro testados, e agrupados segundo o tipo de células utilizadas: condrócitos articulares bovinos (BAC), células estaminais mesenquimais derivadas de medula óssea (hBMSCs) e co-culturas de células primárias humanas: condrócitos articulares (hACs) e células estaminais mesenquimais (hMSCs).

Os primeiros estudos apresentados nesta tese foram desenvolvidos com BACs e dois tipos de malhas de nanofibras: nanofibras de policaprolactona (PCL) ou de amido composto com policaprolactona (SPCL), e também com um "scaffold" com microporosidade. Estes últimos "scaffolds" foram produzidos com uma mistura de quitosano e de polibutileno succinato (CPBS), apresentando dois tamanhos de poros características e morfologias diferentes. Em geral, concluimos que o modelo com BACs permitiu a produção de ECM em todos os "scaffolds" e malhas de nanofibras testados. Não foram observadas diferenças significativas em termos de deposição de ECM entre as malhas de PCL e de SPCL. No entanto, os resultados foram considerados positivos, pois houve deposição de ECM em ambos os substratos. No que

diz respeito aos "scaffolds" de CPBS, a formulação 80 CPBS (com poros maiores de distribuídos aleatoriamente) demonstrou um desempenho biológico mais expressivo quando comparada com a formulação 60 CPBS. Estes resultados mostraram a importância de poros de maior tamanho na estrutura dos "scaffolds" para facilitar a colonização celular e a deposição de ECM.

O segundo modelo explorado é referente ao estudo da diferenciação condrogénica de hBMSCs cultivadas em malhas de nanofibras (PCL) ou de microfibras (formadas por misturas de quitosano), usando para tal um bioreactor de perfusão desenvolvido pelo nosso grupo. Observou-se crescimento e diferenciação das células cultivadas quer nas malhas de nanofibras, quer nas malhas de microfibras. A cultura dinâmica não evidenciou facilitar a diferenciação condrogénica das hBMSCs. Por outro lado, foi demonstrado que as malhas de microfibras são adequadas para este tipo de culturas dinâmicas, pois a diferenciação condrogénica foi potenciada nas culturas destes "scaffolds" no bioreactor, comparativamente com os controlos estáticos. Estes resultados poderão ainda vir a ser melhorados através da optimização das condições de fluxo de meio de cultura utilizadas nestas experiências.

Finalmente, realizaram-se co-culturas de hACs e hMSCs em malhas de microfibras. Foram seleccionados dois tipos de hMSCs: hBMSCs ou células estaminais mesenquimais derivadas da geleia de Wharton (hWJSCs). Comparou-se o potencial condrogénico dos dois tipos de células estaminais quando co-cultivadas em contacto directo com hACs, ou em contacto indirecto, usando para tal meio condicionado proveniente das culturas de hACs. Os resultados demonstraram que as culturas indirectas com meio condicionado promoveram uma maior formação de ECM, usando quer hBMSCs, quer hWJSCs. Adicionalmente, as hWJSCs revelaram um potencial condrogénico mais elevado do que as hBMSCs, que produziram uma ECM rica em colagénio tipo I. O resultado com os meios condicionados é muito interessante porque consideramos que poderá ter um elevado potencial para futuras aplicações clínicas. A utilização de condrócitos heterólogos para obtenção de meios condicionados que promovam a diferenciação condrogénica de células estaminais autólogas parece-nos importante no contexto da engenharia de tecidos da cartilagem.

O trabalho apresentado nesta tese revelou alguns conceitos válidos para a engenharia de cartilagem. Obteve-se tecido cartilagíneo usando quer culturas primárias de células diferenciadas, quer de células indiferenciadas. A deposição de ECM ocorreu em todos os "scaffolds" 3D biodegradáveis testados, quer em condições estáticas, quer em condições dinâmicas. Por fim, demonstraram-se também algumas vantagens relativas à utilização de co-culturas de células diferenciadas com células indiferenciadas para a engenharia de tecidos de cartilagem.

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List of abbreviations

A

ACI – autologous chondrocyte implantation

ACs – Articular chondrocytes

AGC – agreccan

AS - ankylosing spondylitis

α-MEM – Alfa Minimum Essential Medium

B

BAC – bovine articular chondrocytes

bFGF – basic fibroblast growth factor

BMP – bone morphogenic protein

BMSCs – bone marrow derived mesenchymal stem cells

BSA - bovine serum albumin

C

cDNA – complementary DNA

CFU-F – colony forming unit - fibroblast

CPBS – chitosan – poly (butylene succinate)

CPBTA – chitosan – poly (butylene terephthalate adipate)

COL I - collagen type I

COL II - collagen type II

COL X - collagen type X

CV - cryoglobulinemic vasculitis

D

DD – degree of deacetylation

DM – dermatomyositis

DMARDs - disease-modifying anti-rheumatic drugs

DMB - dimethymethylene blue

DMEM - Dulbecco's modified Eagle's medium

DNA – deoxyribonucleic acid

dsDNA – double stranded DNA

E

ECM – extracellular matrix

F

FCM – flow cytometry

FDA – food and drug administration

FITC – fluorescein isothiocyanate

G

GAGs – glycosaminoglycans

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

H

hAC – human articular chondrocytes

hBMSCs – human bone marrow derived mesenchymal stem cells

hWJSCs – human Wharton´s jelly mesenchymal stem cells

H&E – hematoxylin – eosin

I

IL-1α - interleukin-1 alpha

IL-1β - interleukin-1 beta

IVB – in vivo bioreactor

J

JIA - Juvenil idiopatic arthritis

M

MACI - matrix-assisted autologous chondrocyte implantation

MSCs – mesenchymal stem cells

μCT – microcomputed tomography

N

NFM – nanofiber meshes

NSAID – non-steroid anti-inflammatory drugs

O

OA – osteoarthritis

P

PAM - pharmacological active microcarriers

PBS – poly(butylene succinate)

PBT - poly (butylene terephtalate)

PBTA – poly (butylene terephthalate adipate)

PCL – poly (ε-caprolactone)

PE – phycoerythrin

PEGT - poly (ethyleneglycol-terephtalate)

PET - poly(ethylene terephtalate)

PGA - poly (glycolic acid)

PLA - poly(lactic acid)

PLGA – poly (lactic-co-glycolic acid)

PM – polymyositis

PsA - psoriatic arthritis

R

RA - rheumatoid arthritis

RNA – ribonucleic acid

rpm – rotations per minute

Runx2 - runt-related transcription factor 2

RWV - Rotating wall vessel

S

SA - secondary amyloidosis

SCID - severe combined immunodeficiency

SEM - scanning electron microscopy

SLE - systemic lupus erithematosus

SMARDs - symptom-modifying anti-rheumatic drug

Sox9 - sry-type high mobility group box 9

SPCL – starch compounded poly (ε-caprolactone)

T

TBS - tri-buffered saline

TE – tissue engineering

TGF-β1 – transforming growth factor – beta 1

TGF-β3 – transforming growth factor – beta 3

Tm – melting temperature

TNFα - tumour necrosis factor alpha

U

UC – umbilical cord

US – United States

UV – ultra violet

V

VEGF - vascular endothelial growth factor

W

w/v – weight/ volume ratio

WJSCs – Wharton´s jelly mesenchymal stem cells

wt – wet weight

2D – two dimensional

3D – Three dimensional

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Short *Curriculum Vitae*

Marta Alves da Silva was born on May 15, 1979, in Custóias, Portugal. She is currently a PhD student at the 3B´s Research Group (Biomaterials, Biodegradables and Biomimetric) from the School of Engineering of University of Minho, Portugal. Her PhD work has been supervised by Prof. Nuno M. Neves (Assistant Professor at the School of Engineering of University of Minho and member of the 3B´s Research Group), and co-supervised by Prof. Rui L. Reis (also the Director of the 3B´s Research Group) and by Prof. Paul Hatton (Centre for Biomaterials and Tissue Engineering, University of Sheffield, United Kingdom). She has just submitted her PhD thesis on Tissue Engineering, Regenerative Medicine and Stem Cells to the University of Minho, Portugal.

Marta Alves da Silva received her Bachelor in Science degree in Applied Biology from the University of Minho in 2001. She spent 8 months working on her final graduation project at the Department of Genetics and Biotechnology of the University of Trás-os-Montes e Alto Douro, in 2001, under the supervision of Prof. Olinda Carnide (Director of the referred department). In the year 2002, she joined the Master Course in Molecular Genetics at University of Minho, Portugal. She developed her master project at the Biology Department of the University of Minho, under the supervision of Prof. Cândida Lucas (Co-director of the Master Course) and received her Master in Molecular Genetics in 2005.

In January 2006 she formally started her PhD at the 3B´s Research Group where she has been working on the study of in vitro models to test novel scaffolds for cartilage tissue engineering. She was granted with a Marie Curie Short Term Fellowship in February 2006, and spent the next six months at the University of Sheffield, United Kingdom. The work developed during the referred period was based on primary cultures using bovine articular chondrocytes seeded onto different biodegradable scaffolds, and was co-supervised by Prof. Paul Hatton. In June 2006 she attended the Bioreactor Design and Stem Cell Processing Workshop in Buxton, England. In September 2006, Marta Alves da Silva was granted with a scholarship for her doctoral research studies by the Portuguese Foundation for Science and Technology, which

enabled her to perform her studies at the 3B´s Research Group and at the University of Sheffield. At the 3B´s Research Group she has acquired training in cell culture with biomaterials and contacted with several materials processing techniques and explored various cartilage ECM characterization techniques. In October 2006, she attended the Advanced Course "Stem Cells: Pitfalls and Successes" at the School of Health Sciences, University of Minho. In 2008 Marta Alves da Silva participated in the Course on Laboratory Animal Science, at the Institute of Life and Health Sciences of the University of Minho. She was approved for the FELASA category C. In 2009 she spent 9 months at the University of Sheffield, United Kingdom where she explored the use of bovine bone marrow-derived stem cells for chondrogenic differentiation onto chitosan-based scaffolds with different formulations.

Marta Alves da Silva has been involved in the writing of several project proposals, either nationally (Portuguese Foundation for Science and Technology) or internationally (Framework Program VII).

As a result of her research, Marta Alves da Silva attended several international conferences in the field of Biomaterials and Regenerative Medicine. She has been awarded by the School of Engineering of the University of Minho with the prize for Best Oral presentation in October 2007, during the celebrations of the School of Engineering Day. She is the first author of 6 papers in international refereed journals (four published and two submitted).

List of publications

Several publications resulted from the work developed in this PhD programme, from the results that will be presented in this PhD thesis.

International journals with referee

Alves da Silva ML, Crawford A, Mundy JM, Martins A, Araújo JV, Hatton PV, Reis RL and Neves NM. *Evaluation of extracellular matrix formation in PCL and SPCL nanofiber meshes when seeded with bovine articular chondrocytes.* Tissue Engineering: Part A. 2009. 15(2): 377-385.

Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol PC, Battacharya M, Hatton PV, Reis RL and Neves NM. *Chitosan/polyester-based scaffolds for cartilage tissue engineering: assessment of extracellular matrix formation*. Acta Biomaterialia. 2010. 6(3): 1149–1157.

Alves da Silva ML, Martins A, Teixeira AA, Reis RL and Neves NM. *Impact of biological agents and tissue engineering approaches in the treatment of rheumatic diseases*. Tissue Engineering Part B. 2010. 16(3): 331-339.

Alves da Silva ML, Martins A, Costa-Pinto AR, Costa P, Faria S, Gomes ME, Reis RL and Neves NM. *Cartilage tissue engineering using electrospun PCL nanofiber meshes and MSCs*. Biomacromolecules. 2010. 11(12): 3228-3236.

Alves da Silva ML, Martins A, Costa-Pinto AR, Correlo VM, Sol P, Bhattacharya M, Faria S, Reis RL and Neves NM. *Chondrogenic differentiation of human bone marrow mesenchymal stem cells in chitosan based scaffolds using a flow perfusion bioreactor*. Journal of Tissue Engineering and Regenerative Medicine. 2010. DOI: 10.1002/term.372. In press.

Alves da Silva ML, Costa-Pinto AR, Correlo VM, Sol P, Bhattacharya M, Faria S, Reis RL and Neves NM. *Co-culture of human articular chondrocytes with two different*

sources of human mesenchymal stem cells as a new strategy for cartilage tissue engineering. 2010. Submitted.

International conferences

Alves da Silva ML, Oliveira JT, Correlo V, Bhattacharya M, Charbord P, Reis RL, Neves NM. *Comparison between chondrogenic differentiation of human mesenchymal stem cells (hMSCs) and a primary human articular chondrocytes (HAC) culture when seeded onto chitosan/ polyester based scaffolds*. 1st UK Mesenchymal Stem Cell Meeting, York, England. June 2006. (*Poster presentation*).

Alves da Silva ML, Martins A, Costa-Pinto AR, Reis RL and Neves NM. *Human bone marrow stromal cells (hBMSCs) response to different electrospun nanofiber meshes topographies: a study focusing on the osteogenic and chondrogenic differentiation potential*. 1st Annual International Meeting of the Portuguese Society for Stem Cell and Cell Therapy (SPCE-TC), Madeira, Portugal, May 2006. *(Poster presentation).*

Alves da Silva ML, Costa-Pinto AR, Crawford A, Mundy JM, Correlo VM, Sol PC, Bhattacharya M, Hatton PV, Reis RL and Neves NM. *The potential of chitosan-based scaffolds for bone and cartilage tissue engineering.* Genostem 3rd Workshop on Mesenchymal Stem Cells Enginnering for Conective Tissue Disorders. Barcelona, Spain, February 2007. *(Oral presentation).*

Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol PC, Bhattacharya M, Hatton PV, Reis RL and Neves NM. *Evaluation of extracelullar matrix formation on chitosan-based scaffolds using a bovine articular chondrocytes model for articular cartilage*. Genostem 3rd Workshop on Mesenchymal Stem Cells Enginnering for Conective Tissue Disorders, Barcelona, Spain, February 2007. *(Poster presentation).*

Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol PC, Bhattacharya M, Hatton PV, Reis RL and Neves NM. *Evaluation of bovine articular chondrocytes when*

seeded into chitosan/ poly (butylene succinate) scaffolds. Cartilage Biology and Pathology, Ventura, United States of America, March 2007. *(Poster presentation).*

Alves da Silva ML, Crawford A, Mundy JM, Martins A, Araújo JV, Hatton PV, Reis RL and Neves NM. *Evaluation of the potential of polycaprolactone (PCL) and starch polycaprolactone (SPCL) nanofiber meshes for cartilage tissue engineering Approaches*. Society for Biomaterials Annual Meeting, Chicago, United States of America, April 2007. *(Poster presentation).*

Alves da Silva ML, Crawford A, Mundy JM, Martins A, Araújo JV, Hatton PV, Reis RL and Neves NM. *Polycaprolactone (PCL) and starch polycaprolactone (SPCL) nanofiber meshes: an approach to cartilage tissue engineering*. TERMIS-NA, Toronto, Canada, June 2007. *(Oral presentation).*

Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol PC, Bhattacharya M, Hatton PV, Reis RL and Neves NM. *Novel chitosan/ poly (butylene succinate) scaffolds for cartilage tissue engineering.* TERMIS-EU, London, United Kingdom, September 2007. *(Oral presentation).*

Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol PC, Bhattacharya M, Hatton PV, Reis RL and Neves NM. *Cartilage tissue engineering using polycaprolactone (PCL) and starch-polycaprolactone (SPCL) nanofiber meshes.* School of Engineering Day, University of Minho, Braga, Portugal, October 2007. *(Oral presentation).*

Alves da Silva ML, Correlo VM, Sol PC, Bhattacharya M, Charbord P, Delorme B, Reis RL and Neves NM. *Compression moulded based Scaffolds: an approach for cartilage tissue engineering*. Mesenchymal Stem Cells International Symposium, Montpellier, France, November 2007. *(Poster presentation).*

Alves da Silva ML, Martins A, Costa, PF, Correlo VM, Sol PC, Bhattacharya M, Rougier N, Reis RL and Neves NM. *Cartilage tissue engineering using a flow perfusion bioreactor*. TERMIS-EU, Porto, Portugal, June 2008. *(Oral presentation).*

Alves da Silva ML, Martins A, Costa-Pinto AR, Faria S, Reis RL and Neves NM. *Enhanced chondrogenic differentiation of human bone marrow stromal cells (hBMSCs) seeded in nanofiber meshes under flow perfusion stimulation*. 5th SPCE-TC, Guimarães, Portugal, May 2010. *(Poster presentation).*

Alves da Silva ML, Martins A, Costa-Pinto AR, Faria S, Reis RL, Neves NM. *Cartilage tissue engineering using electrospun PCL nanofiber meshes and MSCs*. Final Scientific Meeting of EXPERTISSUES | 1st Annual Meeting of the European Institute of Excellence in Tissue Engineering and Regenerative Medicine. July, 2010, Guimarães, Portugal. *(Oral presentation).*

Alves da Silva ML, Martins A, Costa-Pinto AR, Correlo VM, Sol PC, Bhattacharya M, Faria S, Reis RL and Neves NM. *Chondrogenic differentiation of human bone marrow mesenchymal stem cells in a flow perfusion bioreactor*. TERMIS-AP Meeting. September 2010, Sydney, Australia. *(Oral presentation)*.

Alves da Silva ML, Martins A, Costa-Pinto AR, Faria S, Reis RL and Neves NM. *Polycaprolactone nanofiber meshes for cartilage tissue engineering using dynamic culture*. TERMIS-AP Meeting. September 2010, Sydney, Australia. *(Oral presentation)*.

Alves da Silva ML, Martins A, Costa-Pinto AR, Correlo VM, Sol PC, Bhattacharya M, Faria S, Reis RL and Neves NM. *Chondrogenic differentiation of human bone marrow mesenchymal stem cells in chitosan based scaffolds*. ICRS 2010 Meeting-Barcelona, Spain, September 2010. *(Poster presentation).*

Alves da Silva ML, Martins A, Costa-Pinto AR, Correlo VM, Sol PC, Bhattacharya M, Faria S, Reis RL and Neves NM. *Flow perfusion can stimulate chondrogenic differentiation of human mesenchymal stem cells cultured on chitosan-based scaffolds*. 2nd Scientific meeting of the Institute for Biotechnology and Bioengineering. Braga. 23-24 October 2010. *(Poster presentation).*

Introduction to the thesis format

The present thesis is divided in eight chapters intended to provide a comprehensive report of the progress achieved during this PhD work. The chapters are organized in such a way that show the sequential progress obtained in the various works reported. The obtained results originated five scientific papers presenting new data and one review paper, already published or under submission for publication. The papers were organized in thematic sections, based on their contents and their biological and experimental design. Therefore, the new data presented in the thesis was organized in three sections corresponding to the type of cells used. The first section reports were performed with primary bovine chondrocytes. The second section presents data obtained with primary human mesenchymal stem cells. The last section is based in co-cultures of human chondrocytes and mesenchymal stem cells. The section based in the review paper was adapted to become the thesis introduction, and to provide an extensive overview of cartilage tissue engineering strategies and background information, enabling to analyse in context the progress obtained during this PhD programme.

Section I (Chapter 1)

The first section is adapted from a published review paper and presents an extensive literature survey on the field. This section is focused in discussing in detail the problems related to cartilage diseases or trauma injuries. It also analyses the potential role of tissue engineering in obtaining new solutions and alternative strategies for cartilage regeneration and repair. The literature review presented in this section is intended to provide an in depth perspective of the progress achieved in the community, as well as to stimulate the discussion in context of the works developed in this thesis.

Section II (Chapter 2)

This section provides a detailed description of the materials and methods used. Materials, methods and the basic principles behind the methodologies used in the context of the experimental work developed are all covered. The materials processing and scaffolds production methods are explained in this section. Moreover, the biological assays performed are described thoroughly. The methodologies herein described

provide more details in some specific tests, but more importantly, provide a general framework for the in vitro testing of new scaffolds, and in vitro culturing methods aimed for cartilage tissue engineering. By reading this chapter, it is not required to follow closely the materials and methods sections of chapters 3 to 7.

Section III (Chapters 3 and 4)

The third section includes two published papers using bovine articular chondrocytes as a model to test the biological performance of new scaffolds in vitro. This section describes the influence of scaffolds composed of electrospun nanofiber meshes (chapter 3), or microporous scaffolds obtained by compression moulding and salt leaching (chapter 4), on bovine articular chondrocytes. The two works complement themselves and show that the bovine chondrocytes can be successfully cultured in both scaffolds.

Section IV (Chapters 5 and 6)

This section includes two papers, one published and the other accepted for publication, relating the influence of nanofiber meshes (chapter 5) and microfiber meshes (chapter 6) in the chondrogenic differentiation of human bone marrow derived mesenchymal stem cells (hBMSCs), using a flow perfusion bioreactor. Both works show the positive effect of the flow perfusion provided by the bioreactor in the chondrogenic differentiation of hBMSCs. Both scaffolds support successfully the chondrogenic differentiation of human bone marrow derived mesenchymal stem cells, showing that both the structure and the material composition are adequate for the successful culture of relevant cells.

Section V (Chapter 7)

Section V includes one chapter proposing an advanced co-culture strategy for cartilage repair. After using two types of primary cells in the previous sections, we studied the outcome of co-culturing human articular chondrocytes and mesenchymal stem cells. This chapter describes the use of direct and indirect co-cultures of human chondrocytes and adult mesenchymal stem cells, either isolated from bone marrow or from umbilical cord Wharton´s jelly. The objective was to study the influence of the chondrocyte secreted factors and cell-cell contact over the chondrogenic differentiation of adult mesenchymal stem cells, cultured in 3D microfiber meshes. The results show, somewhat surprisingly, that the indirect co-cultures are more effective, opening new

perspectives for the development of clinically relevant strategies for cartilage tissue engineering.

Section VI (Chapter 8)

The final section of the thesis highlights the contributions and conclusions derived from the presented works, including a discussion on the future perspectives and work to be developed to achieve the aimed clinical progress in the long lasting regeneration of articular cartilage.

SECTION I - GENERAL INTRODUCTION

Chapter 1

Tissue engineering as a remarkable tool for cartilage repair in the context of joint diseases

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This chapter is based on the following publication: Alves da Silva ML, Martins A, Teixeira AA, Reis RL, Neves NM. *Impact of biological agents and tissue engineering approaches on the treatment of rheumatic diseases*. Tissue Engineering: Part B. 2010. 16(3): 331-339

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1. Introduction

Cartilage development is characterized by a series of temporal events, in which the chondrocytes undergo sequential morphological and genetical changes. These modifications involve complex signalling and regulatory networks that link cell-matrix and intercellular interactions with a strongly regulated gene expression pattern [1]. Cellular condensation is a requirement for the beginning of chondrogenesis in the embryonic limb bud and in mesenchymal stem cells (MSCs) cultures [2]. The knowledge of the events that lead to articular cartilage formation is of utmost importance to understand the mechanisms involved in the chondrogenic pathway. Articular cartilage is a very specialized tissue with outstanding load-bearing capacity. It consists mainly of a dense extracellular matrix (ECM) with chondrocytes embedded on it. The ECM binds to a large amount of water that is a key element for the load-bearing capacity of cartilage. Cartilage has very low capacity of self-repair and regeneration after traumatic, degenerative or inflammatory injury. This factor often leads to end-stage osteoarthritis (OA) after repeated damage causing a strong limitation in the mobility of patients. Additionally, these defects are difficult to treat and to fully repair by traditional surgery techniques like mosaicoplasty or microfracture. Tissue engineering (TE) is a very promising approach to achieve articular cartilage repair by obtaining a long lasting and fully functional recovery of the lost tissue.

When a tissue is damaged, the body responds by triggering a cascade of events to repair it, being the vascular access critically important to provide the needed cues. In the case of cartilage lack of vascularisation may prevent this natural response. Cartilage defects can be categorized as chondral or osteochondral, depending on the depth of the defect. Chondral injuries are restricted to the articular cartilage. Osteochondral injuries penetrate deeper into the underlying subchondral bone, opening a path for the inflow of bone marrow mesenchymal cells (BMSCs) into the defect. When this type of injury occurs, the defect is usually filled with fibrocartilage that provides temporary relief [3]. With time, patients will experience pain and an orthopaedic condition will evolve, leading to a cartilage-related disease. The major part of rheumatic disorders is caused by rheumatoid arthritis (RA) or osteoarthritis (OA). Currently, OA affects more than 200 million people worldwide and, just in Europe, 350.000 arthroscopic surgeries are performed annually [4]. The prevalence of arthritis and other rheumatic diseases among

adults over 18, in the United States (US), was of 46.4 million, between 2003 and 2005 [5].

More than half of the unintentional injuries that occur annually in the US are of the musculoskeletal system, corresponding to 60-67% of the total annual injuries [6]. These injuries are caused either by trauma or by diseases related with this system. Orthopaedic conditions affect not only elder people, but also a large number of young people. These conditions are result of sports injuries, car or motorcycle accidents or other traumas. For example, there are annually 1.3 million fractures that require hospital admission, only in US [6].

More than 100 types of rheumatic diseases, arthritis and related conditions were already identified. Examples of inflammatory rheumatic diseases include RA, ankylosing spondylitis (AS), psoriatic arthritis (PsA), systemic lupus erithematosus (SLE), juvenile idiopatic arthritis (JIA) and OA [7, 8]. These diseases affect people of all ages and genders, and until today, its causes remain elusive, as well as its cure. Conventional drug treatment for rheumatic inflammatory diseases combines basically a symptom-modifying anti-rheumatic drug (SMARDs) with disease-modifying anti-rheumatic drugs (DMARDs). The combination of SMARDs with DMARDs can slow down the progression of inflammation and, in some cases, modify the disease progression [9]. Current available surgical treatments for cartilage repair present several drawbacks, such as possible implant rejection or infection, or the need for revision after some years of implantation. Autologous chondrocyte implantation (ACI) is an autologous therapy that was proposed as a basis for TE strategies to repair cartilage [10]. The clinical procedure uses chondrocytes obtained from a biopsy collected from the donor healthy cartilage and then expands autologous cells in vitro. Chondrocytes are further re-implanted at the injured site by injection and using a periosteal flap to close the defect [10]. One of the main criticisms to this technique is the need to perform two surgical interventions in a short period [11]. In ACI, a small biopsy must be collected from a non weight bearing area, and this procedure may lead to donor site morbidity or inflammation [11]. Besides, the technique outcome frequently involves formation of a fibrous tissue rather than the desired and functional articular neo-cartilage [11]. Modifications on various aspects of this surgical technique have been developed, for example the use of alternative patches to close the defect, or the use of biomaterials as supports for chondrocyte expansion. These modifications originated the matrix autologous chondrocytes implantation technique (MACI), recently approved by the

FDA (Food and Drug Administration) in the US. In MACI, a previously cultured chondrocyte/scaffold construct is implanted in the lesion site. This technique comprises a TE strategy, since a biodegradable scaffold is used for supporting cell growth in vitro and in vivo. Hyalograft®C, a hyaluronan-based scaffold has been also proposed and developed for clinical use, associated with MACI procedure [12, 13]. Collagen membranes have also been proposed as a scaffold for clinical application of the MACI technique [14]. An overview of current biological treatments and surgical procedures, as well as their outcomes, will be further discussed in this section.

The challenge behind TE is to coordinate the materials properties and scaffolds production methods, resulting morphologies and structure, with the proper type of cells. Those components should be matured in vitro under adequate culture conditions to generate a construct that can be used to fully repair the injured cartilage tissue. One of the most important aspects for TE is the development of the proper scaffold. The material used for its production, as well as its cytocompatibility and biodegradability, must be considered since they are critical for the strategy success [15, 16]. The type of material is very important, since natural or synthetic polymers have very different properties [17]. Some natural and synthetic polymers will be herein explored, and their outcomes for cartilage regeneration will be studied. We will discuss all those parameters, detailing its role in the general strategy of obtaining cartilage tissue in vitro. Also, in vivo reports from the literature will be discussed. Selection of the proper scaffold structure, porosity and pore size is very important to enable cell attachment and to promote proliferation and differentiation of cells, leading to ECM production. The role of scaffold and cells in the construct, as well as cell types used for cartilage TE strategies will be extensively discussed. Moreover, dynamic culture conditions will be analysed, as well as the influence of different microenvironments for chondrocytes culture. The aim of this review is to provide a general perspective of cartilage TE and its application for the fully functional and long lasting regeneration of this tissue.

2. Basics of cartilage biology

2.1. Chondrogenesis and growth plate

The growth plate formation is one of the major events in skeletal development. The growth plate of long bones is a very organized structure composed by chondrocytes in dissimilar stages of proliferation and differentiation, placed in a complex network of collagens that compose a scaffold of oriented ECM [18].

Cartilage is formed when some mesenchyme cells aggregate to form the blastula, during embryo development. Mesenchymal cells in this structure produce an ECM rich in collagen type I [19]. Blastula cells begin to differentiate into chondrocytes and to secrete cartilage matrix, rich in collagen type II and aggrecan (two of the main components of cartilage ECM) [20]. In general, a specific genetic program driven by *Sox9* and other transcriptional factors is expressed for ECM formation [21]. *Sox9* is critical for all phases of chondrocyte development, from early condensations to conversion of proliferating chondrocytes into hypertrophic chondrocytes [22]. However, *Sox9* is down regulated in hypertrophic chondrocytes [23]. In cultured chondrocytes, *Sox9* stimulates the transcription of a number of cartilage matrix genes, such as *Collagen type II* [21] and *Aggrecan* [1]. Cells in the periphery of the mesenchymal condensations do not differentiate into chondrocytes, they form the perichondrium, continuing to produce collagen type I [24]. Gradually the produced ECM begins to push chondrocytes apart [25]. Eventually, these cells exit the cell cycle and become hypertrophic, producing collagen type X, instead of the usual collagen type II [23] and undergo the mineralization process.

2.2. Joint development

The ends of mammalians long bones are covered by articular cartilage that allows friction and load distribution between bones. During post-natal development, articular cartilage undergoes a reorganization process [26]. At this stage of development, articular cartilage layer undergoes a process of growth and elongation involving the underlying bone [26]. Synovial joints development comprises the formation of the cartilagineous rod followed by the formation of the joint cavity, articular cartilage, synovium and other joint structures such as the meniscus [26]. Some aspects of this

development still remain to be elucidated, such as the events occurring in cavitation to form joints [27]. It is normally assumed that continuous cartilage formation in the early stages of development is interrupted and cartilage specific zones are formed [27]. During maturation of articular cartilage, its components such as collagens and proteoglycans rearrange themselves within the various cartilage zones, and this orientation evolves from an immature arrangement to a fully "arcade" arrangement, present in the mature tissue [28]. Most of the cartilage formed in the early stages of development undergoes segmentation to produce individual bones separated by the synovial joints [28]. Joints formation is initiated with a joint specification phase, where the joint future place is defined. Then, the joint cavity forms in a process called joint cavitation [28]. In the places where joints will develop, chondrocytes flatten and originate transversal stripes called joint interzone [28]. The central layer of the interzone then becomes the joint cavity, while the proximal and distal areas become continuous with the perichondrium of cartilage [28]. The perichondrium will form the articular cartilage superficial layer that coats the ends of long bones. The Hox genes have been reported to have a major role in the limb patterning, as they can specify a cells fate within the limb [29]. Bone morphogenic proteins (BMP) are expressed in the early stages of joint specification and are reported to be involved in limb movement and joint development [30]. There are many signalling proteins involved in these events, and some of the interactions still need to be clarified.

Both growth plate and joint formation events are very important to help to understand the processes involved in cartilage formation in nature, as well as formation of its stratified structure. Their understanding may be valuable for transposing those processes to the laboratory, in an attempt to mimic them in vitro and to produce cartilage tissue.

2.3. Cartilage structure

Articular cartilage is composed only by a single cell type, the chondrocyte. In humans, these cells correspond to 1% of the tissue volume. The remaining 99% is formed by a highly specialized ECM [31]. The chondrocytes are located in small lacunae within the cartilage tissue. These cells synthesize all ECM compounds necessary for tissue structure maintenance (collagen type II, proteoglycans and non-collagenous proteins), and are responsible for matrix stability. Chondrocytes obtain their nutrients through a

double diffusion barrier, survive on low oxygen conditions and thus depend on an anaerobic metabolism [25].

The matrix components are tightly organized in a zone specific way in cartilage. Articular cartilage comprises four zones: superficial, middle, deep and calcified zone. ECM unique viscoelastic properties are due to its nanoarchitecture and to the referred zone-specificity [32]. Cartilage zones of are illustrated in Figure 1.1.

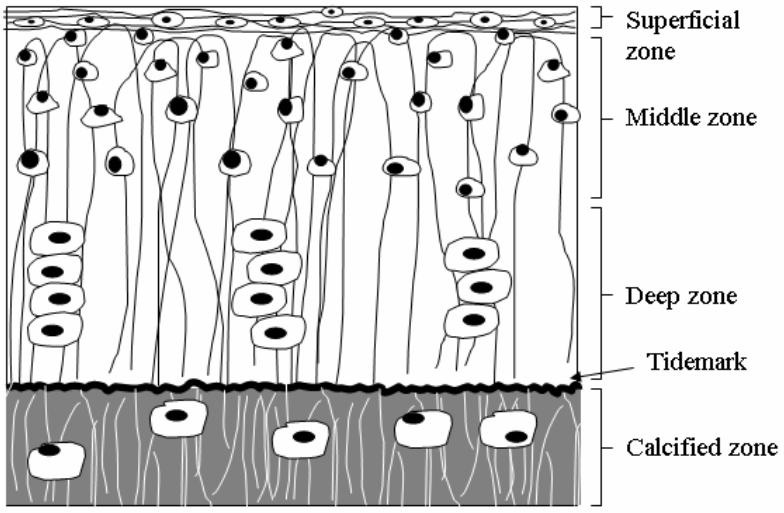

Figure 1.1 – Schematic representation of articular cartilage structure showing the different zones of cartilage. Chondrocytes present in the superficial zone are discoidal and in the deep zones their shape changes to spherical. Tidemark zone separates the deep zone and the calcified zone.

ECM of articular cartilage (as wet mass) is composed by 60-85% of water and dissolved electrolytes [33]. The solid framework is composed by collagens (10-30%), proteoglycans (3-10%) and non-collagenous proteins and glycoproteins [34]. The principal component of the macrofibrilar framework of ECM is composed mainly by collagen type II, which provides tensile strength to the articular cartilage [34]. The different types of collagens present in the articular cartilage are summarized on Table 1.1.

Table 1.1 – Collagens present in the ECM of articular cartilage and their functions.

Collagen type	*Morphological location*	*Function*
II	Principal component of macrofibrils; characteristic of ECM basic architecture	Tensile strength
VI	Pericellular matrix; around cells of the middle zone in small amounts	Forms elastic fibers; attachment of chondrocytes to the matrix
IX	Surface of macrofibrils; characteristic of ECM basic architecture	Inter fibrillar connections; tensile properties
X	Calcified cartilage layer ; associated with hypertrophic cartilage	Aid in cartilage mineralization ; structural support
XI	Within or on macrofibrils; characteristic of ECM basic architecture	Nucleates fibril formation

Table adapted from Bhosale and Richardson, 2008 [25] *and Schultz and Bader, 2007* [4].

Proteoglycans provide compressive strength to the tissue and maintain the electrolyte balance [25]. Chondrocytes produce these protein polysaccharide molecules and secrete them into the matrix [25]. There are two major classes of proteoglycans in the tissue: aggrecans (large monomers) and small proteoglycans such as decorin or biglycan [35]. Proteoglycans subunits are named glycosaminoglycans (GAGs), which are bound to form the larger aggrecan molecule [35].

The cartilage unique structure provides this tissue with the necessary elements for load bearing and movement functions. It is important to understand the way the various elements relate to each other, but also their specific function in the organization of articular cartilage. These elements, such as GAGs or collagens, can be detected by several laboratory methodologies for identification of cartilage like tissue.

3. Cartilage diseases and trauma injuries

Currently, no reliable and durable strategy for complete and fully functional cartilage regeneration has been validated in the clinic, as discussed in the next sections. Thus, there is an urgent need for clinically appropriate strategies that provide a full regeneration of cartilage function. Due to the epidemiological importance and the high

social costs of joint diseases, cartilage engineering holds remarkable potential in orthopaedic surgery as an alternative to current surgical methods.

3.1. Biological agents for the management of cartilage diseases and TE approaches

Advances in biotechnology have led to the development of agents that therapeutically target specific components of the deregulated immune system, known as "biological agents" [36, 37]. Among them, tumour necrosis factor alpha (TNFα), interleukin-1 alpha and beta (IL-1α, IL-1β) have a significant role in the pathogenesis of rheumatic diseases [38, 39]. Biological agents are partially or fully humanized recombinant proteins that target different pathways of the immune response. Further information on the safety and efficacy of TNF blockers can be obtained *e.g.* in reference [40]. The most used biological agents for the treatment of rheumatic diseases, their characteristics and secondary effects are also presented on Table 1.2.

Table 1.2 – Biological agents used in clinical practice for the management of rheumatic diseases.

Trade name	*Type*	*Mechanism of action*	*Application*	*Secondary effects*
Abatacept	CTLA4 Ig	Costimulation inhibitor	RA; CV; SLE	Serious infection
Adalimumab	Anti-TNFα	TNF inhibitor	RA; AS; PsA	Infections, fatal sepsis and demyelinating events
Anakinra	IL-1ra	IL1 inhibitor	RA; AS	Severe infection
Etanercept	TNFα R/Fc fusion protein	TNF-inhibitor	RA;AS ;SA ; PsA; JIA	Infections, bronchitis
Infliximab	Anti-TNFα	TNF inhibitor	RA;AS; SA; PsA; DM/PM	Tuberculosis, Lupus-like syndrome, heart failure, pancreatitis, liver failure
Rituximab	Anti-CD20 mAb	B-cell deplection	RA;CV;SLE	Cardiac arrest, infections, immune toxicity

Legend: RA, rheumatoid arthritis; CV, cryoglobulinemic vasculitis; SLE, systemic lupus erythematosus; AS, ankylosing spondylitis; PsA, psoriatic arthritis; SA, secondary

amyloidosis; JIA, juvenil idiopatic arthritis; DM, dermatomyositis; PM, polymyositis. Table adapted from Bertele *et al*. [41] and Diaz-Borjon *et al*. [40].

From the different biological agents used in clinical practice, TNFα inhibitors have proven to be highly effective in the treatment of advanced rheumatic diseases, such as RA, AS, PsA or JIA [7, 36, 38, 42, 43], reducing their symptoms, improving the physical function of joints and inhibiting joint damage progression [39]. The cost of TNFα inhibitors is one of the main limitations associated with this therapy: they are considerably more expensive than the traditional DMARDs. Considering the high cost of those drugs, several strategies of combination of these agents with DMARDs are being increasingly studied [37, 40, 44]. Also, TE strategies for local administration of these drugs or biological agents have been focus of recent advances. To reach effective drug concentrations in the affected joint tissues, high doses of the therapeutic agent must be administered systemically, which may lead to significant adverse systemic and extra-articular side effects. Currently, most of the available therapies for rheumatic diseases do not have tissue specificity and have a ubiquitous distribution of the molecular targets. Reduction in drug doses may attenuate toxicity, being a plausible solution, but may lead to reduced therapeutic efficacy. To overcome these limitations, targeted drug delivery systems may provide excellent solutions by incorporating the available anti-rheumatic drugs and releasing them at the target sites [45].

Drug delivery systems have been developed in several emerging research fields such as TE and regenerative medicine, as a method to address the problems associated with conventional drugs and improve their pharmacological properties. The means by which a drug is released may have a considerable effect on its efficacy and therapeutics. Drug delivery systems have been designed using different polymers either of synthetic or natural-origin. In order to achieve a better control over the drug release, several technologies have been developed. Most of these procedures are based on a combination of bioactive agents and biomaterials by bonding. Many examples of those strategies were reported in recent literature [46]. Materials used in these processes include poly(lactic-co-glycolic acid) (PLGA) [47], poly(ε-caprolactone) (PCL) [48], chitosan [49] or starch [50]. These materials have also been proposed for controlled release in the context of tissue regeneration [50-53], as described in the previous section. Starch-based materials and composites are among those biomaterials and have shown to induce lower inflammatory cytokines, compared to poly(L-lactide) (PLA)

[51]. Several studies show the efficiency and the utility of starch in different systems for drug delivery, such as fibers [54], microspheres [55] or nanoparticles [56]. Starch-based microparticles were developed in our group for drug delivery systems [57] and showed to be viable as carriers for the incorporation and release of corticosteroids [50], growth factors [48, 58, 59], and as supports for cells [53].

The future generation of regenerative medicine for rheumatic diseases is focused on *in situ* therapies. This strategy consists in cell-free chondroinductive scaffolds implantation combined with chemotactic molecules that allow the recruitment of joint-inherent and –surrounding cells to traumatic or arthritic diseased joints, and their subsequent contribution for growth and differentiation factor-guided joint repair [60]. It is believed that the biological agents and anti-inflammatory drugs will complement the *in situ* therapy of chronic joint diseases enhancing joint tissue regeneration and preventing the degradation of the native and of the neo-tissues formed [61].

3.2. Current surgical treatments for cartilage defects

Numerous treatments have been explored to repair damaged articular cartilage, such as microfracture, mosaicoplasty, grafts and ACI. In the microfracture technique the subchondral bone is disrupted to allow bone marrow to flow into the defect [62]. A blood clot will eventually form and cells from bone marrow may migrate into it, differentiating into chondrocytes [62]. However, these derived chondrocytes are not phenotypically similar to the articular ones, and tend to originate a fibrous tissue rather than the desired articular cartilage tissue [25, 62].

The ACI technique is a technique developed for treatment of large full-thickness chondral defects, where a small biopsy of articular cartilage is collected from a low-weight-bearing area in the joint [63]. Cells are isolated and expanded in vitro, and then a suspension of the cultured chondrocytes is injected underneath a periosteal patch or a by-layer collagen membrane [63]. ACI has been widely used in clinic, but still there are limitations associated to the technique, which do not allow patients to achieve full recovery. This technique has shown promising results for human cartilage defects in early clinical studies, but little difference in the efficacy was observed in a randomized clinical trial designed to obtain a comparison to surgical procedures such as mosaicoplasty [64] There are several complications associated to ACI, such as a non-uniform repair in different areas of the joint, formation of fibrocartilage after cell

implantation, or even loss of chondrocyte phenotype [11]. Furthermore, ACI has been associated with significant donor-site morbidity and initiation of osteoarthritic changes in the joint, simply due to the harvest of donor cartilage, indicating the need for alternative cell sources for tissue-engineered repair of cartilage [65].

Various modifications to ACI are currently being attempted, focusing in a minimal invasive surgery, and combination with three dimensional (3D) scaffolds and growth factors [25], for example the MACI technique. This technique employs 3D biomaterials, different cell sources and growth factors. Chondrocytes are cultured in these structures prior to implantation. Their function is to sustain cells for inducing and maintaining the chondrocyte phenotype and to form ECM before implantation. In the MACI technique, other sources of autologous cells besides chondrocytes may be used. Those sources include MSCs, periosteum-derived cells and synovial fibroblasts [33]. Differentiation of these cells in 3D scaffolds can be further enhanced by using growth factors or anti-inflammatory cytokines, to help phenotype stabilization and protection of the newly formed cartilage [33]. Different resorbable transplants were also tested in clinic and showed promising results [66]. Hyalograft®C was used for MACI, and patients were followed after implantation during 2 years, by magnetic resonance imaging [67]. The post-operative observations showed dynamic processes in cartilage repair over time, with positive effects. Another report of a 5 years follow-up of MACI technology also showed good results: 8 out of 11 patients rated the function of their knees as much better or better than before the surgery [68]. Although the outcomes of MACI have been positive, this is a very cost-intensive alternative for the treatment of knee cartilage defects still showing limitations, thus novel strategies and alternatives should be explored. In the next pages, we will describe some TE based alternatives that in our opinion should be explored to provide other therapeutic solutions for cartilage regeneration.

4. Cartilage tissue engineering

The modern society and evolution on medicine have brought us many improvements in our daily lives and a longer life expectancy. However, problems associated with orthopaedic conditions also increased dramatically, associated with obesity, sports injuries, car accidents and many inflammatory conditions, as referred before on section

3. Therefore, long term orthopaedic repair solutions are needed, since traditional prosthesis require revision after several years post surgery, not providing a long term solution. The previous aim, to replace, has become in the actual need for repair. TE can answer this need. The goal of TE is to design new functional components that can be used to regenerate living tissues and fully restore their function. TE comprises three basic premises: the use of a scaffold to support cell growth and formation of the tissue, a source of cells, and growth factors to induce cells differentiation into the desired tissue (Figure 1.2).

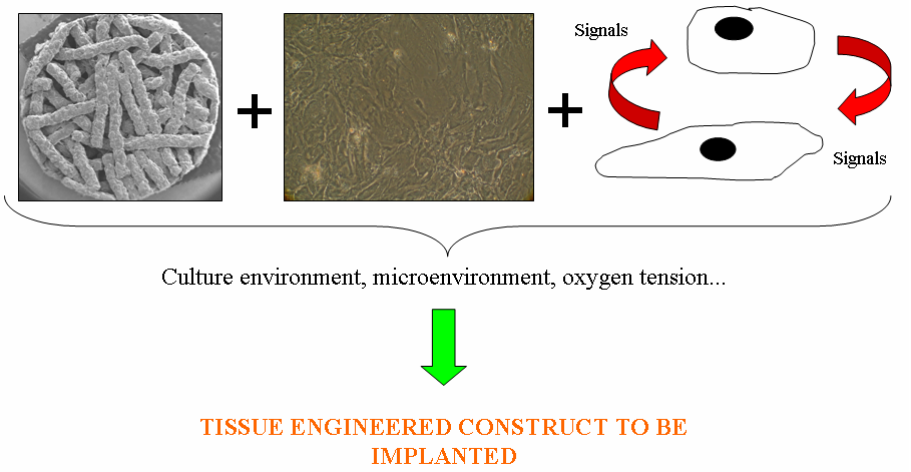

Figure 1.2 – Tissue engineering strategy comprises the use of scaffolds, optimal culture conditions, the maintenance of cell-cell interactions and their microenvironment, as well as the culture parameters.

The understanding of all the mechanisms underlying the interactions between cells and cells-materials is of extreme importance in a TE context. In fact, cell-biomaterial contact is one of the key points for a successful TE approach, and therefore scaffolds surface properties are subject of intense study and modifications [69-71]. The aim of these modifications is to create the most suitable environment for cell attachment, proliferation, phenotype maintenance or cell differentiation. Moreover, the way of culturing constructs has a major influence in the outcome of the neo-formed tissue. In terms of cartilage, dynamic culture conditions have proved to be preferable, as opposed to static ones [72-76] Extensive research effort was recently focused in the development of bioreactors that can stimulate cartilage formation similar to native tissue [74, 77-82]. Another important aspect to consider in cartilage TE approaches is cell-cell interactions. The interest in cells cross-talk and communication has been growing in the past years,

revealing that signalling pathways are pivotal elements when understanding the tissue formation and its repair mechanisms [83]. Chondrocytes release morphogenetic signals that influence the surrounding cells, for example, stem cells, to differentiate into the chondrogenic lineage [83]. Therefore, the study of co-cultures using chondrocytes and undifferentiated cells is a very interesting strategy to develop engineered cartilage. Finally, oxygen levels and the environment in which the culture is performed are a main issue in terms of articular cartilage neo-formation. Chondrocytes are the only cell type present in cartilage, being supplied of oxygen and nutrients by the surrounding synovial fluid. Compared to other cell types, they experience a hypoxic environment in vivo, as local oxygen tension ranges from 1% to 10% [84]. The study of hypoxic conditions to sustain chondrocytes culture and cartilage formation in vitro is therefore another important aspect to be considered in TE.

4.1. Scaffolds for cartilage repair

Biodegradable materials have been widely used for regenerative medicine, because they are degraded and metabolized by the body after repairing the injured site or tissue [85]. These materials can be applied for the most various purposes, such as adhesion and fixation of tissues, shape maintenance, guidance of tissue regeneration or as a temporary scaffolding for tissue developed [85]. An implantable scaffold should ideally recapitulate many of the salient features of native ECM in the target tissue promoting the required conditions for tissues regeneration. Ideally, the scaffold should be biocompatible, have a highly interconnected porous network to allow for cell ingrowth and nutrient and medium exchange [86]. Also the mechanical properties should be compatible with those of the tissue, and allow a degradation rate according to cell and tissue growth in vitro or in vivo [86].

Scaffolds interconnectivity directly influences the diffusion of nutrients to cells and the waste removal of their by-products [87]. Therefore, scaffolds should possess a highly porous and interconnected structure with a large internal surface area to volume ratio to allow cell proliferation, differentiation and even distribution throughout the entire structure [88]. Surface properties are also important parameters to consider, as it is demonstrated that they influence cell adhesion, proliferation and differentiation [89]. Scaffolds mechanical properties may influence tissue formation, as these structures will support the cells while the tissue reorganization occurs, and ideally begin to degrade

when tissue is regenerated [90]. Scaffolds degradation rate affects cell behaviour [91] so it is an important aspect to be considered.

Materials processing techniques have evolved tremendously in the past years, and a large spectrum of methodologies for scaffold development are currently available. Some examples of these methods are solvent casting and salt leaching [92-94], electrospinning [95-99], fiber bonding [100-102], melt-based technologies [103-106] or hydrogels [107-112]. Scaffolds processing techniques are expected to provide a maximum control of the scaffolds structure, without affecting the materials properties.

Natural materials, synthetic or a combination of both types of materials may be used for scaffolds production. Natural polymers used in cartilage TE include for example, chitosan [113-117], starch [118-120] and silk [121-123]. Synthetic polymers for cartilage TE applications include for example, short chain saturated aliphatic polyesters, such as PLA [124, 125] or aliphatic linear polyesters such as PCL [126, 127] and poly(butylene succinate) (PBS) [103, 117]. The work present in this thesis is focused mainly on blends of natural and synthetic materials. The rationale was to test a range of scaffolds produced with different materials, blended or not, and further develop those showing stronger biological performance for the strategy of cartilage tissue formation in vitro.

4.1.1. Natural based materials

The availability in Nature of natural based materials makes them easily accessible, and allows taking advantage of specific properties of interest for the application envisaged. Chitosan is a natural polymer that has interesting properties for TE applications. It is biodegradable, biocompatible and has many structural similarities to GAGs [128]. Chitosan is obtained from chitin, the second most abundant polysaccharide, which is the major element of the shells of many crustaceans, such as shrimps or crabs [128]. Chitosan is obtained by a de-N-deacetylation of chitin [128]. This process is usually made in alkaline conditions, resulting into chitosans with different degrees of deacetylation (DD) and molecular weight [129]. The source of chitosan and the chemical modification may influence both parameters. The DD has been shown to be important for cell biocompatibility, attachment and growth [130]. The importance of DD, ranging between 76 and 90%, in cellular response of two cell lines was studied [130]. This work concluded that, in chitosan scaffolds with high DD, cells were able to attach, grow and proliferate. On the contrary, when seeded onto chitosan

scaffolds with low DD, cells were not able to attach. The amount of N-acetyl groups in chitosan has also influence in its solubility, viscosity and cristallinity [131]. Degrees of deacetylation of chitosan scaffolds ranging from 88 to 95% were tested in a study with buffalo embryonic stem cells and they have promoted a higher cell proliferation and mechanical strength of the scaffold, as well as lower biodegradation compared to the scaffolds with low DD [132]. These results may indicate that higher DD of chitosan scaffolds may be positive for biological performance.

One of the interesting properties of chitosan is its ability to be processed into porous structures, which support cell growth and ECM deposition [103]. Additionally, chitosan is reported to degrade in vivo mainly by enzymatic hydrolysis [129, 133]. Lyzozyme, one enzyme present in the human body, has been used to enhance the formation of pores in situ, in chitosan scaffolds coated with calcium phosphate [134].

Bilayered chitosan-based scaffolds were produced by particle aggregation and cultured with a L929 cell line. The scaffolds were shown to be cytocompatible and biodegradable, supporting cell growth and proliferation, and were proposed as an alternative for osteochondral TE applications [115]. Chitosan-based scaffolds have been studied for cartilage TE, and showed to promote chondrocyte proliferation and metabolic activity [113], as well as MSCs chondrogenesis in vitro [135] and in vivo [136]. Chitosan blends have been explored, combining this material with other natural or with synthetic polymers for cartilage TE. Three dimensional porous scaffolds produced with chitosan hyaluronic acid hybrid fibers were shown to support the culture and ECM deposition by rabbit chondrocytes [137].

Hydrogels have also been extensively studied for cartilage regeneration in the context of developing minimally invasive strategies. New gellan gum injectable hydrogels have been explored recently by our group for treatment of cartilage defects, with very promising results [111, 138, 139]. Our group demonstrated that gellan gum hydrogels were able to support growth of human articular chondrocytes, as well as to promote ECM deposition, when implanted subcutaneously in nude mice [138]. Our group has been developing several natural-based hydrogels, using starch [140], gellan gum [141] or chitosan [142], being the last two specifically intended for cartilage regeneration. Chitosan has also been explored for preparing hydrogels, namely a gene-activated chitosan-gelatin matrix was developed [143]. The referred matrices were capable of releasing in a controlled fashion transforming growth factor-beta 1 (TGF-β1), and promoted chondrocyte proliferation. Moreover, in a study using bovine articular

chondrocytes (BAC) encapsulated in a composite chitosan-hyaluronic hydrogel for injectable TE application, cells were able to retain their chondrocytic morphology [144]. Chondrogenic differentiation has also been observed in a hydrogel composite of oligo(poly(ethylene glycol) fumarate) with encapsulated rabbit bone marrow MSCs and gelatine microparticles loaded with TGF- β1 [145]. The same system was proposed for an intra-articular injection of chondroitin sulphate in the treatment of chondral defects in adult rabbit models [146]. Recently, a novel injectable carrageenan/fibrin/hyaluronic acid-based hydrogel with *in situ* gelling properties was used for cartilage tissue engineering [147]. When seeded with human articular chondrocytes (hACs), this injectable hydrogel was able to regenerate and repair a lesion made in bovine articular cartilage, showing the great potential of this novel delivery system for cartilage tissue engineering [147].

Another natural polymer extensively used in TE is starch. Starch is the major polysaccharide constituent of plants, being its function to store energy in form of carbohydrates. Starch is composed by 10-20% of amylase and 80-90% of amylopectin [148]. The degradation of starch based polymers is mediated by α-amylase, and their main degradation products are glucose and its derivatives, which are proved not to be harmful for cells [149]. Moreover, the degradation kinetics of starch based polymers can be controlled by the concentration of α-amylase present. In a recent work by Azevedo and Reis, 2009, α-amylase was encapsulated into starch –compounded polycaprolactone (SPCL) discs produced by compression moulding, demonstrating that the degradation kinetics of this polymer can be controlled by the amount of encapsulated enzyme into the matrix [150]. This polysaccharide can be processed by several techniques, and blended with other biomaterials and therefore, has been proposed for a wide range of biomedical applications [16, 97, 151-153].

Starch based scaffolds have been studied for bone TE [154, 155], vascularisation and endothelial cells culture [156, 157], or cartilage TE applications [119, 158]. Particularly, different types of starch scaffolds have been used in these applications, namely fiber meshes [155], combined nano- and micro-fibrous scaffolds [156] or injectable hydrogels [158]. The suitability of starch-based scaffolds for cartilage TE has been demonstrated using BAC, using dynamic culture conditions, either using SPCL fiber meshes [119] or SPCL nanofiber meshes [118].

4.1.2. Synthetic polymers

Synthetic polymers are very versatile being conveniently processed into the desired shapes. Also, they present predicable properties that can be modified according to the intended application. Poly(ε-caprolactone) is an aliphatic linear polyester, and is amongst the most utilized synthetic polymers for biomedical applications. It is biodegradable and allows several modifications aiming scaffolds production, such as for drug controlled release. The degradability of PCL is very slow [159]. However, PCL characteristics allow tailoring its degradation rate, for example by combined with natural polymers, such as starch [160]. One of the most common applications of PCL is for electrospun scaffolds, namely for cartilage applications. PCL nanofiber meshes were extensively studied for cartilage TE applications, and showed the suitability of these matrices to support the chondrogenic differentiation and ECM production either using chondrocytes [127, 161] or MSCs [126, 162].

Our group has been exploring PCL nanofiber meshes for cartilage and bone TE applications. The surface of PCL nanofiber meshes has been modified by plasma treatment, and it has been concluded that the modified nanofiber meshes effectively supported cells growth and proliferation, improving cells performance [163]. Moreover, PCL nanofiber meshes were integrated into 3D scaffolds obtained by rapid prototyping. The integration of the nanofiber meshes substantially improved the biological performance of the hierarchical scaffolds, for bone TE [164]. PCL and SPCL nanofiber meshes have also been used for the culture of BAC. It was demonstrated that both nanofiber meshes were able to sustain ECM production [118].

Poly(butylene succinate) is an aliphatic polyester presenting good mechanical properties and melt processability [104]. This polyester has been proposed by our group for the engineering of connective tissues, in form of blends with chitosan [165, 166]. The referred blends were tested for bone TE applications. It has been shown that chitosan-PBS (CPBS) scaffolds produced by compression moulding and salt leaching effectively promoted adhesion, proliferation and osteogenic differentiation of a murine mesenchymal stem cell line [167], as well as the osteogenic differentiation of human MSCs [105]. The same blend was tested for cartilage regeneration, successfully [103]. Bovine ACs have been seeded onto the same CPBS scaffolds, and a cartilagineous ECM deposition was observed [103]. Fiber meshes produced with the same blend proved to sustain BAC culture and enhance ECM deposition [168].

The scaffold is one of the key elements in our strategy for cartilage regeneration, as explained in the next section. However, other elements are essential in the strategy proposed herein, that will be further explored.

4.2. Cell types

The choice of the ideal cell type for cartilage tissue engineering is still a matter of debate in the community. Different strategies based either on chondrocytes or MSCs have been designed for cartilage TE. In the following sections we will discuss these strategies and their reported outcomes.

4.2.1. Chondrocytes

Chondrocytes, as already referred, are the only cell type present in cartilage tissue. Different types of chondrocytes have been used depending on the type of cartilage regenerative application, for example nasal [169, 170] or articular [111, 171-174]. One of the drawbacks of using chondrocytes is that these cells dedifferentiate when expanded in vitro using two-dimensional (2D) cultures [175, 176]. After several days in culture, chondrocytes in monolayer acquire a fibroblastic-like morphology [177]. Biomaterials providing a 3D environment enable the chondrocytes redifferentiation [178], being a valuable support for chondrocytes expansion in vitro.

The use of human chondrocytes isolated from biopsies collected from patients is a current clinical procedure. Some of these biopsies are from patients suffering from cartilage diseases, such as OA. The obtained chondrocytes are therefore osteoarthritic, and this phenotype may have some influence on the outcome of cartilage repair [179]. Human OA chondrocytes have been studied using 3D structures aiming to understand their biology and phenotype development [180-182]. OA chondrocytes retain some ability to undergo chondrogenic differentiation, but it is inferior when compared to age-matched normal chondrocytes, when in pellet cultures [180]. However, when cultured using scaffolds, their response may be different. For example, when human OA chondrocytes were seeded onto collagen scaffolds and implanted in SCID mice, no significant differences were found between the neo-cartilage derived from OA chondrocytes and the one from healthy donors [181]. Relevant results were also obtained when using a Hyaff®-11 membrane with normal and OA chondrocytes that were grown in this membrane [183]. The growth of chondrocytes in Hyaff®-11

membranes in this study seemed to suppress the differences between normal and OA chondrocytes.

Chondrocytes have been extensively studied in cartilage TE approaches, using the most diverse types of scaffolds [107, 110, 122, 127, 161, 184-191]. For example, a cancellous bone matrix gelatine scaffold has been proposed recently for cartilage TE [192]. The scaffold was able to support New Zealand white rabbit's chondrocyte proliferation and sustained their phenotype for up to 6 weeks. As referred previously, blends of synthetic and natural polymers have been used for cartilage TE. PLGA/ collagen hybrid scaffolds with three different structural designs were studied, comparing their effect on in vivo chondrogenesis of BAC [193]. Constructs were implanted subcutaneously in athymic nude mice, and the results showed a spatially even cell distribution, abundant ECM deposition and degradability in vivo, for all the tested scaffolds [193]. A chitosan-alginate-hyaluronate complex whose surface was modified by an Arg-Gly-Asp (RGD)-containing protein and seeded with rabbit chondrocytes was tested [194]. In vitro and in vivo cartilage formation was observed. Moreover, it was observed a complete repair of the cartilage defects in vivo after 6 months of implantation when using those systems.

For cartilage repair, chondrocyte-seeded 3D scaffolds already reached the clinic [195]. The biomaterial ensures initial mechanical stability and an appropriated 3D environment for cell proliferation and differentiation. Thus, scaffolds avoid the need to use chondral sutures, one of the major drawbacks of the ACI technique and provided the possibility of obtaining stable transplant fixation [61]. A second generation ACI TE cartilage graft based on autologous chondrocytes embedded in a 3D bioreasorbable two component gel-polymer scaffold, BioSeed®-C, has been proposed for clinical treatments of cartilage defects [196]. The implantation of this scaffold in focal osteoarthritic defects showed a significant improvement in the knee-related quality of life two years post implantation [196]. Results suggest that the implantation of BioSeed®-C may be an effective treatment option for this kind of pathologies [196].

4.2.2. Mesenchymal stem cells

Stem cells have become one the main cell source for tissue repair, as they present major advantages for regenerative medicine when compared to differentiated cells. MSCs can be isolated from many types of adult tissues, and are characterized by their ability to undergo extensive self-renewal *in vitro* and to go through multilineage

differentiation [197]. Unlike chondrocytes, these cells are available in virtually unlimited numbers, and do not loose their phenotype during expansion [198]. Those cells were already tested successfully in animal models of arthritis [199]. Importantly, they secrete or can be genetically manipulated to secrete immunosuppressive factors and therapeutic agents that target inflammatory tissues [200]. This ability enables MSCs to migrate into a damaged site, supporting their interest to be included in tissue engineered constructs. More details in those issues can be obtained in references [201] and [202]. The challenge in using MSCs as a cell source for articular cartilage repair is to maintain the MSC-derived chondrocytes in the pre-hypertrophic state and prevent them from undergoing terminal differentiation onto the osteogenic phenotype [203].

Different scaffolds have been designed to support and stimulate MSCs chondrogenic differentiation. The scaffold configuration is believed to be very important for the tissue formation and maintenance of its integrity. Recently, three types of scaffolds of poly (ethyleneglycol-terephtalate) - poly (butylene terephtalate) (PEGT/PBT) with various configurations were analysed for the support of adipose derived MSCs [204]. This study showed that manipulation of the scaffolds configuration allows controlling a uniform tissue formation at a microscale. Modification of the scaffold surface can be used to enhance MSCs performance [204]. Recently, a collagen surface modification of PCL-based porous scaffolds was studied for supporting MSCs chondrogenic differentiation [205]. It was shown that surface modification enhanced chondrogenic differentiation of MSCs, when compared to PCL scaffolds without modification. PCL nanofiber scaffolds were tested in vivo for cartilage TE using periosteal cells of rabbits [206]. These membranes were implanted under the periosteum of 6 months old rabbits. After different time periods, constructs were removed, separated from the periosteum and cultured for 6 weeks under chondrogenic conditions. This work showed that is possible to produce engineered cartilage in vitro from PCL nanofibrous scaffolds with periosteal cells, after a period of in vivo pre-culturing [206].

Hydrogels and injectable systems have been a subject of intense investigation for the repair of cartilage and/ or osteochondral defects [109, 111, 112, 114, 139, 207-210]. The rationale of these systems is to provide a minimally invasive technique to repair cartilage defects. Hydrogels or injectable particles combined with MSCs may be introduced into the injured site, avoiding invasive surgery techniques. Microcarriers have been proposed for this approach [211]. Pharmacological active microcarriers (PAM) were prepared from PLGA, coated with fibronectin and engineered to release

transforming growth factor – beta 3 (TGF-β3) [211] and provide an appropriate environment for cartilage formation in vivo. Human MSCs were incubated with these particles, and afterwards were implanted in intramuscular or subcutaneous sites in SCID mice. These particles were shown to provide a suitable environment for MSCs chondrogenic differentiation and to be a feasible carrier for biomedical cartilage repair applications [211].

The type of cell chosen for a TE strategy may influence the outcome of the regenerative strategy. In the case of cartilage both chondrocytes and MSCs are the most used types of cells. The reported results for chondrocytes and/ or MSCs indicate that these cells are able to produce cartilage ECM in vitro, but a long road lies ahead until the objective of regenerating in vitro a fully functional articular cartilage, similar to the native one, is achieved.

5. In vitro enhanced culturing conditions

5.1. Bioreactors

The use of bioreactors may substantially enhance the maturation of constructs in vitro. There are multiple types of bioreactors, aiming different applications such as bone [212-214] or cartilage [79, 215-217]. Bioreactors are very useful in providing biophysical stimulation and facilitating nutrient diffusion for the cells. Additionally, bioreactors provide a closed and controlled environment for cell culture, enhancing the supply of oxygen and nutrients. The mechanical stimulus of a bioreactor may also have beneficial effects over cell growth or ECM production. Different bioreactors have been developed for various TE applications, such as spinner flasks, rotating wall vessels or perfusion bioreactors. Further details on this topic can be found elsewhere in [218], [219] or [220].

5.1.1. Basic bioreactor systems

Spinner flasks are one of the simplest types of bioreactor. They are mainly used for cell seeding of scaffolds [103, 119, 221], although some published reports cover its use for subsequent culture of constructs [73, 204, 222]. In spinner flasks, a magnetic stirrer is used to swirl a cell suspension in a plastic or glass bottle, composed by metallic

harms that hold the scaffolds static in respect to the moving medium (Figure 1.3). Spinner flasks can provide a controlled and reproducible method for cell seeding. However, when using scaffolds with low porosity, cells may have difficulty in penetrate inside the scaffold structure, remaining at its surface and therefore resulting in non-uniform cell distribution [223]. During the seeding stage, cells are driven into the scaffold, and the medium mixing enhances mass transfer of nutrients, but also generates turbulence that may be detrimental for the cultures [224]. The culture of bovine chondrocytes in spinner flasks using poly(glycolic acid) (PGA) scaffolds, compared to static culture conditions resulted in higher fractions of released GAGs, but the net level of accumulated GAGs in the construct was reduced [225]. Some studies show that constructs exposed to turbulent flow originated a fibrous capsule at the construct surface [215, 226]. However, culture of the same constructs in rotating bioreactors having laminar flow, increased the amount of GAGs and showed improved results in terms of cartilage production [226, 227].

Other simple device commonly used to seed cells is the orbital shaker (Figure 1.3C), where petri dishes are placed and constructs are subjected to fluid flow. This device may be used either for cell seeding and/or for dynamic cultures [103, 118, 221, 228, 229], but presents similar limitations to the ones referred for spinner flasks.

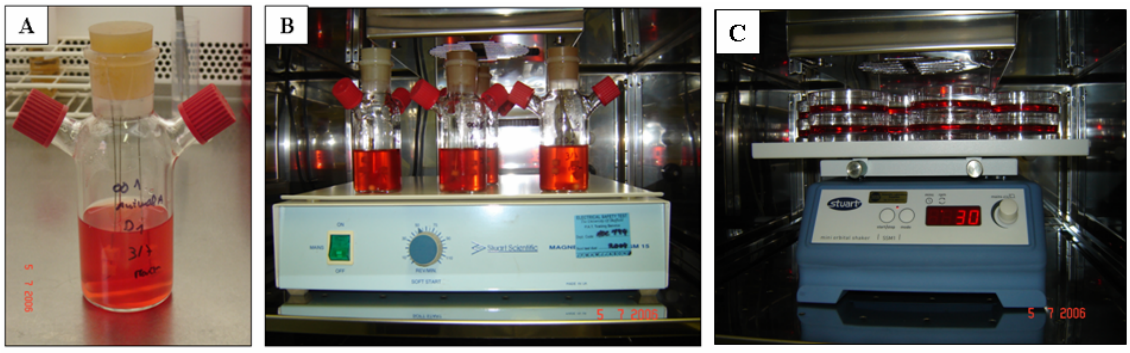

Figure 1.3 – Basic systems for cell seeding and dynamic culture of constructs. A – Individual spinner flask containing a cell suspension in culture medium and holding CPBS scaffolds. B – Spinner flasks in culture inside an incubator. C – Orbital shaker in culture.

Rotating wall vessel (RWV) bioreactors are based in the culture medium rotation coordinated with the scaffold floating in the culture medium. These bioreactors are composed by a rotating cylinder, which is completely filled with culture medium, and

the fluid rotates horizontally by the friction at the cylinder wall (Figure 1.4). The oxygen is provided by a silicone membrane that is wrapped around the central cylinder.

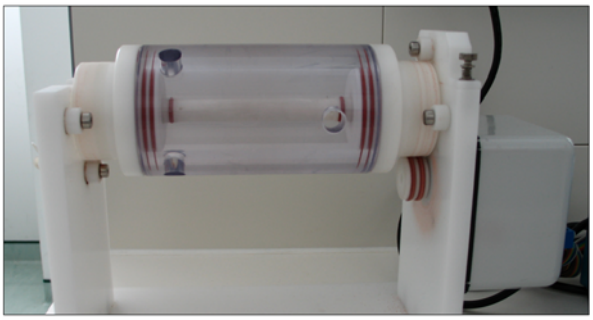

Figure 1.4 – Rotating wall vessel bioreactor.

The RWV presents advantages relatively to spinner flasks systems [215]. The dynamic flow in these bioreactors is laminar, and not turbulent as opposed to the stirring in spinner flasks, eventually supporting the formation of more homogenous cartilagineous tissues [215].

5.1.2. Perfusion bioreactors

Perfusion bioreactor systems apply a direct flow of culture medium across the scaffold porous structure facilitating mass transfer. These systems allow overcoming the limitations of the previously referred systems and provide a continuous exchange of culture medium. Different variants of perfusion bioreactors have been proposed, such as column, hollow-fiber or microfluidic bioreactors [220]. One of the main concerns when using perfusion systems is the washing out of the cells. To avoid this undesired effect, cells are usually previously seeded and cultured in the scaffolds or encapsulated, and when the fluid passes through the construct, cells keep attached to the scaffold. The flow in perfusion bioreactors may be bidirectional or unidirectional, depending on the bioreactor design. Our group has recently developed a bidirectional flow perfusion bioreactor, consisting of a common inlet and outlet, dividing the flowing medium by 20 individual culture chambers. The bioreactor was designed in a way that allows culture medium circulation throughout the chambers and thus, across the scaffolds in culture, ensuring an even distribution by the individual flow chambers (Figure 1.5). The referred bioreactor is currently protected by a Portuguese patent, and an European patent is pending. Detailed information about the device can be found elsewhere [230]. The bidirectional flow perfusion bioreactor was used for cartilage TE applications, using

human MSCs. PCL nanofiber meshes were seeded with human BMSCs for chondrogenic differentiation, by the stimulation provided by the bioreactor, and it was demonstrated that this is a valid model for this type of culture [231]. Additionally, when using chitosan-based scaffolds seeded with human BMSCs for chondrogenic differentiation, the bioreactor samples displayed significantly lower collagen type I expression, compared to the static controls [232]. The expression of several cartilage related genes, as well as the level of ECM deposition were positively affected in the referred samples [232].

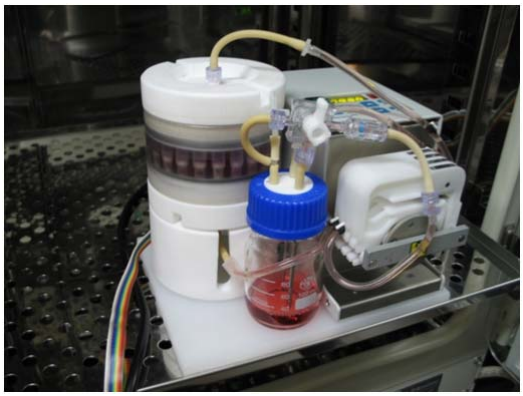

Figure 1.5 – Bidirectional flow perfusion bioreactor developed in the 3B´s Research Group [230].

Dynamic culture conditions were shown in the literature to improve the chondrogenic differentiation and ECM production when compared to static culture conditions [73, 224, 227, 233]. In a work using perfusion-type of recirculation bioreactors operated with periodic medium flow reversal, chondrogenic differentiation of human adipose MSCs in PGA scaffolds was successfully achieved [217]. When compared to pellet cultures, dynamic culture conditions were shown to produce significantly higher amounts of GAGs and collagen [217]. Dynamic culture conditions may also help in maintaining the phenotype of differentiated chondrocytes, without undergoing hypertrophy. Those results were shown in a study using ATDC5 cell line seeded onto chitosan scaffolds and cultured in a rotating bioreactor with perfusion (RCMW) [75]. A rotating wall vessel bioreactor was used for chondrocyte cultures onto nanofibrous scaffolds, showing that constructs grown in the bioreactor produced more GAGs and expressed higher levels of cartilage related genes, compared to static cultures [76]. The applicability of electrospun nanofiber meshes for flow perfusion bioreactors was

recently reported, when using electrospun PCL microfiber scaffolds coated with cartilagineous ECM [234]. Flow perfusion bioreactors are being studied for the scale-up of human cartilage grafts for joint repair [82]. Recent results shown the large-scale production of cartilage grafts in bioreactors for clinical treatments, and that in combination with specific surgical therapies may support the long term development and engrafting of the implant [82].

A different concept for the culture of autologous cartilage was proposed recently, being named as “in vivo bioreactor” (IVB) [235]. The referred strategy for de novo engineering of articular cartilage in the subperiosteal space involves the injection of a biocompatible gel. The obtained results were considered very promising, and authors claim that this can be a valid clinical option for obtaining successful cartilage repair [235].

The importance of the correct stimulus in a cartilage regeneration strategy has been herein described. In our point of view, bioreactors are a source of mechanical stimulus that should be explored for cartilage cultures in vitro.

5.2. Hypoxia

The application of hypoxia has been explored attempting to mimic the in vivo cartilage niche microenvironment. Oxygen levels in this tissue are low and when a change in oxygen levels occurs chondrocytes experience several effects on their phenotype, genetic expression and morphology [236]. Hypoxia conditions have been reported to stimulate chondrogenesis and synthesis of ECM components. It was shown to be beneficial for chondrocytes and augmented ECM deposition, either seeded in scaffolds [237] or not [238]. The same positive effect were also observed for the chondrogenesis of human MSCs [239, 240], as well as for other species MSCs, like rat or bovine [241-244].

Low oxygen levels have been shown to strongly influence the chondrogenic differentiation of stem cells [245]. The manipulation of oxygen levels has proved to be useful in directing stem cell through specific cell lineages, namely chondrocytes and cardiomyocytes [246]. As the oxygen plays a central role in chondrocyte metabolism, exploration of oxygen levels in cultures opens a large window of study. There are still many questions involving the application of low oxygen levels when inducing chondrogenic differentiation of stem cells. Under 5% of oxygen, human adipose MSCs

proliferative potential decreases, while their chondrogenic ability increases [247]. Some studies sustain that a hypoxic pre-conditioning step may be useful to improve stem cell differentiation into the chondrogenic lineage [241, 248, 249]. This aspect is not consistent in the literature, since other authors showed that the chondrogenic ability of human adipose MSCs cultured in pellets did not improve on hypoxic pre-conditioning [250].

6. Co-cultures

Tissues are highly complex and organized structures that comprise cells and matrix, and that interact with each other to maintain the tissue function and integrity. Moreover, cells and matrix are constantly interacting with surrounding tissues and environment. Thus, it is believed that those conditions may help developing a successful TE approach. So, it is relevant to understand and recreate as close as possible this environment, to study those interactions and use them actively to obtain a full tissue repair. Co-cultures allow the study of cell-cell communication and interactions, helping to understand the way different types of cells interact with each other in their niche. Thus, it is important to understand the influence of differentiated cells over undifferentiated cells. Chondrocytes in the cartilage tissue are distributed in four distinct zones or layers, thus their cellular interactions in vivo depend on their spatial location. Cartilage comprises the superficial, transitional, radial and tight zones [251]. Chondrocytes in the superficial zone interact with the synovial fluid and cells, whereas chondrocytes in the transitional and radial zones interact mainly with each other and with the ECM. Chondrocytes in the tide mark zone are located in the cartilage-bone interface, thus contacting with osteoblasts and bone matrix [252]. The cell-cell contact between chondrocytes and other cell types occurs also in the transition with other neighbouring tissues. Understanding the communication mechanisms underlying these contacts may provide valid and new strategies for cartilage regeneration. Table 1.3 describes different co-culture studies for cartilage TE, using several types of cells and diverse ways of co-culturing them. There are two main approaches for performing co-cultures: direct contact of cells (when the culture involves mixing both types of cells), or indirect (using conditioned media). Conditioned medium from chondrocytes has been successfully used to induce undifferentiated cells to follow the chondrogenic lineage

[172, 253-256]. The principle behind using conditioned culture medium is the ability of chondrocytes to release morphogenetic signals to the culture medium, that may be able to induce the chondrogenic differentiation of MSCs [83, 253, 257].

Cartilage inflammatory diseases such as OA or RA have been studied by co-culturing chondrocytes with synovial fluid cells [258-261] or with osteoblasts [80, 262-265]. In RA, formation of the chondrosynovial pannus causes modifications on the chondrosynovial joint, as some layers of cells begin to deposit covering the cartilage surface [266]. Both chondrocytes and synovial cells get in close contact by the deposition of these layers. Evidence of positive direct intercellular communication between these cells was previously reported [267]. Later, it was demonstrated that synoviocytes release factors to the culture medium that can stimulate chondrogenic differentiation [268, 269]. A recent study using direct and indirect co-cultures of bovine synovial fibroblasts and chondrocytes suggests that synovial supernatants and synovial fibroblasts may modulate chondrocytes biosynthetic activity and matrix deposition [259]. Direct contact co-cultures of human BMSCs and articular chondrocytes (ACs) using alginate/chitosan scaffolds in static conditions or using bioreactors have been established for the study of chondrogenesis and osteogenesis [80, 263]. The influence of cells on different degrees of osteogenic differentiation over the chondrogenic differentiation of MSCs was recently demonstrated [262]. The results shown that MSCs in different states of osteogenic preculture and encapsulated in a biodegradable hydrogel could positively influence the chondrogenic differentiation of rat MSCs, both in the presence or in the absence of growth factors [262].

These studies showed the importance of a 3D microenvironment in the communication of different cell types, indicating the potential of controlling these interactions to create specific tissues.

Table 1.3 – Outcome of in vitro co-cultures with several types of cells and different culture systems intended to promote cartilage tissue engineering.

Cells involved	Type of culture	Outcome	Ref.
Human dermal fibroblasts and porcine ACs	Direct contact in PLA/ PGA biphasic scaffolds	Some dermal fibroblasts induced cartilage lacuna structure formation	[124]
Murine myoblasts (C2C12) and rat chondrosarcoma (RCS) cell lines	2D; 3D cultures in collagen gels using conditioned medium from muscle cells	Muscle cells conditioned medium led to increased cartilaginous ECM production	[256]
Human embryonic stem cells and human chondrocytes	Indirect co-culture using well inserts (conditioned medium from chondrocytes)	Culture micro-environment influences embryonic stem cells differentiation to chondrocytes	[270]
Bovine MSCs and ACs	a) Micromass cultures of MSCs incubated with chondrocytes conditioned medium b) MSCs expanded in chondrocytes conditioned medium encapsulated in agarose hydrogels and implanted subcutaneously in athimic mice	a) Incubation with conditioned medium enhanced cartilage production by MSCs b) ECM deposition characteristic of neocartilage formation	[83]
Murine embryonic stem cells and human hepatocarcinoma cell line	Micromass pellets of embryonic stem cells cultured with conditioned medium from cell line	Enhanced chondrogenic differentiation of embryonic stem cells	[271]
Human ACs and human periosteal tissue	Co-cultures of ACs and periosteal tissue, cultures of ACs with conditioned medium from chondrocytes in agarose gels	ACs formed clones in agarose; the periosteum can stimulate ACs clonal growth and differentiation	[172]
Human ACs in different passages	Indirect co-cultures in filter inserts	The addition of primary chondrocytes to passaged cells resulted in an increase in cartilage formation	[272]
Equine MSCs and equine ACs	Direct co-cultures of equine MSCs and ACs	MSCs chondrogenic differentiation was enhanced by coculture with ACs	[273]

7. Concluding remarks

Cartilage regeneration is a demanding task that until now has not been fully accomplished. Some treatments based on TE concepts are available in the market, but still need improvement.

To regenerate fully functional articular cartilage using a TE strategy, several components need to be brought together, such as the scaffold, the culture conditions, cell-cell contacts and signalling pathways or oxygen tension. In this chapter, we highlighted the most important aspects that, in our point of view, need to be considered and optimized. From the literature review we could draw some conclusions and also to identify some key points that should be object of further study, in a cartilage regeneration approach. The scaffold morphology and composition, as well as the material in which it is based were explored herein, and will be studied in the works developed in the present thesis. Moreover, culture conditions have been proved to have positive or negative effects on cell cultures. We will explore dynamic cultures to determine their effect on the models proposed in this thesis. The referred models consist on different types of primary cultures of differentiated or undifferentiated cells, induced for chondrogenesis. The outcomes of using either chondrocytes or MSCs have been highlighted in this chapter.

This literature review is intended to provide a background perspective of the progress of the scientific community, and introducing to the main topics that will be further explored in the experimental work reported in the next sections of this thesis.

References

1 - Kronenberg HM. *Developmental regulation of the growth plate*. Nature. 2003. 423(6937):332-6.

2 - Hall BK, Miyake T. *All for one and one for all: Condensations and the initiation of skeletal development*. Bioessays. 2000. 22(2):138-47.

3 - Evans CH, Ghivizzani SC, Robbins PD. *Orthopedic gene therapy in 2008*. Mol Ther. 2009. 17(2):231-44.

4 - Schulz RM, Bader A. *Cartilage tissue engineering and bioreactor systems for the cultivation and stimulation of chondrocytes*. Eur Biophys J. 2007. 36(4-5):539-68.

5 - Andersson GB, Bouchard J, Bozic KJ, Campbell R, Cisternas MG, Correa A*, et al. Chapter 4 - Arthritis and related conditions.* The burden of musculoskeletal diseases in the United States. Rosemont, IL: American Academy of Orthopaedic Surgeons; 2008. p. 71-96.

6 - Andersson GB, Bouchard J, Bozic KJ, Campbell R, Cisternas MG, Correa A*, et al. Chapter 6 - Musculoskeletal injuries.* The burden of musculoskeletal diseases in the United States. Rosemont, IL: American Academy of Orthopaedic Surgeons; 2008. p. 123-62.

7 - Klareskog L, van der Heijde D, de Jager JP, Gough A, Kalden J, Malaise M*, et al. Therapeutic effect of the combination of Etanercept and Methotrexate compared with each treatment alone in patients with rheumatoid arthritis: Double-blind randomised controlled trial*. Lancet. 2004. 363(9410):675-81.

8 - Rat AC, Boissier MC. *Rheumatoid arthritis: Direct and indirect costs*. Joint Bone Spine. 2004. 71(6):518-24.

9 - Konttinen YT, Seitsalo S, Lehto M, Santavirta S. *Current management: Management of rheumatic diseases in the era of biological anti-rheumatic drugs*. Acta Orthop. 2005. 76(5):614-9.

10 - Brittberg M. *Articular cartilage repair in the knee joint with autologous chondrocytes and periosteal graft*. Orthopedics and Traumatology. 2001. 3:185 - 94.

11 - Drobnic M, Kregar-Velikonja N, Radosavljevic D, Gorensek M, Koritnik B, Malicev E*, et al. The outcome of autologous chondrocyte transplantation treatment of cartilage lesions in the knee*. Cell Mol Biol Lett. 2002. 7(2):361-3.

12 - Friedrich KM, Mamisch TC, Plank C, Langs G, Marlovits S, Salomonowitz E*, et al. Diffusion-weighted imaging for the follow-up of patients after matrix-associated autologous chondrocyte transplantation*. Eur J Radiol. 2010. 73(3):622-8.

13 - Welsch GH, Trattnig S, Hughes T, Quirbach S, Olk A, Blanke M*, et al. T2 and T2* mapping in patients after matrix-associated autologous chondrocyte transplantation: Initial results on clinical use with 3.0-tesla MRI*. Eur Radiol. 2010. 20(6):1515-23.

14 - Steinwachs M. *New technique for cell-seeded collagen-matrix-supported autologous chondrocyte transplantation*. Arthroscopy. 2009. 25(2):208-11.

15 - Gomes ME, Reis RL, Cunha AM, Blitterswijk CA, de Bruijn JD. *Cytocompatibility and response of osteoblastic-like cells to starch-based polymers: Effect of several additives and processing conditions*. Biomaterials. 2001. 22(13):1911-7.

16 - Marques AP, Reis RL, Hunt JA. *The biocompatibility of novel starch-based polymers and composites: In vitro studies*. Biomaterials. 2002. 23(6):1471-8.

17 - Correlo VM, Pinho ED, Pashkuleva I, Bhattacharya M, Neves NM, Reis RL. *Water absorption and degradation characteristics of chitosan-based polyesters and hydroxyapatite composites*. Macromol Biosci. 2007. 7(3):354-63.

18 - Brochhausen C, Lehmann M, Halstenberg S, Meurer A, Klaus G, Kirkpatrick CJ. *Signalling molecules and growth factors for tissue engineering of cartilage-what can we learn from the growth plate?* J Tissue Eng Regen Med. 2009. 3(6):416-29.

19 - Karsenty G, Kronenberg HM, Settembre C. *Genetic control of bone formation*. Annu Rev Cell Dev Biol. 2009. 25:629-48.

20 - Karsenty G. *Transcriptional control of skeletogenesis*. Annu Rev Genomics Hum Genet. 2008. 9:183-96.

21 - Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. *Sox9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene*. Mol Cell Biol. 1997. 17(4):2336-46.

22 - Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. *The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6*. Genes Dev. 2002. 16(21):2813-28.

23 - Okubo Y, Reddi AH. *Thyroxine downregulates Sox9 and promotes chondrocyte hypertrophy*. Biochem Biophys Res Commun. 2003. 306(1):186-90.

24 - Gilbert S. *Paraxial and intermediate mesoderm*. Sixth edition ed. Sunderland (MA): Sinauer Associates; 2000.

25 - Bhosale AM, Richardson JB. *Articular cartilage: Structure, injuries and review of management*. Br Med Bull. 2008. 87:77-95.

26 - Hunziker EB, Kapfinger E, Geiss J. *The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development*. Osteoarthritis Cartilage. 2007. 15(4):403-13.

27 - Pitsillides AA, Ashhurst DE. *A critical evaluation of specific aspects of joint development*. Dev Dyn. 2008. 237(9):2284-94.

28 - Archer CW, Dowthwaite GP, Francis-West P. *Development of synovial joints*. Birth Defects Res C Embryo Today. 2003. 69(2):144-55.

29 - Davis AP, Capecchi MR. *A mutational analysis of the 5' Hoxd genes: Dissection of genetic interactions during limb development in the mouse*. Development. 1996. 122(4):1175-85.

30 - Edwards CJ, Francis-West PH. *Bone morphogenetic proteins in the development and healing of synovial joints*. Semin Arthritis Rheum. 2001. 31(1):33-42.

31 - Heath CA, Magari SR. *Mini-review: Mechanical factors affecting cartilage regeneration in vitro*. Biotechnol Bioeng. 1996. 50(4):430-7.

32 - Schulze-Tanzil G, Muller RD, Kohl B, Schneider N, Ertel W, Ipaktchi K, *et al. Differing in vitro biology of equine, ovine, porcine and human articular chondrocytes derived from the knee joint: An immunomorphological study*. Histochem Cell Biol. 2009. 131(2):219-29.

33 - Schulze-Tanzil G. *Activation and dedifferentiation of chondrocytes: Implications in cartilage injury and repair*. Ann Anat. 2009. 191(4):325-38.

34 - Buckwalter JA, Mankin HJ. *Articular cartilage: Tissue design and chondrocyte-matrix interactions*. Instr Course Lect. 1998. 47:477-86.

35 - Watanabe H, Yamada Y, Kimata K. *Roles of aggrecan, a large chondroitin sulfate proteoglycan, in cartilage structure and function*. J Biochem. 1998. 124(4):687-93.

36 - Kavanaugh A. *Economic consequences of established rheumatoid arthritis and its treatment*. Best Pract Res Clin Rheumatol. 2007. 21(5):929-42.

37 - Yen JH. *Treatment of early rheumatoid arthritis in developing countries. Biologics or disease-modifying anti-rheumatic drugs?* Biomed Pharmacother. 2006. 60(10):688-92.

38 - Finckh A, Gabay C. *At the horizon of innovative therapy in rheumatology: New biologic agents*. Curr Opin Rheumatol. 2008. 20(3):269-75.

39 - Stubgen JP. *Tumor necrosis factor-alpha antagonists and neuropathy*. Muscle Nerve. 2008. 37(3):281-92.

40 - Diaz-Borjon A, Weyand CM, Goronzy JJ. *Treatment of chronic inflammatory diseases with biologic agents: Opportunities and risks for the elderly*. Exp Gerontol. 2006. 41(12):1250-5.

41 - Bertele V, Assisi A, Di Muzio V, Renzo D, Garattini S. *New antirheumatic drugs: Any real added value? A critical overview of regulatory criteria for their marketing approval*. Eur J Clin Pharmacol. 2007. 63(9):879-89.

42 - Mor A, Pillinger MH, Wortmann RL, Mitnick HJ. *Drug-induced arthritic and connective tissue disorders*. Semin Arthritis Rheum. 2007.

43 - Ravindran V, Scott DL, Choy EH. *A systematic review and meta-analysis of efficacy and toxicity of disease modifying anti-rheumatic drugs and biological agents for psoriatic arthritis*. Ann Rheum Dis. 2008. 67(6):855-9.

44 - Meyer O, de Bandt M, Berthelot JM, Cantagrel A, Combe B, Fautrel B, *et al. Clinical practice format for choosing a second-line disease modifying anti-rheumatic drug in early rheumatoid arthritis after failure of 6 months' first-line DMARD therapy*. Joint Bone Spine. 2007. 74(1):73-8.

45 - Wang D, Miller SC, Sima M, Parker D, Buswell H, Goodrich KC*, et al. The arthrotropism of macromolecules in adjuvant-induced arthritis rat model: A preliminary study*. Pharmaceutical research. 2004. 21(10):1741-9.

46 - Anitua E, Sanchez M, Orive G, Andia I. *Delivering growth factors for therapeutics*. Trends Pharmacol Sci. 2008. 29(1):37-41.

47 - Puebla P, Pastoriza P, Barcia E, Fernandez-Carballido A. *PEG-derivative effectively modifies the characteristics of indomethacin-PLGA microspheres destined to intra-articular administration*. J Microencapsul. 2005. 22(7):793-808.

48 - Balmayor ER, Tuzlakoglu K, Azevedo HS, Reis RL. *Preparation and characterization of starch-poly-epsilon-caprolactone microparticles incorporating bioactive agents for drug delivery and tissue engineering applications*. Acta Biomater. 2009. 5(4):1035-45.

49 - Howard KA, Paludan SR, Behlke MA, Besenbacher F, Deleuran B, Kjems J. *Chitosan/siRNA nanoparticle-mediated TNF-alpha knockdown in peritoneal macrophages for anti-inflammatory treatment in a murine arthritis model*. Mol Ther. 2009. 17(1):162-8.

50 - Silva GA, Costa FJ, Neves NM, Coutinho OP, Dias ACP, Reis RL. *Entrapment ability and release profile of corticosteroids from starch-based microparticles*. Journal of Biomedical Materials Research Part A. 2005. 73A(2):234-43.

51 - Marques AP, Reis RL, Hunt JA. *Cytokine secretion from mononuclear cells cultured in vitro with starch-based polymers and poly-L-lactide*. J Biomed Mater Res A. 2004. 71(3):419-29.

52 - Silva GA, Coutinho OP, Ducheyne P, Shapiro IM, Reis RL. *Starch-based microparticles as vehicles for the delivery of active platelet-derived growth factor*. Tissue Engineering. 2007. 13(6):1259-68.

53 - Silva GA, Coutinho OP, Ducheyne P, Shapiro IM, Reis RL. *The effect of starch and starch-bioactive glass composite microparticles on the adhesion and expression of the osteoblastic phenotype of a bone cell line*. Biomaterials. 2007. 28(2):326-34.

54 - Wang Q, Zhang N, Hu X, Yang J, Du Y. *Chitosan/starch fibers and their properties for drug controlled release*. Eur J Pharm Biopharm. 2007. 66(3):398-404.

55 - Elfstrand L, Eliasson AC, Wahlgren M. *The effect of starch material, encapsulated protein and production conditions on the protein release from starch microspheres*. J Pharm Sci. 2009. 98(10):3802-15.

56 - Simi CK, Emilia Abraham T. *Hydrophobic grafted and cross-linked starch nanoparticles for drug delivery*. Bioprocess Biosyst Eng. 2007. 30(3):173-80.

57 - Malafaya PB, Elvira C, Gallardo A, San Roman J, Reis RL. *Porous starch-based drug delivery systems processed by a microwave route*. J Biomater Sci Polym Ed. 2001. 12(11):1227-41.

58 - Balmayor ER, Tuzlakoglu K, Marques AP, Azevedo HS, Reis RL. *A novel enzymatically-mediated drug delivery carrier for bone tissue engineering applications: Combining biodegradable starch-based microparticles and differentiation agents*. J Mater Sci Mater Med. 2008. 19(4):1617-23.

59 - Silva GA, Coutinho OP, Ducheyne P, Shapiro IM, Reis RL. *Starch-based microparticles as vehicles for the delivery of active platelet-derived growth factor*. Tissue Eng. 2007. 13(6):1259-68.

60 - Sittinger M, Burmester GR. *Can engineered cartilage transplants be used for treating rheumatic diseases?* Nature clinical practice. 2006. 2(4):172-3.

61 - Ringe J, Haupl T, Sittinger M. *Future of tissue engineering in rheumatic diseases*. Expert opinion on biological therapy. 2007. 7(3):283-7.

62 - Steadman JR, Rodkey WG, Rodrigo JJ. *Microfracture: Surgical technique and rehabilitation to treat chondral defects*. Clin Orthop Relat Res. 2001. (391 Suppl):S362-9.

63 - Marlovits S, Zeller P, Singer P, Resinger C, Vecsei V. *Cartilage repair: Generations of autologous chondrocyte transplantation*. Eur J Radiol. 2006. 57(1):24-31.

64 - Knutsen G, Drogset JO, Engebretsen L, Grontvedt T, Isaksen V, Ludvigsen TC, *et al. A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years*. The Journal of bone and joint surgery. 2007. 89(10):2105-12.

65 - Estes BT, Wu AW, Guilak F. *Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6*. Arthritis and rheumatism. 2006. 54(4):1222-32.

66 - Ringe J, Sittinger M. *Tissue engineering in the rheumatic diseases*. Arthritis Res Ther. 2009. 11(1):211.

67 - Trattnig S, Pinker K, Krestan C, Plank C, Millington S, Marlovits S. *Matrix-based autologous chondrocyte implantation for cartilage repair with Hyalograft©: Two-year follow-up by magnetic resonance imaging*. Eur J Radiol. 2006. 57(1):9-15.

68 - Behrens P, Bitter T, Kurz B, Russlies M. *Matrix-associated autologous chondrocyte transplantation/implantation (MACT/MACI)-5-year follow-up*. Knee. 2006. 13(3):194-202.

69 - Pashkuleva I, Marques AP, Vaz F, Reis RL. *Surface modification of starch based blends using potassium permanganate-nitric acid system and its effect on the adhesion and proliferation of osteoblast-like cells*. J Mater Sci Mater Med. 2005. 16(1):81-92.

70 - Yu LM, Kazazian K, Shoichet MS. *Peptide surface modification of methacrylamide chitosan for neural tissue engineering applications*. J Biomed Mater Res A. 2007. 82(1):243-55.

71 - Teo WE, He W, Ramakrishna S. *Electrospun scaffold tailored for tissue-specific extracellular matrix*. Biotechnol J. 2006. 1(9):918-29.

72 - Freed LE, Martin I, Vunjak-Novakovic G. *Frontiers in tissue engineering - in vitro modulation of chondrogenesis*. Clinical Orthopaedics and Related Research. 1999. 367S:S46 - S58.

73 - Vunjak-Novakovic G, Obradovic B, Martin I, Bursac PM, Langer R, Freed LE. *Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering*. Biotechnol Prog. 1998. 14(2):193-202.

74 - Griffon DJ, Sedighi MR, Sendemir-Urkmez A, Stewart AA, Jamison R. *Evaluation of vacuum and dynamic cell seeding of polyglycolic acid and chitosan scaffolds for cartilage engineering*. Am J Vet Res. 2005. 66(4):599-605.

75 - Tigli RS, Gumusderelioglu M. *Chondrogenesis on BMP-6 loaded chitosan scaffolds in stationary and dynamic cultures*. Biotechnol Bioeng. 2009. 104(3):601-10.

76 - Li WJ, Jiang YJ, Tuan RS. *Cell-nanofiber-based cartilage tissue engineering using improved cell seeding, growth factor, and bioreactor technologies*. Tissue Eng Part A. 2008. 14(5):639-48.

77 - Démarteau O, Jakob M, Schäfer D, Heberer M, Martin I. *Development and validation of a bioreactor for physical stimulation of engineered cartilage*. Biorheology. 2003. 40:331 – 6.

78 - Tognana E, Padera RF, Chen F, Vunjak-Novakovic G, Freed LE. *Development and remodeling of engineered cartilage-explant composites in vitro and in vivo*. Osteoarthritis Cartilage. 2005. 13(10):896-905.

79 - Mahmoudifar N, Doran PM. *Effect of seeding and bioreactor culture conditions on the development of human tissue-engineered cartilage*. Tissue Eng. 2006.

80 - Pound JC, Green DW, Roach HI, Mann S, Oreffo RO. *An ex vivo model for chondrogenesis and osteogenesis*. Biomaterials. 2007. 28(18):2839-49.

81 - Mahmoudifar N, Doran PM. *Tissue engineering of human cartilage in bioreactors using single and composite cell-seeded scaffolds*. Biotechnol Bioeng. 2005. 91(3):338-55.

82 - Santoro R, Olivares AL, Brans G, Wirz D, Longinotti C, Lacroix D, *et al. Bioreactor based engineering of large-scale human cartilage grafts for joint resurfacing*. Biomaterials. 2010.

83 - Hwang NS, Varghese S, Puleo C, Zhang Z, Elisseeff J. *Morphogenetic signals from chondrocytes promote chondrogenic and osteogenic differentiation of mesenchymal stem cells*. J Cell Physiol. 2007. 212(2):281-4.

84 - Henderson JH, Ginley NM, Caplan AI, Niyibizi C, Dennis JE. *Low oxygen tension during incubation periods of chondrocyte expansion is sufficient to enhance postexpansion chondrogenesis*. Tissue Eng Part A. 2010. 16(5):1585-93.

85 - Suzuki M, Ikada Y. *Biodegradable polymers in medicine.* In: Reis R, San Roman J, editors. Biodegradable systems in tissue engineering and regenerative medicine. Boca Raton, Florida, USA: CRC Press; 2005. p. 3-12.

86 - Hutmacher DW. *Scaffolds in tissue engineering bone and cartilage*. Biomaterials. 2000. 21:2529 - 43.

87 - Mikos AG, Sarakinos G, Lyman MD, Ingber DE, Vacanti JP, Langer R. *Prevascularization of porous biodegradable polymers*. Biotechnol Bioeng. 1993. 42(6):716-23.

88 - Leong KF, Cheah CM, Chua CK. *Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs*. Biomaterials. 2003. 24(13):2363-78.

89 - Yan LP, Wang YJ, Ren L, Wu G, Caridade SG, Fan JB*, et al. Genipin-cross-linked collagen/chitosan biomimetic scaffolds for articular cartilage tissue engineering applications*. J Biomed Mater Res A. 2010. 95(2):465-75.

90 - Karageorgiou V, Kaplan D. *Porosity of 3D biomaterial scaffolds and osteogenesis*. Biomaterials. 2005. 26(27):5474-91.

91 - Wang Y, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL. *In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells*. Biomaterials. 2005. 26(34):7082-94.

92 - Lee CT, Huang CP, Lee YD. *Biomimetic porous scaffolds made from poly(L-lactide)-G-chondroitin sulfate blend with poly(L-lactide) for cartilage tissue engineering*. Biomacromolecules. 2006. 7(7):2200-9.

93 - Subramanian A, Vu D, Larsen GF, Lin HY. *Preparation and evaluation of the electrospun chitosan/PEO fibers for potential applications in cartilage tissue engineering*. J Biomater Sci Polym Ed. 2005. 16(7):861-73.

94 - Suh SW, Shin JY, Kim J, Kim J, Beak CH, Kim DI*, et al. Effect of different particles on cell proliferation in polymer scaffolds using a solvent-casting and particulate leaching technique*. Asaio J. 2002. 48(5):460-4.

95 - Li W-J, Laurencin CT, Caterson E, Tuan RS, Ko FK. *Electrospun nanofibrous structure: A novel scaffold for tissue engineering*. J Biomed Mater Res. 2002. 60:613 – 21.

96 - Yoshimoto H, Shin YM, Terai H, Vacanti JP. *A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering*. Biomaterials. 2003. 24(12):2077-82.

97 - Tuzlakoglu K, Bolgen N, Salgado AJ, Gomes ME, Piskin E, Reis RL. *Nano- and micro-fiber combined scaffolds: A new architecture for bone tissue engineering*. J Mater Sci Mater Med. 2005. 16(12):1099-104.

98 - Pinho ED, Martins A, Araujo JV, Reis RL, Neves NM. *Degradable particulate composite reinforced with nanofibres for biomedical applications*. Acta Biomater. 2009. 5(4):1104-14.

99 - Pham QP, Sharma U, Mikos AG. *Electrospun poly(epsilon-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: Characterization of scaffolds and measurement of cellular infiltration*. Biomacromolecules. 2006. 7(10):2796-805.

100 - Gomes ME, Holtorf HL, Reis RL, Mikos AG. *Influence of the porosity of starch-based fiber mesh scaffolds on the proliferation and osteogenic differentiation of bone marrow stromal cells cultured in a flow perfusion bioreactor*. Tissue Eng. 2006. 12(4):801-9.

101 - Holloway JL, Lowman AM, Palmese GR. *Mechanical evaluation of poly(vinyl alcohol)-based fibrous composites as biomaterials for meniscal tissue replacement*. Acta Biomater. 2010.

102 - Kang SW, Son SM, Lee JS, Lee ES, Lee KY, Park SG*, et al. Regeneration of whole meniscus using meniscal cells and polymer scaffolds in a rabbit total meniscectomy model*. J Biomed Mater Res A. 2006. 77(4):659-71.

103 - Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol P, Bhattacharya M*, et al. Chitosan/polyester-based scaffolds for cartilage tissue engineering: Assessment of extracellular matrix formation*. Acta Biomater. 2010. 6(3):1149-57.

104 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, Neves NM*, et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromol Biosci. 2010.

105 - Costa-Pinto AR, Correlo VM, Sol PC, Bhattacharya M, Charbord P, Delorme B*, et al. Osteogenic differentiation of human bone marrow mesenchymal stem cells seeded on melt based chitosan scaffolds for bone tissue engineering applications*. Biomacromolecules. 2009. 10(8):2067-73.

106 - Sarazin P, Roy X, Favis BD. *Controlled preparation and properties of porous poly(L-lactide) obtained from a co-continuous blend of two biodegradable polymers*. Biomaterials. 2004. 25(28):5965-78.

107 - Bryant SJ, Anseth KS. *Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly(ethylene glycol) hydrogels*. J Biomed Mater Res. 2002. 59:63 – 72.

108 - Anseth KS, Metters AT, Bryant SJ, Martens PJ, Elisseeff JH, Bowman CN. *In situ forming degradable networks and their application in tissue engineering and drug delivery*. J Control Release. 2002. 78(1-3):199-209.

109 - Tachaboonyakiat W, Furubayashi T, Katoh M, Ooya T, Yui N. *Novel biodegradable cholesterol-modified polyrotaxane hydrogels for cartilage regeneration*. J Biomater Sci Polym Ed. 2004. 15(11):1389-404.

110 - Nicodemus GD, Bryant SJ. *The role of hydrogel structure and dynamic loading on chondrocyte gene expression and matrix formation*. J Biomech. 2008. 41(7):1528-36.

111 - Oliveira JT, Gardel LS, Rada T, Martins L, Gomes ME, Reis RL. *Injectable gellan gum hydrogels with autologous cells for the treatment of rabbit articular cartilage defects*. J Orthop Res. 2010. 28(9):1193-9.

112 - Zheng L, Fan HS, Sun J, Chen XN, Wang G, Zhang L*, et al. Chondrogenic differentiation of mesenchymal stem cells induced by collagen-based hydrogel: An in vivo study*. J Biomed Mater Res A. 2010. 93(2):783-92.

113 - Griffon DJ, Sedighi MR, Schaeffer DV, Eurell JA, Johnson AL. *Chitosan scaffolds: Interconnective pore size and cartilage engineering*. Acta Biomater. 2006. 2(3):313-20.

114 - Hoemann CD, Sun J, Legare A, McKee MD, Buschmann MD. *Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle*. Osteoarthritis Cartilage. 2005. 13(4):318-29.

115 - Malafaya PB, Reis RL. *Bilayered chitosan-based scaffolds for osteochondral tissue engineering: Influence of hydroxyapatite on in vitro cytotoxicity and dynamic bioactivity studies in a specific double-chamber bioreactor*. Acta Biomater. 2009. 5(2):644-60.

116 - Malafaya PB, Santos TC, van Griensven M, Reis RL. *Morphology, mechanical characterization and in vivo neo-vascularization of chitosan particle aggregated scaffolds architectures*. Biomaterials. 2008. 29(29):3914-26.

117 - Oliveira JT, Crawford A, Mundy JL, Sol PC, Correlo VM, Bhattacharya M*, et al. Novel melt-processable chitosan-polybutylene succinate fibre scaffolds for cartilage tissue engineering*. J Biomater Sci Polym Ed. 2010.

118 - Alves da Silva ML, Crawford A, Mundy JM, Martins A, Araujo JV, Hatton PV*, et al. Evaluation of extracellular matrix formation in PCL and SPCL nanofiber meshes when seeded with bovine articular chondrocytes*. Tissue Eng. 2009. 15(2):377-85.

119 - Oliveira JT, Crawford A, Mundy JM, Moreira AR, Gomes ME, Hatton PV*, et al. A cartilage tissue engineering approach combining starch-polycaprolactone fibre mesh scaffolds with bovine articular chondrocytes*. J Mater Sci Mater Med. 2007. 18(2):295-302.

120 - Silva SS, Motta A, Rodrigues MT, Pinheiro AF, Gomes ME, Mano JF*, et al. Novel genipin-cross-linked chitosan/silk fibroin sponges for cartilage engineering strategies*. Biomacromolecules. 2008. 9(10):2764-74.

121 - Meinel L, Hoffmann S, Karageorgiou V, Zichner L, Langer R, Kaplan DL*, et al. Engineering cartilage-like tissue using human mesenchymal stem cells and silk protein scaffolds*. Biotechnology and Bioengineering. 2004. 88(3):379 - 91.

122 - Wang Y, Blasioli DJ, Kim HJ, Kim HS, Kaplan DL. *Cartilage tissue engineering with silk scaffolds and human articular chondrocytes*. Biomaterials. 2006. 27(25):4434-42.

123 - Seda Tigli R, Ghosh S, Laha MM, Shevde NK, Daheron L, Gimble J*, et al. Comparative chondrogenesis of human cell sources in 3d scaffolds*. J Tissue Eng Regen Med. 2009. 3(5):348-60.

124 - Liu X, Zhou G, Liu W, Zhang W, Cui L, Cao Y. *In vitro formation of lacuna structure by human dermal fibroblasts co-cultured with porcine chondrocytes on a 3D biodegradable scaffold*. Biotechnol Lett. 2007. 29(11):1685-90.

125 - Thorvaldsson A, Stenhamre H, Gatenholm P, Walkenstrom P. *Electrospinning of highly porous scaffolds for cartilage regeneration*. Biomacromolecules. 2008. 9(3):1044-9.

126 - Li W-J, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ*, et al. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells*. Biomaterials. 2005. 26:599 – 609.

127 - Li W-J, Danielson KG, Alexander PG, Tuan RS. *Biological response of chondrocytes cultured in threedimensional nanofibrous poly(epsylon-caprolactone) scaffolds*. J Biomed Mater Res. 2003. 67(A):1105 - 14.

128 - Brown CD, Hoffman AL. *Modification of natural polymers: Chitosan.* In: Atala A, Lanza R, editors. Methods of tissue engineering. San Diego: Academic Press; 2002. p. 565-74.

129 - Tuzlakoglu K, Reis RL. *Chitosan-based scaffolds in orthopeadic applications.* In: Reis RL, editor. Natural-based polymers for biomedical applications. Cambridge, England: Woodhead Publishing Limited and CRC Press LLC; 2008. p. 357-73.

130 - Prasitsilp M, Jenwithisuk R, Kongsuwan K, Damrongchai N, Watts P. *Cellular responses to chitosan in vitro: The importance of deacetylation*. J Mater Sci Mater Med. 2000. 11(12):773-8.

131 - Thein-Han WW, Kitiyanant Y, Misra RDK. *Chitosan as a scaffold matrix for tissue engineering*. Materials Science & Technology. 2008. 24(9):1062-75.

132 - Thein-Han WW, Kitiyanant Y. *Chitosan scaffolds for in vitro buffalo embryonic stem-like cell culture: An approach to tissue engineering*. J Biomed Mater Res B Appl Biomater. 2007. 80(1):92-101.

133 - Kim IY, Seo SJ, Moon HS, Yoo MK, Park IY, Kim BC*, et al. Chitosan and its derivatives for tissue engineering applications*. Biotechnol Adv. 2008. 26(1):1-21.

134 - Martins AM, Pereira RC, Leonor IB, Azevedo HS, Reis RL. *Chitosan scaffolds incorporating lysozyme into CaP coatings produced by a biomimetic route: A novel concept for tissue engineering combining a self-regulated degradation system with in situ pore formation*. Acta Biomater. 2009. 5(9):3328-36.

135 - Ragetly GR, Griffon DJ, Lee HB, Fredericks LP, Gordon-Evans W, Chung YS. *Effect of chitosan scaffold microstructure on mesenchymal stem cell chondrogenesis*. Acta Biomater. 2010. 6(4):1430-6.

136 - Mrugala D, Bony C, Neves N, Caillot L, Fabre S, Moukoko D*, et al. Phenotypic and functional characterization of ovine mesenchymal stem cells: Application to a cartilage defect model*. Ann Rheum Dis. 2007.

137 - Yamane S, Iwasaki N, Kasahara Y, Harada K, Majima T, Monde K*, et al. Effect of pore size on in vitro cartilage formation using chitosan-based hyaluronic acid hybrid polymer fibers*. J Biomed Mater Res A. 2007. 81(3):586-93.

138 - Oliveira JT, Santos TC, Martins L, Alves da Silva ML, Marques AP, Castro AG*, et al. Performance of new gellan gum hydrogels combined with human articular chondrocytes for cartilage regeneration when subcutaneously implanted in nude mice*. J Tissue Eng Regen Med. 2009. 3(7):493-500.

139 - Oliveira JT, Santos TC, Martins L, Picciochi R, Marques AP, Castro AG*, et al. Gellan gum injectable hydrogels for cartilage tissue engineering applications: In vitro studies and preliminary in vivo evaluation*. Tissue Eng Part A. 2010. 16(1):343-53.

140 - Pereira CS, Cunha AM, Reis RL, Vazquez B, San Roman J. *New starch-based thermoplastic hydrogels for use as bone cements or drug-delivery carriers*. J Mater Sci Mater Med. 1998. 9(12):825-33.

141 - Oliveira JT, Picciochi R, Santos TC, Martins L, Pinto LG, Malafaya PB*, et al. Injectable gellan gum hydrogels as supports for cartilage tissue engineering applications*. Tissue Eng. 2008. 14(5):748-.

142 - Lima HC, Araujo JV, Neves N, Mano JF, Reis RL. *Cell encapsulation in thermoresponsive chitosan based hydrogels for cartilage tissue engineering*. Tissue Eng. 2008. 14(5):709-10.

143 - Guo T, Zhao J, Chang J, Ding Z, Hong H, Chen J*, et al. Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-beta1 for chondrocytes proliferation*. Biomaterials. 2006. 27(7):1095-103.

144 - Tan H, Chu CR, Payne KA, Marra KG. *Injectable in situ forming biodegradable chitosan-hyaluronic acid based hydrogels for cartilage tissue engineering*. Biomaterials. 2009. 30(13):2499-506.

145 - Park H, Temenoff JS, Tabata Y, Caplan AI, Mikos AG. *Injectable biodegradable hydrogel composites for rabbit marrow mesenchymal stem cell and growth factor delivery for cartilage tissue engineering*. Biomaterials. 2007. 28(21):3217-27.

146 - Hui JH, Chan SW, Li J, Goh JC, Li L, Ren XF*, et al. Intra-articular delivery of chondroitin sulfate for the treatment of joint defects in rabbit model*. J Mol Histol. 2007. 38(5):483-9.

147 - Pereira RC, Scaranari M, Castagnola P, Grandizio M, Azevedo HS, Reis RL*, et al. Novel injectable gel (system) as a vehicle for human articular chondrocytes in cartilage tissue regeneration*. J Tissue Eng Regen Med. 2009. 3(2):97-106.

148 - Pawar R, Jadhav W, Bhusare S, Borade R. *Sources, properties, modification and processing of natural-based polymers.* In: Reis Rl, editor. Natural-based polymers for biomedical applications. Cambridge, England: Woodhead Publishing Limited and CRC Press LLC; 2008. p. 3-53.

149 - Martins AM, Pham QP, Malafaya PB, Sousa RA, Gomes ME, Raphael RM*, et al. The role of lipase and alpha-amylase in the degradation of starch/poly(epsilon-caprolactone) fiber meshes and the osteogenic differentiation of cultured marrow stromal cells*. Tissue Eng Part A. 2009. 15(2):295-305.

150 - Azevedo HS, Reis RL. *Encapsulation of alpha-amylase into starch-based biomaterials: An enzymatic approach to tailor their degradation rate*. Acta Biomater. 2009. 5(8):3021-30.

151 - Marques AP, Reis RL, Hunt JA. *An in vivo study of the host response to starch-based polymers and composites subcutaneously implanted in rats*. Macromol Biosci. 2005. 5(8):775-85.

152 - Salgado AJ, Coutinho OP, Reis RL. *Novel starch-based scaffolds for bone tissue engineering: Cytotoxicity, cell culture, and protein expression*. Tissue Engineering. 2004. 10(3/4):465 - 74.

153 - Santos MI, Fuchs S, Gomes ME, Unger RE, Reis RL, Kirkpatrick CJ. *Response of micro- and macrovascular endothelial cells to starch-based fiber meshes for bone tissue engineering*. Biomaterials. 2007. 28(2):240-8.

154 - Fuchs S, Ghanaati S, Orth C, Barbeck M, Kolbe M, Hofmann A*, et al. Contribution of outgrowth endothelial cells from human peripheral blood on in vivo vascularization of bone tissue engineered constructs based on starch polycaprolactone scaffolds*. Biomaterials. 2009. 30(4):526-34.

155 - Martins AM, Saraf A, Sousa RA, Alves CM, Mikos AG, Kasper FK*, et al. Combination of enzymes and flow perfusion conditions improves osteogenic differentiation of bone marrow stromal cells cultured upon starch/poly(epsilon-caprolactone) fiber meshes*. J Biomed Mater Res A. 2010. 94(4):1061-9.

156 - Santos MI, Tuzlakoglu K, Fuchs S, Gomes ME, Peters K, Unger RE*, et al. Endothelial cell colonization and angiogenic potential of combined nano- and micro-fibrous scaffolds for bone tissue engineering*. Biomaterials. 2008. 29(32):4306-13.

157 - Santos MI, Unger RE, Sousa RA, Reis RL, Kirkpatrick CJ. *Crosstalk between osteoblasts and endothelial cells co-cultured on a polycaprolactone-starch scaffold and the in vitro development of vascularization*. Biomaterials. 2009. 30(26):4407-15.

158 - Ngoenkam J, Faikrua A, Yasothornsrikul S, Viyoch J. *Potential of an injectable chitosan/starch/beta-glycerol phosphate hydrogel for sustaining normal chondrocyte function*. Int J Pharm. 2010. 391(1-2):115-24.

159 - Puppi D, Chiellini F, Piras AM, Chiellini E. *Polymeric materials for bone and cartilage repair*. Progress in Polymer Science. 2010. 35:403-40.

160 - Wang Y, Rodriguez-Perez MA, Reis RL, Mano JF. *Thermal and thermomechanical behaviour of polycaprolactone and starch/polycaprolactone blends for biomedical applications*. Macromol Mater Eng. 2005. 290:792-801.

161 - Li WJ, Jiang YJ, Tuan RS. *Chondrocyte phenotype in engineered fibrous matrix is regulated by fiber size*. Tissue Eng. 2006. 12(7):1775-85.

162 - Li W-J, Tuli R, Huang X, Laquerriere P, Tuan RS. *Multilineage differentiation of human mesenchymal stem cells in three-dimensional nanofibrous scaffold*. Biomaterials. 2005. 26:5158 - 66.

163 - Martins A, Pinho ED, Faria S, Pashkuleva I, Marques AP, Reis RL*, et al. Surface modification of electrospun polycaprolactone nanofiber meshes by plasma treatment to enhance biological performance*. Small. 2009. 5(10):1195-206.

164 - Martins A, Chung S, Pedro AJ, Sousa RA, Marques AP, Reis RL*, et al. Hierarchical starch-based fibrous scaffold for bone tissue engineering applications*. J Tissue Eng Regen Med. 2009. 3(1):37-42.

165 - Correlo VM, Boesel LF, Pinho E, Costa-Pinto AR, Alves da Silva ML, Bhattacharya M*, et al. Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties*. J Biomed Mater Res A. 2009. 91(2):489-504.

166 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, N.M. N*, et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromolecular Bioscience. 2010. accepted for publication.

167 - Costa-Pinto AR, Salgado AJ, Correlo VM, Sol P, Bhattacharya M, Charbord P*, et al. Adhesion, proliferation, and osteogenic differentiation of a mouse mesenchymal stem cell line (BMC9) seeded on novel melt-based chitosan/polyester 3D porous scaffolds*. Tissue Eng Part A. 2008. 14(6):1049-57.

168 - Oliveira JT, Crawford A, Mundy JL, Sol PC, Correlo VM, Bhattacharya M*, et al. Novel melt-processable chitosan-polybutylene succinate fibre scaffolds for cartilage tissue engineering*. J Biomater Sci Polym Ed - In press. 2010.

169 - Miot S, Woodfield TB, Daniels AU, Suetterlin R, Peterschmitt I, Heberer M*, et al. Effects of scaffold composition and architecture on human nasal chondrocyte redifferentiation and cartilaginous matrix deposition*. Biomaterials. 2005. 26:2479 – 89.

170 - Woodfield TBF, Miot S, Martin I, Van Blitterswijk CA, Riesle J. *The regulation of expanded human nasal chondrocyte re-differentiation capacity by substrate composition and gas plasma surface modification*. Biomaterials. 2006. 27(7):1043 -53

171 - Ahmed N, Taylor DW, Wunder J, Nagy A, Gross AE, Kandel RA. *Passaged human chondrocytes accumulate extracellular matrix when induced by bovine chondrocytes*. J Tissue Eng Regen Med. 2010. 4(3):233-41.

172 - Brittberg M, Sjögren-Jansson E, Thornemo M, Faber B, Tarkowski A, Peterson L*, et al. Clonal growth of human articular cartilage and the functional role of the periosteum in chondrogenesis*. Osteoarthritis and Cartilage. 2005. 13:146 - 53.

173 - Jakob M, Dèmarteau O, Suetterlin R, Heberer M, Martin I. *Chondrogenesis of expanded adult human articular chondrocytes is enhanced by specific prostaglandins*. Rheumatology. 2004. 43:852 – 7.

174 - Verdonk PCM, Forsythz RG, Wangy J, Almqvist KE, Verdonk R, Veysy EM*, et al. Characterisation of human knee meniscus cell phenotype*. Osteoarthritis and Cartilage. 2005. 13:548 - 60.

175 - Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M*, et al. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro*. J Cell Biochem. 2001. 81(2):368-77.

176 - Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vecsei V*, et al. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture*. Osteoarthritis Cartilage. 2002. 10(1):62-70.

177 - Ahmed N, Gan L, Nagy A, Zheng J, Wang C, Kandel RA. *Cartilage tissue formation using redifferentiated passaged chondrocytes in vitro*. Tissue Eng Part A. 2009. 15(3):665-73.

178 - Chaipinyo K, Oakes BW, van Damme MI. *Effects of growth factors on cell proliferation and matrix synthesis of low-density, primary bovine chondrocytes cultured in collagen i gels*. Journal of Orthopaedic Research. 2002. 20:1070 – 8.

179 - Sandell LJ, Aigner T. *Articular cartilage and changes in arthritis. An introduction: Cell biology of osteoarthritis*. Arthritis Res. 2001. 3:107 – 13.

180 - Garvican ER, Vaughan-Thomas A, Redmond C, Clegg PD. *Chondrocytes harvested from osteochondritis dissecans cartilage are able to undergo limited in vitro chondrogenesis despite having perturbations of cell phenotype in vivo*. J Orthop Res. 2008. 26(8):1133-40.

181 - Stoop R, Albrecht D, Gaissmaier C, Fritz J, Felka T, Rudert M*, et al. Comparison of marker gene expression in chondrocytes from patients receiving autologous chondrocyte transplantation versus osteoarthritis patients*. Arthritis Res Ther. 2007. 9(3):R60.

182 - Stove J, Schneider-Wald B, Scharf HP, Schwarz ML. *Bone morphogenetic protein 7 (BMP-7) stimulates proteoglycan synthesis in human osteoarthritic chondrocytes in vitro*. Biomed Pharmacother. 2006. 60(10):639-43.

183 - Grigolo B, Lisignoli G, Desando G, Cavallo C, Marconi E, Tschon M*, et al. Osteoarthritis treated with mesenchymal stem cells on hyaluronan-based scaffold in rabbit*. Tissue Eng Part C Methods. 2009. 15(4):647-58.

184 - Brun P, Abatangelo G, Radice M, Zacchi V, Guidolin D, Gordini DD*, et al. Chondrocyte aggregation and reorganization into three-dimensional scaffolds*. J Biomed Mater Res. 1999. 46:337 – 46.

185 - Davisson T, Sah RL, Ratcliffe A. *Perfusion increases cell content and matrix synthesis in chondrocyte three-dimensional cultures*. Tissue Eng. 2002. 8(5):807-16.

186 - Freyria AM, Yang Y, Chajra H, Rousseau CF, Ronziere MC, Herbage D*, et al. Optimization of dynamic culture conditions: Effects on biosynthetic activities of chondrocytes grown in collagen sponges*. Tissue Eng. 2005. 11(5-6):674-84.

187 - Kino-Oka M, Maeda Y, Yamamoto T, Sugawara K, Taya M. *A kinetic modeling of chondrocyte culture for manufacture of tissue-engineered cartilage*. Journal of Bioscience and Bioengineering. 2005. 99(3):197 – 207.

188 - van der Kraan PM, Buma P, van Kuppevelt T, van den Berg WB. *Interaction of chondrocytes, extracellular matrix and growth factors: Relevance for articular cartilage tissue engineering*. Osteoarthritis and Cartilage. 2002. 10:631 – 7.

189 - Vavken P, Arrich F, Pilz M, Dorotka R. *An in vitro model of biomaterial-augmented microfracture including chondrocyte-progenitor cell interaction*. Arch Orthop Trauma Surg. 2010. 130(5):711-6.

190 - Wolf F, Candrian C, Wendt D, Farhadi J, Heberer M, Martin I*, et al. Cartilage tissue engineering using pre-aggregated human articular chondrocytes*. Eur Cell Mater. 2008. 16:92-9.

191 - Xu X, Urban JP, Tirlapur U, Wu MH, Cui Z, Cui Z. *Influence of perfusion on metabolism and matrix production by bovine articular chondrocytes in hydrogel scaffolds*. Biotechnol Bioeng. 2006. 93(6):1103-11.

192 - Yang B, Yin Z, Cao J, Shi Z, Zhang Z, Song H*, et al. In vitro cartilage tissue engineering using cancellous bone matrix gelatin as a biodegradable scaffold*. Biomed Mater. 2010. 5(4):045003.

193 - Dai W, Kawazoe N, Lin X, Dong J, Chen G. *The influence of structural design of PLGA/collagen hybrid scaffolds in cartilage tissue engineering*. Biomaterials. 2010. 31(8):2141-52.

194 - Hsu SH, Whu SW, Hsieh SC, Tsai CL, Chen DC, Tan TS. *Evaluation of chitosan-alginate-hyaluronate complexes modified by an rgd-containing protein as tissue-engineering scaffolds for cartilage regeneration*. Artif Organs. 2004. 28(8):693-703.

195 - Marlovits S, Striessnig G, Kutscha-Lissberg F, Resinger C, Aldrian SM, Vecsei V*, et al. Early postoperative adherence of matrix-induced autologous chondrocyte implantation for the treatment of full-thickness cartilage defects of the femoral condyle*. Knee Surg Sports Traumatol Arthrosc. 2005. 13(6):451-7.

196 - Ossendorf C, Kaps C, Kreuz PC, Burmester GR, Sittinger M, Erggelet C. *Treatment of posttraumatic and focal osteoarthritic cartilage defects of the knee with autologous polymer-based three-dimensional chondrocyte grafts: 2-year clinical results*. Arthritis Res Ther. 2007. 9(2):R41.

197 - Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD*, et al. Multilineage potential of adult human mesenchymal stem cells*. Science. 1999. 284(5411):143-7.

198 - Noth U, Steinert AF, Tuan RS. *Technology insight: Adult mesenchymal stem cells for osteoarthritis therapy*. Nat Clin Pract Rheumatol. 2008. 4(7):371-80.

199 - Jorgensen C, Djouad F, Fritz V, Apparailly F, Plence P, Noel D. *Mesenchymal stem cells and rheumatoid arthritis*. Joint Bone Spine. 2003. 70(6):483-5.

200 - Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E*, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease*. Eur J Immunol. 2008. 38(6):1745-55.

201 - Sundelacruz S, Kaplan DL. *Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine*. Semin Cell Dev Biol. 2009. 20(6):646-55.

202 - van der Kraan PM, Davidson EN, van den Berg WB. *Bone morphogenetic proteins and articular cartilage: To serve and protect or a wolf in sheep clothing's?* Osteoarthritis Cartilage. 2010. 18(6):735-41.

203 - Fischer J, Dickhut A, Rickert M, Richter W. *Human articular chondrocytes secrete parathyroid hormone-related protein and inhibit hypertrophy of mesenchymal stem cells in coculture during chondrogenesis*. Arthritis Rheum. 2010. 62(9):2696-706.

204 - Wang H, van Blitterswijk CA. *The role of three-dimensional polymeric scaffold configuration on the uniformity of connective tissue formation by adipose stromal cells*. Biomaterials. 2010. 31(15):4322-9.

205 - Kim HJ, Lee JH, Im GI. *Chondrogenesis using mesenchymal stem cells and pcl scaffolds*. J Biomed Mater Res A. 2010. 92(2):659-66.

206 - Casper ME, Fitzsimmons JS, Stone JJ, Meza AO, Huang Y, Ruesink TJ*, et al. Tissue engineering of cartilage using poly-epsilon-caprolactone nanofiber scaffolds seeded in vivo with periosteal cells*. Osteoarthritis Cartilage. 2010. 18(7):981-91.

207 - Holland TA, Bodde EW, Cuijpers VM, Baggett LS, Tabata Y, Mikos AG*, et al. Degradable hydrogel scaffolds for in vivo delivery of single and dual growth factors in cartilage repair*. Osteoarthritis Cartilage. 2007. 15(2):187-97.

208 - Liao E, Yaszemski M, Krebsbach P, Hollister S. *Tissue-engineered cartilage constructs using composite hyaluronic acid/collagen I hydrogels and designed poly(propylene fumarate) scaffolds*. Tissue Eng. 2007. 13(3):537-50.

209 - Noth U, Rackwitz L, Heymer A, Weber M, Baumann B, Steinert A*, et al. Chondrogenic differentiation of human mesenchymal stem cells in collagen type I hydrogels*. J Biomed Mater Res A. 2007. 83(3):626-35.

210 - Park H, Temenoff JS, Holland TA, Tabata Y, Mikos AG. *Delivery of TGF-beta1 and chondrocytes via injectable, biodegradable hydrogels for cartilage tissue engineering applications*. Biomaterials. 2005. 26(34):7095-103.

211 - Bouffi C, Thomas O, Bony C, Giteau A, Venier-Julienne MC, Jorgensen C*, et al. The role of pharmacologically active microcarriers releasing TGF-beta3 in cartilage formation in vivo by mesenchymal stem cells*. Biomaterials. 2010. 31(25):6485-93.

212 - Gomes ME, Bossano CM, Johnston CM, Reis RL, Mikos AG. *In vitro localization of bone growth factors in constructs of biodegradable scaffolds seeded with marrow stromal cells and cultured in a flow perfusion bioreactor*. Tissue Eng. 2006. 12(1):177-88.

213 - Gomes ME, Sikavitsas VI, Behravesh E, Reis RL, Mikos AG. *Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds*. J Biomed Mater Res A. 2003. 67(1):87-95.

214 - Sailon AM, Allori AC, Davidson EH, Reformat DD, Allen RJ, Warren SM. *A novel flow-perfusion bioreactor supports 3d dynamic cell culture*. J Biomed Biotechnol. 2009. 2009:873816.

215 - Vunjak-Novakovic G, Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R*, et al. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage*. J Orthop Res. 1999. 17(1):130-8.

216 - Pazzano D, Mercier KA, Moran JM, Fong SS, DiBiasio DD, Rulfs JX, *et al. Comparison of chondrogensis in static and perfused bioreactor culture*. Biotechnol Prog. 2000. 16(5):893-6.

217 - Mahmoudifar N, Doran PM. *Chondrogenic differentiation of human adipose-derived stem cells in polyglycolic acid mesh scaffolds under dynamic culture conditions.* Biomaterials. 2010. 31(14):3858-67.

218 - Darling EM, Athanasiou K. *Articular cartilage bioreactors and bioprocesses*. Tissue Engineering. 2003. 9(1):9 - 26.

219 - Martin I, Wendt D, Hereber M. *The role of bioreactors in tissue engineering*. TRENDS in Biotechnology. 2004. 22(2):80 - 6.

220 - Godara P, McFarland CD, Nordon RE. *Design of bioreactors for mesenchymal stem cell tissue engineering*. Journal of Chemical Technology and Biotechnology. 2008. 83:408-20.

221 - Wang L, Sheshareddy K, Weiss ML, Detamore MS. *Effect of initial seeding density on human umbilical cord mesenchymal stromal cells for fibrocartilage tissue engineering*. Tissue Eng Part A. 2009. 15(5):1009-17.

222 - Carver SE, Heath CA. *Influence of intermittent pressure, fluid flow, and mixing on the regenerative properties of articular chondrocytes*. Biotechnol Bioeng. 1999. 65(3):274-81.

223 - Wendt D, Marsano A, Jakob M, Heberer M, Martin I. *Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity*. Biotechnol Bioeng. 2003. 84:205 – 14.

224 - Freyria AM, Cortial D, Ronziere MC, Guerret S, Herbage D. *Influence of medium composition, static and stirred conditions on the proliferation of and matrix protein expression of bovine articular chondrocytes cultured in a 3-D collagen scaffold*. Biomaterials. 2004. 25(4):687-97.

225 - Gooch KJ, Kwon JH, Blunk T, Langer R, Freed LE, Vunjak-Novakovic G. *Effects of mixing intensity on tissue-engineered cartilage*. Biotechnol Bioeng. 2001. 72(4):402-7.

226 - Martin I, Obradovic B, Freed LE, Vunjak-Novakovic G. *Method for quantitative analysis of glycosaminoglycan distribution in cultured natural and engineered cartilage*. Ann Biomed Eng. 1999. 27(5):656-62.

227 - Freed LE, Hollander AP, Martin I, Barry JR, Langer R, Vunjak-Novakovic G. *Chondrogenesis in a cell-polymer-bioreactor system*. Exp Cell Res. 1998. 240(1):58-65.

228 - Bouchet BY, Colon M, Polotsky A, Shikani AH, Hungerford DS, Frondoza CG. *Beta-1 integrin expression by human nasal chondrocytes in microcarrier spinner culture*. J Biomed Mater Res. 2000. 52(4):716-24.

229 - Frondoza C, Sohrabi A, Hungerford D. *Human chondrocytes proliferate and produce matrix components in microcarrier suspension culture*. Biomaterials. 1996. 17(9):879-88.

230 - Costa P, Martins A, Gomes M, Neves NM, Reis RL. *Multichamber bioreactor with bidirectional perfusion integrated in a culture system for tissue engineering strategies*. European Patent Office. 2010. EP 2151491 A2(09009863.3):11.

231 - Alves da Silva ML, Martins A, Costa-Pinto AR, Costa P, Faria S, Gomes M*, et al. Cartilage tissue engineering using electrospun PCL nanofiber meshes and MSCs*. Biomacromolecules. 2010. 11(12):3228-36.

232 - Alves da Silva M, Martins A, Costa-Pinto A, Correlo V, Sol P, Bhattacharya M*, et al. Chondrogenic differentiation of human bone marrow mesenchymal stem cells in chitosan based scaffolds using a flow perfusion bioreactor*. Journal of Tissue Engineering and Regenerative Medicine. 2010. Epub ahead of print.

233 - Tarng YW, Casper ME, Fitzsimmons JS, Stone JJ, Bekkers J, An KN*, et al. Directional fluid flow enhances in vitro periosteal tissue growth and chondrogenesis on poly-epsilon-caprolactone scaffolds*. J Biomed Mater Res A. 2010. 95(1):156-63.

234 - Liao J, Guo X, Grande-Allen KJ, Kasper FK, Mikos AG. *Bioactive polymer/extracellular matrix scaffolds fabricated with a flow perfusion bioreactor for cartilage tissue engineering*. Biomaterials. 2010.

235 - Emans PJ, van Rhijn LW, Welting TJ, Cremers A, Wijnands N, Spaapen F*, et al. Autologous engineering of cartilage*. Proc Natl Acad Sci U S A. 2010. 107(8):3418-23.

236 - Gibson JS, Milner PI, White R, Fairfax TP, Wilkins RJ. *Oxygen and reactive oxygen species in articular cartilage: Modulators of ionic homeostasis*. Pflugers Arch. 2008. 455(4):563-73.

237 - Coyle CH, Izzo NJ, Chu CR. *Sustained hypoxia enhances chondrocyte matrix synthesis*. J Orthop Res. 2009. 27(6):793-9.

238 - Das RH, van Osch GJ, Kreukniet M, Oostra J, Weinans H, Jahr H. *Effects of individual control of pH and hypoxia in chondrocyte culture*. J Orthop Res. 2010. 28(4):537-45.

239 - Khan WS, Adesida AB, Tew SR, Lowe ET, Hardingham TE. *Bone marrow-derived mesenchymal stem cells express the pericyte marker 3G5 in culture and show enhanced chondrogenesis in hypoxic conditions*. J Orthop Res. 2010. 28(6):834-40.

240 - Markway BD, Tan GK, Brooke G, Hudson JE, Cooper-White JJ, Doran MR. *Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures*. Cell Transplant. 2010. 19(1):29-42.

241 - Hu X, Yu SP, Fraser JL, Lu Z, Ogle ME, Wang JA*, et al. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via*

enhanced survival of implanted cells and angiogenesis. J Thorac Cardiovasc Surg. 2008. 135(4):799-808.

242 - Kanichai M, Ferguson D, Prendergast PJ, Campbell VA. *Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: A role for akt and hypoxia-inducible factor (HIF)-1alpha*. J Cell Physiol. 2008. 216(3):708-15.

243 - Zscharnack M, Poesel C, Galle J, Bader A. *Low oxygen expansion improves subsequent chondrogenesis of ovine bone-marrow-derived mesenchymal stem cells in collagen type I hydrogel*. Cells Tissues Organs. 2009. 190(2):81-93.

244 - Zuscik MJ, Hilton MJ, Zhang X, Chen D, O'Keefe RJ. *Regulation of chondrogenesis and chondrocyte differentiation by stress*. J Clin Invest. 2008. 118(2):429-38.

245 - Abdollahi H, Harris LJ, Zhang P, McIlhenny S, Srinivas V, Tulenko T*, et al. The role of hypoxia in stem cell differentiation and therapeutics*. J Surg Res. 2009.

246 - Koay EJ, Athanasiou KA. *Hypoxic chondrogenic differentiation of human embryonic stem cells enhances cartilage protein synthesis and biomechanical functionality*. Osteoarthritis Cartilage. 2008. 16(12):1450-6.

247 - Khan WS, Adesida AB, Hardingham TE. *Hypoxic conditions increase hypoxia-inducible transcription factor 2alpha and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients*. Arthritis Res Ther. 2007. 9(3):R55.

248 - Chen L, Fink T, Ebbesen P, Zachar V. *Hypoxic treatment inhibits insulin-induced chondrogenesis of ATDC5 cells despite upregulation of DEC1*. Connect Tissue Res. 2006. 47(3):119-23.

249 - Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T. *Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs*. J Cell Physiol. 2006. 207(2):331-9.

250 - Pilgaard L, Lund P, Duroux M, Lockstone H, Taylor J, Emmersen J*, et al. Transcriptional signature of human adipose tissue-derived stem cells (hASCs) preconditioned for chondrogenesis in hypoxic conditions*. Exp Cell Res. 2009. 315(11):1937-52.

251 - Kuettner KE, Pauli BU, Gall G, Memoli VA, Schenk RK. *Synthesis of cartilage matrix by mammalian chondrocytes in vitro. I. Isolation, culture characteristics, and morphology*. J Cell Biol. 1982. 93(3):743-50.

252 - Hendriks J, Riesle J, van Blitterswijk CA. *Co-culture in cartilage tissue engineering*. J Tissue Eng Regen Med. 2007. 1(3):170-8.

253 - Maxson S, Burg KJ. *Conditioned media enhance osteogenic differentiation on poly(l-lactide-co-epsilon-caprolactone)/hydroxyapatite scaffolds and chondrogenic differentiation in alginate*. J Biomater Sci Polym Ed. 2010. 21(11):1441-58.

254 - Korecki CL, Taboas JM, Tuan RS, Iatridis JC. *Notochordal cell conditioned medium stimulates mesenchymal stem cell differentiation toward a young nucleus pulposus phenotype*. Stem Cell Res Ther. 2010. 1(2):18.

255 - Kariya H, Yoshihara Y, Nakao Y, Sakurai N, Ueno M, Hashimoto M, *et al. Carboxymethyl-chitin promotes chondrogenesis by inducing the production of growth factors from immune cells*. J Biomed Mater Res A. 2010. 94(4):1034-41.

256 - Cairns DM, Lee PG, Uchimura T, Seufert CR, Kwon H, Zeng L. *The role of muscle cells in regulating cartilage matrix production*. J Orthop Res. 2010. 28(4):529-36.

257 - Lee JS, Im GI. *Influence of chondrocytes on the chondrogenic differentiation of adipose stem cells*. Tissue Eng Part A. 2010.

258 - Lee CH, Cook JL, Mendelson A, Moioli EK, Yao H, Mao JJ. *Regeneration of the articular surface of the rabbit synovial joint by cell homing: A proof of concept study*. Lancet. 2010. 376(9739):440-8.

259 - Steinhagen J, Bruns J, Niggemeyer O, Fuerst M, Ruther W, Schunke M, *et al. Perfusion culture system: Synovial fibroblasts modulate articular chondrocyte matrix synthesis in vitro*. Tissue Cell. 2010. 42(3):151-7.

260 - Varshney RR, Zhou R, Hao J, Yeo SS, Chooi WH, Fan J, *et al. Chondrogenesis of synovium-derived mesenchymal stem cells in gene-transferred co-culture system*. Biomaterials. 2010. 31(26):6876-91.

261 - Wu Y, Liu J, Feng X, Yang P, Xu X, Hsu HC, *et al. Synovial fibroblasts promote osteoclast formation by RANKL in a novel model of spontaneous erosive arthritis*. Arthritis Rheum. 2005. 52(10):3257-68.

262 - Guo X, Liao J, Park H, Saraf A, Raphael RM, Tabata Y, *et al. Effects of TGF-beta3 and preculture period of osteogenic cells on the chondrogenic differentiation of rabbit marrow mesenchymal stem cells encapsulated in a bilayered hydrogel composite*. Acta Biomater. 2010. 6(8):2920-31.

263 - Pound JC, Green DW, Chaudhuri JB, Mann S, Roach HI, Oreffo RO. *Strategies to promote chondrogenesis and osteogenesis from human bone marrow cells and articular chondrocytes encapsulated in polysaccharide templates*. Tissue Eng. 2006. 12(10):2789-99.

264 - Prasadam I, van Gennip S, Friis T, Shi W, Crawford R, Xiao Y. *ERK-1/2 and P38 in the regulation of hypertrophic changes of normal articular cartilage chondrocytes induced by osteoarthritic subchondral osteoblasts*. Arthritis Rheum. 2010. 62(5):1349-60.

265 - Thompson AD, Betz MW, Yoon DM, Fisher JP. *Osteogenic differentiation of bone marrow stromal cells induced by coculture with chondrocytes encapsulated in three-dimensional matrices*. Tissue Eng Part A. 2009. 15(5):1181-90.

266 - Bromley M, Woolley DE. *Chondroclasts and osteoclasts at subchondral sites of erosion in the rheumatoid joint*. Arthritis Rheum. 1984. 27(9):968-75.

267 - D'Andrea P, Calabrese A, Grandolfo M. *Intercellular calcium signalling between chondrocytes and synovial cells in co-culture*. Biochem J. 1998. 329 (Pt 3):681-7.

268 - Huch K, Stove J, Gunther KP, Puhl W. *Interactions between human osteoarthritic chondrocytes and synovial fibroblasts in co-culture*. Clin Exp Rheumatol. 2001. 19(1):27-33.

269 - Kurz B, Steinhagen J, Schunke M. *Articular chondrocytes and synoviocytes in a co-culture system: Influence on reactive oxygen species-induced cytotoxicity and lipid peroxidation*. Cell Tissue Res. 1999. 296(3):555-63.

270 - Vats A, Bielby RC, Tolley N, Dickinson SC, Boccaccini AR, Hollander AP*, et al. Chondrogenic differentiation of human embryonic stem cells: The effect of the micro-environment*. Tissue Eng. 2006. 12(6):1687-97.

271 - Hwang Y-S, Bishop AE, Polak JM, Mantalaris A. *Enhanced in vitro chondrogenesis of murine embryonic stem cells*. biotechnology and Bioprocesses Engineering. 2007. 12:696-706.

272 - Gan L, Kandel RA. *In vitro cartilage tissue formation by co-culture of primary and passaged chondrocytes*. Tissue Eng. 2007. 13(4):831-42.

273 - Lettry V, Hosoya K, Takagi S, Okumura M. *Coculture of equine mesenchymal stem cells and mature equine articular chondrocytes results in improved chondrogenic differentiation of the stem cells*. Jpn J Vet Res. 2010. 58(1):5-15.

SECTION II – DETAILED DESCRIPTION OF EXPERIMENTAL TESTING AND MATERIALS

Chapter 2

Materials and Methods

Materials and Methods

The objective of this chapter is to describe the materials and methods used in the experimental work presented in this thesis. Each chapter contains the materials and methods used, but herein we intend to explain in more detail the experiments that were performed, as well as the techniques used.

1. Materials

One transversal issue in the work presented in this thesis is the use of three dimensional (3D) structures as scaffolds for primary cell cultures. Those structures are intended to promote the attachment, proliferation and chondrogenic differentiation or chondrocyte phenotype maintenance. The objective was to understand the way different materials and structures, either natural or synthetic, processed with various techniques (either in blends or alone), would interact with the established primary cultures and influence formation of tissue engineered cartilage in vitro.

1.1. Chitosan-based blends

Chitosan is a natural polymer derived by the deacetylation of chitin. Chitin is usually extracted from crustaceous exoskeleton, like crabs or shrimps. Chitosan is obtained by a de-N-deacetylation of chitin, and is composed of $\beta(1\rightarrow4)$-linked glucosamine and N-acetyl-D-glucosamine (Figure 2.1) [1]. Chitosan used for blends production was supplied by France Chitin (Orange, France) and had a degree of deacetylation of 85 %. The deacetylation process is usually made in alkaline conditions, resulting in different chitosans depending on the degrees of deacetylation and on the molecular weight [2].

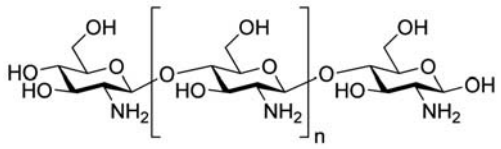

Figure 2.1 – Representative chemical structure of chitosan.

Most of chitosan properties of are affected by its molecular weight and the degree of deacetylation, as was already discussed in section I. In the present thesis, chitosan was blended with synthetic biodegradable polymers to enhance the processability of chitosan and provide better mechanical properties to the final material. The synthetic biodegradable material enables melt processing and acts as the continuous phase of the blend, enhancing its mechanical properties. In previous works by our group, several innovative blends were produced, with different percentages of natural and synthetic polymer but always keeping the biodegradability [3, 4]. The synthetic polymers used in the referred works were not previously tested for biomedical applications, only for environmental ones. This innovative approach resulted in several blends of chitosan with synthetic aliphatic polyesters, produced by compression moulding followed by particulate leaching [3, 5]. A biological screening was performed to test the blends cytocompatibility and biological performance when in cell culture [3]. From this study, two chitosan – polyester blends were selected for the work herein presented: chitosan and poly(butylene succinate) (CPBS) (50/50 % by weight), and chitosan and poly(butylene terephthalate adipate) (CPBTA) (50/50 % by weight).

Poly(butylene succinate) is a biodegradable aliphatic polyester commercialized under the trade name Bionolle™ 1000 (Showa Highpolymer, Japan), and its chemical structure is shown in Figure 2.2. It possesses a crystallinity of 35-45 % and a melting temperature of 114-115 ºC [6]. PBS is a semi-rigid polyester (elongation at break of 170-250 %) that can be processed under conventional melt processing techniques, and its applications are mainly for industry purposes, such as food packaging, clothing or industrial trays [7]. The biodegradation of the material is achieved in composting and also by hydrolysis of the ester link, eventually mediated by lipase [8] (an enzyme found in the human body).

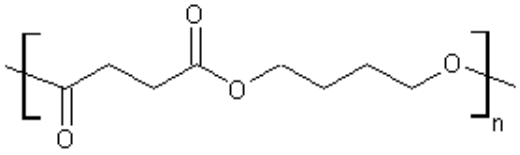

Figure 2.2 – Chemical structure of poly(butylene succinate).

Poly(butylene terephthalate adipate) is another biodegradable aliphatic polyester used for the production of chitosan-based blends, but presenting elastomeric mechanical properties. PBTA is an Eastar Bio Copolyester 14766™ butanediol, adipate and terephthalate copolymer obtained from Eastman Chemical Company, Kingsport, Tennessee. It presents elastic properties similar to those of a rubber (elongation at break of 600 %), being more flexible than PBS [7]. PBTA is formed by the reaction of butanediol with adipic and terephthalic acids [6]. PBTA is degraded into various aliphatic and aromatic oligomers by the action of microorganisms in compost [9]. The material, being a polyester, is also degraded by the action of enzymes.

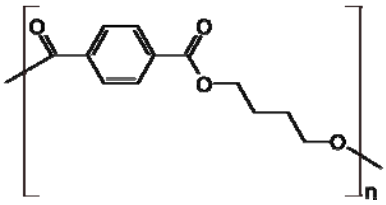

Figure 2.3 – Chemical structure of poly(butylene terephthalate) that will be modified to produce PBTA.

1.2. Poly(ε-caprolactone)

Poly(ε-caprolactone) is also an aliphatic polyester, semicrystalline and with a melting temperature (Tm) of 59-64 ºC (Figure 2.4), which degrades at slow rates, being used to produce resorbable sutures (for example Monoderm®) or for implantable drug delivery systems [6]. PCL is water stable and can undergo hydrolysis [10]. It is a very versatile polymer, being proposed for several tissue engineering applications [11-15], either alone or in blends with other polymers.

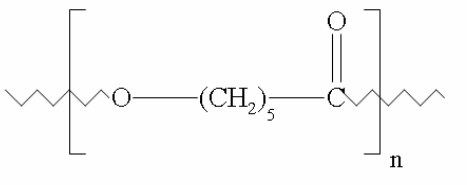

Figure 2.4 – Chemical structure of poly(ε-caprolactone).

The PCL used for scaffolds production was acquired from Union Carbide Chemicals and Plastic Division, Bound Brook, New Jersey, and it is commercially available as TONE™ polymer.

1.3. Starch – polycaprolactone blend

Starch is the major polysaccharide used to store energy by many types of plants. It occurs in nature in the form of non-soluble granules. It is composed of a variable mixture of amylase and amylopectin. Amylase is a linear polysaccharide whereas amylopectin is a branched polysaccharide (Figure 2.5). Depending on the type of plant, the biosynthesis mechanisms differ, as well as their shape, size or composition [10]. Starch is transformed into a thermoplastic starch by disruption of its natural structure by the hot processing in water in the presence of glycerol. It has been blended with other synthetic polymers, for example polyhydroxybutyrate, cellulose or PCL [16]. Starch blends with PCL, poly-L-lactic acid or ethylene vinyl alchool have been studied in our group for tissue engineering applications, either in vitro or in vivo, showing low cytotoxicity and good cytocompatibility [17-23].

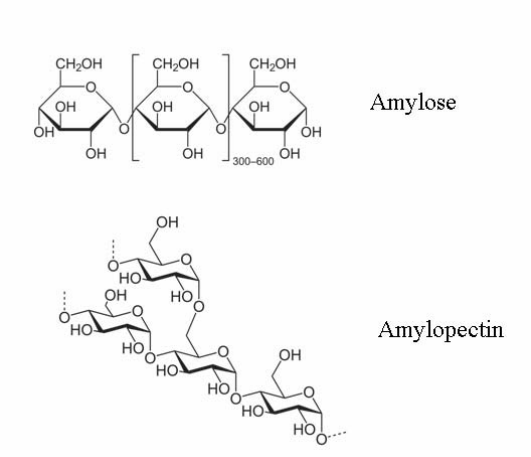

Figure 2.5 – Chemical structure of amylase and amylopectin. Their ratio in starch granules varies according to their origin.

The starch-polycaprolactone blend used in the framework of this thesis was supplied by Novamont (Novara, Italy), in the ratio of 30 % starch (Mater-BI ZI01U, Novamont, Novara, Italy) and 70 % PCL.

2. Scaffolds production

2.1. Compression moulding and particulate leaching

Depending on the scaffold material and its intended application, different production techniques and methodologies may be used. The procedure used should not modify the materials chemical properties or its cytocompatibility. However, it should allow for tailoring in a controlled and reproducible way its pores and structure to meet the intended requirements of the TE application. Several techniques have been used, such as solvent casting combined with salt leaching, freeze drying, fiber bonding, melt bonding or combination of these techniques [24, 25].

The scaffold porosity plays a very important role in tissue formation in vitro, as the pores will potentially enhance cell-cell interactions. In the particular case of cartilage, pores may provide an ECM like structure that mimics cartilage environment. Therefore, scaffold porosity and consequent pore shape and size were studied in the context of the present thesis to assess interactions between cells and porous scaffolds. Our group has been exploring melt based techniques for porous scaffolds production, namely for chitosan and starch blends. CPBS (50/50 % wt) blends were produced and processed into scaffolds, using a methodology based in hot compression moulding followed by NaCl particle leaching [5, 26]. The porous structure of these scaffolds was created by solid powder mixing with salt particles. The solid blend was loaded into a mould, heated and compression-moulded into large and thick discs. Those discs were sliced to obtain cubes of 5 mm. These cubes were further immersed in distilled water to leach out the NaCl particles, during 6 days (Figure 2.6). The time period was previously optimized and is the time required to ensure that salt particles are dissolved.

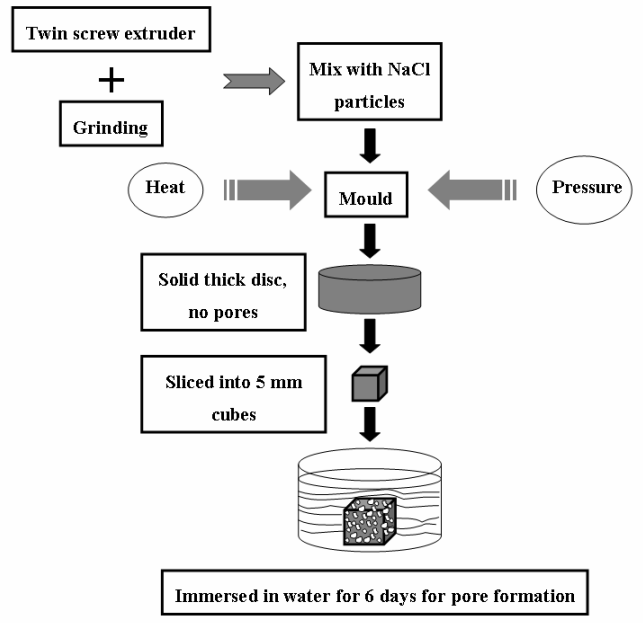

Figure 2.6 – Illustration of the procedure followed for the production of scaffolds by compression moulding followed by NaCl particle leaching.

In the present work, a CPBS (50/50 % wt) blend was first compounded and processed into scaffolds using this methodology. For achieving a high degree of mixing, the blends were produced using a twin screw extruder (Leistriz LSM 36). More detailed information about the characteristics of the blends can be found elsewhere [3, 4, 27]. Common salt (NaCl) was used as the porogen agent. Two different groups of scaffolds were produced: one using 80 % by weight (wt) of salt with particle size ranging between 63-125 μm (80 CPBS). The second group of scaffolds was produced using 60 % by weight of salt with a particle size range of 250-500 μm (60 CPBS). In both cases, large discs were processed from where cubes of 5 mm were sliced. These cubes were immersed in distilled water to leach out the salt, in a process optimized to take 6 days. Water was changed every day to facilitate the complete dissolution of the porogen agent. By the end of this procedure, cubes were dried until a constant weight was obtained.

2.2. Fiber bonding

CPBS scaffolds produced by compression moulding and salt leaching were shown to be suitable for cartilage TE, when seeded with bovine articular chondrocytes [28]. However, the cells show some difficulty in penetrating inside the scaffolds porous

structure, suggesting that an optimization of the porous structure was needed. The same blend was used for the production of fiber meshes, by fiber bonding technique. This technique allows the production of highly interconnected and larger scaffolds having a large surface area to allow cell attachment and proliferation [25].

The previously compounded chitosan blends were processed by fiber extrusion, using a prototype single screw micro-extruder coupled to a capillary die. Two blends were herein used: CPBS and CPBTA, both in the ratio of 50/50 % (wt). Extruded fibers were chopped into 1 cm sections and then were loaded into a mould. The mould was heated above the thermoplastic blend Tm during 10 minutes. Immediately after removing the moulds from the oven, fibers were slightly compressed by a Teflon cylinder to pack and promote bonding between the fibers. Finally, standardized scaffolds of 2 mm thick discs, with diameter of 8 mm were obtained by cutting the fiber bonded meshes with a circular die (8 mm in inner diameter). Scaffolds were further sterilized by ethylene oxide.

2.3. Electrospinning

In recent years, the electrospinning technique has been extensively explored for producing fibrous mesh scaffolds that are composed of fibers with diameters ranging from micro to nanoscale. The characteristic morphology of those meshes resembles the native ECM of many tissues. It is a very versatile technique that allows a fine control over the scaffold geometry, allows obtaining different fiber diameters and meshing morphologies, and also enables processing different materials. The individual fiber diameter can be adjusted by optimizing the spinning parameters and the polymeric solution properties. The obtained nanofiber meshes have a high surface area volume that promote cell attachment, and also allows a higher density of cells [29].

Over the past decade, electrospinning has gained popularity as a means of fabricating scaffolds with micro to nanoscale features similar to the hierarchical structure of extracellular matrix (ECM). The ability to mimic the ECM structural organization is an important consideration in rational design of a cell-responsive scaffold platform upon which additional functionalities can be incorporated. Electrospinning offers great flexibility in terms of choice of scaffold material, as well as fine control over the scaffold morphology. Fibers with diameters ranging from tens to hundreds of nanometers can be easily produced. The diameter is adjusted empirically via modulation

of spinning parameters such as the voltage used, flow rate, collecting distance and polymer solution properties. A schematic of the electrospinning apparatus is shown in Figure 2.7.

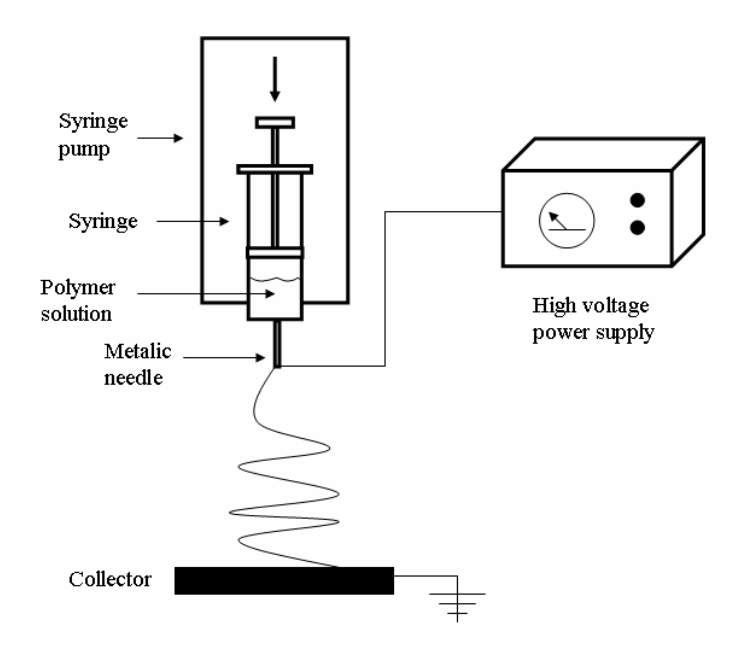

Figure 2.7 – Electrospinning setup composed by an electric power supply, a syringe pump and a collector. Different collectors can be used, depending on the intended fiber mesh morphology.

2.3.1. SPCL and PCL nanofiber meshes production

In the present work, PCL and SPCL nanofiber meshes were produced. Polymeric solutions of PCL and SPCL were prepared by dissolving the polymer or the blend into an organic solvent mixture of chloroform/dimethylformamide (70:30) (Sigma-Aldrich, USA), at concentrations of 17 % and 24 % (w/v), respectively. Polymeric solutions were placed into syringes with blunted metallic needles attached to it. The selected needles had an internal diameter of 0.8 mm. Furthermore, the syringe was coupled to a syringe pump (model KDS100, KD Scientific, USA) to control the flow rate. A flat aluminium foil connected to the ground was used as the fiber mesh collector. The high voltage power supply (0-25 kV) was applied in the needle, generating the electric field. The capillary tip-to-collector distance and the flow rate were fixed at 20 cm and 1.0 mL/h, respectively. Nanofiber meshes production lasted 1 hour, and for that it was needed 1 mL of polymeric solution. The applied voltage was kept at 9 kV. Nanofiber meshes were collected and cut in squares with 1 cm^2. Experiments were performed at room temperature and the conditions were optimized for the two materials. After

production and prior to the seeding procedures, nanofiber meshes were sterilized under ultra-violet (UV) light.

2.3.2. PCL nanofiber meshes clamping system for culture in the bioreactor

A clamping system to fixate the produced PCL nanofiber meshes in the multichamber flow perfusion bioreactor used in chapters 5 and 6 had to be assembled, as shown in Figure 2.8. The PCL nanofiber meshes meshes were clamped in between two silicon rings (with 8 mm outside diameter and 5 mm inside diameter), which were then clamped with nylon stitches [30]. The membranes were then sterilized under UV light for 1 hour in each side, with the meshes already mounted between the rings.

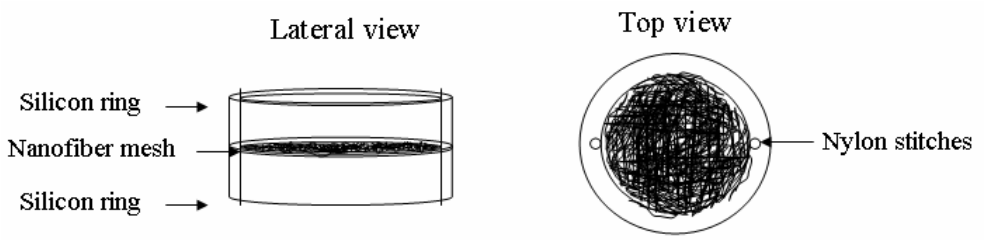

Figure 2.8 – Clamping system used for culturing PCL nanofiber meshes in the bioreactor.

3. Scaffolds characterization

3.1. Scaffolds morphology

As referred previously, we intended to work herein with different types of scaffolds morphology. Therefore, to clarify about the different porous morphologies of scaffolds, we include a figure with an example of each structure used: microporous scaffolds, nanofiber meshes and microfiber meshes (Figure 2.9).

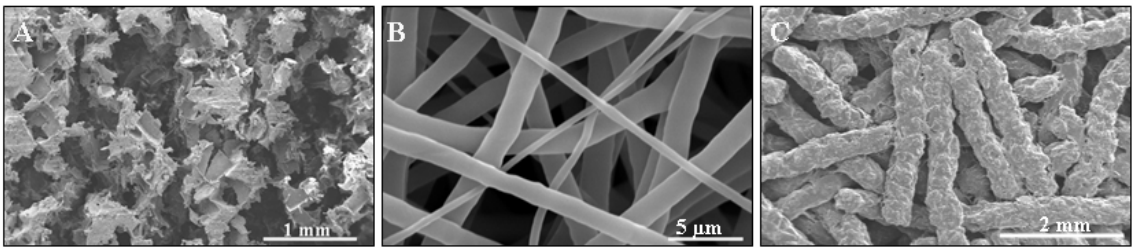

Figure 2.9 – Morphology of the scaffolds used in this thesis. A – Microporous scaffolds. B – Nanofiber meshes. C – Microfiber meshes.

3.2. Scanning electron microscopy for morphological analysis

Scanning electron microscopy (SEM) is a technique that allows analysing the surface structure and topography of a specimen at very large magnifications. It is possible by this technique to analyse details at submicron level. SEM provides a direct assessment of pore size and structure. Interconnectivity of a scaffold can also be estimated, as well as examination of scaffold interior by sectioning the specimens. Usually it is necessary to coat the samples with conductive materials such as gold or carbon, to provide conductivity and avoid charge accumulation or damaging the specimen surface. Herein, we used SEM for morphological analysis of the scaffolds surface. The different scaffolds were sputter-coated with gold (Fisons Instruments, model SC502; England). Then, samples were analyzed using a Leica Cambridge electron microscope, model S360 (Cambridge, England).

PCL and SPCL nanofibers diameter was assessed by quantification based in SEM micrographs with the software ImageJ (version 1.38X, Wayne Rasband Nacional Institutes of Health, USA). Pore sizes were also characterized using this software, and SEM micrographs.

3.3. Microcomputed tomography

Microcomputed tomography (µCT) is a non-destructive technique for the characterization of the structures with micrometer resolution, allowing the study of the interior of the structure in detail. Samples used in this technique can be used for futher tests because the technique is non destructive, providing a clear advantage relative to SEM analysis. Additionally, µCT provides a 3D imaging of the specimen and a close up

view of its interior and surface. Detailed information about scaffold characterization using μCT technique may be obtained *e.g.* in reference [31].

In the present work, a μCT equipment (SkyScan, Belgium) was used for a very detailed analysis of the morphology of the developed CPBS porous scaffolds. Four scaffolds of each condition were scanned in high-resolution mode of 8.7 μm x/y/z and an exposure time of 1792 ms. The energy parameters defined in the scanner were 63 keV with a current of 157 μA. Isotropic slice data were obtained by the system and captured into 2D images. These slice images were compiled and analyzed to render 3D images and obtain quantitative architecture parameters. A μCT analyser and a μCT Volume Realistic 3D Visualization, both from SkyScan, were used as image processing tools for both μCT reconstruction and to create/visualize the 3D images. Regions of interest (square of 4.5 x 4.5 mm^2) were selected in each slice image and a threshold was set to eliminate background noise. This threshold (to distinguish polymer material from pore voids) was chosen and maintained constant for all the scanned specimens and samples. The threshold was also inverted to obtain the volume of the pore and to analyze both the pore morphology and interconnectivity. The analysis was performed to obtain quantitative data on the level of porosity and interconnectivity of the studied scaffolds.

4. Cell isolation and expansion

4.1. Culture medium

In the context of the work developed during the PhD, several types of primary cultures were used, both from bovine and human origin samples. Chondrocytes and mesenchymal stem cells (MSCs) isolation procedures were studied and optimized, for both bovine and human samples. The culture medium used was similar for both types of cells, as we tested and validated the efficiency of the medium cocktail in both cultures.

4.1.1. Culture medium for chondrocytes primary cultures

For chondrocytes, two culture media were used, having a common basic cocktail named basic medium. For chondrocyte expansion in monolayer cultures basic medium supplemented with basic Fibroblast Growth Factor (bFGF) was used. For chondrocyte

culture in the 3D structures and to enhance cartilage ECM deposition, basic medium was supplemented with insulin and ascorbic acid. The culture media composition used for bovine and human chondrocytes in monolayer expansion and in 3D culture was prepared as follows:

- Basic Medium: Dulbecco's modified Eagle's medium (DMEM) (Sigma, D5671), containing 10 mM Hepes buffer (Sigma, H0887), L-alanyl-L-glutamine (Sigma, G8541), Non Essential Aminoacids (Sigma, M7145) 10000 units/ml penicillin, 10000 μg/ml streptomycin (Sigma, P0781), 10 % foetal calf serum (Biosera, S1810).

- Expansion medium for articular chondrocytes: medium composed by basic medium supplemented with 10 ng/ml of bFGF (PeproTech, 100-18B).

- Differentiation medium articular chondrocytes: this medium is composed by basic medium supplemented with 1 mg/ml of insulin (Sigma, I5500) and 1 mg/ml of ascorbic acid (Sigma, A4544).

4.1.2. Culture medium for MSCs primary cultures

Different culture media were used for the isolation and expansion of MSCs, and for chondrogenic differentiation of these cells.

- Expansion medium for MSCs: this medium was composed by Alfa Minimum Essential Medium (α-MEM) (Invitrogen, 12000-063), supplemented with antibiotic/ antimycotic solution (Gibco, 15240062), 20 % fetal bovine serum (Baptista Marques, BSC0115/0943k).

- Chondrogenic differentiation medium: this medium consisted of a mixture of α-MEM supplemented with antibiotic/antimycotic solution, ITS liquid media supplement (Sigma, I2521), dexamethasone 1 mM (Sigma, D1756), sodium pyruvate 0.1M (Sigma, P4562), ascorbate-2-phosphate 17 mM (Sigma, A4544), L-proline 35 mM (Sigma, P5607) and 1 ng/ml of human recombinant TGF-β3 (PeproTech, 100-36).

4.2. Articular chondrocytes isolation and expansion

Isolation of articular chondrocytes was performed from bovine (chapters 3 and 4) or from human samples (chapter 7). The method applied for isolation and expansion of articular chondrocytes was performed according to the method previously reported [32]. This method is based on the enzymatic digestion of cartilage ECM for release of chondrocytes, using collagenase type II for the degradation of ECM. The procedure was similar for both types of samples.

Bovine legs were acquired at the local abattoir, in Sheffield, UK. Full thickness hyaline cartilage was harvested from the metacarpophalangeal joint (Figure 2.10A). Human samples were collected at the local hospital, in Braga (Figure 2.10B). Articular cartilage was isolated from human patellae collected under informed consent from patients undergoing knee arthroplasties at the Hospital de São Marcos, Braga, Portugal. During surgeries, the knee was collected into a container with phosphate buffer saline (Sigma, D8537) and maintained in ice until the isolation procedure.

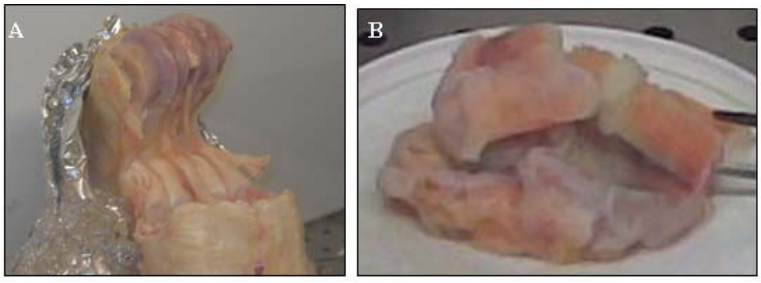

Figure 2.10 – Example of bovine and human samples used for isolation of articular chondrocytes. A – Bovine leg exposing the metacarpophalangeal joint. B – Human knee sliced in pieces.

Cartilage was dissected in small full-depth pieces and washed twice with phosphate saline buffer. Then, it was digested with 0.25 % (w/v) of trypsin solution (Sigma, E5134) for 30 minutes at 37 ºC on a rotator. The solution was removed, cartilage was washed again in phosphate saline buffer and then incubated in a collagenase type II solution (2 mg/mL) (Sigma, C6885) overnight at 37ºC on a rotator (Stuart mini orbital shaker SSM1). The following day, cells were washed twice with phosphate saline

buffer, counted and platted at a density of $2x10^6$ cells per Petri dish in the case of bovine cells and in culture flasks, in the case of human cells. Cells were cultivated with expansion medium prepared as described previously.

4.3. Human bone marrow mesenchymal stem cells isolation and expansion

Isolation of human bone marrow derived mesenchymal stem cells (hBMSCs) was based on these cells ability to adhere to plastic. The referred cells present a spindle-shape morphology and colony forming unit capacity (CFUs). The procedure employed in this thesis was based on a method previously described in the literature [33]. Human BMSCs were isolated from bone marrow aspirates collected under informed consent from patients undergoing knee arthroplasties in Hospital de São Marcos, Braga, Portugal (Figure 2.11). During surgeries, bone marrow was collected into a container with α-MEM medium, supplemented with antibiotic/ antimycotic solution and 5000 units of heparin (Sigma, H3393) and maintained in ice until the isolation procedure. Aspirates were homogenised, diluted in phosphate buffer saline (1:1) and incubated for 5 minutes at room temperature. Then, bone marrow was diluted in lyses buffer (1:10) and left under agitation for 10 minutes. Lyses buffer was prepared with 10 mM of Tris-HCl (Sigma, T3253), 1.21 g of Tris Base (Sigma, T1503) and 8.3 g of NH_4Cl (Merck, 1011455000), in 1L of distilled water. Afterwards, the suspension was centrifuged at 1200 rpm, for 15 minutes at room temperature. Cells were ressuspended in α-MEM medium, supplemented with antibiotic/ antimycotic solution and 20 % fetal bovine serum. Cell suspension was filtered for disposal of debris, using 100 μm and 70 μm Cell Strainer (BD Falcon™, 352360 and BD Falcon™, 352350). Cells were counted and plated at the density of $4.7x10^3$ cells/cm^2. Cells were expanded in expansion culture medium for MSCs as described previously.

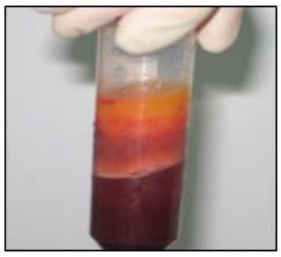

Figure 2.11 – Human bone marrow sample for isolation of mesenchymal stem cells.

4.4. Human Wharton´s jelly mesenchymal stem cells isolation and expansion

Human umbilical cords (UC) were obtained from informed consent full-term caesarian sections, under the cooperation agreement established between the 3B´s Research Group and the Obstetrics Department of the Hospital de São Marcos, Braga, Portugal and approved by the Ethics Committee of the same hospital (Figure 2.12). Human WJSCs (hWJSCs) were isolated according to the procedure originally described by Sarugaser *et al*. [34]. Briefly, UC were cut into 3–5 cm segments. These segments were then dissected by separation of the UC section epithelium along its length, exposing the underlying Wharton's jelly. Each vessel, with its surrounding Wharton's jelly matrix, was pulled away, and the ends of each dissected vessel were tied together with a suture creating "loops". Following, the loops were digested using a 1 mg/ml collagenase type I solution (Sigma, C0130) prepared with phosphate buffer saline (Sigma, D8537). After 18–24 hours, the loops were removed. The remaining suspension was diluted with phosphate buffer saline to reduce its viscosity, followed by a centrifugation at 1200 rpm for 5 minutes. Cells were ressuspended in 10 mL of expansion culture medium for MSCs and counted in a hemocytometer. Finally, cells were plated and expanded until reaching 80–90 % confluence.

Figure 2.12 – Example of an umbilical cord sample for isolation of human Wharton´s jelly mesenchymal stem cells.

4.5. Analysis of the "stemness" of isolated mesenchymal stem cells

Flow cytometry (FCM) is a technique that allows counting of cells or particles, suspending them in a fluid and passing them in a flow cytometer. Flow cytometry analysis was used herein to characterize surface markers of the isolated potential MSCs. It is well established that MSCs show non-haematopoietic surface markers such as

CD29, CD73 or CD166 and present colony forming unit –fibroblast (CFU-F) ability [35, 36].

To evaluate cell-surface marker expression, and the stemness of the isolated cells, some studies were performed, namely FCM and cell differentiation into several lineages. Cultured hBMSCs and hWJSCs were incubated for 20 min at 4 ºC with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies specific for human markers associated with mesenchymal or haematopoietic lineages. All the antibodies were purchased from BD Pharmingen. The samples were analysed using a BD FACSCalibur flow cytometer (BD Biosciences, Portugal). The stemness character of both of these cells was studied in a previous work by our group [37]. Cells were studied by flow cytometry for MSCs markers (CD31, CD34, CD45-negative and CD13, CD29, CD73, CD90, CD105, CD166-positive cells), and by differentiation studies into osteogenic, chondrogenic, and adipogenic lineage, being considered stem cells.

5. Cell and materials culture

5.1. Bovine articular chondrocytes (BAC) culture under static and dynamic conditions

In this first approach to cartilage tissue formation in vitro (corresponding to chapters 3 and 4), we employed a model using primary cultures of BAC, comparing static and dynamic (stirred) culture conditions. Those studies used nano or macro structures as scaffolds for support and enhancement of cartilagineous ECM deposition.

5.1.1. BAC seeding and culture onto SPCL and PCL nanofiber meshes

Dynamic cell seeding was performed using Petri dishes placed in a rotator inside the incubator. Three nanofiber meshes were placed in each Petri dish, and the cell suspension was added to obtain $6.5x10^6$ cells per nanofiber mesh. We used the expansion culture medium for articular chondrocytes described previously for this step. We used 20 nanofiber meshes per experimental condition, for each material. Petri dishes were placed in the rotator, inside the incubator, at 60 rpm, for 72 hours. Afterwards, cell seeding was complete, and half of the constructs were kept in the rotator inside the incubator (dynamic conditions), whereas the others were placed in the incubator (static

conditions). Culture medium was changed for differentiation medium prepared as described in 4.1.1. The medium was changed every three days.

5.1.2. BAC seeding and culture onto CPBS scaffolds

Bovine AC were harvested, counted and ressuspended in expansion medium. We used this method for dynamic seeding of CPBS scaffolds with different porosities, as described in chapter 4 of this thesis. Twenty scaffolds were used in each experiment, for each type of scaffold. Materials were seeded with a cell suspension containing $6.5x10^6$ cells per scaffold. Seeding was performed in spinner flasks in order to allow the cell penetration into the porous structure of the scaffolds. After 72 hours, the constructs were removed from the spinner flasks and divided by static and dynamic cultures, as described for PCL and SPCL nanofiber meshes.

5.2. Human BMSCs culture in a flow perfusion bioreactor

In the second part of the present PhD (corresponding to chapters 5 and 6), we used primary cultures of human mesenchymal stem cells, in static and dynamic culture (bioreactor) conditions to assess chondrogenic differentiation of hMSCs. The aim was to verify if the bioreactor would enhance ECM production and accumulation when using 3D structures produced with nano or microfibrous as scaffolds for cells.

5.2.1. Human BMSCs seeding onto PCL nanofiber meshes

Human BMSCs were harvested, counted and ressuspended in basic medium for MSCs in order to obtain a cell suspension of 200.000 cells/nanofiber mesh. The seeding method was previously described in detail and published by our group [30]. Each scaffold was seeded with a 10 µL drop of the prepared cell suspension in 24 well plates. Afterwards, constructs were maintained at 37 ºC and 5 % CO_2, during 4 hours in an incubator to allow cell attachment to the nanofiber meshes. After this time period, 1 mL of expansion medium for MSCs was added to each culture well and left in the incubator for 24 hours. The constructs were then transferred to the bioreactor. In the next day, the flow perfusion bioreactor was fully sterilized in the autoclave. Then, it was assembled inside the hood and the constructs to be cultured were transferred into the bioreactor. The bidirectional flow perfusion bioreactor (PT patent nº 104155; European patent pending [38]) has a maximum of 20 individual culture chambers. According to the

study plan, 20 constructs were transferred to the bioreactor. The flow rate was set to 70 µl/min/nanofiber mesh. The bioreactor was transferred into the incubator and kept in those conditions during the whole experiment.

Another set of 20 identical constructs was transferred to new culture plates and maintained in static conditions to be used as control. Chondrogenic differentiation medium was added to the bioreactor and to the new culture plates for static culture, for promoting chondrogenic differentiation. Culture medium in the bioreactor was completely changed every week (100 mL each time). Control samples were cultured in 24 well plates, and were fed every other day with 1 mL of culture medium, until the end of the experiment (28 days).

5.2.2. Human BMSCs seeding onto CPBTA fiber meshes

Human BMSCs were harvested from culture and counted. A cell suspension of $1x10^6$ cells/ fiber mesh was prepared to seed each scaffold. Cell seeding was performed in static conditions, inside the incubator, at 37ºC, during 24 hours. All the procedure follows the previous description presented in section 5.2.1, except for the bioreactor flow rate. For this work, the established flow rate was 100 µl/min/microfiber mesh.

5.3. Co-cultures of human ACs and MSCs onto CPBS fiber meshes

In the last part of this thesis (corresponding to the chapter 7), we studied the influence of chondrocytes onto the chondrogenic differentiation of human MSCs, using a 3D structure to facilitate the cell differentiation. We performed these experiments in static culture conditions, as we did not obtain conclusive results herein on the positive influence of dynamic culture conditions on chondrogenic differentiation and ECM production, compared to static conditions (chapters 5 and 6). We selected in this case the scaffold morphology that, based in previous results, showed the highest performance in having a positive influence over the chondrogenic differentiation of MSCs (CPBS microfibrous structure).

Cells were expanded, split, counted and then seeded onto the CPBS fiber meshes. Two study groups were established: direct co-culture using with each source of hMSCs (hBMSCs or hWJSCs) and hACs; indirect co-culture using conditioned medium obtained from the culture of hACs. This indirect co-culture method herein described is a variation of the transwell inserts technique [39, 40]. In this case, instead of separating

the two cultures with a transwell, we cultured separately the chondrocytes and the stem cells. As discussed in the introduction of this thesis, it is known that the scaffolds 3D structure can positively influence the chondrogenic commitment of MSCs. We hypothesised that by providing the microenvironment and the soluble factors present in chondrocytes medium we would be able to influence the chondrogenic differentiation of both types of MSCs used in the present work. Thus, as the medium provided was previously consumed by chondrocytes, we used conditioned medium mixed (50/50 in volume) with fresh medium. By diluting the factors secreted by the chondrocytes in fresh medium, it was intended to provide the required nutrients to the MSCs indirect co-cultures, avoiding the risk of nutrient deprivation.

5.3.1 Direct co-cultures

A suspension of $1x10^6$ cells ($5x10^5$ hBMSCs and $5x10^5$ hACs) per scaffold was used for seeding the fiber meshes using the dynamic culture method in a rotator, for 24 hours. Afterwards, constructs were changed into new culture plates and fed with chondrocytes differentiation medium. Constructs were placed inside the incubator in static culture conditions and cultured for 28 days. Medium was changed every other day. The same procedure was used for the direct co-cultures of hWJSCs.

5.3.2 Indirect co-cultures

Indirect co-cultures were performed using conditioned medium, obtained from hACs in culture. First, a cell suspension containing $1x10^6$ cells per scaffold was prepared for each type of cells (hBMSCs or hACs). Each cell type was seeded onto different scaffolds, in different culture plates. The seeding procedure was similar to the one described above for the direct co-cultures. Afterwards, cells were placed in new culture plates containing differentiation medium. After two days, medium was changed. The human BMSCs medium was completely removed, and replaced by conditioned medium. This consisted of a mixture of the medium which was removed from the chondrocytes culture, mixed with fresh differentiation medium (50/50 in volume). Medium of these cultures (hBMSCs) was changed every other day, using this mixed conditioned medium. For the hACs cultures, fresh differentiation medium was used, and changed every other day. The two types of cultures were maintained for 28 days, in an incubator, at 37ºC and 5% CO_2. The same procedure was used for the indirect co-cultures of hWJSCs.

6. Biological assays

6.1. Cell proliferation assay

Viability and proliferation of cells is an important parameter to consider when using cell cultures. Cell proliferation can be assessed indirectly by quantifying its metabolic activity. The PicoGreen dsDNA assay allows the detection of double stranded DNA. The number of cells is directly correlated with the quantity of protein or DNA present in cells. The kit used herein is composed with fluorescent solutions that bind to DNA, allowing its indirect detection by fluorescence, using a microplate reader. The evaluation of the cell proliferation was performed using the PicoGreen dsDNA quantification Kit (Molecular Probes, P7589), according to the manufacturer instructions. Samples were collected at several time points. Triplicates of every time point were used. A standard curve ranging from 0.0 to 1.5 μg/ml was established. Fluorescence of both samples and standard curve was read with an excitation of 485/20 nm and an emission of 528/20 nm. DNA concentration was extrapolated from the standard curve. This method was used to evaluate cell proliferation in chapters 5, 6 and 7.

6.2. Evaluation of cell morphology and distribution

Evaluation of cell morphology and distribution was performed using SEM. In the present thesis we used two different microscopes for constructs analysis: a Philips XL-20 and a Leica Cambridge S360, Leica Cambridge, Cambridge, UK. Preparation of samples was different, depending on which SEM used. All samples were collected at 7, 14, 21 and 28 days of experiments. Samples from chapters 3 and 4 were prepared as follows. Samples were washed in sterile phosphate buffer saline and immersed in 3 % glutaraldehyde (Sigma, G5882) with 0.1 M cacodylate buffer at pH 7.4 (AGAR R1103) at room temperature for one hour. Afterwards, they were washed in phosphate buffer saline and osmium tetra oxide was added and left for 2 hours. Finally, samples were dehydrated in increasing alcohol concentrations and let to dry. Then, they were sputter coated with gold and analysed.

Samples from chapters 5 and 6 were washed in sterile phosphate buffer saline and immersed in 3 % glutaraldehyde (Sigma, G5882) at room temperature for one hour.

Samples were washed in phosphate buffer saline, dehydrated in increasing alcohol concentrations and the procedure continued as described above.

6.3. Glycosaminoglycans quantification

Glycosaminoglycans (GAGs) quantification was based on the use of proteolytic enzymes able to degrade cartilage ECM and a colorimetric assay to measure the quantity of GAGs released [41]. Enzymes will degrade proteoglycans and the released GAGs will be quantified indirectly. The basic die 1,9 dimethylmethylene blue will link to the negatively charged GAGs and allow their detection by measuring the solution absorbance. Herein, GAGs quantification was performed using two different methods, differing mostly in the enzyme used for digestion of the produced ECM.

6.3.1. GAGs quantification using papain digestion

Samples were collected, freeze/dried overnight and then digested. Digestion solution was prepared by adding papain (Sigma, P4762) and N-acetyl cysteine (Sigma, A8199) at the concentrations of 0.05 % and 0.096 %, respectively, to 50 ml of digestion buffer (200 mM of phosphate buffer containing 1 mM EDTA (Sigma, E5134), pH 6.8). Samples were incubated with 600 µl of the referred solution, overnight at 60ºC. Afterwards, they were centrifuged at 13000 rpm for 10 minutes. Supernatant was collected and stored at -20 ºC until the assay was performed. Dimethymethylene Blue (DMB) stock solution was prepared dissolving 16 mg of DMB powder in 900 ml of distilled water containing 3.04 g of glycine and 2.73 g of NaCl. pH was adjusted to 3.0 with HCl and volume adjusted to 1L. The solution was stored at room temperature covered by alumin foil. Chondroitin sulphate (Sigma, C8529) solution was prepared in water, in a 5 mg/ml stock solution and kept refrigerated. Dilutions of this solution were performed in order to make a standard curve. Samples were also diluted as appropriated, with distilled water. Optical density was measured in a microplate reader, at 530 nm. Unseeded nanofiber meshes and CPBS scaffolds (chapters 3 and 4) were used as controls. They were treated in the same way than the seeded ones for the GAG assay procedure. We used three samples per each GAG assay.

6.3.2. GAGs quantification using proteinase K digestion

DMB assay was performed according to the method described elsewhere [42]. Briefly, samples (Chapter 7) were collected at 1 and 4 weeks of culture, frozen overnight and then digested. This digestion allowed separating the formed ECM from the scaffold. Digestion solution was prepared by adding 1 mg/mL of proteinase K (Sigma, P8044) diluted in 50 mM Tris-HCl, at pH of 7.6. Tris-HCl solution was prepared using the appropriate amount of Tris-HCl, adding sodium azide (Sigma, 13412) to a final concentration of 0.05 % (w/v). The pH was corrected for the final value of 7.6. Samples were placed in 1.5 ml tubes and incubated with 1 mL of the referred solution, overnight at 60 ºC. The procedure followed the same steps before mentioned in 6.3.1.

6.4. Histological analysis

Prior to sectioning, samples need to be processed. In the case of cells or tissues, several embedding media are available for covering the structure and preserving its integrity. Depending on the sample its characteristics, various embedding media can be selected, such as paraffin, OCT compound or glycol methacrylate. The main difference between these media is on the processing technique. Paraffin samples are processed using high temperatures (between 60-80 ºC) whereas OCT samples are processed in cold conditions using liquid nitrogen. Glycol methacrylate embedded samples are processed using intermediate temperatures (4 ºC).

Herein, due to the different materials used, two inclusion procedures were applied for processing samples for histology. Nanofiber meshes and CPBS constructs (from chapters 3, 4 and 5, respectively) were included in OCT compound. This compound is used for embedding sections prior to be frozen and sectioned in cryogenic conditions. Samples from chapters 6 and 7 were collected at the end of the experiment and processed for histology using Technovit, which is an embedding kit based in glycol methacrylate.

6.4.1. Samples inclusion and processing using OCT compound

Samples were collected at the end of the experiment, included in OCT (OCT compound BDH, Gurr®) and stored at -20 ºC. Eight µm thick sections were cut and placed in microscopy slides. They were further fixed during 30 minutes at 4 ºC, in a

fresh 4 % paraformaldehyde (Sigma, P6148) solution in phosphate buffer saline. The samples were futher washed twice in distilled water and let overnight to air-dry. Slides were stored at 4 ºC until they were used for the staining procedures.

6.4.2. Samples inclusion and processing using Technovit

Samples were fixed in 10 % neutral buffered formalin and then dehydrated through increasing ethanol concentrations. The samples were immersed in infiltration solutions and embedded in Technovit® glycol methacrylate (kit 8100, Heraeus Kulzer, EBSciences, CO, USA) and polymerized. Specimens were cut to obtain longitudinal cross-sections of 7 µm of thickness, using a modified microtome equipped with a tungsten blade (Leica RM 2155).

6.4.3. Staining

Hematoxylin-eosin (H&E) staining is a method widely used for the analysis of cells distribution and morphology. The combination of two stains (hematoxilyn and eosin) allows detecting cells nuclei and cytoplasm. This stain was performed to evaluate the cells distribution within the scaffolds. H&E staining was conducted in an automatic machine (Fume Cupboard; X219/E11/LEV1) Sections were washed in running water for 5 minutes, and then dipped in 1 % acid alcohol for 5-10 seconds. They were washed again in water and stained in eosin for 10 minutes. Another washing with water was performed, for 5 minutes, and sections were afterwards dehydrated through crescent concentrations of alcohol. After staining, slides were cleared in xylene and mounted in DPX (BDH 36029 2F).

Toluidine blue staining was also performed. This method allows a metachromatic staining of cartilage matrix and specifically of its proteoglycan content. It stains proteoglycans and GAGs. This procedure was used for all samples in the results chapters. Staining solution was prepared by adding 1 % of toluidine blue (Sigma, T0394) dissolved in distilled water containing 0.5 g of sodium borate, followed by filtering. One drop of this solution was added to each section for 2-3 seconds. Then, sections were rinsed with distilled water and let to air dry. They were cleared in xylene and mounted in DPX.

Alcian blue stain was performed for samples studied in chapters 3 and 4 to detect cartilage formation. This dye stains acid mucopolysacharides and GAGs. Alcian Blue stain was performed by rinsing the sections in 3 % acetic acid and keeping them in 1 %

alcian blue solution prepared as described in the referred chapters. Sections were counterstained with aqueous neutral red (Sigma, N6634) and then washed with water, let to dry and then rinsed in absolute alcohol. Mounting of slides was processed as previously described for the other staining procedures.

Safranin O staining is another technique to detect cartilage components. This is a cationic dye that in dehydrated cartilage sections gets its orthochromatic form, and is in this form that safranin O links to tissues GAGs. This stain was performed was used for samples in chapters 5 to 7. A detail description of the procedure can be found in the referred chapters. By the end of the stain, sections were cleared in xylene and mounted as previously described.

6.5. Immunolocalisation of collagens type I and type II

Immunolocalisation is a technique performed to detect and identify specific protein markers on in vitro cultures cells and in tissues. The methods used in the present thesis were performed according to the instructions of the manufacturer of the Vectastain and DAB kits, but some of the solutions employed in the procedure were different. Therefore, two methods are described in this section. The principle of this method relies on the incubation of the sample section with a specific primary antibody for the antigen of interest. Then, the secondary antibody is added. This antibody is biotin-labeled, introducing many biotin molecules in the section and in the location of the primary antibody. An avidin-biotinylated enzyme complex in then added that binds to the secondary antibody. Localisation of the antigen is obtained by adding an enzyme substrate.

6.5.1. Immunolocalisation method I

Immunolocalisation of type I and type II collagens was performed in frozen sections of samples in chapters 3 and 4. Sections were pre-treated in 10 mg/ml hyaluronidase (Sigma, H4272) for 30 minutes at 37 ºC and in 2 mg/ml pronase (Sigma, P6911), for 30 minutes, at 37 ºC. Sections were washed in phosphate buffer saline and endogenous peroxidase activity was quenched with 3 % hydrogen peroxide in 50 % methanol (BDH, 101586 6B) for 5 minutes. This step is justified by the need to block tissues intrinsic endogenous peroxidases that produce a reaction product from the substrate alone. Sections were washed in Tri-buffered saline (TBS) and blocked with 3 % bovine serum

albumin (BSA) (Sigma, A2153) in TBS/ Tween 20 (Sigma, P7949) for 1 hour to avoid non-specific staining. Sections were further incubated with primary antibodies (collagen type I and collagen type II) (UNLB 1310-01 and UNLB 1320-01) overnight at 4 ºC, in a humidified atmosphere. Then, sections were washed once with high salt wash solution and twice in TBS/Tween 20, 10 minutes each and then incubated with the secondary antibody from the kit during 1 hour at room temperature, again in a humidified atmosphere. The remaining protocol is similar to the one described in the Vectastain Elite ABC Kit PK-6105 (Vector Laboratories Ltd, UK) and in the Vector DAB Kit (Vector Laboratories Ltd, UK). Slides were washed in water for 5 minutes and then counterstained with hematoxylin for nuclei visualization. Finally, slides were mounted in DPX. Controls were performed using normal goat serum instead of primary antibodies, which was also included in the kit.

6.5.2. Immunolocalisation method II

For chapters 5 to 7, a variant of this method was used. Immunolocalisation of type I and type II collagens was performed in technovit sections. Endogenous peroxidase activity was quenched with 0.3 % hydrogen peroxide solution for 30 minutes. Sections were rinsed in phosphate buffer saline for 5 minutes. R.T.U. Vectastain® Universal Elite ABC Kit (Vector, VCPK-7200) was used for antibody incubation, according to the instructions of the manufacturer. Shortly, sections were incubated with primary antibodies (collagen type I and collagen type II) (UNLB 1310-01 and UNLB 1320-01) overnight at 4 ºC, in a humidified atmosphere. Incubation was revealed by using the Peroxidase Substrate Kit DAB (Vector, VCSK-4100). Slides were washed in water for 5 minutes and then counterstained with hematoxylin for nuclei visualization. Finally, slides were mounted in Histo-clear®. Controls were performed using normal goat serum instead of primary antibodies, which was also included in the kit.

6.6. RNA isolation

The first and often most critical step in performing fundamental molecular biology experiments such as RT-PCR, is the RNA isolation. The method herein used for RNA extraction and isolation was based on the Trizol reagent. This reagent is ready-to-use for isolation of total RNA from cells and tissues. Trizol disrupts cells and dissolves their components while maintaining RNA integrity. Then, chloroform is added, which allows

the separation of the solution into two distinct phases: aqueous and organic. RNA will remain in the aqueous phase and it is recovered by precipitation with isopropyl alcohol.

Three samples were collected for each time point and each condition used herein, in chapters 5, 6 and 7. Samples were washed in phosphate buffer saline, immersed in TRIzol reagent (Invitrogen, 15596-018) and kept at -80 ºC for posterior RNA extraction. To perform the RNA extraction, samples were taken from the freezer, and kept in ice until complete thawing. Chloroform (Sigma, C2432) was added; samples were vigorously agitated for 15 seconds and then incubated in ice for 15 minutes. After that incubation, samples were centrifuged at 13000 rpm, for 15 minutes, at 4 ºC. Afterwards, the supernatant was collected for a sterile 1.5 mL tube, and an equivalent volume of isopropanol (Sigma, I9516) was added. Samples were incubated at -20 ºC overnight, to precipitate the RNA. In the next day, samples were centrifuged at 13000 rpm, for 15 minutes, at 4 ºC. Then, the supernatant was removed and 800 µl of ethanol 70 % was added, in order to wash away the isopropanol. The ethanol 70 % solution was prepared from absolute ethanol (Merck, 1.00983.2511) and ultra pure water. The 1.5 mL tubes were agitated vigorously and centrifuged again, at 9000 rpm for 5 minutes, at 4 ºC. The supernatant was again removed, and the pellet was left to air dry. Finally, the pellet was ressuspended in 50 µl of DNase, RNase free water (Gibco, 10977-015). The concentration and purity of the extracted RNA was evaluated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA).

6.7. Real-Time Polymerase Chain Reaction (RT-PCR)

Real-time PCR (RT-PCR) is a quantitative technique based in the Polymerase Chain Reaction (PCR) procedure. PCR is a widely used technique that allows the amplification of a copy of a DNA fragment million of times amplifying its number to detectable quantities (amplicon). This technique relies on thermal cycling and enzyme (DNA polymerase) replication of DNA. One major drawback of PCR is that the amplicon can only be visualized when the reaction finishes. In RT-PCR this does not happen, since this technique allows the amplification and simultaneous quantification of the amplicon. RT-PCR is usually combined with reverse transcription to quantify messenger RNA in cells or tissues. In the present thesis, we used this combination for detecting gene expression of cartilage related genes. We used a reverse transcriptase RT-PCR, consisting of a two step fluorogenic assay. In the first step, isolated RNA was

reversed transcribed into complementary DNA (cDNA), followed by RT-PCR for detection of cartilage related genes.

Samples from chapters 5 and 6 were studied using the SyberGreen system (Bio-Rad). All the reagents used in this procedure were from Bio-Rad, following the instructions of the manufacturer. Thermocycler reaction conditions used were also the ones proposed in the kits. In the first step, RNA was reversed transcribed into cDNA, using the iScript cDNA synthesis kit (1708891). A MiniOpticon real-time PCR detection system (BioRad Laboratories, USA) was used to perform the reaction. Afterwards, the cDNA obtained was used as template for the amplification of the target genes (aggrecan, collagen type I, II and X, Sox9 and Runx2), with the Syber Green Kit (1708884). The number of amplification cycles used for every reaction was of 45.

Samples in chapter 7 were analysed using a different thermocycler and different kits. In the Real Time-PCR procedure the PerfeCta™ SYBR® Green system (Quanta Biosciences) was used. All reagents used in this procedure were purchased from Quanta Biosciences, following the instructions of the manufacturer. Thermocycler reaction conditions used were also the ones mentioned in the kits. In the first step, RNA was reversed transcribed into cDNA, using the qScript cDNA Synthesis Kit (95047-500). A MasterCycler EP Gradient detection system (Eppendorf, USA) was used to perform the reaction. Afterwards, the obtained cDNA was used as template for the amplification of the targets genes (Table 2.1), with the PerfeCta™ SYBR® Green FastMix™ kit (95072-05K).

Table 2.1 – Primer sequences used for RT-PCR procedures.

Gene[a]	Forward (5´-3´)	Reverse (5´-3´)
AGC	TGAGTCCTCAAGCCTCCTGT	TGGTCTGCAGCAGTTGATTC
COL II	CGGTGAGAAGGGAGAAGTTG	GACCGGTCACTCCAGTAGGA
COL I	AGCCAGCAGATCGAGAACAT	ACACAGGTCTCACCGGTTTC
COL X	CCAGGTCTCGATGGTCCTAA	GTCCTCCAACTCCAGGATCA
Runx2	TTCCAGACCAGCAGCACTC	CAGCGTCAACACCATCATTC
Sox9	TTCATGAAGATGACCGACGC	GTCCAGTCGTAGCCCTTGAG
GAPDH	ACAGTCAGCCGCATCTTCTT	ACGACCAAATCCGTTGACTC

[a]AGC = Agreccan; COL II = Collagen type II; COL I = Collagen type I; COL X = Collagen type X; Runx2 = Runt-related transcription factor 2; Sox9 = Sry-type high mobility group box 9; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

GAPDH was used as reference gene, and the expression of all the target genes was normalized to the GAPDH expression of that sample. All the primer sequences were generated using Primer3 software [43] and acquired from MWG Biotech AG, Germany. Primers sequences used are shown in Table 2.1. Obtained results were analyzed with CFX Manager Software – version 1.5 (BioRad Laboratories, USA).

6.8. Statistical analysis

Statistical analysis was performed for data of GAGs quantification in chapters 3 and 4 using One-way analysis of variance in conjunction with Tukey's test. Data from triplicates of GAG quantification are present as averages ± standard errors.

Statistical analysis for chapters 5 and 6 was performed using the SPSS statistic software (Release 15.0.0 for Windows). Firstly, a Shapiro-Wilk test was used to ascertain about the data normality and variance equality. The normality was rejected and, consequently, nonparametric tests were used in further comparisons between static and dynamic culture conditions. A Mann-Whitney U-test was applied to compare independent groups of samples for each variable. P values lower than 0.01 were considered statistically significant in the analysis of the results. A Kruskal-Wallis test was applied to compare data between the different time points in each group. P values lower than 0.01 were considered statistically significant in the analysis of the results.

For chapter 7, statistical analysis was performed as described above using the SPSS statistic software. Nonparametric tests were used in comparisons between direct and indirect co-cultures. A Kruskal-Wallis test followed by Tukey's HSD test was applied to compare data between the different time points. P values lower than 0.001 were considered statistically significant in the analysis of the results.

References

1 - Brown CD, Hoffman AL. *Modification of natural polymers: Chitosan.* In: Atala A, Lanza R, editors. Methods of tissue engineering. San Diego: Academic Press; 2002. p. 565-74.

2 - Tuzlakoglu K, Reis RL. *Chitosan-based scaffolds in orthopeadic applications.* In: Reis RL, editor. Natural-based polymers for biomedical applications. Cambridge, England: Woodhead Publishing Limited and CRC Press LLC; 2008. p. 357-73.

3 - Correlo VM, Boesel LF, Pinho E, Costa-Pinto AR, Alves da Silva ML, Bhattacharya M*, et al. Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties*. J Biomed Mater Res A. 2009. 91(2):489-504.

4 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, Neves NM*, et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromol Biosci. 2010.

5 - Correlo VM, Boesel LM, Bhattacharya M, Mano JF, Neves NM, Reis RL. *Properties of melt processed chitosan and aliphatic polyester blends*. Materials Science and Engineering A. 2005. 403:57–68.

6 - Clarinval A-M, Halleux J. *Classification of biodegradable polymers.* In: Smith R, editor. Biodegradable polymers for industrial applications Cambridge, England: Woodhead publishing limited; 2005. p. 3-31.

7 - Bhattacharya M, Reis RL, Correlo VM, Boesel L. *Material properties of biodegradable polymers.* In: Smith R, editor. Biodegradable polymers for industrial applications. Cambridge, England: Whoodhead Publishing limited; 2005. p. 336-56.

8 - Khan MA, Ali KM, Yoshii F, Makuuchi K. *Enzymatic degradation of Bionolle and Bionolle–rubber blends* Polymer Degradation and Stability. 1999. 63(2):261-4.

9 - Witt U, Einig T, Yamamoto M, Kleeberg I, Deckwer WD, Muller RJ. *Biodegradation of aliphatic-aromatic copolyesters: Evaluation of the final biodegradability and ecotoxicological impact of degradation intermediates*. Chemosphere. 2001. 44(2):289-99.

10 - Halley PJ. *Thermoplastic starch biodegradable polymers.* In: Smith R, editor. Biodegradable polymers for industrial applications. Cambridge, England: Whoolhead Publishing limited; 2005. p. 140-62.

11 - Shao X, Hutmacher DW, Ho ST, Goh CH, Lee EH. *Evaluation of a hybrid scaffold/cell construct in repair of high-load-bearing osteochondral defects in rabbits*. Biomaterials. 2006. 27(7):1071 - 80.

12 - Yoshimoto H, Shin YM, Terai H, Vacanti JP. *A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering*. Biomaterials. 2003. 24(12):2077-82.

13 - Venugopal J, Ma LL, Yong T, Ramakrishna S. *In vitro study of smooth muscle cells on polycaprolactone and collagen nanofibrous matrices*. Cell Biol Int. 2005. 29(10):861-7.

14 - Tillman BW, Yazdani SK, Lee SJ, Geary RL, Atala A, Yoo JJ. *The in vivo stability of electrospun polycaprolactone-collagen scaffolds in vascular reconstruction*. Biomaterials. 2009. 30(4):583-8.

15 - Kim HJ, Lee JH, Im GI. *Chondrogenesis using mesenchymal stem cells and PCL scaffolds*. J Biomed Mater Res A. 2010. 92(2):659-66.

16 - Neves NM, Mano J, Reis RL. *Biodegradable composites for biomedical applications.* In: Reis RL, San Roman J, editors. Biodegradable systems in tissue engineering and regenerative medicine. Boca Raton, Florida: CRC Press LLC; 2005. p. 91-113.

17 - Oliveira JT, Crawford A, Mundy JM, Moreira AR, Gomes ME, Hatton PV*, et al. A cartilage tissue engineering approach combining starch-polycaprolactone fibre mesh scaffolds with bovine articular chondrocytes*. J Mater Sci Mater Med. 2007. 18(2):295-302.

18 - Salgado AJ, Coutinho OP, Reis RL. *Novel starch-based scaffolds for bone tissue engineering: Cytotoxicity, cell culture, and protein expression*. Tissue Engineering. 2004. 10(3/4):465 - 74.

19 - Santos MI, Fuchs S, Gomes ME, Unger RE, Reis RL, Kirkpatrick CJ. *Response of micro- and macrovascular endothelial cells to starch-based fiber meshes for bone tissue engineering*. Biomaterials. 2007. 28(2):240-8.

20 - Marques AP, Reis RL, Hunt JA. *An in vivo study of the host response to starch-based polymers and composites subcutaneously implanted in rats*. Macromol Biosci. 2005. 5(8):775-85.

21 - Martins AM, Pham QP, Malafaya PB, Sousa RA, Gomes ME, Raphael RM*, et al. The role of lipase and alpha-amylase in the degradation of starch/poly(epsilon-caprolactone) fiber meshes and the osteogenic differentiation of cultured marrow stromal cells*. Tissue Eng Part A. 2009. 15(2):295-305.

22 - Santos MI, Unger RE, Sousa RA, Reis RL, Kirkpatrick CJ. *Crosstalk between osteoblasts and endothelial cells co-cultured on a polycaprolactone-starch scaffold and the in vitro development of vascularization*. Biomaterials. 2009. 30(26):4407-15.

23 - Fuchs S, Ghanaati S, Orth C, Barbeck M, Kolbe M, Hofmann A*, et al. Contribution of outgrowth endothelial cells from human peripheral blood on in vivo vascularization of bone tissue engineered constructs based on starch polycaprolactone scaffolds*. Biomaterials. 2009. 30(4):526-34.

24 - Pawar R, Jadhav W, Bhusare S, Borade R. *Sources, properties, modification and processing of natural-based polymers.* In: Reis Rl, editor. Natural-based polymers for biomedical applications. Cambridge, England: Woodhead Publishing Limited and CRC Press LLC; 2008. p. 3-53.

25 - Puppi D, Chiellini F, Piras AM, Chiellini E. *Polymeric materials for bone and cartilage repair*. Progress in Polymer Science. 2010. 35:403-40.

26 - Correlo VM, Boesel LF, Pinho E, Costa-Pinto AR, Alves da Silva ML, Bhattacharya M*, et al. Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties*. J Biomed Mater Res A. 2008.

27 - Correlo VM. *Development of new chitosan based biodegradable blends for bone and cartilage tissue engineering*. Guimarães: University of Minho, Portugal; 2009.

28 - Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol P, Bhattacharya M*, et al. Chitosan/polyester-based scaffolds for cartilage tissue engineering: Assessment of extracellular matrix formation*. Acta Biomater. 2010. 6(3):1149-57.

29 - Lim SH, Mao HQ. *Electrospun scaffolds for stem cell engineering*. Adv Drug Deliv Rev. 2009. 61(12):1084-96.

30 - Guimaraes A, Martins A, Pinho ED, Faria S, Reis RL, Neves NM. *Solving cell infiltration limitations of electrospun nanofiber meshes for tissue engineering applications*. Nanomedicine (Lond). 2010. 5(4):539-54.

31 - Ho ST, Hutmacher DW. *A comparison of micro CT with other techniques used in the characterization of scaffolds*. Biomaterials. 2006. 27(8):1362-76.

32 - Crawford A, Dickinson S. *Chondrocyte isolation, expansion, and culture in polymer scaffolds.* In: Hollander AP, Hatton PV, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 147-57.

33 - Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D*, et al. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo*. J Bone Miner Res. 1997. 12(9):1335-47.

34 - Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. *Human umbilical cord perivascular (hUCPV) cells: A source of mesenchymal progenitors*. Stem Cells. 2005. 23(2):220-9.

35 - Delorme B, Charbord P. *Culture and characterization of human bone marrow mesenchymal stem cells*. Methods Mol Med. 2007. 140:67-81.

36 - Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR*, et al. Characterization and functionality of cell surface molecules on human mesenchymal stem cells*. J Biomed Sci. 2003. 10(2):228-41.

37 - Costa-Pinto A. R., Frias AM, Alves da Silva ML, Barros A., Fernandes S., RL R*, et al. Comparative study of human Wharton's jelly and amniotic fluid derived stem cells differentiation potential*. Tissue Engineering: . 2008. 14:713.

38 - Costa P, Martins A, Gomes M, Neves NM, Reis RL. *Multichamber bioreactor with bidirectional perfusion integrated in a culture system for tissue engineering strategies*. European Patent Office. 2010. EP 2151491 A2(09009863.3):11.

39 - Hwang NS, Varghese S, Elisseeff J. *Derivation of chondrogenically-committed cells from human embryonic cells for cartilage tissue regeneration*. PLoS ONE. 2008. 3(6):e2498.

40 - Vats A, Bielby RC, Tolley N, Dickinson SC, Boccaccini AR, Hollander AP*, et al. Chondrogenic differentiation of human embryonic stem cells: The effect of the micro-environment*. Tissue Eng. 2006. 12(6):1687-97.

41 - Billington CJ. *Cartilage proteoglycan release assay*. Methods Mol Biol. 2001. 151:451-6.

42 - Kafienah W, Sims TS. *Biochemical methods for the analysis of tissue engineered cartilage.* In: Hollander AP, Hatton PV, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 217 - 29.

43 - http://frodo.wi.mit.edu/

SECTION III – BOVINE ARTICULAR CHONDROCYTES CULTURE UNDER STATIC AND DYNAMIC CONDITIONS USING NANOFIBER MESHES AND MICROPOROUS SCAFFOLDS AS CELL SUPPORT

Chapter 3

Evaluation of extracellular matrix formation in PCL and SPCL nanofiber meshes when seeded with bovine articular chondrocytes

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This chapter is based on the following publication: Alves da Silva ML, Crawford A, Mundy J, Martins A, Araújo JV, Hatton PV, Reis RL, Neves NM. *Evaluation of extracellular matrix formation in PCL and SPCL nanofiber meshes when seeded with bovine articular chondrocytes*. Tissue Engineering: Part A. 2009. 15(2): 377-385

Abstract

Repair of cartilage defects is still a major health problem. Tissue engineering has developed different strategies and several biomaterials morphologies, including natural-based ones, for repairing these defects. We used electrospun polycaprolactone (PCL) and starch-compounded polycaprolactone (SPCL) nanofiber meshes to evaluate extracellular matrix (ECM) formation by bovine articular chondrocytes (BAC). The main aim of this work is to evaluate the suitability of PCL and SPCL nanofiber meshes in chondrocyte cultures, and their capability to produce ECM when seeded onto these nanostructured materials. Furthermore, the effect of culture conditions (static vs. dynamic) over ECM formation was also assessed. BAC were seeded onto PCL and SPCL nanofiber meshes using a dynamic cell seeding procedure and cultured under static or dynamic conditions for 4 weeks. Constructs were characterized by scanning electron microscopy (SEM), histology, immunolocalisation of collagen types I and II, and glycosaminoglycans (GAG) quantification. Results show an extensive cell colonization of the entire nanofiber meshes, for both materials, and that chondrocytes presented typical spherical morphology. It is noticeable some degree of cell infiltration inside nanofiber meshes, again for both materials. ECM formation throughout the materials and GAG were detected, evidencing typical construct maturation. It is shown that PCL and SPCL nanofiber meshes are suitable as supports for ECM formation, and therefore, adequate for cartilage tissue engineering approaches.

1. Introduction

Joint diseases due to cartilage degeneration are a major health problem concerning people of all ages, but particularly elder people. Osteoarthritis, for example, affects 80% of people over 60 years. One therapeutic option is non steroid ant-inflammatory drugs (NSAID) treatment, aiming the delay and control of cartilage loss [1]. Other options involve surgical procedures, like arthroplasty with implantation of joint prosthesis [2]. These procedures are able to improve joint function considerably. However, patients´ mobility may be limited, and some problems concerning the interface of bone and implant may arise, causing the need for revision [2]. Although surgical outcome is very good at the beginning, durability of solutions and improvement of patients' quality of

life is still far from ideal. Tissue engineering represents an alternative route to current treatments. Its strategy may be based in cells injection into the defect and fixation with a membrane [3] or implantation of combinations of cells, scaffolds and growth factors [4] that may result in ECM production.

Articular hyaline cartilage is an avascular tissue that covers the joint and acts as a load-bearing and wear-control structure. As cartilage is subjected to mechanical stimulus in its natural environment, it is typically followed a strategy to regenerate cartilage under the action of similar stimulus. It has been shown *in vitro* that the culture environment deeply affects chondrocytes proliferation in a 3D scaffold [5]. Bioreactors including spinner-flasks, rotating-wall vessels or direct perfusion are designed to provide a controlled environment for tissue engineered cartilage production [6]. Biomaterial morphology [7], as well as culture conditions and cell source [8], can also influence the quality of tissue engineered cartilage constructs.

Biodegradable biomaterials, either natural or synthetic, have been processed into scaffolds for tissue engineering [9, 10]. PCL is a biodegradable polymer, belonging to the aliphatic polyester family. Currently, this class of materials is among the most attractive polymers which meet various medical and physical demands for safe clinical applications. PCL is used in clinical practice as a biomaterial due to its good mechanical properties, ability to form compatible blends and copolymers with a wide range of other polymers, including natural-based polymers, low toxicity, biocompatibility and biodegradability [11-13]. Nevertheless, its composition with starch offers new opportunities for controlling its degradation kinetics [14], release of growth factors and new surface chemistry [15, 16]. SPCL combines the affinity of starch and the processability and mechanical properties of the synthetic polymer (PCL) [17]. Several works with SPCL have been conducted with cells, for instance, in osteoblast-like cells [18], MSCs [19, 20], or leucocytes [21]. Dynamic culture conditions [22] and *in vivo* implantation in murine animal models [23] were also explored.

Electrospinning allows producing polymeric ultrafine fibres with diameters ranging from few microns down to tens of nanometers [24]. Due to their very small diameter, polymeric nanofibers exhibit unusual properties like high specific surface area, flexibility in surface functionality and superior mechanical properties [25]. Additionally, nonwoven mesh structures produced by electrospinning physically mimic the structure and morphology of ECM components of a great variety of native tissues, including bone and cartilage [26-28]. The aim of the present study is to assess ECM

formation in PCL and SPCL electrospun nanofiber meshes, evaluating the effect of starch in the material formulation and also the effect of static or dynamic culture conditions.

2. Materials and Methods

2.1. Nanofiber meshes processing

Polymeric solutions of PCL (PCL 787, TONE^{TM} polymer, Union Carbide Chemicals and Plastics Division, Bound Brook, New Jersey, with 80kD) and SPCL ((30% starch, 70% PCL); Novamont: SPCL (Mater-BI ZI01U, Novamont, Novara, IT)) were prepared by dissolving the polymer or the blend into an organic solvent mixture of chloroform/dimethylformamide (70:30) (Sigma-Aldrich, USA), at concentrations of 17% and 24% (w/v), respectively. Polymeric solutions were placed into syringes with blunted metallic needles attached to it. Used needles had an internal diameter of 0.8 mm. Furthermore, the syringe was coupled to a syringe pump (model KDS100, KD Scientific, USA) to control the solution flow rate. A high voltage power supply (0-25 kV) was applied to the needle to generate the electric field. An aluminium foil connected to the ground was used as the fiber mesh collector. The capillary tip-to-collector distance and the flow rate were fixed at 20 cm and 1.0 ml/h, respectively. Nanofiber meshes production lasted 1 hour, and for that it was needed 1 mL of polymeric solution. The applied voltage was maintained at 9 kV. Nanofiber meshes were collected and cut in squares with 1 cm^2. Experiments were all performed at room temperature and the conditions were optimized for the two materials.

2.2. Isolation of BAC and culture on nanofiber meshes

Isolation and expansion of BAC was performed according to the method previously reported [29]. Cells were cultured with expansion medium: Dulbecco's modified Eagle's medium (Sigma, D5671), containing 10 mM Hepes buffer (Sigma, H0887), L-alanyl-L-glutamine (Sigma, G8541), Non Essential AminoAcids (Sigma, M7145) 10000 units/ml penicillin, 10000 μg/ml streptomycin (Sigma, P0781), 10% foetal calf serum (Biosera, S1810) and 10 ng/ml of basic Fibroblast Growth Factor (bFGF)

(PeproTech, 100-18B). Dynamic cell seeding was performed using Petri dishes. Three nanofiber meshes were placed in each Petri dish, and cells suspension added in order to have $6,5x10^6$ cells per nanofiber mesh. We used 20 nanofiber meshes per experimental condition, for each material. Petri dishes were placed in a rotator, inside the incubator, at 60 rpm, for 72 hours.

After 72 hours, cell seeding was complete, and half of the constructs were kept in the rotator (dynamic conditions), whereas the others were placed in the incubator (static conditions). Differentiation medium was changed every 3 days, and was composed of expansion medium without bFGF and with 1 mg/ml of insulin (Sigma, I5500) and 1 mg/ml of ascorbic acid) (Sigma, A4544). Samples were taken in different time points: 1, 2, 3 and 4 weeks of culture.

2.3. Scanning Electron Microscopy (SEM)

For SEM analysis, nanofiber meshes were collected at each time point: 1, 2, 3 and 4 weeks. They were washed in sterile PBS and immersed in 3% glutaraldehyde (Sigma, G5882) with 0.1 M cacodylate buffer at pH 7.4 (AGAR, R1103) at room temperature for one hour. Afterwards, they were washed in PBS, osmium tetra oxide was added and left for 2 hours. Finally, samples were dehydrated in crescent alcohol concentrations and let to dry. SEM micrographs were taken at different time periods and with several magnifications.

2.4. Histological analysis

Samples were collected at the end of the experiment, included in O.C.T. (OCT compound BDH, Gurr®) and stored at -20ºC. Sections were cut 8 μm thick and placed in microscopy slides. Then, they were fixed during 30 minutes at 4ºC, in a fresh 4% paraformaldehyde (Sigma, P6148) solution in PBS buffer, washed twice in distilled water and let overnight to air-dry. Slides were stored at 4ºC until they were used for staining procedures. Hematoxylin-eosin staining was conducted in an automatic machine, Fume Cupboard; X219/E11/LEV1. Sections were washed in running water for 5 minutes, and then dipped in 1% acid alcohol for 5-10 seconds. They were washed again in water and stained in eosin for 10 minutes. Another washing with water was performed, for 5 minutes, and sections were afterwards dehydrated through crescent

concentrations of alcohol. They were cleared in xylene and mounted in DPX (BDH, 36029 2F). Toluidine blue staining was also performed. Staining solution was prepared by adding 1% of toluidine blue (Sigma, T0394) dissolved in distilled water containing 0.5g of sodium borate, followed by filtering. One drop of this solution was added to each section for 2-3 seconds. Then, sections were rinsed with distilled water and let to air dry. They were cleared in xylene and mounted in DPX. Alcian Blue staining was performed by rinsing the sections in 3% acetic acid and keeping them in 1% alcian blue solution (Sigma, A3157) for 18 hours. After that, stain was poured off and sections were counterstained with aqueous neutral red (Sigma, N6634) for 1 min. Sections were washed with water, let to dry and then rinsed in absolute alcohol, cleared in xylene and mounted in DPX.

2.5. Immunolocalisation of type I and type II collagens

Immunolocalisation of type I and type II collagens was performed in fixed sections. Sections were pre-treated in 10 mg/ml hyaluronidase (Sigma, H4272) for 30 minutes at 37ºC and in 2 mg/ml pronase (Sigma, P6911), for 30 minutes, at 37ºC. Sections were washed in PBS and endogenous peroxidase activity was quenched with 3% hydrogen peroxide in 50% methanol (BDH, 101586 6B) for 5 minutes. Sections were washed in Tri-buffered saline (TBS) and blocked with 3% BSA (Sigma, A2153) in TBS/ Tween 20 (Sigma, P7949) for 1 hour to avoid non-specific staining. Sections were further incubated with primary antibodies (collagen type I and collagen type II) (Goat anti-type I collagen UNLB 1310-01 and Goat anti-type II collagen UNLB 1320-01) overnight at 4ºC, in a humidified atmosphere. Then, sections were washed once with high salt wash solution and twice in TBS/Tween 20, 10 minutes each and then incubated with secondary antibody from the kit during 1 hour at room temperature, again in a humidified atmosphere. The remaining protocol is as described in the Vectastain Elite ABC Kit PK-6105 (Vector Laboratories Ltd, UK) and in the Vector DAB Kit (Vector Laboratories Ltd, UK). Slides were washed in water for 5 minutes and then counterstained with hematoxylin for nuclei visualization. Finally, slides were mounted in DPX. Controls were performed using normal goat serum instead of primary antibodies, which was also included in the kit.

2.6. Dimethylmethylene blue (DMB) assay for glycosaminoglycans quantification

Samples were collected at 4 weeks of culture, freeze/dried overnight and then digested. Digestion solution was prepared by adding papain (Sigma, P4762) and N-acetyl cysteine (Sigma, A8199) at the concentrations of 0.05% and 0.096%, respectively, to 50 ml of digestion buffer (200 mM of phosphate buffer containing 1 mM EDTA (Sigma, E5134), pH 6.8). Samples were incubated with 600 µl of the referred solution, overnight at 60ºC. Afterwards, they were centrifuged at 13000 rpm for 10 minutes. Supernatant was collected and stored at -20ºC until the assay was performed. Dimethymethylene Blue (DMB) stock solution was prepared dissolving 16 mg of DMB powder in 900 ml of distilled water containing 3.04 g of glycine and 2.73 g of NaCl. pH was adjusted to 3.0 with HCl and volume adjusted to 1L. The solution was stored at room temperature covered by alumin foil. Chondroitin sulphate (Sigma, C8529) solution was prepared in water, in a 5 mg/ml stock solution and kept refrigerated. Dilutions of this solution were performed in order to make a standard curve. Samples were also diluted as appropriated, with distilled water. Optical density was measured in a microplate reader, at 530 nm. Unseeded nanofiber meshes were used as controls. They were treated in the same way than the seeded ones for the GAG assay procedure. We used three per each GAG assay.

2.7. Statistical analysis

Data from triplicates of GAG quantification are present as averages ± standard errors. One-way analysis of variance in conjunction with Turkey's test was also performed.

3. Results

3.1. Electrospun nanofiber meshes

SEM observations of produced nanofiber meshes evidence a random distribution of nanofibers, as expected. This is the typical result of electrospining process, caused by the electric filed generated in the equipment (Figure 3.1). PCL nanofiber meshes were composed by nanofibers with diameters in the submicron range, from 0.4-1.4 µm

(Figure 3.1A). The average pore sizes of PCL nanofiber meshes were 72.67±31.48 μm. Conversely, the SPCL nanofiber meshes presented a larger dispersion of nanofiber diameters (the thinner had diameters around 0.3-20 μm), probably due to the insolubility of the starch phase in the organic solvent mixture used. Indeed, a PCL solution containing a suspension of colloidal starch particles is obtained, and this has important implications in fibers diameter and in meshes morphology. The average pore sizes of SPCL, evaluated from SEM micrographs of the nanofiber meshes, were 65.21±22.07 μm.

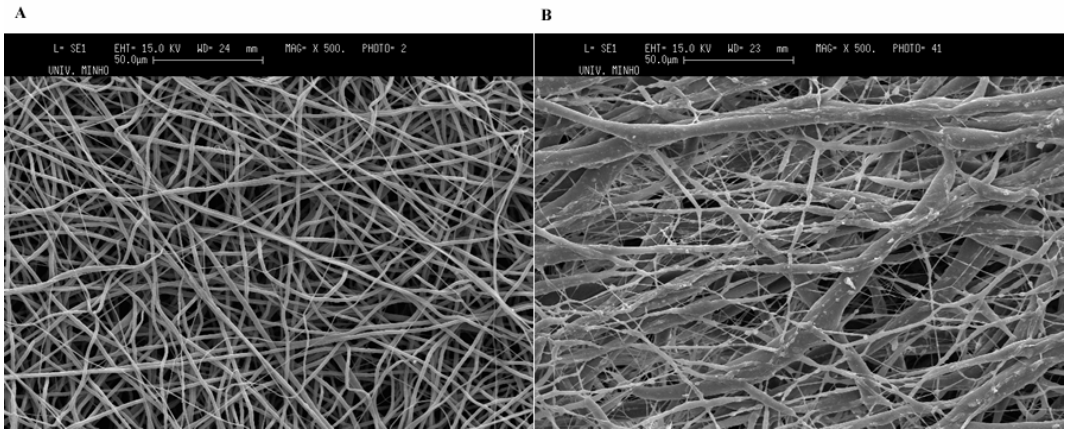

Figure 3.1 – Electron micrographs of (A) poly(ε-caprolactone) (PCL) and (B) starch-poly(ε -caprolactone) (SPCL) nanofiber meshes processed by electrospinning.

3.2. SEM analysis

SEM micrographs (Figure 3.2) show that cells colonize both types of nanofiber meshes, in both culture conditions, covering their surface extensively and homogeneously. PCL nanofiber meshes keep their membrane like shape (Figures 3.2A-C; 3.2G-I), while SPCL nanofiber meshes tend to curl in a tubular structure (Figures 3.2D-F; 3.2J-L). This structure trapped a considerable number of cells inside. Chondrocytes kept their round morphology throughout the experiment, either in PCL (Figures 3.2A, B, G-I) or SPCL (Figures 3.2E, F, K) nanofiber meshes, for both culture conditions.

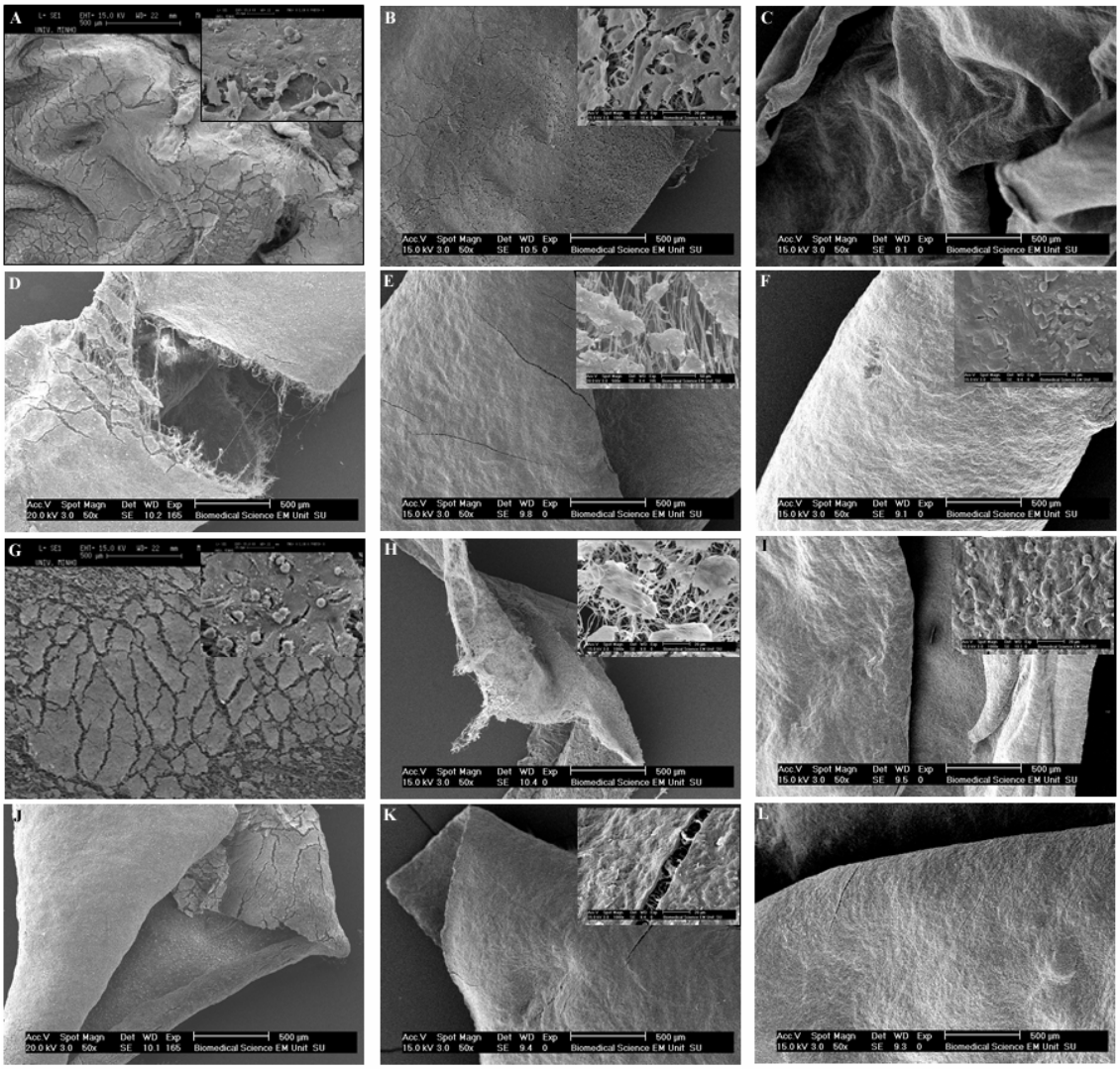

Figure 3.2 – BAC growth morphology in PCL and SPCL nanofiber meshes, in different time points of the experiment, either in static (A-F) or dynamic (G-L) culture conditions. Different magnifications were used to highlight cell morphology. Cell growth was identical in static culture conditions either for PCL (A-C) or SPCL (D-F) nanofiber meshes. The same was observed in dynamic culture conditions, both for PCL (G-I) and SPCL (J-L).

3.3. Histological staining

BAC attached and spread not only to the nanofiber meshes surface, but also in the inner regions of the mesh structure, as it can be observed with the H&E staining (Figures 3.3A, D, G, J). Both PCL and SPCL nanofiber meshes showed similar cell

distribution, as well as proteoglycans presence, as shown by toluidine blue (Figure 3.3B, E, H, K) and alcian blue stainings (Figures 3.3C, F, I, L). Darker staining of alcian blue is observed in static culture conditions histological sections (Figure 3.3C, F) than in dynamic conditions (Figures 3.3I, L). This result indicates the presence of more sulphated proteoglycans in static conditions. SPCL nanofiber meshes curled and formed tubes, entrapping considerable amounts of cells inside.

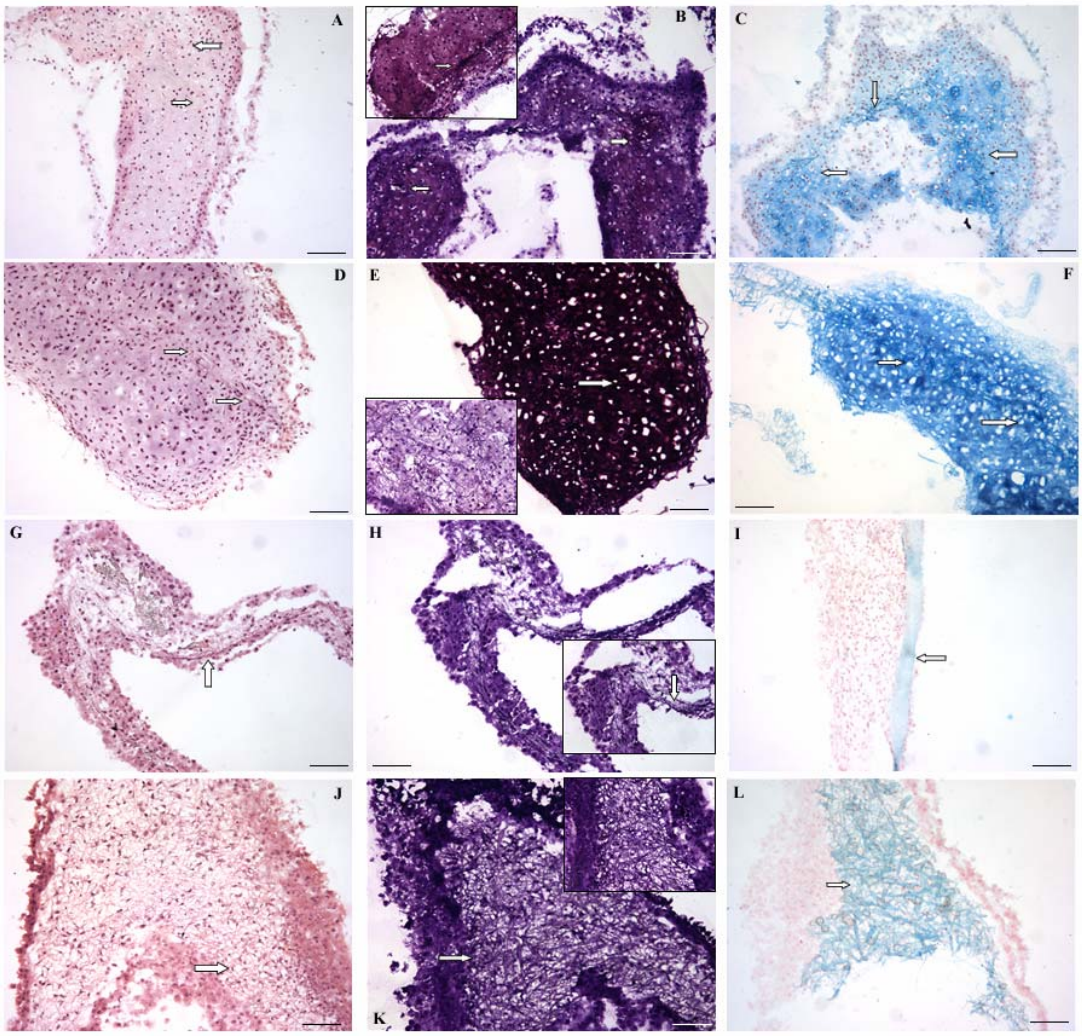

Figure 3.3 – Production of extracellular matrix in PCL and SPCL nanofiber meshes, at 4 weeks of culture, either in static (A-F) or dynamic (G-L) culture conditions. Cells were able to attach to the nanofibers structure, as showed in hematoxylin-eosin staining, for PCL (A, G) and SPCL (D, J). Cells penetrated in this structure and produced proteoglycans, detected by toluidine blue staining, either in PCL (B, H) or SPCL (E, K) nanofiber meshes. Sulphated proteoglycans were detected by alcian blue staining, again for both PCL (C, I) and SPCL (F, L) nanofiber meshes. Two magnifications (10x and

20x) were used at each staining for microscopic observation. Scale bar = 100 μm. Arrows highlight nanofiber mesh structure.

3.4. Immunolocalisation of type I and type II collagens

There was a larger amount of proteoglycans in these protected inner regions, as well as collagen type I and type II (data not shown). Both collagens types were also detected either in static (Figure 3.4A-F) or dynamic culture conditions (Figure 3.4G-L). Interestingly, collagen type I was more evident on sections prepared from SPCL nanofiber meshes (both culture conditions) (Figures 3.4E, K) than on sections from PCL nanofiber meshes (Figure 3.4B, H), indicating a predominance of fibrocartilage being generated by this combination of structure and material.

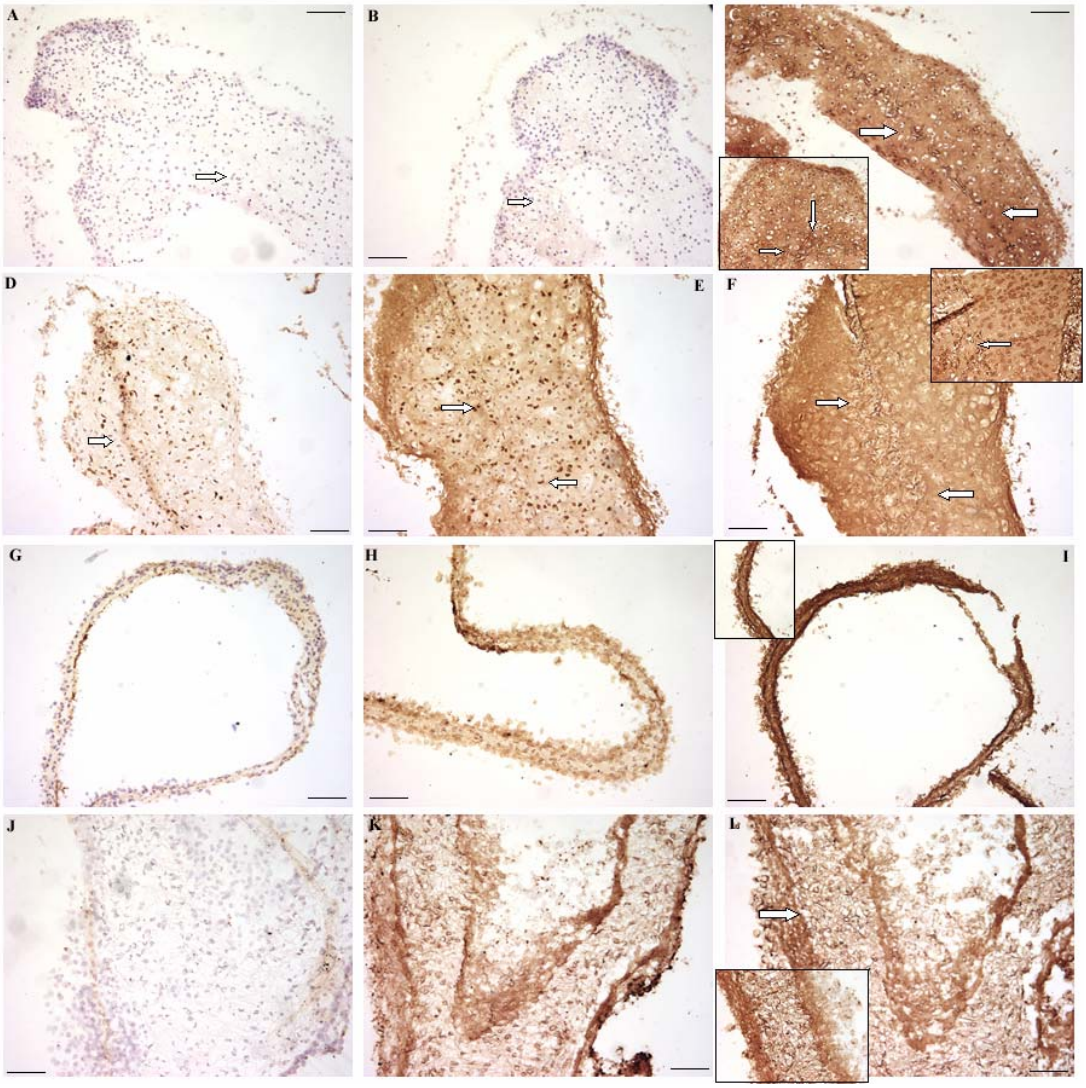

Figure 3.4 – Immunolocalisation of collagens in PCL and SPCL nanofiber meshes, at 4 weeks of culture, either in static (A-F) or dynamic (G-L) culture conditions. Controls

(A, D, G, J) were performed with normal goat serum. In static conditions, collagen type I was only detected for SPCL (E) nanofiber meshes. Collagen type II, on the other hand, was detected for both PCL (C) and SPCL (F) nanofiber meshes. In dynamic conditions, collagen type I and type II were detected in PCL (H, I) and SPCL (K, L) nanofiber meshes. Two magnifications (10x and 20x) were used at each staining for microscopic observation. Scale bar = 100 μm. Arrows highlight nanofiber mesh structure.

3.5. GAGs quantification

Quantification of GAGs present in PCL nanofiber meshes revealed the presence of approximately 2.0% of total GAGs in the ECM in static culture conditions, versus 0.8% of total GAGs found in dynamic conditions (Figure 3.5). No statistically significant difference was found between these results ($P>0.05$). As for the SPCL nanofiber meshes, results revealed 2.4% of total GAGs for the static cultures versus 1.1% for the dynamic ones. Again, there was not a statistically significant difference between static and dynamic cultures ($P>0.05$). The same lack of statistical significance was found on differences between results obtained with two types of materials ($P>0.05$).

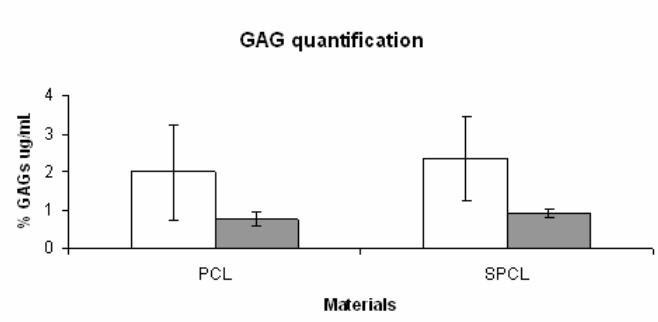

Figure 3.5 –GAGs quantification for PCL and SPCL nanofiber meshes, at 4 weeks of culture. Static culture conditions are represented in white, dynamic culture conditions in grey.

4. Discussion

The main goal of designing and producing a new scaffold is to provide enhanced microenvironment for cells, in an attempt to substitute the natural ECM milieu while they expand and repair the tissue. Many materials were already proposed for scaffolds production. Among those, natural polymers such as starch [14, 16-23, 30-38] or biodegradable polyesters, for example, PCL [11, 13, 17, 39-41] have been developed. In this case, electrospinning technique was used to explored those materials and consequently obtain a new SPCL fibrous structure. Electrospinning was used to produce meshes that were intended to provide good conditions for BAC to produce ECM. Recent applications of polymer nanofibers include fields of biotechnology and biomedicine [42]. Several tissue engineering approaches, aiming bone [11, 37, 38] and cartilage [40, 41] regeneration have also been explored. SEM micrographs confirmed that cells were able to colonize both nanofiber meshes, reinforcing the idea that PCL and SPCL are suitable polymers for the aimed purpose [17]. BAC attached and spread not only at the surface of the nanofiber meshes, but also in the inner regions of the mesh structure, as it can be observed with the H&E staining. Our present results indicate that BAC penetrates the structure, colonizing it and using it as a support for cell activity. This is, to our best knowledge a very encouraging result since most of the studies with nanofiber meshes obtained by electrospinning do not show a so clear penetration of cells into the mesh structure [25]. Cells were able to produced ECM, as showed by toluidine and alcian blue stainings, detecting GAGs accumulation. Both collagen type I and II were detected, expression of collagen type I being larger in SPCL meshes. Collagen type I is associated with fibrocartilage tissue [43], being undesirable when articular cartilage is the goal of a regeneration strategy. PCL electrospun nanofiber meshes have been proposed as novel scaffolds for tissue engineering [44], and specially for cartilage repair. Li *et al.*, (2003) showed the production of cartilaginous matrix with fetal bovine chondrocytes seeded onto PCL nanofibrous scaffolds, highlighting the importance of these materials for proliferation and maintenance of the chondrocyte phenotype [41]. Recent work from the same authors also show the versatility of these nanofibrous scaffolds, in sustaining chondrogenic differentiation of human MSCs [40, 45].

GAG production was not statistically different, comparing both types of materials. There was a slightly difference between them, being higher in SPCL than in PCL (2.4%

versus 2.0%). SPCL is a blend composed by corn starch and polycaprolactone, that has been extensively studied as biomaterial for different biomedical applications [17, 27, 37, 46]. It was recently reported to support BAC adhesion, proliferation and differentiation, in SPCL fiber based scaffolds [46]. It was unexpected to find more GAGs in static cultures than in dynamic ones, because chondrocytes are naturally subjected to a fluid flow microenvironment, and therefore, could be stimulated by these culture conditions [47]. The use of a mechanical force can be employed in their culturing process to produce a phenotypically correct tissue [48]. However, it needs to replicate relevant physiologic conditions. The intention of using agitation during culture was to produce a phenotipically adequate tissue. However, it may be concluded that these conditions were not sufficient. We can hypothesize that the higher GAG content in static cultures observed in this study was due to some GAGs release to the culture medium during the experiment, originated by mixing of culture medium. Furthermore, seeding in spinner flasks may have somehow caused some cell damage, due to the turbulent flow, generated by the magnetic stirring [49]. Similar results were obtained with bovine calf chondrocytes seeded onto fibrous polyglycolic acid meshes, where constructs exposed to similar mixing synthesised and released more GAGs into the culture medium, and resulted in lower fractions of GAGs [50]. Another study points out that mixing may increase the loss of GAGs from the construct, decreasing pericellular GAG concentration [51].

Data did not allow us to substantiate clearly which one of the materials is best for the approach used in this work. It is known that a suitable scaffold for tissue engineering is due to have good surface properties, which will allow cell attachment and efficient transport of nutrients to growing cells. In that sense, a highly hydrophilic material would allow a higher water uptake and consequently, more nutrient supply to cells [52, 53]. PCL has a more hydrophobic character, whereas SPCL is more hydrophilic, being more advantageous, in this perspective, for cell growth comparing to PCL. PCL is a homopolymer with very slow degradation kinetics. It is in clinical use for many applications, and could be the biodegradable material of choice. However, a wide range of options for development of PCL have been already explored in the past. One additional alternative to develop its performance being proposed herein is to compound it with other biodegradable materials, those compounds offering a new range of tunable properties. If we accept that there are advantages of working with a natural-based polymer, the combination of starch with PCL may lead to an attractive combination of

properties that may be tailored for specific applications, including cartilage tissue engineering. This material has been extensively studied for biomedical applications because of its biocompatibility and biodegradability [14, 16, 18, 27, 31, 54] and potential of inducing low levels of inflammatory response [21]. Its degradation kinetics can be controlled by material composition, having a more hydrophilic character and enabling designing controlled release systems for growth factors [55]. Results indicate that SPCL may be slightly more effective in sustaining ECM production, but it was also shown that higher levels of expression of collagen type I may be detrimental in obtaining hyaline cartilage by BAC cultured in these meshes. Further optimization of this material will be addressed in order to overcome this limitation.

5. Conclusions

The aim of this work was to assess if a blend of starch with polycaprolactone (SPCL) would have similar performance in sustaining BAC growth and ECM production when comparing with PCL. Both PCL and SPCL electrospun nanofiber meshes revealed to be interesting structures for cartilage tissue engineering strategies. PCL and SPCL nanofiber meshes seeded dynamically in spinner flasks evidenced a good response for this purpose. A very interesting result was the evidence that chondrocytes proliferated in external regions, and showed some capacity of migrating into inner regions of both types of fiber meshes. BAC were able to produce ECM, either in static or dynamic culture conditions. Static culture conditions lead to enhanced ECM production. These observations were confirmed by staining of proteoglycans, immunolocalisation of collagens type I and type II, and GAGs detection.

In this work we confirmed evidences from the literature that sustain the claim that PCL nanofiber meshes are suitable for tissue engineering approaches, and also propose a new scaffold for cartilage tissue engineering (SPCL nanofiber meshes). Data did not show obvious qualitative nor quantitative differences between the two materials, nevertheless, we consider advantageous to use SPCL, not only because of their natural-based composition, but also because it allows a greater control of the degradation kinetics and further functionalization of its structure. It is our understanding that both materials have a role to play in cartilage regeneration strategies, and that in particular SPCL nanofiber meshes can be further enhanced, not only in terms of degradability but

also providing opportunities for local release of growth factors for articular cartilage tissue engineering strategies.

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References

1 - Brandt KD. *The role of analgesics in the management of osteoarthritis pain*. Am J Ther. 2000. 7(2):75-90.

2 - Ringe J, Haupl T, Sittinger M. *Future of tissue engineering in rheumatic diseases*. Expert Opin Biol Ther. 2007. 7(3):283-7.

3 - Hoemann CD, Sun J, Legare A, McKee MD, Buschmann MD. *Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle*. Osteoarthritis Cartilage. 2005. 13(4):318-29.

4 - Martin I, Miot S, Barbero A, Jakob M, Wendt D. *Osteochondral tissue engineering*. J Biomech. 2007. 40(4):750-65.

5 - Freed LE, Martin I, Vunjak-Novakovic G. *Frontiers in tissue engineering - in vitro modulation of chondrogenesis*. Clinical Orthopaedics and Related Research. 1999. 367S:S46 - S58.

6 - Martin I, Wendt D, Hereber M. *The role of bioreactors in tissue engineering*. TRENDS in Biotechnology. 2004. 22(2):80 - 6.

7 - Crawford A, Frazer A, Dickinson S, Sims T, Hollander AP, Hatton PV. *Effect of biomaterial morphology on the deposition of collagen in tissue engineered cartilage constructs*. Transactions - 7th World Biomaterials Congress 2004 May; Sydney; 2004. p. 594.

8 - Hatton PV, Crawford A, Frazer A, Fraser S, Dickinson S, Hollander AP. *Effect of culture conditions and cell source on the quality of tissue engineered cartilage*

constructs. Transactions - 7th World Biomaterials Congress 2004 May; Sydney; 2004. p. 1621.

9 - Agrawal C, Ray R. *Biodegradable polymeric scaffolds for musculoskeletal tissue engineering*. J Biomed Mater Res. 2001. 55:141-50.

10 - Azevedo HS, Gomes ME, Malafaya PB, Marques AP, Salgado AJ, Reis RL. *Natural origin degradable polymers in biomedical applications*. California, USA: American Scientific Publishers 2006.

11 - Yoshimoto H, Shin YM, Terai H, Vacanti JP. *A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering*. Biomaterials. 2003. 24(12):2077-82.

12 - Venugopal J, Ma LL, Yong T, Ramakrishna S. *In vitro study of smooth muscle cells on polycaprolactone and collagen nanofibrous matrices*. Cell Biol Int. 2005. 29(10):861-7.

13 - Zhang YZ, Venugopal J, Huang ZM, Lim CT, Ramakrishna S. *Characterization of the surface biocompatibility of the electrospun PCL-collagen nanofibers using fibroblasts*. Biomacromolecules. 2005. 6(5):2583-9.

14 - Azevedo HS, Gama FM, Reis RL. *In vitro assessment of the enzymatic degradation of several starch based biomaterials*. Biomacromolecules. 2003. 4(6):1703-12.

15 - Alves CM, Yang Y, Carnes DL, Ong JL, Sylvia VL, Dean DD, *et al. Modulating bone cells response onto starch-based biomaterials by surface plasma treatment and protein adsorption*. Biomaterials. 2007. 28(2):307-15.

16 - Costa SA, Reis RL. *Immobilisation of catalase on the surface of biodegradable starch-based polymers as a way to change its surface characteristics*. J Mater Sci Mater Med. 2004. 15(4):335-42.

17 - Wang Y, Rodriguez-Perez MA, Reis RL, Mano JF. *Thermal and thermomechanical behaviour of polycaprolactone and starch/polycaprolactone blends for biomedical applications*. Macromol Mater Eng. 2005. 290:792-801.

18 - Marques AP, Cruz HR, Coutinho OP, Reis RL. *Effect of starch-based biomaterials on the in vitro proliferation and viability of osteoblast-like cells*. J Mater Sci Mater Med. 2005. 16(9):833-42.

19 - Gomes ME, Holtorf HL, Reis RL, Mikos AG. *Influence of the porosity of starch-based fiber mesh scaffolds on the proliferation and osteogenic differentiation of bone marrow stromal cells cultured in a flow perfusion bioreactor*. Tissue Eng. 2006. 12(4):801-9.

20 - Gomes ME, Sikavitsas VI, Behravesh E, Reis RL, Mikos AG. *Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds*. J Biomed Mater Res A. 2003. 67(1):87-95.

21 - Marques AP, Reis RL, Hunt JA. *The effect of starch-based biomaterials on leukocyte adhesion and activation in vitro*. J Mater Sci Mater Med. 2005. 16(11):1029-43.

22 - Gomes ME, Bossano CM, Johnston CM, Reis RL, Mikos AG. *In vitro localization of bone growth factors in constructs of biodegradable scaffolds seeded with marrow stromal cells and cultured in a flow perfusion bioreactor*. Tissue Eng. 2006. 12(1):177-88.

23 - Marques AP, Reis RL, Hunt JA. *An in vivo study of the host response to starch-based polymers and composites subcutaneously implanted in rats*. Macromol Biosci. 2005. 5(8):775-85.

24 - Reneker D, Chun I. *Nanometre diameter fibers of polymer, produced by electrospinning*. Nanotechnology. 1996. 7:216-23.

25 - Huang Z-M, Zhang Y-Z, Kotaki M, Ramakrishna S. *A review on polymer nanofibers by electrospinning and their applications in nanocomposites*. Composites Science and Technology. 2003. 63:2223 – 53.

26 - Ma Z, Kotaki M, Inai R, Ramakrishna S. *Potential of nanofiber matrix as tissue-engineering scaffolds*. Tissue Engineering. 2005. 11:101-9.

27 - Oliveira AL, Reis RL. *Pre-mineralisation of starch/polycrapolactone bone tissue engineering scaffolds by a calcium-silicate-based process*. J Mater Sci Mater Med. 2004. 15(4):533-40.

28 - Smith L, Ma P. *Nano-fibrous scaffolds for tissue engineering*. Colloids and Surfaces B: Biointerfaces. 2004. 39:125-31.

29 - Crawford A, Dickinson S. *Chondrocyte isolation, expansion, and culture in polymer scaffolds.* In: Hollander AP, Hatton PV, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 147-57.

30 - Alves CM, Reis RL, Hunt JA. *Preliminary study on human protein adsorption and leukocyte adhesion to starch-based biomaterials*. J Mater Sci Mater Med. 2003. 14(2):157-65.

31 - Gomes ME, Reis RL, Cunha AM, Blitterswijk CA, de Bruijn JD. *Cytocompatibility and response of osteoblastic-like cells to starch-based polymers: Effect of several additives and processing conditions*. Biomaterials. 2001. 22(13):1911-7.

32 - Marques AP, Reis RL, Hunt JA. *The biocompatibility of novel starch-based polymers and composites: In vitro studies*. Biomaterials. 2002. 23(6):1471-8.

33 - Marques AP, Reis RL, Hunt JA. *Evaluation of the potential of starch-based biodegradable polymers in the activation of human inflammatory cells*. J Mater Sci Mater Med. 2003. 14(2):167-73.

34 - Marques AP, Reis RL, Hunt JA. *Cytokine secretion from mononuclear cells cultured in vitro with starch-based polymers and poly-l-lactide*. J Biomed Mater Res A. 2004. 71(3):419-29.

35 - Pashkuleva I, Marques AP, Vaz F, Reis RL. *Surface modification of starch based blends using potassium permanganate-nitric acid system and its effect on the adhesion and proliferation of osteoblast-like cells*. J Mater Sci Mater Med. 2005. 16(1):81-92.

36 - Salgado AJ, Coutinho OP, Reis RL. *Novel starch-based scaffolds for bone tissue engineering: Cytotoxicity, cell culture, and protein expression*. Tissue Engineering. 2004. 10(3/4):465 - 74.

37 - Santos MI, Fuchs S, Gomes ME, Unger RE, Reis RL, Kirkpatrick CJ. *Response of micro- and macrovascular endothelial cells to starch-based fiber meshes for bone tissue engineering*. Biomaterials. 2007. 28(2):240-8.

38 - Tuzlakoglu K, Bolgen N, Salgado AJ, Gomes ME, Piskin E, Reis RL. *Nano- and micro-fiber combined scaffolds: A new architecture for bone tissue engineering*. J Mater Sci Mater Med. 2005. 16(12):1099-104.

39 - Shao X, Hutmacher DW, Ho ST, Goh CH, Lee EH. *Evaluation of a hybrid scaffold/cell construct in repair of high-load-bearing osteochondral defects in rabbits*. Biomaterials. 2006. 27(7):1071 - 80.

40 - Li W-J, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, *et al. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells*. Biomaterials. 2005. 26:599 – 609.

41 - Li W-J, Danielson KG, Alexander PG, Tuan RS. *Biological response of chondrocytes cultured in threedimensional nanofibrous poly(epsylon-caprolactone) scaffolds*. J Biomed Mater Res. 2003. 67(A):1105 - 14.

42 - Venugopal J, Ramakrishna S. *Applications of polymer nanofibers in biomedicine and biotechnology*. Appl Biochem Biotechnol. 2005. 125(3):147-58.

43 - Freemont AJ, Hoyland J. *Lineage plasticity and cell biology of fibrocartilage and hyaline cartilage: Its significance in cartilage repair and replacement*. European Journal of Radiology. 2006. 57:32 - 6.

44 - Li W-J, Laurencin CT, Caterson E, Tuan RS, Ko FK. *Electrospun nanofibrous structure: A novel scaffold for tissue engineering*. J Biomed Mater Res. 2002. 60:613 – 21.

45 - Li W-J, Tuli R, Huang X, Laquerriere P, Tuan RS. *Multilineage differentiation of human mesenchymal stem cells in three-dimensional nanofibrous scaffold*. Biomaterials. 2005. 26:5158 - 66.

46 - Oliveira JT, Crawford A, Mundy JM, Moreira AR, Gomes ME, Hatton PV, *et al. A cartilage tissue engineering approach combining starch-polycaprolactone fibre mesh*

scaffolds with bovine articular chondrocytes. J Mater Sci Mater Med. 2007. 18(2):295-302.

47 - Gaissmaier C, Fritz J, Krackhardt T, Flesch I, Aicher WK, Ashammakhi N. *Effect of human platelet supernatant on proliferation and matrix synthesis of human articular chondrocytes in monolayer and three-dimensional alginate cultures*. Biomaterials. 2005. 26:1953 – 60.

48 - Darling EM, Athanasiou K. *Articular cartilage bioreactors and bioprocesses*. Tissue Engineering. 2003. 9(1):9 - 26.

49 - Vunjak-Novakovic G, Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R, *et al. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage*. J Orthop Res. 1999. 17(1):130-8.

50 - Gooch KJ, Kwon JH, Blunk T, Langer R, Freed LE, Vunjak-Novakovic G. *Effects of mixing intensity on tissue-engineered cartilage*. Biotechnol Bioeng. 2001. 72(4):402-7.

51 - Sah RL, Grodzinsky AJ, Plaas AH, Sandy JD. *Effects of tissue compression on the hyaluronate-binding properties of newly synthesized proteoglycans in cartilage explants*. Biochem J. 1990. 267(3):803-8.

52 - Ivirico JL, Martinez EC, Sanchez MS, Criado IM, Ribelles JL, Pradas MM. *Structure and properties of methacrylate-endcapped caprolactone networks with modulated water uptake for biomedical applications*. J Biomed Mater Res B Appl Biomater. 2007. 83(1):266-75.

53 - Hutcheon GA, Messiou C, Wyre RM, Davies MC, Downes S. *Water absorption and surface properties of novel poly(ethylmethacrylate) polymer systems for use in bone and cartilage repair*. Biomaterials. 2001. 22(7):667-76.

54 - Mendes SC, Reis RL, Bovell YP, Cunha AM, van Blitterswijk CA, de Bruijn JD. *Biocompatibility testing of novel starch-based materials with potential application in orthopaedic surgery: A preliminary study*. Biomaterials. 2001. 22(14):2057-64.

55 - Malafaya PB, Elvira C, Gallardo A, San Roman J, Reis RL. *Porous starch-based drug delivery systems processed by a microwave route*. J Biomater Sci Polym Ed. 2001. 12(11):1227-41.

Chapter 4

Chitosan/ polyester – based scaffolds for cartilage tissue engineering: assessment of extracellular matrix formation

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Abstract

Natural-based polymers have been extensively used in scaffolds production for cartilage tissue engineering. The present work aims at evaluating and characterizing extracellular matrix (ECM) formation in two types of chitosan-based scaffolds, using bovine articular chondrocytes (BAC). The influence of these scaffolds porosity, as well as pore size and geometry in cartilagineous tissue formation was studied. The effect of stirred conditions on ECM formation was also assessed. Chitosan-Poly (Butylene Succinate) (PBS) scaffolds where produced by compression moulding and salt leaching, using a blend of 50% of each material. Different porosities and pore sizes structures were obtained. BAC were seeded onto CPBS scaffolds using spinner flasks. After that period, constructs were transferred to the incubator, where half were cultured under stirred conditions, and the other half under static conditions, during 4 weeks. Constructs were characterized by scanning electron microscopy, histology procedures, immunolocalisation of collagen type I and collagen type II and dimethylmethylene blue (DMB) assay for glycosaminoglycans (GAG) quantification. Both materials showed good affinity for cell attachment. Cells colonized the entire scaffolds and were able to produce ECM. Large pores with random geometry improved proteoglycans and collagen type II production. However, that structure has the opposite effect on GAG production. Stirred culture conditions indicate to enhance GAG production, in both types of scaffolds.

1. Introduction

There are four major types of cartilage, which can be distinguished by their specific constitutive components: hyaline cartilage, fibrocartilage, elastic cartilage and costochondral cartilage [1]. Hyaline articular cartilage is the most abundant type in the body, composed of one cell type (the chondrocyte) dispersed in an abundant extracellular matrix (ECM). ECM is composed mainly of collagen type II and a large proteoglycan, aggrecan [1, 2]. The ECM provides most of the functional properties associated with hyaline cartilage, including resistance to compression and provision of low friction articulating surfaces in the joints. Injuries to cartilage are often painful and may severely affect movement. Unfortunately, articular cartilage has a relatively poor

capacity for self-repair (related with the lack of a direct blood supply), and cartilage injury is frequently associated with the onset of chronic problems including osteoarthritis [1, 3].

Currently, there is no agreed method to restore fully damaged cartilage [4]. Current therapies include abrasion arthroplasty, subchondral drilling, prosthetic joint replacement, and ultimately transplantation of autologous chondrocytes or tissues [4, 5]. However, these treatments do not constitute a complete recovery for the patient, and in most of the cases persistent problems of donor site morbidity, limitations of patient mobility and consequent disability, loss of implants and limited durability of the prosthetics [2, 4-6] are observed.

Tissue Engineering represents a promising approach for the repair of articular cartilage, but there is still no ideal scaffold for this approach. Chitosan/polyesters appear to offer several advantages in this field.

In recent years, natural based polymers have been extensively studied, as for example, chitosan [7-11]. Chitosan is a derivative of chitin, obtained by deacetylation of this biopolymer [12]. Chitin is the second most abundant polysaccharide in nature, being found usually in crustaceans' shells. Chitosan is a linear polysaccharide with a structure similar to glycosaminoglycans present in native cartilage ECM [13]. This property is extremely important for cartilage tissue engineering, since it allows the development of several types of scaffolds. Another interesting property of chitosan is that it can be moulded into various shapes [14] and allows for the formation of different pore sizes structures [12]. It has an intrinsic antibacterial activity and high biocompatibility [13]. Chitosan has been used in several blends to produce many types of scaffolds, for example hydrogels [15, 16], porous scaffolds [11], amino acid immobilization [9] or drug delivery [17]. Our group has developed innovative blends of synthetic polymers with chitosan [7, 8, 18, 19], which have been developed for biomedical applications, such as bone [20-22] or cartilage [23, 24]. Studies have been conducted preparing chitosan based scaffolds aimed at cartilage regeneration, which showed evidence of favourable responses *in vitro* [20, 21, 24-26]. As for *in vivo* studies, a high degree of biocompatibility of chitosan scaffolds has been shown in mice [13]. Another study, conducted in rabbits, observed hyaline-like tissue after 24 weeks of implantation [15]. Recently, the basis for a cartilage engineering model in a large animal has been established, using Merino Sheep and chitosan-based scaffolds [27]. Using ovine MSCs combined with chitosan and TGF-β3, histological analysis revealed chondrocyte-like

cells surrounded by a hyaline-like cartilaginous matrix that was well integrated with the host cartilage [27]. The bio functionality of chitosan scaffolds produced by particle aggregation was tested in rats *in vivo*, which showed promising results in terms of connective tissues ingrowth and neo-vascularization [28].

In the current study, a bovine chondrocyte model was used to establish primary cultures and the formation of ECM in chitosan/ polyester based scaffolds was evaluated. Two types of scaffolds with different pore sizes and pore geometries were studied, and ECM deposition on both types of scaffolds was evaluated. In addition, different culture conditions were used, namely static versus dynamic, in order to establish the most suitable method to obtain hyaline cartilage tissue *in vitro*.

2. Materials and Methods

2.1. Scaffolds production

A chitosan-poly(butylene succinate) (50/50% wt) blend was produced and processed into scaffolds using a methodology based on compression moulding followed by salt leaching, as previously described [8, 18]. Common salt (NaCl) was used as the porogen agent. Two different groups of scaffolds were produced: one using 80% weight (wt) of salt with particles size between 63-125 μm (80 CPBS). The second group of scaffolds was produced using 60% wt of salt with particle size between 250-500 μm (60 CPBS). In both cases salt and blend were loaded into a mould, heated and compression-moulded into large discs. Discs were sliced to obtain cubes of 5 mm. These cubes were immersed in distilled water to leach out the salt, over 6 days. Water was changed every day to facilitate the complete dissolution of the porogen agent. By the end of the procedure, cubes were dried until a constant weight was obtained. The resulting scaffolds were characterized, and will be further referred as 80% porosity or 60% porosity.

2.2. Isolation of bovine articular chondrocytes

Isolation of bovine articular chondrocytes was performed according to a method previously reported [29]. Full thickness hyaline cartilage was harvested from bovine

metacarpophalangeal joint. The skin was removed and the joint was transferred to a laminar air hood. Cartilage was dissected in small full-depth pieces and washed twice with PBS buffer (Sigma, D8537). Then, it was digested with 0.25% (w/v) of tripsin solution (Sigma, E5134) for 30 minutes at 37ºC on a rotator. The solution was removed, cartilage was washed again in PBS buffer and then incubated in a collagenase type I solution (2 mg/ml) (Sigma, E0130) overnight at 37ºC on a rotator (Stuart mini orbital shaker SSM1). The following day, cells were washed twice with PBS, counted and platted at a density of 2 x 10^6 cells per Petri dish. Cells were cultivated with expansion medium: Dulbecco's modified Eagle's medium (Sigma, D5671), containing 10 mM Hepes buffer (Sigma, H0887), L-alanyl-L-glutamine (Sigma, G8541), Non Essential Aminoacids (Sigma, M7145) 10000 units/ml penicillin, 10000 μg/ml streptomycin (Sigma, P0781), 10% foetal calf serum (Biosera, S1810) and 10 ng/ml of basic Fibroblast Growth Factor (bFGF) (PeproTech, 100-18B). Culture medium was changed twice a week, and cells were expanded until passage 2.

2.3. BAC culture on chitosan-based scaffolds

BAC seeding was performed dynamically, in spinner flasks. Cells were harvested, counted and resuspended in expansion medium. Twenty scaffolds were used in each experiment, for each type of scaffold. Materials were seeded with a cell suspension containing 6,5x10^6 cells per scaffold. Seeding was performed in spinner flasks, using a magnetic stirrer placed inside an incubator, at 37ºC, in order to allow cells penetration into the material's porous structure. After cell seeding was complete, constructs were removed from spinner flasks. Two experimental conditions were set: dynamic and static. For dynamic culture conditions, half of the constructs were incubated at 37ºC on a rotator at 60 rpm. For static conditions, constructs were left to culture statically inside the incubator. Culture medium was changed every 3 days. At this stage, the culture medium used was differentiation medium (expansion medium without bFGF and with 1 mg/ml of insulin (Sigma, I5500) and 1 mg/ml of ascorbic acid) (Sigma, A4544). Samples were taken at different time points: 1, 2, 3 and 4 weeks of culture.

2.4. Microcomputed tomography (μCT)

Microcomputed tomography (μCT) equipment (SkyScan, Belgium) was used as a nondestructive technique for a very detailed analysis of the morphology of the developed scaffolds. Four scaffolds of each condition were scanned in high-resolution mode of 8,7 μm x/y/z and an exposure time of 1792 ms. The energy parameters defined in the scanner were 63 keV with a current of 157 μA. Isotropic slice data were obtained by the system and reconstructed into 2D images. These slice images were compiled and analyzed to render 3D images and obtain quantitative architecture parameters. A μCT analyser and a μCT Volume Realistic 3D Visualization, both from SkyScan, were used as image processing tools for both μCT reconstruction and to create/visualize the 3D representation. Regions of interest (square of 4,5 x 4,5 mm^2) were selected in each slice image and a threshold was set to eliminate background noise. This threshold (to distinguish polymer material from pore voids) was chosen and maintained constant for all the scanned specimens and samples. The threshold was also inverted to obtain pore volume and to analyze both the pore morphology.

2.5. Scanning Electron Microscopy (SEM)

For scanning electron microscopy analysis, constructs were collected at every time point. They were washed in sterile PBS and immersed in 3% glutaraldehyde (Sigma, G5882) with 0.1 M cacodylate buffer pH 7.4 (AGAR, R1103) at room temperature for 30 minutes. Afterwards, they were washed in PBS buffer three times to remove all gluteraldehyde from the surface. Then constructs were submerged in osmium tetra oxide and left for 2 hours. Finally, they were dehydrated in alcohols and left to air dry. The samples were splutter coated with gold and analysed by scanning electron microscopy. For this work we used two different scanning electron microscopes: a Philips XL-20 and a Leica Cambridge S360, Leica Cambridge, Cambridge, UK.

2.6. Histological analysis (H&E, Toluidine Blue, Alcian Blue)

Samples were collected at the end of the experiment (4 weeks of culture) and embedded in O.C.T. (OCT compound BDH, Gurr®), in order to make them suitable for cryosectioning, and stored at -20ºC. Sections were cut at 8 μm and placed on

microscopy slides. A fresh 4% paraformaldehyde (Sigma, P6148) solution in PBS buffer was prepared, filtered and cooled down for fixation of the slides. Slides were fixed for 30 minutes at 4ºC, washed twice in distilled water and left to air-dry overnight. Slides were subsequently stored at 4ºC until used for staining procedures. Hematoxylin-eosin staining was conducted in an automatic machine, Fume Cupboard; X219/E11/LEV1. In this procedure, sections were washed in running tap water for 5 minutes, and then dipped in 1% acid alcohol for 5-10 seconds. Sections were washed again in tap water and stained in eosin for 10 minutes. Another wash with tap water was performed for 5 minutes and sections were afterwards dehydrated through alcohols. They were cleared in xylene and mounted in DPX (BDH, 36029 2F). Toluidine blue stain was performed on a selection of the sections. The staining solution was prepared by dissolving 1% of toluidine blue (Sigma, T0394) in distilled water containing 0.5g of sodium borate, followed by filtering. One drop of this solution was added to each section for 2-3 seconds. Then, sections were rinsed with distilled water and let to air dry overnight. Sections were cleared and mounted as described previously. Alcian Blue stain was performed by rinsing the sections in 3% acetic acid and incubating them in 1% alcian blue solution (Sigma, A3157) for 18 hours. After that stage, stain was poured off and sections were counterstained with aqueous neutral red (Sigma, N6634) for 1 min. Sections were washed with water, left to air dry and then rinsed in absolute alcohol, cleared and mounted as described previously.

2.7 Immunolocalisation of type I and type II collagens

Immunolocalisation of type I and type II collagens was performed in fixed sections. Sections were washed in PBS and pre-treated with 10 mg/ml hyaluronidase (Sigma, H4272) in PBS for 30 minutes at 37ºC and with 2 mg/ml pronase (Sigma, P6911) again for 30 minutes at 37ºC. Then, sections were washed in PBS and endogenous peroxidase activity was quenched with 3% hydrogen peroxide in 50% methanol (BDH, 101586 6B) for 5 minutes. Sections were washed in Tri-buffered saline (TBS) solution and blocked with 3% BSA (Sigma, A2153) in TBS/ Tween 20 (Sigma, P7949) for 1 hour to avoid non-specific staining. Sections were then incubated with primary antibodies (collagen type I and collagen type II) (Goat anti-type I collagen UNLB 1310-01 and Goat anti-type II collagen UNLB 1320-01) overnight at 4ºC, in a humidified atmosphere. The next day, sections were washed once with high salt wash solution and twice in

TBS/Tween 20 for 10 minutes each and then incubated with secondary antibody from the kit during 1 hour at room temperature, again in a humidified atmosphere. The remaining protocol is as described in the Vectastain Elite ABC Kit PK-6105 (Vector Laboratories Ltd, UK) and in the Vector DAB Kit (Vector Laboratories Ltd, UK). Slides were washed in water for 5 minutes and then counterstained with hematoxilyn for nuclei visualization. Then slides were mounted in DPX. Controls were performed using normal goat serum instead of primary antibodies, which was also included in the kit.

2.8. Dimethylmethylene blue (DMB) assay for glycosaminoglycans quantification

For this assay samples were collected at 4 weeks of culture, freeze/dried overnight and then digested. This digestion allowed the separation of the formed extracelular matrix from the scaffold. Digestion solution was prepared by adding papain (Sigma, P4762) and N-acetyl cysteíne (Sigma, A8199), to obtain the final concentrations of 0.05% and 0.096%, respectively, to 50 ml of digestion buffer (200 mM of phosphate buffer containing 1 mM EDTA (Sigma, E5134), pH 6.8). Samples were placed in 1.5 ml tubes and incubated with 600 µl of the referred solution, overnight at 60ºC. Afterwards, samples were centrifuged in a bench centrifuge at 13000 rpm for 10 minutes. Supernatant was collected and stored at -20ºC until the GAG assay was performed. Solutions for this assay were prepared as follows. Dimethymethylene Blue (DMB) stock solution was prepared dissolving 16 mg of DMB powder in 900 ml of distilled water containing 3.04 g of glycine and 2.73 g of NaCl. This was mixed for 2 hours, covered with aluminium foil. pH was adjusted to 3.0 with HCl and volume to 1L. The solution was stored at room temperature covered with aluminium foil. Chondroitin sulphate (Sigma, C8529) solution was prepared in water, in a 5 mg/ml stock solution and kept refrigerated. This solution was diluted with water resulting in 5 unit increments from 0 µg/ml to 50 µg/ml, in order to make a standard curve. Samples were also diluted as appropriate with distilled water. To a 96 well plate, 20 µl of water was added as a blank. The same quantity of chondroitin sulphate diluted solutions was added, in duplicate, and the same was performed with all the samples. DMB solution was added, 250 µl of the solution to each well, in a multichannel pipette, and the optical density was measured in a microplate reader, at 530 nm. We used scaffolds without seeded cells as controls.

2.9. Reproducibility of the experiments

Each scaffold seeding was performed 3 times with cells isolated from a different animal.

2.10. Statistical analysis

Data from triplicates of glycosaminoglycans quantification are present as averages ± standard errors. One-way analysis of variance in conjunction with Turkey's test was performed on the glycosaminoglycans assays results.

3. Results

3.1. Microcomputed tomography

Bi-dimensional and tri-dimensional μCT images from both groups of scaffolds show differences between their morphology (Figure 4.1 and 4.2). 60 CPBS scaffolds (4.1A) show less pores than 80 CPBS scaffolds (4.1B), as expected. 60 CPBS scaffolds present smaller pores. Correlo *et al.*, (2008) recently produced and characterized these two types of scaffolds in terms of porosity, pore size and mechanical properties [18]. 80 CPBS scaffolds were shown to have 78.6±2.5% porosity, and a pore size of 276.8±52.5μm. 60 CPBS scaffolds, on the other hand, were shown to have a lower porosity (57.7±6.6%), as well as a smaller pore size (199.3±5.3 μm) [18].

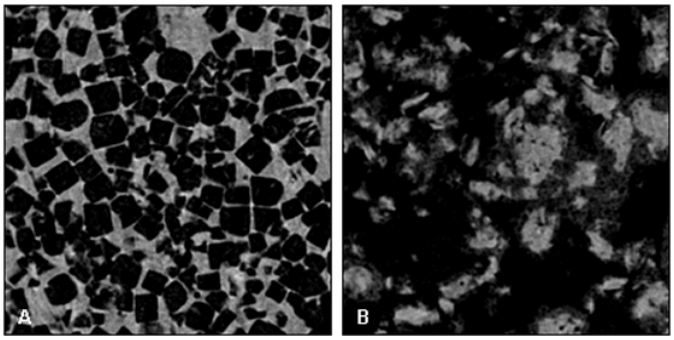

Figure 4.1 – Bi-dimensional microCT images of CPBS scaffolds, 60 CPBS scaffolds (A) 80 CPBS scaffolds (B), obtained by compression moulding and salt leaching. The images highlight the geometry of the obtained pores.

Pore geometry was also different in both compositions. As can be observed in the bi-dimensional images of μCT, pores of the 60 CPBS scaffolds have a geometrically defined shape (similar to the salt structure), and seem to be homogeneously distributed throughout the scaffolds. On the other hand, the pores of the 80 CPBS scaffolds are randomly distributed and do not present a specific shape.

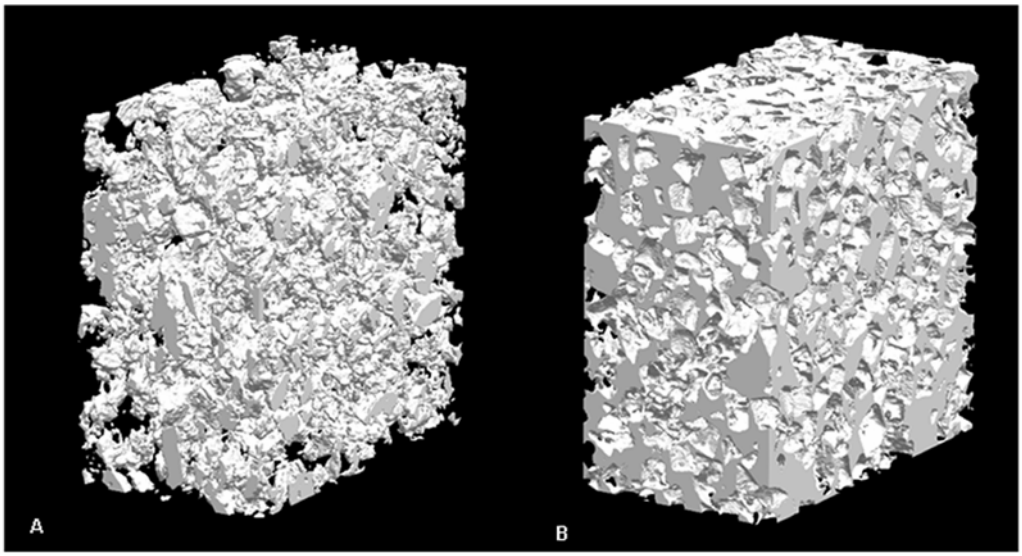

Figure 4.2 – Tri-dimensional microCT images of CPBS scaffolds, 80 CPBS scaffolds (A) 60 CPBS scaffolds (B), obtained by compression moulding and salt leaching. The images highlight the porosity of both scaffolds.

3.2. SEM analysis

The scanning electron microscopy micrographs presented in Figure 4.3 show the distribution of cells in the seeded scaffolds throughout the period of the experiment. It can be observed that the chondrocytes colonized the entire scaffold, in both types of materials. Cells kept their round shaped morphology, typical of chondrocytes, either in static culture (Figure 4.3A-4.3D; 4.3I-4.3L), or in stirred culture conditions (Figure 4.3E-4.3H; 4.3M-4.3P). Nevertheless, it was observed that the colonization of 60 CPBS scaffolds appeared slower than in 80 CPBS scaffolds, in both culture conditions.

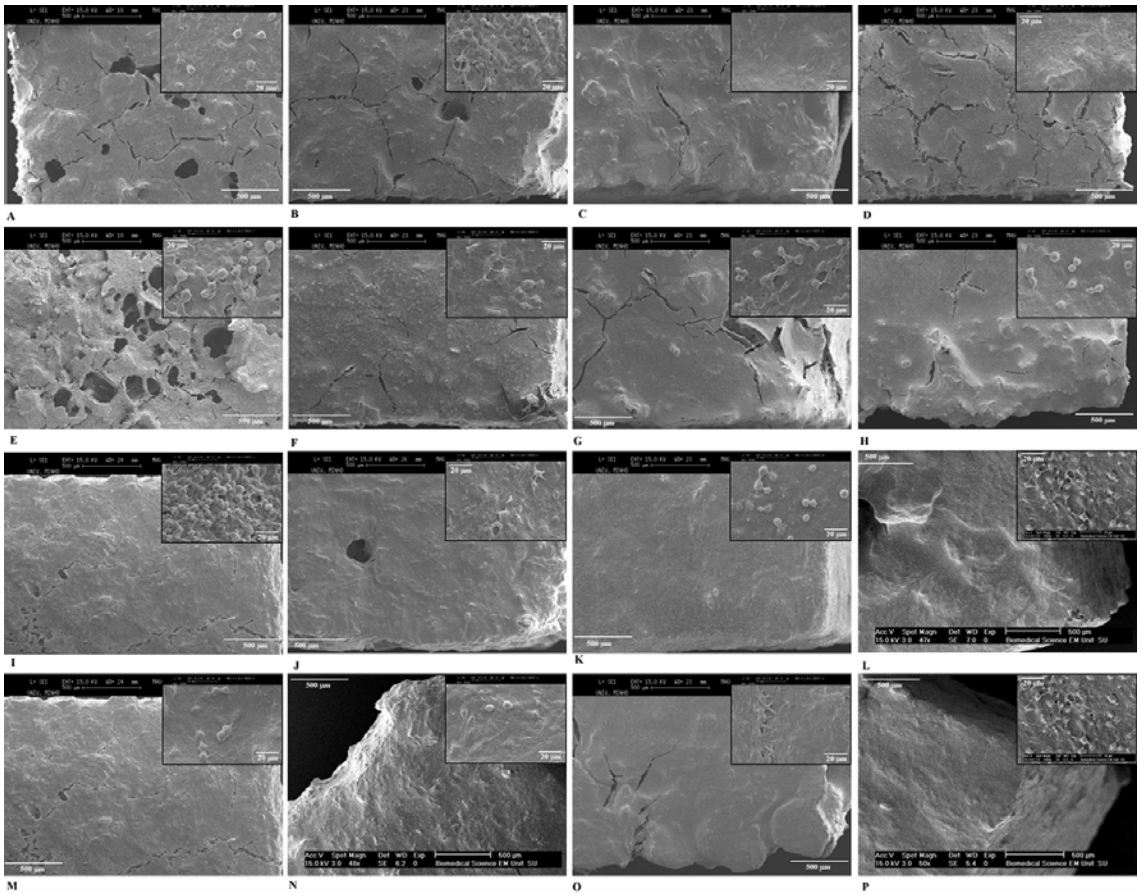

Figure 4.3 – BAC growth morphology in CPBS scaffolds, throughout the time course of the experiment. A-H shows results for static (A-D) and dynamic (E-H) cultures in 60% porosity scaffolds. I-P shows results for static (I-L) and dynamic (M-P) cultures in 80% porosity scaffolds. Different magnifications were used to highlight cell morphology.

3.3. Histological staining

The colonization of the scaffolds by cells was shown by H&E staining (Figure 4.4A, D, G, J). Concerning the 80 CPBS scaffolds, H&E staining shows more cells covering the surface of the scaffold than in the 60 CPBS ones. Consequently, more proteoglycans are observed, as stated by results of toluidine blue (Figures 4.4H and 4K) and alcian blue (Figures 4.4I and 4.4L) stainings. This observation applies for both culture conditions (static and dynamic). It is important to highlight the alcian blue staining results. The staining in the 80 CPBS scaffolds seems to be stronger and again covering more surface area than the obtained for 60 CPBS scaffolds, in both culture conditions.

Those results indicate a higher concentration of sulphated proteoglycans in these samples.

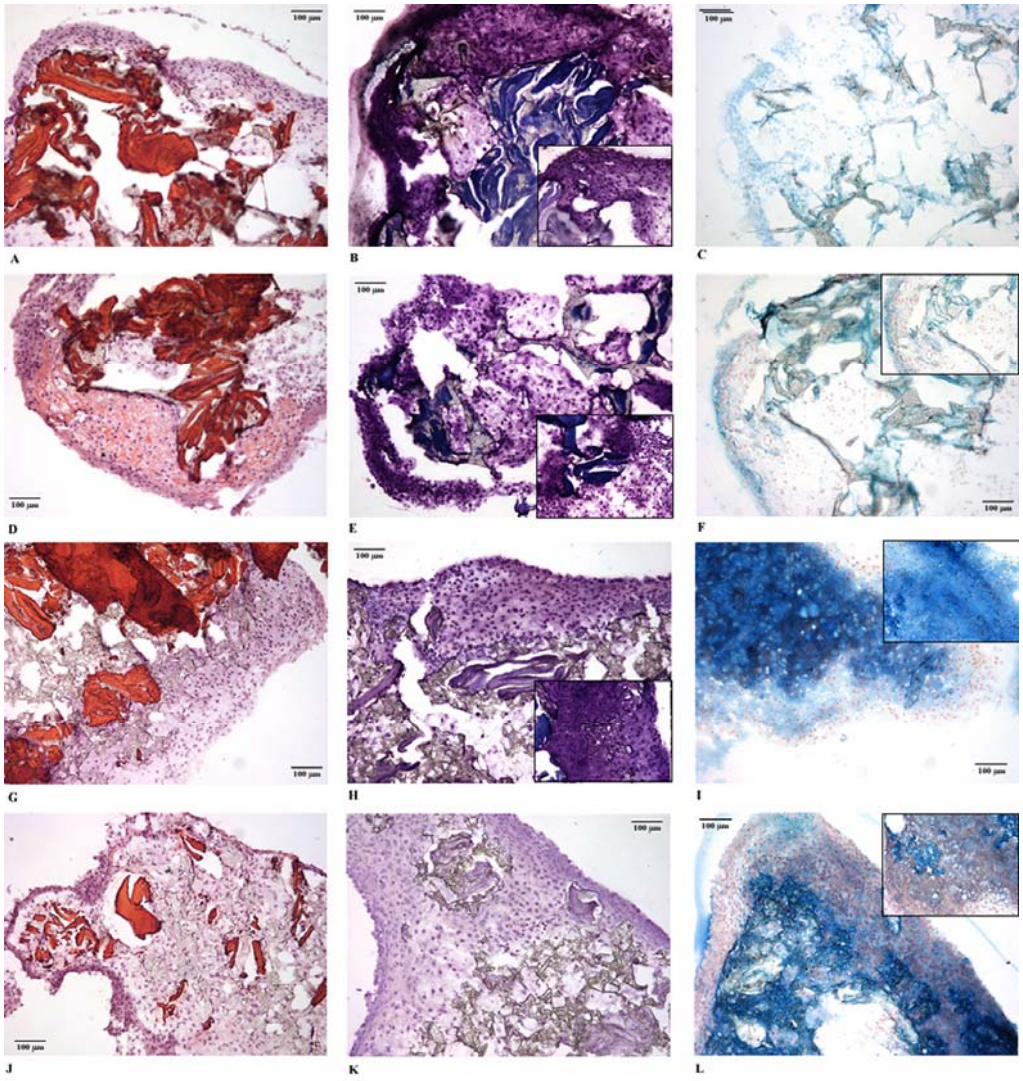

Figure 4.4 – Production of extracellular matrix in CPBS scaffolds at 4 weeks of culture. A-F shows results for static (A-C) and dynamic (D-F) cultures in 60% porosity scaffolds. G-L shows results for static (G-I) and dynamic (J-L) cultures in 80% porosity scaffolds. Cells were able to attach to scaffolds as showed in hematoxylin-eosin staining for 60% porosity (A, D) and 80% porosity scaffolds (G, J). Cells produced proteoglycans, detected by toluidine blue staining, either in 60% porosity (B, E) or 80% porosity (H, K) scaffolds. Sulphated proteoglycans were detected by alcian blue staining, again for both 60% porosity (C, F) and 80% porosity scaffolds (I, L). Two magnifications (10x and 20x) were used at each staining for microscopic observation. Scale bar = 100 μm.

3.4. Immunolocalisation of type I and type II collagens

Results of immunolocalisation of collagens type I and type II in both groups of scaffolds are presented in Figure 4.5. Cells seeded into 60 CPBS scaffolds produced both collagen type I and type II (Figure 4.5A-F), either in static (Figure 4.5B and 4.5C) or in stirred culture conditions (Figure 4.5D and 4.5F). Contrarily, collagen type I was discrete in the section of 80 CPBS scaffolds (Figures 4.5H and 4.5K), whereas collagen type II synthesis was revealed in all samples (Figures 4.5I and 4.5L).

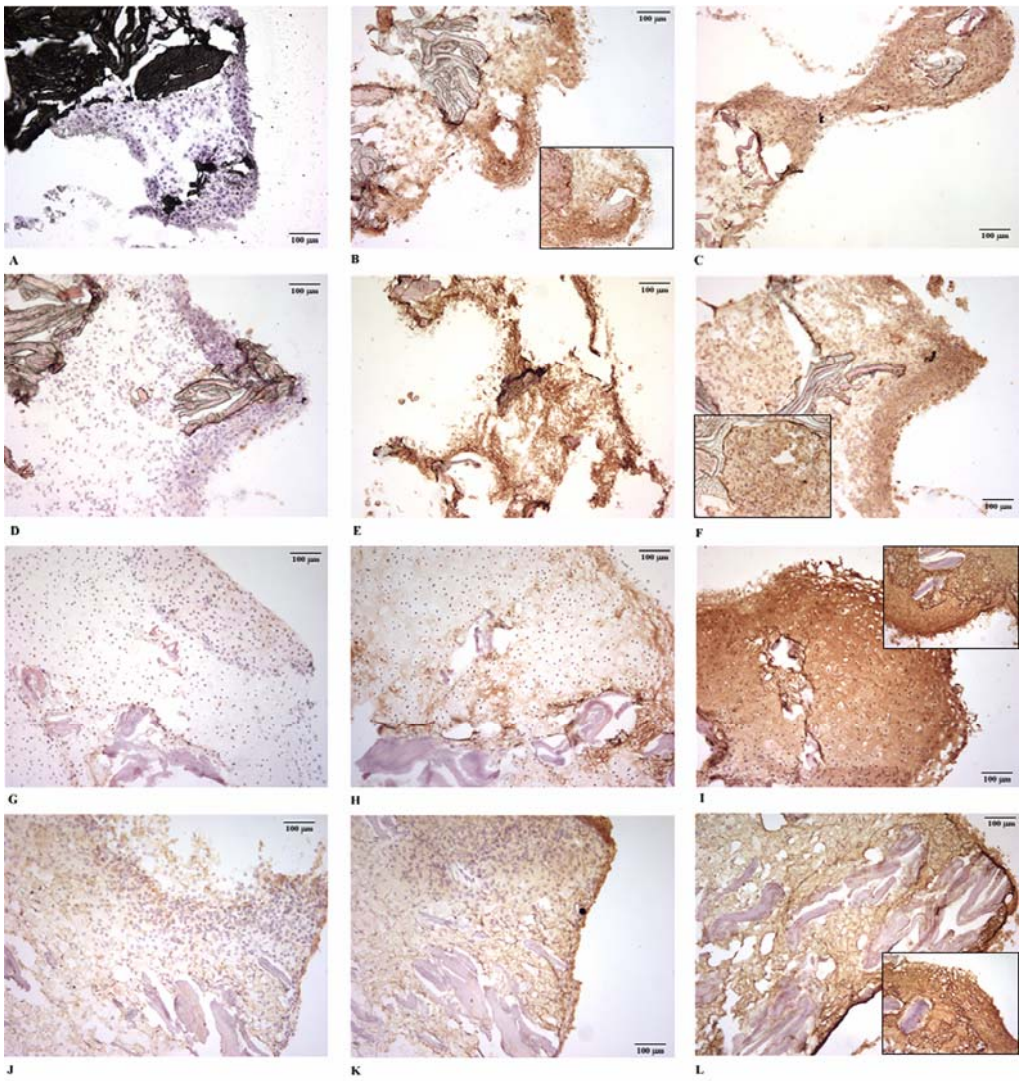

Figure 4.5 – Immunolocalisation of collagens in CPBS scaffolds, at 4 weeks of culture. A-F shows results for static (A-C) and dynamic (D-F) cultures in 60% porosity scaffolds. G-L shows results for static (G-I) and dynamic (J-L) cultures in 80% porosity scaffolds. Controls (A, D, G, J) were performed with normal goat serum. Collagen type

I (B-K) and collagen type II (C-L) were detected. Two magnifications (10x and 20x) were used at each staining for microscopic observation. Scale bar = 100 μm.

3.5. GAGs quantification

Glycosaminoglycans presented in both types of scaffolds were quantified by the DMB assay, and results are presented in Figure 4.6. A significant higher glycosaminoglycans production in 60 CPBS scaffolds was detected in comparison with 80 CPBS scaffolds. Stirred culture conditions significantly ($P<0.05$) enhance glycosaminoglycans production in both types of scaffolds when compared to static culture conditions. There was a statistical difference in glycosaminoglycans production in 60 CPBS scaffolds when cultured in stirred conditions compared to the data obtained for static culture conditions. For 80 CPBS scaffolds, glycosaminoglycans production in stirred culture conditions is higher, but not statistically different.

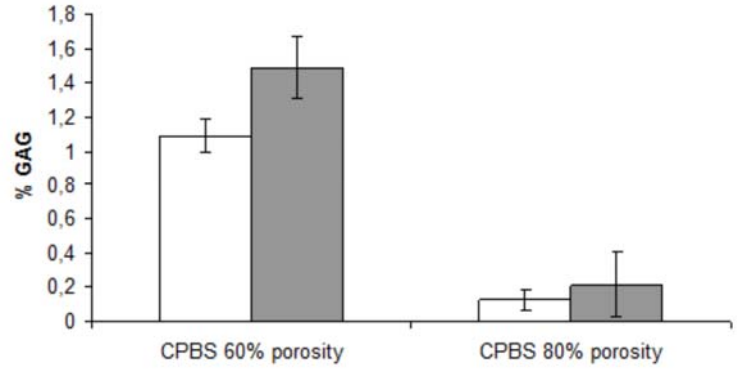

Figure 4.6 –GAGs quantification for CPBS scaffolds, at 4 weeks of culture. Static culture conditions are represented in white, dynamic culture conditions in grey.

4. Discussion

Structure of pores is one important factor in tissue regeneration. The growth of some specific cell types in injured sites is dependent on optimal pore size and geometry [30]. Pore size affects cells response in terms of attachment, growth and proliferation [31]. Variation in pore size also affects the mechanical stability of constructs, and this is an important factor when implanting them in load bearing areas, as well as to evaluate the

construct response to mechanical stimuli in bioreactors [32]. In particular, permeability affects the shear stresses inside the construct [33], which is a stimulus for cellular differentiation or functional adaptation of the construct during the implant. Furthermore, mechanical environment in the tissue has a controlling influence on tissue differentiation [33]. For in vivo implantation, cells need to be expanded in vitro to a sufficient number in order to generate a construct with certain mechanical stability [34]. Furthermore, upon implantation the scaffolds and cells will be subjected to the action of synovial fluid flow in the joint. The fluid flow is believed to facilitate cell migration and promote positive signals by mechanotransduction to the cells. Therefore, the dynamic culture conditions may support high seeding densities and help cells to grow, proliferate and produce ECM. Those conditions are also believed to lead to more stable constructs, and therefore, may enhance the construct integration within the tissue at the implant site. In a work by Gotterbarm *et al.,* (2006), two layered biomaterials were implanted in osteochondral defects created in the trochlear groove of Göttinger Minipigs. They observed cellular migration and cell attachment, as well as matrix production. These authors claim that this cellular filling and attachment was fostered by the porous character of the biomaterials, and led to an increased amount of repaired tissue [35].

In a previous work, Correlo *et al.*, (2008) produced and characterized several scaffolds produced from different blends of chitosan and synthetic polyesters, including the CPBS scaffolds studied in the present work [18]. A higher range of different types of scaffolds were produced by compression moulding and salt leaching, using different salt sizes to induce small and large pores. Curiously, small salt sizes originated larger pores, due to the aggregation of the salt particles [18]. We choose the scaffolds from the blend CPBS (50/50) based on cytotoxicity and direct contact tests previously performed (data not shown). We intended to determine the effect of the pore size and shape on the production of ECM by bovine articular chondrocytes.

In a work by Spiteri *et al*. (2006) it has been shown that substrate porosity enhanced bovine articular chondrocytes attachment, growth and formation of cartilage *in vitro* [31]. In our present work, scaffold pore morphology seems to equally affect BAC attachment and colonization. Globally, we can observe that cells attached and colonized both scaffolds. This observation is similar to the one obtained when rabbit chondrocytes were seeded onto chitosan-based hyaluronic acid hybrid polymer fibers. In the referred study, cell proliferation had no significant difference between 3 groups of scaffolds with different pore sizes [26].

Some studies have focused on the effect of pore size on chondrocytes performance. Cartilaginous tissue obtained on porous titanium alloy discs with the smaller pore size was thicker and had greater amount of proteoglycans, in comparison to tissue obtained form discs with larger pore sizes [36]. Another study by Nehrer *et al.*, (1997) showed that biosynthetic activity and chondrocyte phenotype were improved in collagen matrices with smaller pores [37]. It has been suggested that cell-cell interactions are enhanced in scaffolds with small pore sizes, thus resulting in improved chondrocyte proliferation [37]. These studies lead to the conclusion that small pores improve chondogenesis. However, several other studies point to the opposite direction. Griffon *et al.,* (2006) determined that large interconnective pores improve the cellularity and matrix content within chitosan scaffolds [38]. In a recent work, Lien *et al.* (2009) also showed that articular chondrocytes of Wistar rats performed better in the group of scaffolds with pore size between 250-500 μm in terms of proliferation and ECM production [39]. In fact, our findings support these observations. Cells seeded into 60 CPBS produced both collagen type I and type II (Figure 5A-F), indicative of a fibrous cartilage tissue, either in static (Figures 5B and 5C), or stirred culture conditions (Figures 5C and 5F). Fibrocartilage is aa mixed matrix consisting of fibrous tissue and hyaline cartilage. It contains type I and type II collagen and aggrecan [40]. On the other hand, hyaline cartilage contains type II collagens and proteoglycans, being the major one aggrecan molecule [40]. Cartilage-like tissue formed in 80 CPBS scaffolds did not presented significant staining of collagen type I (5H and 5K) yet showed a marked staining for collagen type II, indicating that these tissue properties are more similar to hyaline-like cartilage tissue, than those obtained with 60 CPBS scaffolds. However, glycosaminoglycans content present in 80 CPBS scaffolds were lower compared with obtained for 60 CBPS scaffolds (Figure 6). This result seems to contradict the previous ones, going in the direction of the first series of results that were referred, where smaller pores enhance the proteoglycans deposition [36, 37]. In fact, the opposite effects of pore size in this work, was observed. On one hand, large pores induced cell proliferation, production of proteoglycans and collagens. On the other hand, glycosaminoglycans production was significantly lower in scaffolds with large pore sizes. Additionally, stirred culture conditions seem to also affect glycosaminoglycans production, for both types of scaffolds. In general, we observed that stirred conditions enhance glycosaminoglycans production. A similar observation was reported by Freyria *et al.* (2004), when using 3D collagen scaffolds seeded with BAC and cultured under static or

stirred conditions. Thus, authors state that these conditions allow obtaining a homogeneous distribution of cells and ECM within scaffolds structure [41]. It is described in the literature that substrate geometry and porosity may both influence chondrocyte behaviour [36, 37, 42]. Understanding the impact of the biomaterial geometry is very important to enhance the in vivo ECM formation. Many factors may act in concert, such as fluid flow to enhance the nutrition and waste product removal, as well as number of attached cells and their ability to produce ECM [31]. Therefore, the optimal conditions for each scaffold must be determined, because each scaffold characteristics determine the cell attachment, growth, maintenance of phenotype and ECM production [34]. The referred characteristics include the scaffolds texture, porosity (size, structure, distribution) and surface free energy [34]. In summary, every parameter by itself is important and should be regarded, but the overall of the referred parameters makes the scaffold be functional and suitable for cartilage tissue engineering.

5. Conclusions

Chitosan-PBS scaffolds were produced by compression moulding followed by salt leaching. Two different types of NaCl salt particles were used as porogens, and two types of scaffolds with different porosities, pore geometry and size were produced. 80 CPBS scaffolds presented larger, randomly structured pores opposite to 60 CPBS scaffolds that showed smaller pores with a cubic structure. By our observations, we can state that 80 CPBS scaffolds seem more effective in inducing ECM production by BAC. Pore size and geometry of the pore had an effect on cell proliferation and ECM production. Proteoglycans and collagen type II were detected in larger quantities in 80 CPBS scaffolds (large pores, random structure), when compared with 60 CPBS scaffolds (small pores, cubic structure). Nevertheless, glycosaminoglycan amount was lower in the 80 CPBS scaffolds. Large pores affected GAG production, either in static or dynamic culture conditions. Concerning culture conditions, stirred conditions improved ECM production in both types of scaffolds, thus being preferable than static culture conditions.

Acknowledgements

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References

1 - Bonassar LJ. *Cartilage reconstruction.* In: Atala A, Lanza R, editors. Methods of tissue engineering. San Diego: Academic Press; 2002. p. 1027-39.

2 - Nesic D, Whiteside R, Brittberg M, Wendt D, Martin I, Mainil-Varlet P. *Cartilage tissue engineering for degenerative joint disease*. Adv Drug Deliv Rev. 2006. 58(2):300-22.

3 - Magne D, Vinatier C, Julien M, Weiss P, Guicheux J. *Mesenchymal stem cell therapy to rebuild cartilage*. Trends Mol Med. 2005. 11(11):519-26.

4 - Steinert AF, Ghivizzani SC, Rethwilm A, Tuan RS, Evans CH, Noth U. *Major biological obstacles for persistent cell-based regeneration of articular cartilage*. Arthritis Res Ther. 2007. 9(3):213.

5 - Martin I, Miot S, Barbero A, Jakob M, Wendt D. *Osteochondral tissue engineering*. J Biomech. 2007. 40(4):750-65.

6 - Goessler UR, Hormann K, Riedel F. *Tissue engineering with chondrocytes and function of the extracellular matrix (review).* Int J Mol Med. 2004. 13(4):505-13.

7 - Correlo VM, Boesel LM, Bhattacharya M, Mano JF, Neves NM, Reis RL. *Hydroxyapatite reinforced chitosan and polyester blends for biomedical applications*. Macromol Mater Eng. 2005. 290:1157–65.

8 - Correlo VM, Boesel LM, Bhattacharya M, Mano JF, Neves NM, Reis RL. *Properties of melt processed chitosan and aliphatic polyester blends*. Materials Science and Engineering A. 2005. 403:57–68.

9 - Ho M-H, Wang D-M, Hsieh H-J, Liu H-C, Hsien T-Y, Lai J-Y, *et al. Preparation and characterization of rgd-immobilized chitosan scaffolds*. Biomaterials. 2005. 26:3197–206.

10 - Medrado GCB, Machado CB, Valerio P, Sanches MD, Goes AM. *The effect of a chitosan–gelatin matrix and dexamethasone on the behavior of rabbit mesenchymal stem cells*. Biomedical Materials. 2006. 1:155-61.

11 - Zhao L, Chang J. *Preparation and characterization of macroporous chitosan/wollastonite composite scaffolds for tissue engineering*. J Mater Sci Mater Med. 2004. 15(5):625-9.

12 - Di Martino A, Sittinger M, Risbud MV. *Chitosan: A versatile biopolymer for orthopaedic tissue-engineering*. Biomaterials. 2005. 26:5983–90.

13 - VandeVord PJ, Matthew HW, DeSilva SP, Mayton L, Wu B, Wooley PH. *Evaluation of the biocompatibility of a chitosan scaffold in mice*. J Biomed Mater Res. 2002. 59(3):585-90.

14 - Hu Q, Li B, Wang M, Shen J. *Preparation and characterization of biodegradable chitosan/hydroxyapatite nanocomposite rods via in situ hybridization: A potential material as internal fixation of bone fracture*. Biomaterials. 2004. 25(5):779-85.

15 - Frenkel SR, Bradica G, Brekke JH, Goldman SM, Ieska K, Issack P*, et al. Regeneration of articular cartilage - evaluation of osteochondral defect repair in the rabbit using multiphasic implants*. Osteoarthritis and Cartilage. 2005. 13:798 - 807.

16 - Montembault A, Tahiri K, Korwin-Zmijowska C, Chevalier X, Corvol MT, Domard A. *A material decoy of biological media based on chitosan physical hydrogels: Application to cartilage tissue engineering*. Biochimie. 2006. 88(5):551-64.

17 - Hoemann CD, Sun J, Legare A, McKee MD, Buschmann MD. *Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle*. Osteoarthritis Cartilage. 2005. 13(4):318-29.

18 - Correlo VM, Boesel LF, Pinho E, Costa-Pinto AR, Alves da Silva ML, Bhattacharya M*, et al. Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties*. J Biomed Mater Res A. 2008.

19 - Correlo VM, Pinho ED, Pashkuleva I, Bhattacharya M, Neves NM, Reis RL. *Water absorption and degradation characteristics of chitosan-based polyesters and hydroxyapatite composites*. Macromol Biosci. 2007. 7(3):354-63.

20 - Costa-Pinto AR, Correlo VM, Sol P, Bhattacharya M, Charbord P, Delorme B, Reis RL, Neves NM. *Adhesion, proliferation and osteogenic differentiation of a mouse mesenchymal stem cell line (BMC9) seeded on novel melt based chitosan/polyester 3D porous scaffolds*. Tissue Eng. 2008. 14(6):1049-57.

21 - Malafaya PB, Reis RL. *Bilayered chitosan-based scaffolds for osteochondral tissue engineering: Influence of hydroxyapatite on in vitro cytotoxicity and dynamic bioactivity studies in a specific double-chamber bioreactor*. Acta Biomater. 2008.

22 - Oliveira JM, Rodrigues MT, Silva SS, Malafaya PB, Gomes ME, Viegas CA, *et al. Novel hydroxyapatite/chitosan bilayered scaffold for osteochondral tissue-engineering applications: Scaffold design and its performance when seeded with goat bone marrow stromal cells*. Biomaterials. 2006. 27(36):6123-37.

23 - Oliveira J, Correlo VM, Sol P, Costa-Pinto A, Salgado A, Bhattacharya M, *et al. Assessment of the suitability of chitosan/polybutylene succinate scaffolds seeded with mouse mesenchymal progenitor cells for a cartilage tissue engineering approach* Tissue Eng. 2008. 14(10):1651-61.

24 - Silva SS, Motta A, Rodrigues MT, Pinheiro AF, Gomes ME, Mano JF, *et al. Novel genipin-cross-linked chitosan/silk fibroin sponges for cartilage engineering strategies*. Biomacromolecules. 2008. 9(10):2764-74.

25 - Kuo YC, Lin CY. *Effect of genipin-crosslinked chitin-chitosan scaffolds with hydroxyapatite modifications on the cultivation of bovine knee chondrocytes*. Biotechnol Bioeng. 2006. 95(1):132-44.

26 - Yamane S, Iwasaki N, Kasahara Y, Harada K, Majima T, Monde K, *et al. Effect of pore size on in vitro cartilage formation using chitosan-based hyaluronic acid hybrid polymer fibers*. J Biomed Mater Res A. 2007. 81(3):586-93.

27 - Mrugala D, Bony C, Neves N, Caillot L, Fabre S, Moukoko D, *et al. Phenotypic and functional characterization of ovine mesenchymal stem cells: Application to a cartilage defect model*. Ann Rheum Dis. 2007.

28 - Malafaya PB, Santos TC, van Griensven M, Reis RL. *Morphology, mechanical characterization and in vivo neo-vascularization of chitosan particle aggregated scaffolds architectures*. Biomaterials. 2008. 29(29):3914-26.

29 - Crawford A, Dickinson S. *Chondrocyte isolation, expansion, and culture in polymer scaffolds.* In: Hollander AP, Hatton PV, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 147-57.

30 - Cheung H-Y, Lau K-T, Lu T-P, Hui D. *A critical review on polymer-based bio-engineered materials for scaffold development*. Composites: Part B. 2007. 38:291-300.

31 - Spiteri CG, Pilliar RM, Kandel RA. *Substrate porosity enhances chondrocyte attachment, spreading, and cartilage tissue formation in vitro*. J Biomed Mater Res A. 2006. 78(4):676-83.

32 - Al-Munajjed AA, Hien M, Kujat R, Gleeson JP, Hammer J. *Influence of pore size on tensile strength, permeability and porosity of hyaluronan-collagen scaffolds*. J Mater Sci Mater Med. 2008. 19(8):2859-64.

33 - Prendergast PJ, Huiskes R, Soballe K. *ESB research award 1996. Biophysical stimuli on cells during tissue differentiation at implant interfaces*. J Biomech. 1997. 30(6):539-48.

34 - Mainil-Varlet P, Rieser F, Grogan S, Mueller W, Saager C, Jakob RP. *Articular cartilage repair using a tissue-engineered cartilage-like implant: An animal study*. Osteoarthritis Cartilage. 2001. 9 Suppl A:S6-15.

35 - Gotterbarm T, Richter W, Jung M, Berardi Vilei S, Mainil-Varlet P, Yamashita T, *et al. An in vivo study of a growth-factor enhanced, cell free, two-layered collagen-tricalcium phosphate in deep osteochondral defects*. Biomaterials. 2006. 27(18):3387-95.

36 - Bhardwaj T, Pilliar RM, Grynpas MD, Kandel RA. *Effect of material geometry on cartilagenous tissue formation in vitro*. J Biomed Mater Res. 2001. 57(2):190-9.

37 - Nehrer S, Breinan HA, Ramappa A, Young G, Shortkroff S, Louie LK, *et al. Matrix collagen type and pore size influence behaviour of seeded canine chondrocytes*. Biomaterials. 1997. 18(11):769-76.

38 - Griffon DJ, Sedighi MR, Schaeffer DV, Eurell JA, Johnson AL. *Chitosan scaffolds: Interconnective pore size and cartilage engineering*. Acta Biomater. 2006. 2(3):313-20.

39 - Lien SM, Ko LY, Huang TJ. *Effect of pore size on ECM secretion and cell growth in gelatin scaffold for articular cartilage tissue engineering*. Acta Biomater. 2009. 5(2):670-9.

40 - Freemont AJ, Hoyland J. *Lineage plasticity and cell biology of fibrocartilage and hyaline cartilage: Its significance in cartilage repair and replacement*. European Journal of Radiology. 2006. 57:32 - 6.

41 - Freyria AM, Cortial D, Ronziere MC, Guerret S, Herbage D. *Influence of medium composition, static and stirred conditions on the proliferation of and matrix protein expression of bovine articular chondrocytes cultured in a 3-D collagen scaffold*. Biomaterials. 2004. 25(4):687-97.

42 - Li WJ, Jiang YJ, Tuan RS. *Chondrocyte phenotype in engineered fibrous matrix is regulated by fiber size*. Tissue Eng. 2006. 12(7):1775-85.

SECTION IV – HUMAN BONE MARROW MESENCHYMAL STEM CELLS CHONDROGENIC DIFFERENTIATION IN A BIDIRECTIONAL FLOW PERFUSION BIOREACTOR USING NANOFIBER AND MICROFIBER MESHES AS CELL SUPPORT

Chapter 5

Cartilage tissue engineering using electrospun PCL nanofiber meshes and MSCs

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This chapter is based on the following publication: Alves da Silva ML, Martins A, Costa-Pinto AR, Costa P, Faria S, Gomes M, Reis RL, Neves NM. *Cartilage tissue engineering using electrospun PCL nanofiber meshes and MSCs*. Biomacromolecules. 2010. 11(12): 3228-3236.

Abstract

Mesenchymal Stem Cells (MSCs) have been recognized for their ability to differentiate into cells of different tissues such as bone, cartilage or adipose tissue, and therefore are of great interest for potential therapeutic strategies. Adherent, colony-forming, fibroblastic-cells were isolated from human bone marrow aspirates, from patients undergoing knee arthroplasties, and the MSCs phenotype characterized by flow cytometry. Afterwards, cells were seeded onto electrospun polycaprolactone nanofiber meshes and cultured in a multichamber flow perfusion bioreactor, in order to determine their ability to produce cartilagineous extracellular matrix. Results indicate that the flow perfusion bioreactor increased the chondrogenic differentiation of hBM-MSCs, as confirmed either by morphological and RT-PCR analysis. Cartilage-related genes such as aggrecan, collagen type II and Sox9 were expressed. ECM deposition was also detected by histological procedures. Collagen type II was present in the samples, as well as collagen type I. Despite no statistically significant values were obtained for gene expression, the other results support the choice of the bioreactor for this type of culture.

1. Introduction

Due to the epidemiological importance and the high social costs of joint diseases, cartilage engineering holds remarkable potential in orthopedic surgery as an alternative to current surgical methods. Traditional implantable materials frequently fail its biofunction, due to implant loosening, inflammation, infection, rejection, wear debris and tissue inflammation or infection [1]. Tissue Engineering can provide the answers to many of these problems. One of the challenges of TE is to mimic the natural extracellular matrix (ECM) of connective tissues, including cartilage. Nanoscale is very important in this approach, as the ECM substratum that interacts with cells includes fibrils at submicron level [2]. Electrospinning process has been used to produce nanofibrous scaffolds that can mimic tissues' ECM by imitating its fibrils morphology and distribution [3]. These nanofibrous structures present many advantages, such as high specific surface area for cell attachment, higher micro-porous structure and a 3D microenvironment for cell-cell and cell-biomaterial contact [1, 4]. In the last few years, nanomaterials and nanofibers have been explored as new functional structures for tissue

regeneration, in bone [5], cartilage [6], veins or nerves [7]. Some materials intended for cartilage tissue engineering have been produced using electrospinning of several synthetic and natural materials, such as PCL [8], PLGA [9], chitosan-based materials [10] or starch based materials [11].

Cartilage is an avascular tissue composed by chondrocytes entrapped in an ECM rich in proteoglycans and collagens [12]. The low self-regeneration potential of cartilage is due to the absence of vascular networks and progenitor cells in the tissue, as well as the non-mobility of chondrocytes in the dense ECM [13]. Chondrocytes have been used to generate engineered cartilage tissue [11, 14], and are usually isolated from articular cartilage tissues. Stem cells are also commonly proposed for cartilage tissue engineering. Chondrocytes are developmentally derived from stem cells [15]. Compared with adult chondrocytes, stem cells are easier to obtain and manipulate, as they can undergo several passages before loosening their differentiation potential [16]. Mesenchymal stem cells (MSCs) can be isolated from several tissues, including bone marrow [17], fat tissue [18] or synovium [19]. Selecting the ideal source of cells for a cartilage tissue engineering approach is a demanding and challenging task, as there are numerous options. Additionally, the choice of the scaffold is very important. In a recent study it was shown dissimilar chondrogenic differentiation behaviour of MSCs derived from different tissues, namely human embryonic stem cells, bone marrow and adipose tissue. Additionally, their behaviour also differ among the tested silk and chitosan scaffolds [16].

Dynamic culture systems or bioreactors have been widely studied for cartilage tissue engineering approaches [20-22]. Results often show that they enhance ECM formation when compared with static cultures. In some bioreactor studies for cartilage tissue engineering approaches low levels of shear stress were used successfully, as they promoted good mass transfer properties [23]. As chondrocytes are surrounded by an environment influenced by mechanical forces, it is logical that the formation of cartilagineous tissue is also heavily influenced by the environment. Therefore, if cells are surrounded by an appropriated environment, and they have access to the correct levels of nutrients, it is believed that they will act like native chondrocytes and secrete ECM [23].

In a recent work using a bovine articular chondrocytes model, we showed that polycaprolactone (PCL) and SPCL (starch-compounded PCL) nanofiber meshes are suitable for cartilage tissue engineering [11]. In that study, we used a high initial

concentration of bovine articular chondrocytes, and cultured the nanofiber meshes in a lab rotator bioreactor. The results were encouraging, but from that work several other questions were raised, namely the aspects involved in the future application of this model in human therapies. In that study it was used a high number of cells, which most of the times are not readily available for clinical application. The dynamic culture system used in that previous study did not allow create a controlled environment for the culture of the constructs, and it is important to obtain a pre-implant with similar characteristics to the native cartilage. Finally, when going to clinic, the access to articular chondrocytes is more limited than it is for MSCs. Having all these issues in mind, we designed this study to validate if, starting from low seeding densities of human bone marrow-derived MSCs in PCL nanofiber meshes cultured in an in-house developed multichamber flow perfusion bioreactor (PT patent nº 104155; European patent pending [24]), we could induce chondrogenic differentiation of the MSCs and consequent ECM deposition. Also, we aimed to determine whether the ECM deposition would result in cartilage-like tissue, whose characteristics would resemble native articular cartilage.

2. Materials and Methods

2.1. Nanofiber meshes processing

A polymeric solution of PCL (PCL 787, TONE polymer, Union Carbide Chemical and Plastics Division, Bound Brook, NJ, with 80 kDa), at the concentration of 17% (w/v), was prepared by dissolving the polymer into an organic solvent mixture of chloroform/ dimethylformamide (70:30) (Sigma-Aldrich, USA). The polymeric solution was placed into a syringe, which is coupled to a syringe pump (model KDS100, KD Scientific, USA) for flow rate control. A blunted metallic needle with an internal diameter of 0.8 mm was attached to the syringe. A grounded aluminium foil was used as the fiber mesh collector. A high voltage of 9kV was applied to the needle tip to generate the electric field; a needle tip-to-collector distance of 20 cm and the flow rate of 1.0 ml/h were established. The applied voltage was maintained at 9 kV. All the experiments were performed at room temperature and the conditions were optimized for the production of PCL nanofiber meshes. The produced PCL nanofiber meshes presented a

thickness between 40-60 μm, 70-80% of porosity, determined by nCT (data not shown) and an average pore size of 2.7 μm. For more details on the PCL nanofiber meshes, please refer to Guimarães *et al.,* 2010 [25]. After collection, nanofiber meshes were cut into 1 cm^2 squares.

A clamping system to fixate the produced nanofiber meshes in the multichamber flow perfusion bioreactor (PT patent nº 104155; European patent pending [24]) had to be assembled. The meshes were fixated in between two silicon rings, which were then clamped with nylon stitches [25]. Using this clamping system, the available surface of the nanofiber meshes to be cultured in the bioreactor was a circle of 5 mm in diameter. For the static cultures, the referred 1 cm^2 squares were used. The membranes were then sterilized under UV light for 1hour in each side.

2.2. Isolation of hBM-MSCs

HBM-MSCs were isolated from bone-marrow aspirates collected under informed consent from patients undergoing knee arthroplasties in Hospital de S. Marcos, Braga, Portugal. Samples were collected from a 55 years old female donor, isolated, expanded and frozen in several passages. Briefly, during the surgery, bone marrow was collected to a container with α-MEM medium (Invitrogen, 12000-063), supplemented with antibiotic/ antimycotic solution (Gibco, 15240062) and 5000 units of heparin (Sigma, H3393) and maintained in ice until the isolation procedure. Aspirates were homogenised, diluted in PBS (Sigma, P4417) (1:1) and incubated for 5 minutes at room temperature. Then, bone marrow was diluted in lyses buffer (1:10) and left under agitation for 10 minutes. Lyses buffer was prepared with 10 mM of Tris-HCl (Sigma, T3253), 1.21g of Tris Base (Sigma, T1503) and 8.3g of NH_4Cl (Merck, 1011455000), in 1L of distilled water. Afterwards, the suspension was centrifuged at 1200 rpm, for 15 minutes at room temperature. Cells were ressuspended in α-MEM medium, supplemented with antibiotic/ antimycotic solution and 20% FBS (Biochrom, BSC0115/0943k). Cell suspension was filtered for disposal of debris, using 100μm and 70μm Cell Strainer (BD Falcon™, 352360 and BD Falcon™, 352350). Cells were counted and plated at the density of $4.7x10^3$ cells/cm^2. Cells were expanded in the referred culture medium until passage 5, and then used for seed the nanofiber meshes.

2.3. Flow-cytometry analysis

To evaluate cell-surface marker expression, cultured cells were incubated for 20 min at 4ºC with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies specific for human markers associated with mesenchymal and haematopoietic lineages. The antibodies used were: CD29, CD34, CD45, CD73, CD90, CD105, CD116. All the antibodies were purchased from BD Pharmingen. The samples were analysed on a FACS Calibur (BD Biosciences).

2.4. hBM-MSCs culture on the multichamber flow perfusion bioreactor

hBM-MSCs were detached from the culture flasks by treatment with Trypsin/EDTA solution (Invitrogen, 25300-062). Cells were counted and a cell suspension of 200.000 cells/nanofiber mesh was prepared to seed each of the nanofiber meshes. Cell seeding was performed using the "drop" method. The seeding method has been described previously in more detail by our group, in Guimarães *et al.*, 2010 [25]. Briefly, each scaffold was seeded with a 10 μL drop of α-MEM culture medium containing 200.000 cells, in 24 well plates. Afterwards, the constructs were maintained at 37ºC and 5% CO_2, during 4 hours in an incubator to allow cell attachment to the scaffolds. After this time period, culture medium was added to each culture well and left in the incubator for 24 hours. The constructs were then transferred to the bioreactor.

This is the first work reporting the use of the multichamber bioreactor designed by our group. The bioreactor has currently a PT patent and the European patent is under submission. For detailed information please refer to Costa *et al.*, 2010 [24]. Briefly, the bioreactor apparatus is composed of a central part with 20 individual culture chambers, with the diameter of 8 mm each, and two lids attached to the central part. These lids form a common inlet and outlet of medium. These chambers were designed in a way which allows the circulation of the culture medium through them, and assures the evenly distribution of it between the several flow chambers. For bioreactor cultures, 20 constructs were transferred for the apparatus and placed one in each chamber. A flow velocity of 70 μl/min/nanofiber mesh was established. The bioreactor apparatus herein used can be observed in Figure 1. The clamping system has been previously reported also by a work in our group by Guimarães *et al.*, 2010 [25].

Another set of 20 constructs was transferred to new culture plates and maintained in static conditions to be used as control. Chondrogenic differentiation medium consisting of α-MEM supplemented with antibiotic/antimycotic solution, ITS liquid media supplement (Sigma, I2521), dexamethasone 1mM (Sigma, D1756), Sodium Pyruvate 0,1M (Sigma, P4562), Ascorbate-2-phosphate 17 mM (Sigma, A4544), L-Proline 35 mM (Sigma, P5607) and 1 ng/ml of human recombinant TGF-β3 (PeproTech, 100-36) was used for the 28 days of the experiment. Culture medium in the bioreactor was completely changed every week (100 mL each time). Control samples were cultured in 24 well plates, and were fed every other day with 1 mL of culture medium.

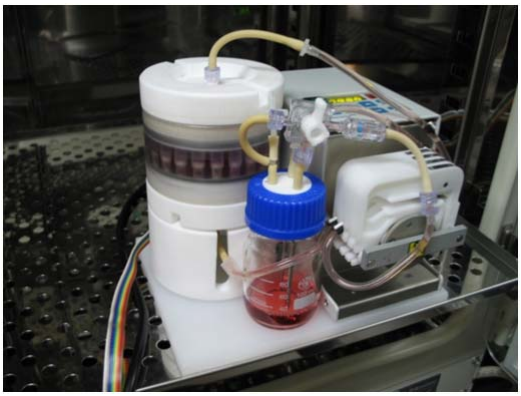

Figure 5.1 – Multichamber flow perfusion bioreactor (PT Patent nº 104155; European patent pending [24]) assembled and the culture system inside the incubator.

Samples were collected at every time point, both from the bioreactor and from static controls. For DNA analysis, 2 samples were collected at 14, 21 and 28 days of the experiment. For SEM analysis, 1 sample was collected on every time point. Three samples at 14, 21 and 28 days were collected for PCR analysis. For histological procedures, 1 sample was collected at the end of the experiment. Experiments were repeated 3 times.

2.5. Proliferation assay (DNA quantification)

Evaluation of cell proliferation was performed using the PicoGreen dsDNA quantification Kit (Molecular Probes, P7589), according to the manufacturer instructions. Samples from the flow perfusion bioreactor and from the static control were collected at 14, 21 and 28 days. Triplicates of every time point were used. A

standard curve ranging from 0.0 to 1.5 µg/ml was established. Fluorescence of both samples and standard curve was read with an excitation of 485/20 nm and an emission of 528/20 nm. DNA concentration was extrapolated from the standard curve.

2.6. Scanning Electron Microscopy (SEM)

For SEM analysis, one hMSCS-nanofiber meshes construct was collected at each time point: 1, 2, 3 and 4 weeks, for each culture condition. They were washed in sterile PBS and immersed in 3% glutaraldehyde (Sigma, G-5882) at room temperature for one hour. Samples were washed in PBS, dehydrated in increasing ethanol concentrations and let to dry at room temperature. Samples were sputter coated with gold and analysed in a scanning electron microscope (model S360, Leica Cambridge,UK).

2.7. RNA isolation

Three samples were collected from the flow perfusion bioreactor and from the static control. Samples were washed in PBS, immersed in TRIzol reagent (Invitrogen, 15596-018) and kept at -80ºC for posterior RNA extraction. To perform the RNA extraction, samples were taken from the freezer, and kept in ice until complete thawing. Chloroform (Sigma, C-2432) was added; samples were vigorously agitated for 15 seconds and then incubated in ice for 15 minutes. After that incubation, samples were centrifuged at 13000 rpm, for 15 minutes, at 4ºC. Afterwards, the supernatant was collected for a sterile 1.5 mL tube, and an equivalent volume of isopropanol (Sigma, I-9516) was added. Samples were incubated at -20ºC overnight, to precipitate the RNA. In the next day, samples were centrifuged at 13000 rpm, for 15 minutes, at 4ºC. Then, the supernatant was removed and 800 µl of ethanol 70% was added, in order to wash away the isopropanol. The ethanol 70% solution was prepared from absolute ethanol (Merck, 1.00983.2511) and ultra pure water. The 1.5 mL tubes were agitated vigorously and centrifuged again, at 9000 rpm for 5 minutes, at 4ºC. The supernatant was again removed, and the pellet was left to air dry. Finally, the pellet was ressuspended in 50 µl of DNase, RNase free water (Gibco, 10977-015). The concentration and purity of the extracted RNA was evaluated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA).

2.8. Real-Time PCR

The Real-Time PCR procedure used in the present work consisted of a two step fluorogenic assay using the SyberGreen system (Bio-Rad). All the reagents used in this procedure were from Bio-Rad, following the instructions of the manufacturer. Thermocycler reaction conditions used were also the mentioned in the kits. In the first step, RNA was reversed transcribed into cDNA, using the iScript cDNA synthesis kit (1708891). A MiniOpticon real-time PCR detection system (BioRad Laboratories, USA) was used to perform the reaction. Afterwards, the cDNA obtained was used as template for the amplification of the target genes (aggrecan, collagen type I, II and X, Sox9 and Runx2), with the Syber Green Kit (1708884). The number of amplification cycles used for every reaction was of 45. GAPDH was used as reference gene, and the expression of all the target genes was normalized to the GAPDH expression of that sample. All the primer sequences were generated using Primer3 software [26] and acquired from MWG Biotech AG, Germany. Primers sequences used are shown in Table 5.1. Obtained results were analyzed with CFX Manager Software – version 1.5 (BioRad Laboratories, USA).

Table 5.1 – Primer sequences used for RT-PCR procedures [a].

Gene	Forward (5´-3´)	Reverse (5´-3´)
AGC	TGAGTCCTCAAGCCTCCTGT	TGGTCTGCAGCAGTTGATTC
COL II	CGGTGAGAAGGGAGAAGTTG	GACCGGTCACTCCAGTAGGA
COL I	AGCCAGCAGATCGAGAACAT	ACACAGGTCTCACCGGTTTC
COL X	CCAGGTCTCGATGGTCCTAA	GTCCTCCAACTCCAGGATCA
Runx2	TTCCAGACCAGCAGCACTC	CAGCGTCAACACCATCATTC
Sox9	TTCATGAAGATGACCGACGC	GTCCAGTCGTAGCCCTTGAG
GAPDH	ACAGTCAGCCGCATCTTCTT	ACGACCAAATCCGTTGACTC

[a]AGC = Agreccan; COL II = Collagen type II; COL I = Collagen type I; COL X = Collagen type X; Runx2 = Runt-related transcription factor 2; Sox9 = Sry-type high mobility group box 9; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

2.9. Histological analysis

Samples were collected at the end of the experiment, included in O.C.T. (Gurr® OCT compound, BDH) and stored at -20ºC. Sections with 8 μm thickness were placed in microscopy slides, fixed in a fresh 4% paraformaldehyde (PF) (Sigma, P-6148) solution in PBS buffer, during 30 minutes at 4ºC, washed twice in distilled water and let overnight to air-dry. Slides were stored at 4ºC until they were further used for staining procedures. Toluidine blue staining was performed. Staining solution was prepared by adding 1% of toluidine blue (Sigma, T0394) dissolved in distilled water containing 0.5 g of sodium borate, followed by filtering. One drop of this solution was added to each section for 2-3 seconds. Then, the sections were rinsed with distilled water and let to air dry overnight. Sections were cleared in xylene substitute (Sigma, A5597) and mounted in Histo clear (Frilabo, HS200). Safranin O staining was performed by washing slides in tap water, then immersed in 0.02% fast green (Fluka, 44715) for 3 minutes. Then, samples were immersed in 1% acetic acid (Panreac, 131008) solution for 30 seconds. After, slides were immersed in 0.1% safranin O (Fluka, 84120) solution for 5 minutes. By the end, slides were washed in tap water and let to air dry. Sections were cleared in xylene and mounted as previously described.

2.10. Immunolocalisation of type I and type II collagens

Immunolocalisation of type I and type II collagens was performed in fixed sections. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide solution for 30 minutes. Sections were rinsed in PBS for 5 minutes. R.T.U. Vectastain® Universal Elite ABC Kit (Vector, VCPK-7200) was used for antibody incubation, according to the instructions of the manufacturer. Shortly, sections were incubated with primary antibodies (collagen type I and collagen type II) (UNLB, Goat anti-type I collagen 1310-01 and UNLB, Goat anti-type II collagen 1320-01) overnight at 4ºC, in a humidified atmosphere. Incubation was revealed by using the Peroxidase Substrate Kit DAB (Vector, VCSK-4100). Slides were washed in water for 5 minutes and then counterstained with haematoxylin for nuclei visualization. Finally, slides were mounted in Histo clear. Controls were performed using normal goat serum instead of primary antibodies, which was also included in the kit.

2.11. Statistical analysis

Statistical analysis was performed using the SPSS statistic software (Release 15.0.0 for Windows). Firstly, a Shapiro-Wilk test was used to ascertain about the data normality and variance equality. The normality is strongly rejected and, consequently, nonparametric tests were used in further comparisons between static and dynamic culture conditions. A Mann-Whitney U-test was applied to compare the two independent groups of samples for each variable (*i.e.* DNA quantification and real-time PCR). P values lower than 0.01 were considered statistically significant in the analysis of the results. A Kruskal-Wallis test was applied to compare differences in between the days in each group. P values lower than 0.01 were considered statistically significant in the analysis of the results.

3. Results

3.1. Electrospun PCL nanofiber meshes

SEM observations of the produced nanofiber meshes show a random distribution as expected. This is the most typical morphology obtained in the electrospinning process, caused by the electric field generated in the equipment (Figure 5.2). PCL nanofiber meshes were composed by nanofibers with diameters in the submicron range, from 0.4 - 1.4 μm.

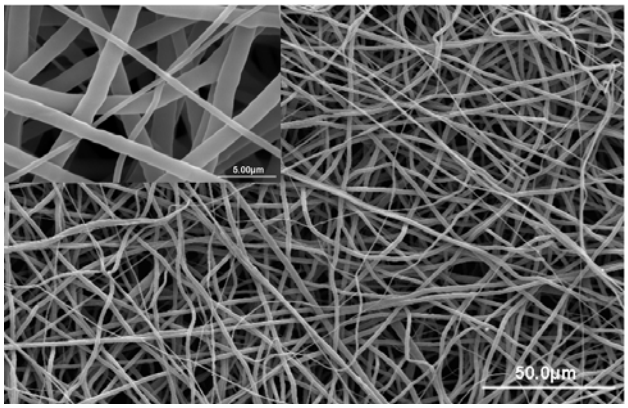

Figure 5.2 – SEM micrografs of poly(ε-caprolactone) (PCL) nanofiber meshes processed by electrospinning. Scale bars: 50.0 μm= 5 cm; 5.0 μm= 2 cm.

3.2. FCM analysis of the isolated cells

Isolated bone marrow cells were expanded and characterized for specific surface antigen expression by flow cytometry analysis. hBM-MSCs were analysed for hematopoietic marker expression (CD34 and CD45): isolated cells were negative for these two markers (not detected, less than 5%). Cells were positive for CD29 (95%), CD73 (98.3%), CD90 (97.9%), CD105 (85%) and CD166 (85%) surface markers, which are characteristic of mesenchymal stem cells. Based in this data we are very confident that the cell fraction isolated from bone marrow contains mostly MSCs.

3.3. DNA quantification

Results show a higher DNA content in samples collected from the flow perfusion bioreactor, compared to the static control conditions, in terms of DNA concentration (Figure 5.3). In this condition, DNA contents decreased a little at 21 days, but then increased by 28 days. In static conditions samples, there is a continuous decrease of the DNA contents along the time. In order to find significant differences in DNA contents between static and bioreactor culture conditions, the Mann–Whitney U-test was performed. No significant differences between both culture conditions were found at 14 ($p=0.18$) or 21 days of experiment ($p=1.00$). However, bioreactor samples displayed a significantly higher DNA quantification than the static ones at 28 days of experiment ($p=0.002$).

Concerning statistic differences between the days of culture, we found none either in static (Kruskal-Wallis test, $p=0.149$), either in the bioreactor (Kruskal-Wallis test, $p=0.261$).

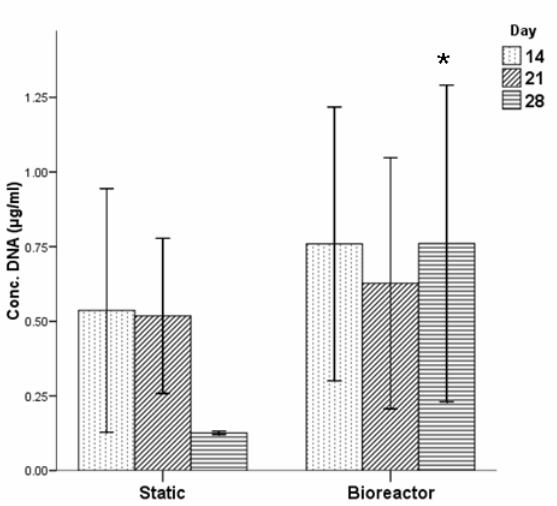

Figure 5.3 - Box plot of the DNA quantification in static and bioreactor cultures after 14, 21 and 28 days of culture. Data were analyzed by nonparametric way of a Mann–Whitney U-test (* $p<0.01$, vs Static).

3.4. SEM analysis

SEM observations of the cultured hBM-MSC in electrospun PCL nanofiber meshes shows some differences in their morphology along the experiment (Figure 5.4). At 7 days of culture, samples from the bioreactor showed a round-shaped morphology, whereas the cells from the static culture samples presented a fibroblast-like morphology, very stretched. The round-shaped morphology of cells in the bioreactor samples is maintained throughout the time course of the experiment (Figure 5.4A, C, E, G). In the two last time points, the cells morphology is not that evident. In figures 5.4E and 5.4G, referring to the bioreactor cultures at 21st and 28th days, respectively, we can observe that cells form a sheet that covers the entire meshes surface. Our interpretation of these two figures is that the production of ECM component and consequent deposition led to a thin film of cells entrapped in this matrix. Therefore, there are less "visible" cells, as they are now part of the matrix. On the other hand, in static cultures we can observe individual cells, as the matrix production here seems to be delayed, in comparison to the bioreactor cultures. Cells in static samples seem to be acquiring the round shaped morphology along the time, but by the 28th day of culture they are still not as round-shaped as the bioreactor (Figure 5.4G and 5.4H).

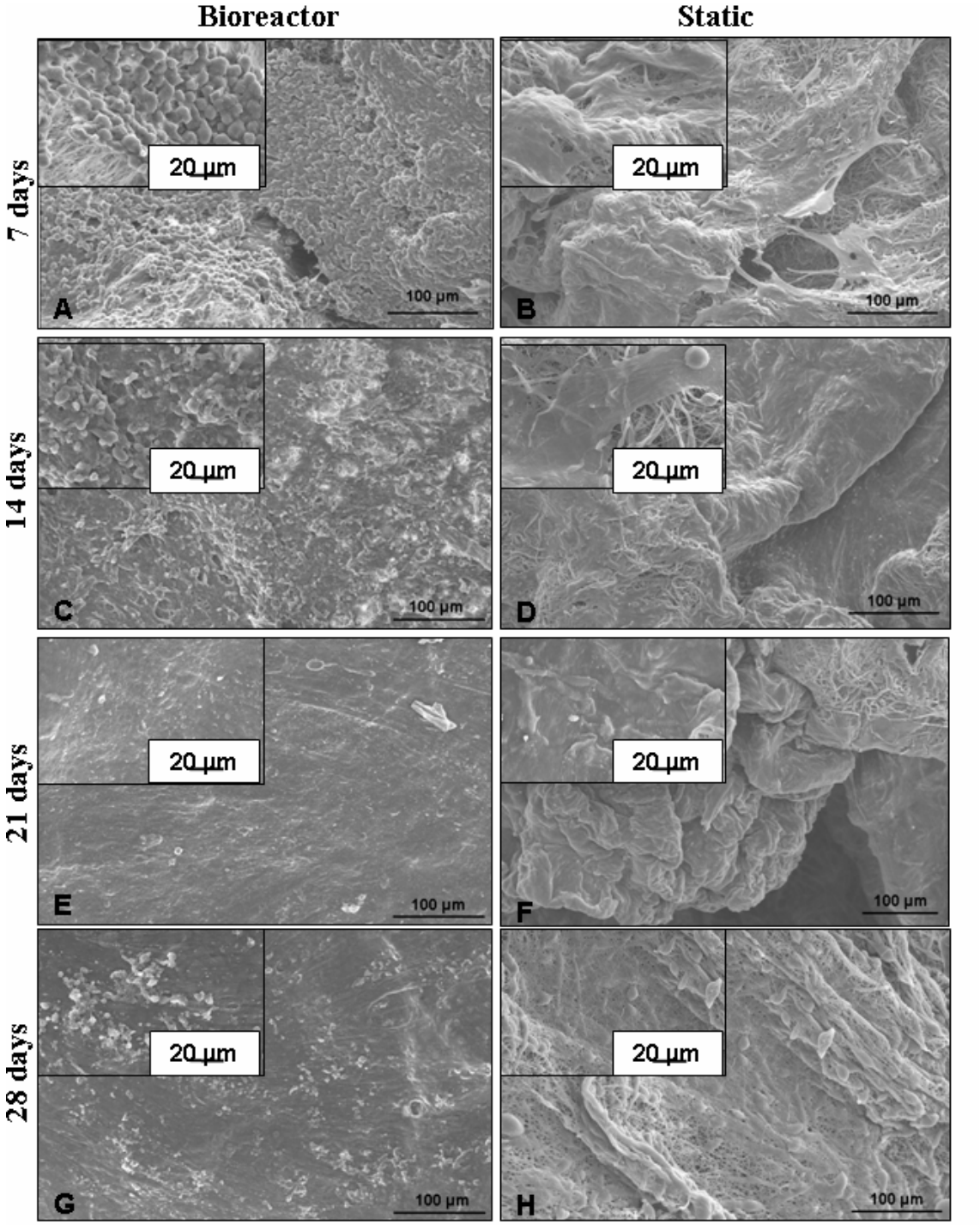

Figure 5.4 – Morphology of hBM-MSCs cultured in electrospun PCL nanofiber meshes, in the flow perfusion bioreactor (A, C, E, G) and in static control conditions (B, D, F, H) along the time course of the experiment: A, B-7 days; C, D-14 days; E, F- 21 days; G, H- 28 days. Different magnifications were used to highlight cell morphology. Scale bars: 100 µm= 2 cm; 20 µm= 0.5 cm.

3.5. Real-time PCR

Samples were collected for real-time PCR at 14, 21 and 28 days of experiment, both from the bioreactor and static control culture conditions. The aim was to determine the expression of several cartilage-related genes. All the tested genes were being expressed. The expression of the *Aggrecan* transcript was detected for both culture conditions, in all time points. The statistical analysis performed, using a Mann–Whitney U-test, confirmed the absence of significant difference between culture conditions at 14 ($p=1.000$), 21 ($p=0.762$) and at 28 days of experiment ($p=0.800$). The *Collagen type II* expression was detected, but no significant difference was found between static and bioreactor culture conditions in the 14th day ($p=0.233$), in the 21st day ($p=0.413$) or in the 28th day of experiment ($p=0.400$). The *Sox9* transcript is observed in both culture conditions, following a similar expression pattern. Statistical analysis of the results for 14 days of culture ($p=1.000$), 21 ($p=0.800$) and 28 days ($p=0.333$) showed no significant difference between both culture conditions. The expression of *Collagen type I* showed no significant difference between bioreactor and static culture conditions was found in the 14th day ($p=0.563$), in the 21st day ($p=0.075$) or in the 28th day ($p=0.071$). *Collagen type X* is expressed in slightly equal values for both types of cultures, as well as *Runx2*. Statistical analysis for *Collagen type X* confirmed that no significant difference was found between culture conditions in the 14th day ($p=1$), 21st day ($p=0.4$) or 28th day ($p=1$). For *Runx2*, the same was observed: no significant difference between the two tested conditions was found in the 21st day ($p=0.400$) or in the 28th day of experiment ($p=1.00$).

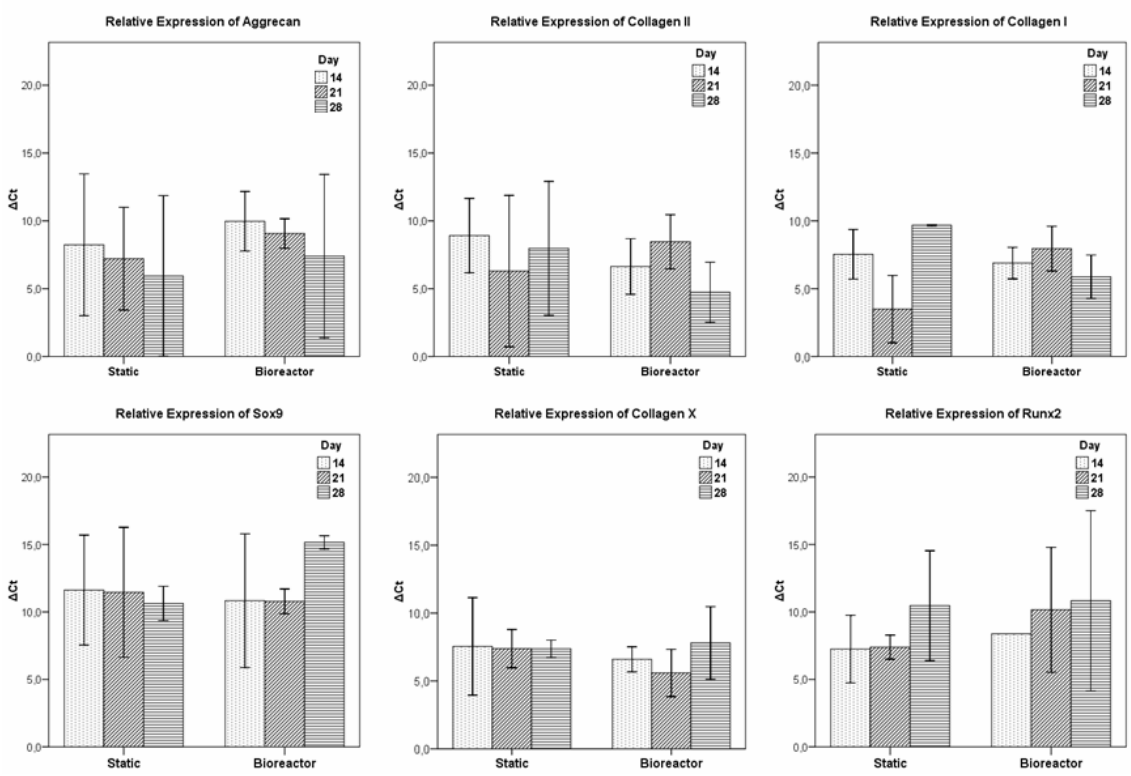

Figure 5.5 – Bar plots of chondrogenic markers of flow perfusion bioreactor samples and static control conditions normalized for the reference gene *GAPDH*, after 14, 21 and 28 days of culture. Data were analyzed with a Mann–Whitney U-test.

3.6. Histological staining

Histological sections of electrospun PCL nanofiber meshes seeded and cultured with hBMSCs, at 28 days of culture in the multichamber bioreactor or in static conditions were stained for cartilagineous ECM using toluidine blue and safranin O assays (Figure 5.6). Toluidine blue staining detected the presence of glycosaminoglycans in those sections, and safranin O staining confirmed this observation. It is also possible to observe a dense concentration of staining on sections from the bioreactor samples (Figure 5.6A and B) compared to the static culture sections, on both toluidine blue and safranin O staining.

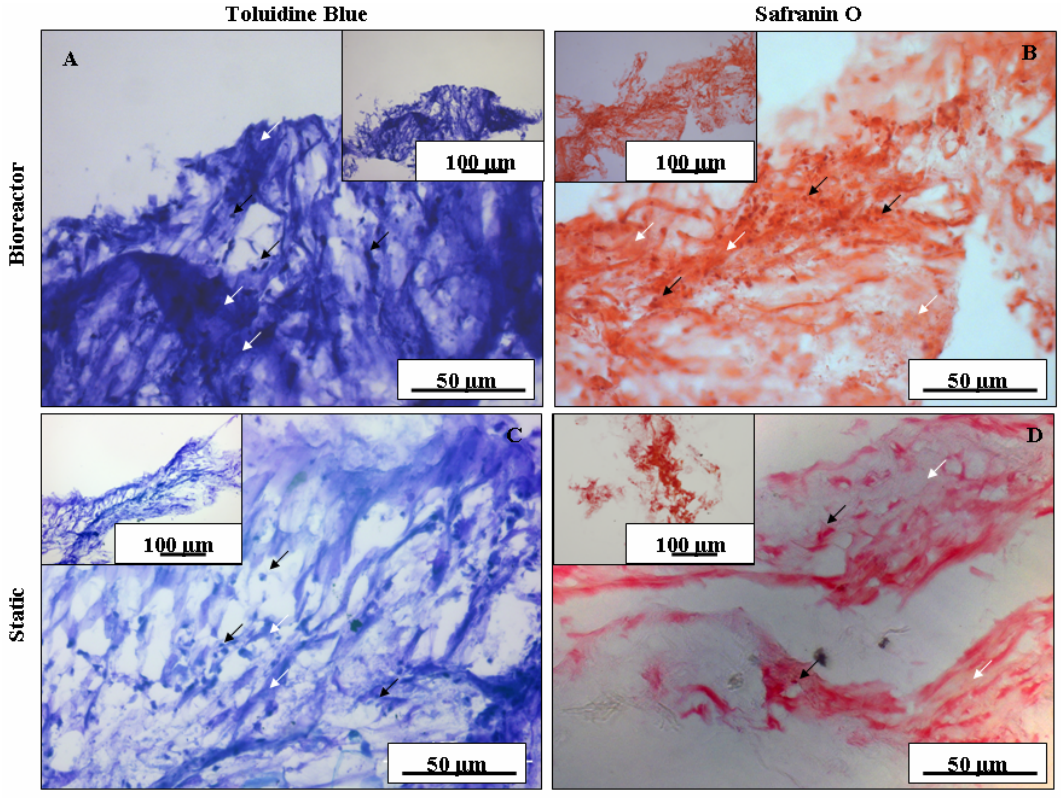

Figure 5.6 – Histological section of hBMSCs seeded onto PCL nanofiber meshes for chondrogenic differentiation, at 28 days, from bioreactor constructs (A, B) and static control cultures (C, D). A, C – Toluidine blue staining; B, D –Safranin O staining. Black arrows show the nuclei of the cells. White arrows show ECM. Scale bars: 100 µm= 1 cm; 50 µm= 2.5 cm.

3.7. Immunolocalisation of type I and type II collagens

Histological sections of electrospun PCL nanofiber meshes cultured with hBMSCs, at 28 days of culture in the multichamber bioreactor or in static conditions were stained for imunolocalisation of collagens type I and II (Figure 5.7). For the bioreactor cultures, both types of collagens were detected. Collagen type I stain (Figure 5.7A) appears to be slightly clear than the collagen type II staining (Figure 5.7B). On the other hand, there is a heavy unspecific staining on the control sections of both types of cultures (Figures 5.7A and 5.7D), which may have in part been due to dye trapped in folded regions of the sections. In static cultures, a marked staining for collagen type I (Figure 5.7E) can be observed, when compared to the collagen type II section (Figure 5.7F).

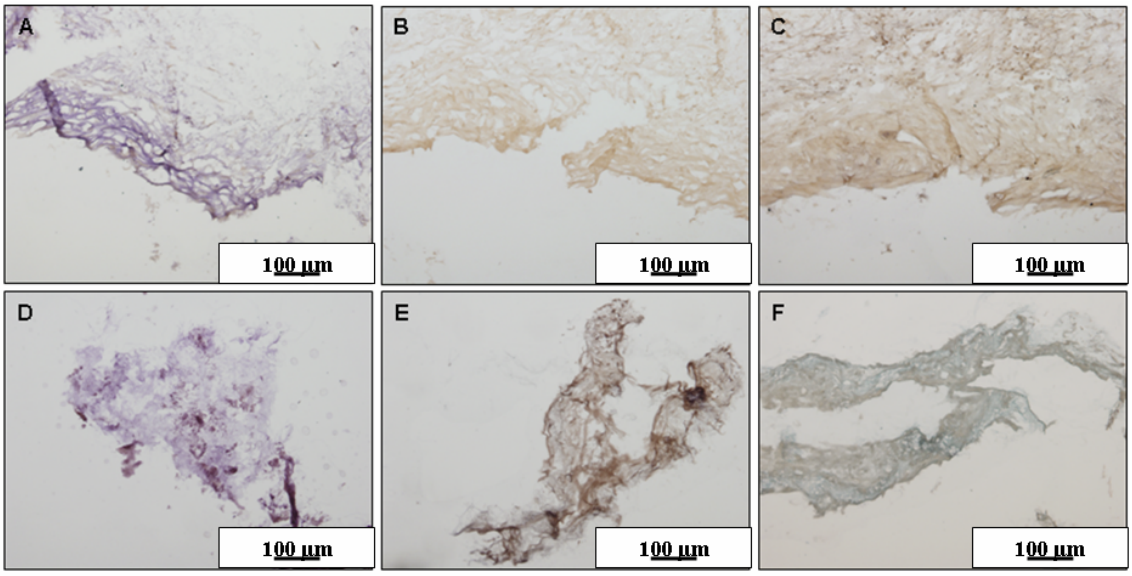

Figure 5.7 – Immunolocalisation of collagen type I (B, E) and type II (C, F) in electrospun PCL nanofiber meshes, after 28 days of culture in the bioreactor (A-C) and in static cultures (D-F). Controls of the immunohistochemistry assay (A) were performed with normal horse serum. Scale bar: 100 µm= 1 cm.

4. Discussion

Bioreactors are a powerful tool in several areas of tissue engineering. In cartilage tissue engineering, they can provide mechanical stimulus to cells, as well as better access of nutrients, enhancing the production of ECM. In the present work, we aimed to study the effect of a new multichamber flow perfusion bioreactor in the chondrogenic differentiation of hBM-MSCs when seeded onto electrospun PCL nanofiber meshes. The present work is one of the first reports that we release concerning the utilization of the multichamber bioreactor and the present data is starting point for optimization of the culture conditions used, as several new questions were raised from the obtained results. It has been demonstrated that the quality of tissue-engineered cartilage can be manipulated depending on the type and seeding density of cells and bioreactor culture conditions [27]. Several perfusion bioreactors have been developed in recent years, aiming to enhance tissue growth in in vitro constructs [27-29], for example for bone [30], dermal tissues [31] and cartilage [32]. Dynamic flow conditions are known to enhance cartilage development compared to static cultures [32-36]. For detailed review please refer to Godara *et al.*, 2008 and Butler *et al.*, 2009 [37, 38]. Fluid flow enhances

nutrient and waste exchange in vitro, improving mass transport and delivering shear stress within the construct [39]. In perfusion bioreactors, the level of shear stress and of nutrient transfer can be easily changed by modifying the medium flow rate, and thus, the quality of the tissue engineered construct can be improved, depending on the media flow velocity [33]. In the present work, we used a media flow of 70 µl/min/ nanofiber mesh. We based our choice of the flow velocity in the literature. However, references using nanofiber meshes are scarce, if not inexistent. Reported flow velocity for 3D constructs for cartilage tissue engineering range from 0.01 ml/min to 5ml/min [27, 28, 39, 40]. We decided to choose a low flow velocity as a starting point to allow the deposition of the ECM components. As we performed a low density seeding, the chosen perfusion velocity would allow, in our perspective, to provide cells the nutrients and oxygen needed. We would expect cell proliferation, differentiation and consequent enhanced deposition of ECM components along the time. As for cell numbers, the amount of cellular DNA varied along the experiment, being observed a high decrease in static culture conditions at the 28th day. In this time point, bioreactor samples displayed a significantly higher DNA quantification than static ($p=0.002$). It is the fair to state that, in terms of cell proliferation, the chosen fluid flow was beneficial for the constructs cultured in the bioreactor, enhancing cell proliferation. The beneficial effect of directional fluid flow has been demonstrated by Tarng and co-workers, using periosteal explants secured onto PCL scaffolds and cultured in spinner flasks. The authors showed that fluid flow enhanced cell proliferation, chondrogenic differentiation and cell organization [41].

Morphological observation of cells showed some differences between the two conditions. Cells in the bioreactor samples have acquired a round-shaped morphology at early stages of culture (*i.e.* 7th and 14th day), whereas the cells cultured in static conditions are stretched and flat until at least the 21st day of culture. These observations may be related to the fluid flow in the bioreactor samples. The beneficial effect of the perfusion can be observed, as the chondrogenic morphology seems to be highlighted in these samples, whereas in the static ones, the morphology change can be observed only after 21 days in culture. TGF-β3 is the essential growth factor for promoting chondrogenesis both *in vivo* and *in vitro* conditions [42]. It has been shown that TGF-β3 induced hBMSCs having increased *Sox9* expression with the presence of collagen type II and aggrecan [43]. Although the cartilage obtained in this study showed some signs of hypertrophy, induced either by the flow perfusion or by the utilization of TGF-β3, the

conjugation of both factors may have contributed for the increased chondrogenesis at the early stages of culture, for the bioreactor samples. Chondrogenesis at early stages of cultures have been also observed when culturing chitosan-BMP6 loaded scaffolds with ATDC5 chondrocytes cell line in RCMW™ reactors [44]. Additionally, cells cultured in static cultures showed hypertrophy, contrarily to the observed in the cultures from the bioreactor. This is not the case of our results, as the presence of hypertrophy related genes was detected in both static and dynamic cultures. Hypertrophy was also observed by Jung and co-authors, when they applied continuous compressive deformation for 10 or 24 days to PLCL scaffolds seeded with chondrocytes. These constructs were then implanted subcutaneously in nude mice. The observations showed hypertrophic forms in the implants stimulated for 24 days, demonstrating that the proper periodical application of dynamic compression can enhance GAGs production [45].

No statistical differences were found between the expression levels in the bioreactor samples and the static control cultures. Cartilage-related genes expression such as *Aggrecan*, *Collagen type II* and *Sox9*, was detected for both conditions. *Sox9* is highly expressed, stimulating the production of these genes. The high values obtained for standard deviations may be related to the flow velocity inside the fiber meshes. The dissimilar geometry of the scaffolds or of the flow perfusion may lead to different local shear stressed in each sample, even for the same input flow velocity [39]. Thus, the random morphology of the nanofiber meshes may be influencing the results within the same replicates. This fact, along with the common inlet and outlet of the bioreactor, could have resulted in non uniform flow velocity, and therefore could have influenced the ECM deposition and generate the large standard deviations observed in the experiments.

hBM-MSCs cultured in the bioreactor were able to produce proteoglycans, as showed by the toluidine blue and safranin O staining. The presence of proteoglycans was also detected in the static samples. However, these sections revealed less amount of staining when compared to the bioreactor sections. These staining, together with the results of RT-PCR for the expression of *Aggrecan*, indicate the deposition of ECM. Furthermore, collagen type I and type II presence in both types of samples at 28 days was also detected by immunolocalisation, which is consistent with the RT-PCR results. A different result was obtained by Li *et al.*, 2008, using PLLA nanofibrous scaffolds cultured in a rotating wall vessel bioreactor for cartilage tissue engineering. Compared to the constructs obtained in static cultures, the bioreactor grown constructs produced

more total collagen and GAGs, and expressed higher levels of cartilage related genes [46].

The flow perfusion bioreactor used in this work provides a continuous flow of media through the construct, enhancing the mass transfer and the nutrients availability. However, the mechanical forces can have a detrimental effect in terms of damaging the neo-tissue formation [40]. In the present work, the bioreactor allowed the proliferation and chondrogenic differentiation of hBM-MSCs, but no significant differences were observed compared to the static cultures. Similar results were obtained for the chondrogenic differentiation of human adipose stem cells when cultured in PGA scaffolds using a recirculating bioreactor [40]. The authors showed that the chondrogenic differentiation, as well as the ECM formation, was not detrimentally affected by the bioreactor culture. The obtained results do not allow us to draw any conclusion concerning the enhanced effect of the bioreactor over chondrogenic differentiation of hBM-MSCs when seeded onto PCL nanofiber meshes. Some of the parameters of the experiment here reported have to be modified, as for example the number of initial cell seeding, nanofiber meshes structure or even the fluid flow. We do sustain that this bioreactor is suitable for this type of culture, and further optimizations will be performed. The concept of nanofiber meshes cultured in bioreactors for cartilage tissue engineering has been proved to be applicable to this field, combined with efficient cell loading and bioreactor technology [46].

5. Conclusions

In the overall, we would like to highlight that the new flow perfusion bioreactor is suitable for culturing hBM-MSCs and electropun PCL nanofiber meshes. The culture in a flow perfusion bioreactor supported the attachment, proliferation and chondrogenic differentiation of hBM-MSCs. The MSCs were able to produce ECM on the electrospun PCL nanofiber meshes, as stated by the staining and imunolocalisation results. The media fluid flow may be influencing these results, as well as the nanofiber meshes random morphology. Therefore, some modifications in the experimental design should be considered in future works. The fluid flow should be extensively studied, as there are scarce literature references using nanofiber meshes in perfusion bioreactors. In doing this, we will try to improve the ECM deposition. Additionally, as one of the factors that

may also be influencing the results is the random morphology of the nanofiber meshes, different production techniques may be studied in order to obtain fully controlled morphologies.

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References

1 - Zhang L, Webster TJ. *Nanotechnology and nanomaterials: Promisses for improved tissue regeneration*. Nano Today. 2009. (4):66-80.

2 - Chew SY, Wen Y, Dzenis Y, Leong KW. *The role of electrospinning in the emerging field of nanomedicine*. Curr Pharm Des. 2006. 12(36):4751-70.

3 - Teo WE, He W, Ramakrishna S. *Electrospun scaffold tailored for tissue-specific extracellular matrix*. Biotechnol J. 2006. 1(9):918-29.

4 - Huang Z-M, Zhang Y-Z, Kotaki M, Ramakrishna S. *A review on polymer nanofibers by electrospinning and their applications in nanocomposites*. Composites Science and Technology. 2003. 63:2223 – 53.

5 - Martins A, Chung S, Pedro AJ, Sousa RA, Marques AP, Reis RL*, et al. Hierarchical starch-based fibrous scaffold for bone tissue engineering applications*. J Tissue Eng Regen Med. 2009. 3(1):37-42.

6 - Thorvaldsson A, Stenhamre H, Gatenholm P, Walkenstrom P. *Electrospinning of highly porous scaffolds for cartilage regeneration*. Biomacromolecules. 2008. 9(3):1044-9.

7 - Venugopal J, Low S, Choon AT, Ramakrishna S. *Interaction of cells and nanofiber scaffolds in tissue engineering*. J Biomed Mater Res B Appl Biomater. 2008. 84(1):34-48.

8 - Pham QP, Sharma U, Mikos AG. *Electrospun poly(epsilon-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: Characterization of scaffolds and measurement of cellular infiltration*. Biomacromolecules. 2006. 7(10):2796-805.

9 - Park JS, Park K, Woo DG, Yang HN, Chung HM, Park KH. *PLGA microsphere construct coated with TGF-beta 3 loaded nanoparticles for neocartilage formation*. Biomacromolecules. 2008. 9(8):2162-9.

10 - Pinho ED, Martins A, Araujo JV, Reis RL, Neves NM. *Degradable particulate composite reinforced with nanofibres for biomedical applications*. Acta Biomater. 2009. 5(4):1104-14.

11 - Alves da Silva ML, Crawford A, Mundy JM, Martins A, Araujo JV, Hatton PV*, et al. Evaluation of extracellular matrix formation in PCL and SPCL nanofiber meshes when seeded with bovine articular chondrocytes*. Tissue Eng. 2009. 15(2):377-85.

12 - Chung C, Burdick JA. *Engineering cartilage tissue*. Adv Drug Deliv Rev. 2008. 60(2):243-62.

13 - Vasita R, Katti DS. *Nanofibers and their applications in tissue engineering*. Int J Nanomedicine. 2006. 1(1):15-30.

14 - Wang Y, Blasioli DJ, Kim HJ, Kim HS, Kaplan DL. *Cartilage tissue engineering with silk scaffolds and human articular chondrocytes*. Biomaterials. 2006. 27(25):4434-42.

15 - DeLise AM, Fischer L, Tuan RS. *Cellular interactions and signaling in cartilage development*. Osteoarthritis Cartilage. 2000. 8(5):309-34.

16 - Seda Tigli R, Ghosh S, Laha MM, Shevde NK, Daheron L, Gimble J*, et al. Comparative chondrogenesis of human cell sources in 3D scaffolds*. J Tissue Eng Regen Med. 2009. 3(5):348-60.

17 - Loken S, Jakobsen RB, Aroen A, Heir S, Shahdadfar A, Brinchmann JE*, et al. Bone marrow mesenchymal stem cells in a hyaluronan scaffold for treatment of an osteochondral defect in a rabbit model*. Knee Surg Sports Traumatol Arthrosc. 2008. 16(10):896-903.

18 - Lee RH, Kim BC, Choi S, Kim H, Choi HS, Suh KT*, et al. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue*. Cellular Physiology and Biochemistry. 2004. 14:311 - 24.

19 - Teramura T, Fukuda K, Kurashimo S, Hosoi Y, Miki Y, Asada S, *et al. Isolation and characterization of side population stem cells in articular synovial tissue*. BMC Musculoskelet Disord. 2008. 9:86.

20 - Janjanin S, Li WJ, Morgan MT, Shanti RM, Tuan RS. *Mold-shaped, nanofiber scaffold-based cartilage engineering using human mesenchymal stem cells and bioreactor*. J Surg Res. 2008. 149(1):47-56.

21 - Mahmoudifar N, Doran PM. *Tissue engineering of human cartilage and osteochondral composites using recirculation bioreactors*. Biomaterials. 2005. 26:7012 – 24.

22 - Martin I, Miot S, Barbero A, Jakob M, Wendt D. *Osteochondral tissue engineering*. J Biomech. 2007. 40(4):750-65.

23 - Darling EM, Athanasiou K. *Articular cartilage bioreactors and bioprocesses*. Tissue Engineering. 2003. 9(1):9 - 26.

24 - Costa P, Martins A, Gomes M, Neves NM, Reis RL. *Multichamber bioreactor with bidirectional perfusion integrated in a culture system for tissue engineering strategies*. European Patent Office. 2010. EP 2151491 A2(09009863.3):11.

25 - Guimaraes A, Martins A, Pinho ED, Faria S, Reis RL, Neves NM. *Solving cell infiltration limitations of electrospun nanofiber meshes for tissue engineering applications*. Nanomedicine (Lond). 2010. 5(4):539-54.

26 - http://frodo.wi.mit.edu/.

27 - Mahmoudifar N, Doran PM. *Effect of seeding and bioreactor culture conditions on the development of human tissue-engineered cartilage*. Tissue Eng. 2006.

28 - Devarapalli M, Lawrence BJ, Madihally SV. *Modeling nutrient consumptions in large flow-through bioreactors for tissue engineering*. Biotechnol Bioeng. 2009. 103(5):1003-15.

29 - Lawrence BJ, Devarapalli M, Madihally SV. *Flow dynamics in bioreactors containing tissue engineering scaffolds*. Biotechnol Bioeng. 2009. 102(3):935-47.

30 - Sailon AM, Allori AC, Davidson EH, Reformat DD, Allen RJ, Warren SM. *A novel flow-perfusion bioreactor supports 3D dynamic cell culture*. J Biomed Biotechnol. 2009. 2009:873816.

31 - Aulin C, Foroughi F, Brown R, Hilborn J. *Extracellular matrix-polymer hybrid materials produced in a pulsed-flow bioreactor system*. J Tissue Eng Regen Med. 2009. 3(3):188-95.

32 - Pazzano D, Mercier KA, Moran JM, Fong SS, DiBiasio DD, Rulfs JX, *et al. Comparison of chondrogensis in static and perfused bioreactor culture*. Biotechnol Prog. 2000. 16(5):893-6.

33 - Davisson T, Sah RL, Ratcliffe A. *Perfusion increases cell content and matrix synthesis in chondrocyte three-dimensional cultures*. Tissue Eng. 2002. 8(5):807-16.

34 - Freed LE, Hollander AP, Martin I, Barry JR, Langer R, Vunjak-Novakovic G. *Chondrogenesis in a cell-polymer-bioreactor system*. Exp Cell Res. 1998. 240(1):58-65.

35 - Freyria AM, Cortial D, Ronziere MC, Guerret S, Herbage D. *Influence of medium composition, static and stirred conditions on the proliferation of and matrix protein expression of bovine articular chondrocytes cultured in a 3-D collagen scaffold*. Biomaterials. 2004. 25(4):687-97.

36 - Vunjak-Novakovic G, Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R, *et al. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage*. J Orthop Res. 1999. 17(1):130-8.

37 - Butler DL, Goldstein SA, Guldberg RE, Guo XE, Kamm R, Laurencin CT, *et al. The impact of biomechanics in tissue engineering and regenerative medicine*. Tissue Eng Part B Rev. 2009. 15(4):477-84.

38 - Godara P, McFarland CD, Nordon RE. *Design of bioreactors for mesenchymal stem cell tissue engineering*. Journal of Chemical Technology and Biotechnology. 2008. 83:408-20.

39 - Porter B, Zauel R, Stockman H, Guldberg R, Fyhrie D. *3-D computational modeling of media flow through scaffolds in a perfusion bioreactor*. J Biomech. 2005. 38(3):543-9.

40 - Mahmoudifar N, Doran PM. *Chondrogenic differentiation of human adipose-derived stem cells in polyglycolic acid mesh scaffolds under dynamic culture conditions.* Biomaterials. 2010. 31(14):3858-67.

41 - Tarng YW, Casper ME, Fitzsimmons JS, Stone JJ, Bekkers J, An KN, *et al. Directional fluid flow enhances in vitro periosteal tissue growth and chondrogenesis on poly-epsilon-caprolactone scaffolds*. J Biomed Mater Res A. 2010. 95(1):156-63.

42 - Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. *In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells*. Experimental Cell Research. 1998. 238:265 - 72.

43 - Indrawattana N, Chen G, Tadokoro M, Shann LH, Ohgushi H, Tateishi T, *et al. Growth factor combination for chondrogenic induction from human mesenchymal stem cell*. Biochemical and Biophysical Research Communications. 2004. 320:914 – 9.

44 - Tigli RS, Gumusderelioglu M. *Chondrogenesis on BMP-6 loaded chitosan scaffolds in stationary and dynamic cultures*. Biotechnol Bioeng. 2009. 104(3):601-10.

45 - Jung Y, Kim SH, Kim SH, Kim YH, Xie J, Matsuda T, *et al. Cartilaginous tissue formation using a mechano-active scaffold and dynamic compressive stimulation*. J Biomater Sci Polym Ed. 2008. 19(1):61-74.

46 - Li WJ, Jiang YJ, Tuan RS. *Cell-nanofiber-based cartilage tissue engineering using improved cell seeding, growth factor, and bioreactor technologies*. Tissue Eng Part A. 2008. 14(5):639-48.

Chapter 6

Chondrogenic differentiation of human bone marrow mesenchymal stem cells in chitosan based scaffolds using a flow perfusion bioreactor

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Abstract

Native articular cartilage is subjected to synovial fluid flow during normal joint function. Thus, it is believed that the morphogenesis of articular cartilage may be positively regulated by the application of similar stimulation in vitro.

In the present work, the effect of fluid flow over the chondrogenic differentiation of human bone marrow derived mesenchymal stem cells (hBM-MSCs) was studied. We intended to find out if the shear stress caused by the medium perfusion trough the constructs was capable of augmenting the differentiation process. Human BMSCs were isolated from bone marrow aspirates and were characterized by flow cytometry. After expansion, hBM-MSCs were seeded statically onto fiber meshes scaffolds, consisting of a blend of 50/50 chitosan and Poly(Butylene Terephtalate Adipate) – CPBTA. Constructs were cultured in a flow perfusion bioreactor for 28 days, using complete medium for chondrogenesis supplemented with TGF-β3. An enhanced ECM deposition and collagen type II production was observed in the bioreactor samples, when compared to the static controls. Moreover, it was observed that hBM-MSCs, in static cultures, take longer to differentiate. ECM accumulation in these samples is lower than in the bioreactor sections, and there is a significant difference in the expression of collagen type I. We found that the flow induced shear stress has a beneficial effect on the chondrogenic differentiation of hMSCs.

1. Introduction

Mesenchymal Stem Cells (MSCs) have been recognized for their ability to differentiate into cells of different tissues such as bone, cartilage or adipose tissue, and therefore might be of interest for therapeutic strategies. These cells may be induced to differentiate in vitro by growth factors supplementation in the culture medium to trigger the differentiation into the desired cell type. Particularly, several studies showed that MSCs derived from different tissues behave in different ways when induced to differentiate in the chondrogenic lineage [1-3]. The chondrogenic potential of adipose tissue-derived MSCs over bone marrow-derived MSCs has been compared in various works, using different animals. These studies seem to indicate a higher chondrogenic

potential of bone marrow-derived MSCs over adipose tissue-derived MSCS [1-3], isolated from different animals.

Materials and fabrication technologies are critically important in designing temporary, artificial extracellular matrices (scaffolds) for tissue engineering. There is still a quest for the ideal scaffold for cartilage regeneration, either using synthetic or natural origin materials. In recent years, natural based polymers have been extensively studied, including hyaluronic acid, collagen or chitosan [4-8]. Chitosan is a derivative of chitin, obtained by the deacetylation of this biopolymer [9]. It is a linear polysaccharide with a structure similar to the glycosaminoglycans (GAGs) present in the extracellular matrix (ECM) of native cartilage [10]. This property is extremely important for cartilage tissue engineering, since it allows the development of several types of scaffolds. Another interesting property of chitosan is that it can be moulded into various shapes [11] and allows different pore sizes structures formation [9]. Chitosan has been used in several blends for matrices or scaffolds production, such as hydrogels [12, 13], porous scaffolds [8], amino acid immobilization in scaffolds [6] or drug delivery system [14]. In recent years, chitosan-based scaffolds were found to be a remarkable tool for tissue engineering of various tissues [4, 15-18]. Chitosan has also proved to be suitable for improving the in vivo performances of natural-based scaffolds, in particular of soy-based biomaterials [19]. Chitosan scaffolds have been developed in blends with synthetic materials for cartilage tissue engineering, for example chitosan/polybutylene succinate (PBS) [20] or chitosan/polycaprolactone [21]. Particularly, the chitosan/ PBS blend has proved to be suitable for cartilage tissue engineering, either using cell lines or primary cells [20, 22, 23]. Chitosan is frequently referred as a positive influence in promoting ECM formation by cells. In a study comparing chitosan and PGA scaffolds, it was found that matrix production by chondrocytes was improved in chitosan constructs, mainly on the ones with smaller fibers [24]. The beneficial effect of microfibers structure in chitosan scaffolds was demonstrated more recently by the same authors. They compared the effect of chitosan scaffolds structure (macroporous sponges and microfibers) in the chondrogenesis of MSCs, and concluded that it is superior in the microfibers [24], indicating the effect of the morphology in cell activity.

Chondrocytes are responsible for the maintenance and turnover of cartilage ECM, by producing collagen type II and aggrecan. The combination of these two proteins provides the tensile, shear and compressive stiffness of cartilage. It is very difficult to determine a detailed causal relationship between the type of mechanical stimulation

used in the cell culture and the resulting ECM structure and mechanical properties of the tissue. It was reported, however, that detailed stress limits exist in the application of mechanical stimuli, leading to pathological changes in cartilage explants or tissue engineering constructs [25]. Flow-induced shear stress has been found to produce a significant stimulatory effect [26]. Dynamic shear increases the transcription of matrix proteins and, at the same time, augments matrix biosynthesis, preferentially collagen synthesis [27, 28].

At early stages of skeletogenesis, mesenchymal condensation occurs, which is a key event to determine cell shape and cell fate. This will be determined by the balance of forces in the cell during condensation. The role of shape-changing deviatory shear stresses and developmental context in the modulation of gene transcription prior to cell commitment was recently studied [29]. It was found that the magnitude and duration of multipotent embryonic stem cells to shear stress affects significantly the genes activity of key genes to musculoskeletal development, at mesenchymal condensation [29]. Therefore, mechanical stimuli must be studied as one of the key elements for a tissue engineering strategy, in the sense that it can affect the structure and quality of the neo-formed tissue.

In the present work, our aim was to determine the outcome of an in-house developed flow perfusion bioreactor (PT patent nº 104155; European patent pending [30]) in the chondrogenic differentiation of hBM-MSCs. We intended to find out if the shear stress caused by the medium perfusion through the constructs was capable of augmenting the differentiation process.

2. Materials and Methods

2.1. CPBTA fiber meshes

The chitosan-poly(butylene terephthalate adipate) (CPBTA) (50/50 wt%) fiber mesh scaffolds were compounded as described elsewhere [31]. Briefly, the CPBTA fibers were obtained using a prototype single screw micro-extruder coupled to a capillary die. The extruded fibers were chopped and then were loaded into a mould. The mould was heated above the melting temperature (Tm) of the thermoplastic blend during 10 min. Finally, immediately after removing the moulds from the oven, the fibers are slightly

compressed by a Teflon cylinder to pack and bond the fibers. Finally, standardized scaffolds of 2 mm thick discs, with diameter of 8 mm were obtained by cutting the fiber bonded meshes with a circular die (8 mm in diameter).

2.2. Isolation of hBM-MSCs

HBM-MSCs were isolated from bone marrow aspirates collected under informed consent from patients undergoing knee arthroplasties in the Hospital de São Marcos, in Braga, Portugal. During the surgeries, bone marrow is collected to a container with α-MEM medium (Invitrogen, 12000-063), supplemented with an antibiotic/antimycotic solution (Gibco, 15240062) and 5000 units of heparin (Sigma, H3393) and maintained in ice until the isolation procedure. Aspirates were homogenised, diluted in PBS (Sigma, P4417) (1:1) and incubated for 5 minutes at room temperature. Then, bone marrow was diluted in lyses buffer (1:10) and left under agitation for 10 minutes. Lyses buffer was prepared with 10 mM of Tris-HCl (Sigma, T3253), 1.21 g of Tris Base (Sigma, T1503), 8.3 g of NH_4Cl (Merck, 1011455000), in 1L of distilled water. Afterwards, cells suspension was centrifuged at 1200 rpm, for 15 minutes at room temperature. Cells were ressuspended in α-MEM medium, supplemented with antibiotic/antimycotic solution and 20% of foetal bovine serum - FBS (Biochrom, Berlin, Germany; Heat Inactivated). Cell suspension was filtered for disposal of debris, using 100 μm and 70 μm Cell Strainer (BD Falcon™, 352360 and BD Falcon™, 352350). Cells were counted and plated at the density of $4.7x10^3$ cells/cm^2. Cells were expanded in the referred culture medium until the numbers needed for this study.

2.3. Flow-cytometry (FCM) analysis

To evaluate cell-surface marker expression, cultured cells were incubated for 20 min at 4ºC with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies specific for human markers associated with mesenchymal or haematopoietic lineages. The antibodies used were: CD29, CD34, CD44, CD45, CD73, CD90, CD105, CD106. All the antibodies were purchased from BD Pharmingen. The samples were analysed using a BD FACSCalibur flow cytometer (Enzifarma, Portugal).

2.4. hBM-MSCs culture on the bidirectional flow perfusion bioreactor

hBM-MSCs were detached from the culture flasks by treatment with Trypsin/EDTA solution (Invitrogen, 25300-062). Cells were counted and a cell suspension of $1x10^6$ cells/ fiber mesh was prepared to seed each scaffold. Cell seeding was performed in static conditions, inside the incubator, at 37ºC, during 24 hours. On the next day, the bidirectional flow perfusion bioreactor was fully sterilized in the autoclave. Then, it was assembled inside the hood and the constructs to be cultured within were transferred. The bidirectional flow perfusion bioreactor (PT patent nº 104155; European patent pending [30]) has a maximum of 20 individual culture chambers; according to the study plan, 20 constructs were transferred for each chamber. The bioreactor was closed and the flow rate was set to 100 µl/min/fiber mesh. The bioreactor was transferred into an incubator and kept in those conditions during the whole experiment.

Similar numbers of species was transferred for new 24 wells culture plates and were maintained in static conditions as a control, inside the same incubator. Chondrogenic differentiation medium consisting of α-MEM supplemented with antibiotic/antimycotic solution, ITS liquid media supplement (Sigma, I2521), dexamethasone 1Mm (Sigma, D1756), Sodium Pyruvate 0.1 M (Sigma, P4562), Ascorbic acid-2-phosphate 17 mM (Sigma, A4544), L-Proline 35 mM (Sigma, P5607) and 1 ng/ml of human recombinant TGF-β3 (PeproTech, 100-36) was used for the 28 days duration of the experiment. Culture medium in the bioreactor was completely changed every week (100 mL each time). Control samples were cultured in 24 well plates, and were fed every other day with 1 mL of culture medium.

2.5. Proliferation assay (DNA quantification)

Evaluation of cell proliferation was performed using the PicoGreen dsDNA quantification Kit (Molecular Probes, P-7589), according to the instructions. Samples from the flow perfusion bioreactor and from the static control were collected in every time point. Triplicates of every time point were produced. A standard curve was established. Fluorescence of both samples and standard curve was read with an excitation of 485/20 nm and an emission of 528/20 nm. DNA concentration was extrapolated from the standard curve.

2.6. Scanning Electron Microscopy (SEM)

For SEM analysis, fiber meshes were collected at each time point: 1, 2, 3 and 4 weeks. They were washed in sterile PBS and immersed in 3% glutaraldehyde (Sigma, G-5882) at room temperature for one hour. Samples were washed in PBS, dehydrated in increasing alcohol concentrations and let to dry. Samples were sputter coated with gold and analysed. A scanning electron microscope (Cambridge S360, Leica Cambridge, Cambridge, UK) was used to observe cells distribution and morphologies.

2.7. RNA isolation

Samples were collected from the flow perfusion bioreactor and from the static control, by the end of the experiment. Samples were washed in PBS, immersed in TRIzol reagent (Invitrogen, 15596-018) and kept at -80ºC for posterior RNA extraction. When the extraction was performed, samples were taken from the freezer and thawed. Chloroform (Sigma, C2432) was added and samples were vigorously agitated for 15 seconds and further incubated in ice for 15 minutes. After that incubation, samples were centrifuged at 13000 rpm, during 15 minutes, at 4ºC. Afterwards, the supernatant was collected into a sterile 1.5 mL tube, and an equivalent volume of isopropanol (Sigma, I9516) was added. Samples were incubated at -20ºC overnight, to precipitate the RNA. The next day, samples were centrifuged at 13000 rpm, during 15 minutes, at 4ºC. The supernatant was taken and 800 µl of ethanol 70% was added, to wash away the isopropanol. The ethanol 70% solution was prepared from absolute ethanol (Merck, 1.00983.2511) and ultra pure water. Tubes were agitated vigorously and centrifuged again, at 9000 rpm during 5 minutes, at 4ºC. The supernatant was again removed, and the pellet was left to air dry. Finally, the pellet was ressuspended in 50 µl of distilled water DNase, RNase free (Gibco, 10977-015). The concentration and purity of the extracted RNA was evaluated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA).

2.8. Real-Time PCR

The Real-Time PCR procedure used in the present work consisted of a two step fluorogenic assay using the SyberGreen system (Bio-Rad). All the reagents used in this

procedure were from Bio-Rad, following the instructions of the manufacturer. Thermocycler reaction conditions were also performed according with those referred in the kits. In the first step, RNA was reversed transcribed into cDNA, using the iScript cDNA synthesis kit (1708891). A BioRad CFX96 real-time PCR detection system (BioRad Laboratories, USA) was used to perform the reaction. Afterwards, the cDNA obtained was used as template for the amplification of the target genes (Table 6.1), with the Syber Green Kit (1708884). GAPDH was used as the house-keeping gene, and the expression of all the target genes was normalized to the GAPDH of that sample. All the primer sequences were generated using Primer3 software (http://frodo.wi.mit.edu/) and acquired from MWG Biotech AG, Germany. Table I shows the primers sequences used in the analysis. The obtained results were analyzed with CFX Manager Software – version 1.5 (BioRad Laboratories, USA).

Table 6.1 – Primer sequences used for RT-PCR procedures *.

Gene	Forward (5´-3´)	Reverse (5´-3´)
AGC	TGAGTCCTCAAGCCTCCTGT	TGGTCTGCAGCAGTTGATTC
COL II	CGGTGAGAAGGGAGAAGTTG	GACCGGTCACTCCAGTAGGA
COL I	AGCCAGCAGATCGAGAACAT	ACACAGGTCTCACCGGTTTC
COL X	CCAGGTCTCGATGGTCCTAA	GTCCTCCAACTCCAGGATCA
Runx2	TTCCAGACCAGCAGCACTC	CAGCGTCAACACCATCATTC
Sox9	TTCATGAAGATGACCGACGC	GTCCAGTCGTAGCCCTTGAG
GAPDH	ACAGTCAGCCGCATCTTCTT	ACGACCAAATCCGTTGACTC

*AGC = Agreccan; COL II = Collagen type II; COL I = Collagen type I; COL X = Collagen type X; Runx2 = Runt-related transcription factor 2; Sox9 = Sry-type high mobility group box 9; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

2.9. Histological analysis

Samples were collected at the end of the experiment and processed for histology. Samples were fixed in 10% neutral buffered formalin and then dehydrated through increasing concentrations of ethanol, immersed in infiltration solutions, embedded in Technovit glycol methacrylate (kit 8100, Heraeus Kulzer, EBSciences, CO, USA) and then polymerized. Specimens were cut to obtain longitudinal 7 μm thick sections, using a modified microtome equipped with a tungsten blade (Leica RM 2155). Hematoxylin-

eosin staining was performed to evaluate the cells distribution within the scaffolds. Toluidine blue stain was also performed to detect the presence of proteoglycans. Staining solution was prepared by adding 1% of toluidine blue (Sigma, T-0394) dissolved in distilled water containing 0.5g of sodium borate, followed by filtering. One drop of this solution was added to each section during 1 minute. The sections were further rinsed with distilled water and let to air dry overnight. Sections were cleared in xylene substitute (Sigma, A5597) and mounted in Histo clear (Frilabo, HS200). Safranin O staining was performed by washing slides in tap water, then immersed in 0.02% fast green (Fluka, 44715) during 6 minutes. Then, they were immersed in 1% acetic acid (Panreac, 131008) solution during 1 minute. After, slides were immersed in 0.1% safranin O (Fluka, 84120) solution for 10 minutes. By the end, slides were washed in tap water and let to air dry. Sections were cleared in xylene and mounted as previously described for further analysis.

2.10. Immunolocalisation of type I and type II collagens

Immunolocalisation of type I and type II collagens was performed. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide solution during 30 minutes. Sections were rinsed in PBS during 5 minutes. R.T.U. Vectastain® Universal Elite ABC Kit (Vector, VCPK-7200) was used for antibody incubation, according to the instructions of the manufacturer. Shortly, sections were incubated with primary antibodies (collagen type I and collagen type II) (UNLB, Goat anti-type I collagen 1310-01 and UNLB, Goat anti-type II collagen 1320-01) overnight at 4ºC, in a humidified atmosphere. Incubation was revealed by using the Peroxidase Substrate Kit DAB (Vector, VCSK-4100), according to the instructions of the manufacturer. Slides were washed in water during 5 minutes and counterstained with hematoxilin for nuclei visualization. Finally, slides were mounted in Histo clear. Controls were performed using normal goat serum instead of primary antibodies, which was also included in the kit.

2.11. Statistical analysis

Statistical analysis was performed using the SPSS statistic software (Release 15.0.0 for Windows). Firstly, a Shapiro-Wilk test was used to ascertain about the data

normality and variance equality. The result of the normality was negative and, consequently, nonparametric tests were used in further comparisons between static and dynamic culture conditions. A Mann-Whitney U-test was applied to compare the two independent groups of samples for each variable (i.e. DNA quantification and real-time PCR). P values lower than 0.01 were considered statistically significant in the analysis of the results.

3. Results

3.1. FCM analysis of the isolated cells

Isolated bone marrow cells were expanded and characterized for specific surface antigen expression by flow cytometry analysis. hBM-MSCs were analysed for hemopoietic marker expression (CD34 and CD45): isolated cells were negative for these two markers. Cells were positive for CD29, CD44, CD73, CD90, CD105 and CD106 surface markers typically detected in mesenchymal stem cells. Based on this data we are confident that the bone marrow isolated cells are effectively MSCs.

3.2. DNA quantification

Samples were collected in different time points of the experiment from both culture conditions, and processed for DNA quantification. Results are shown in Figure 6.1. Concerning the bioreactor samples, DNA contents increased at 21 days of culture, decreasing in the last time point. In static culture conditions, DNA content increased continuously during the whole period of the experiment. In order to find significant differences in DNA quantification among static and bioreactor conditions, the Mann–Whitney U-test was performed. In terms of DNA quantification, no significant difference between static and bioreactor cultures was found at 14 days ($p=0.18$), but static cultures displayed a significantly higher DNA quantification than the bioreactor ones at 21 and 28 days of culture ($p=0.002$).

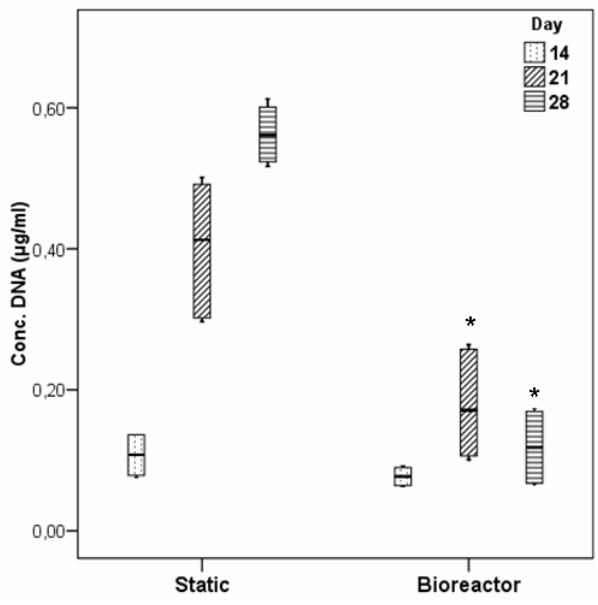

Figure 6.1 - Box plot of the DNA quantification in static and bioreactor cultures after 14, 21 and 28 days of culture. Data were analyzed by nonparametric way of a Mann–Whitney U-test (* $p<0.01$, vs Static).

3.3. SEM analysis

SEM analysis of the cultured hBM-MSC in CPBTA fiber meshes show differences in their morphology during the time course of the experiment (Figure 6.2). Micrographs of the bioreactor samples show that cells were located at the center of the scaffold, and that they proliferated. Cells show a round-shaped morphology at early stages of culture (*e.g.* Figures 6.2A and 6.2C), which remains unchanged until the end of the experiment. On the other hand, cells in static culture samples maintained their stretched morphology until the 21st day of experiment (Figures 6.2B, 6.2D and 6.2F). At 21 days, cells begin to acquire a round-shaped morphology (Figures 6.2F and 6.2H), but not as evident as in the bioreactor samples (Figures 6.2A, 6.2C, 6.2E, 6.2G). By the observation of the micrographs, it seems that cells cultured under static conditions proliferated more than the ones cultured in the bioreactor.

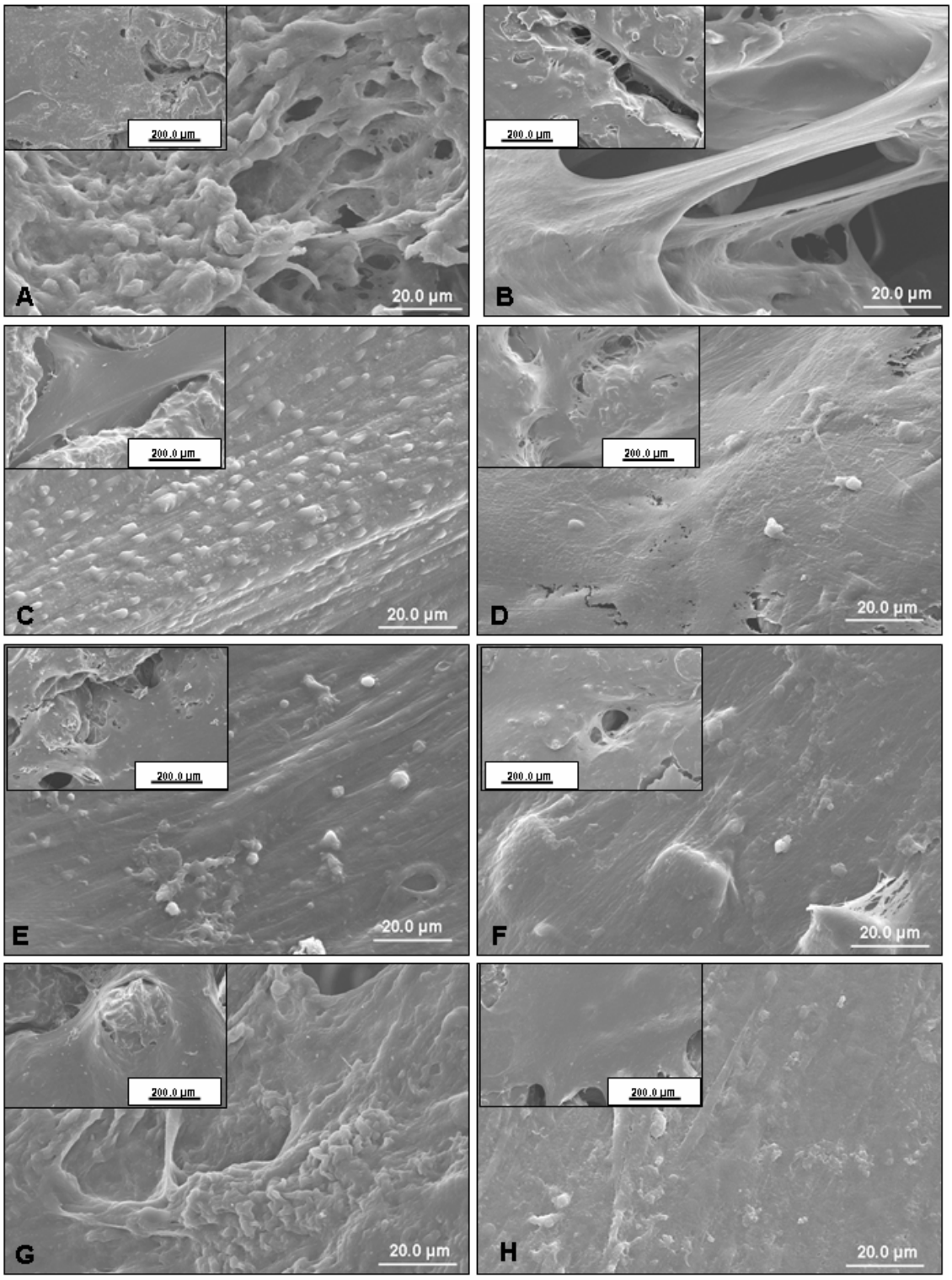

Figure 6.2 – Morphology of hBM-MSCs cultured in CPBTA fiber meshes, in the flow perfusion bioreactor (A, C, E, G) and in static control conditions (B, D, F, H) during the time course of the experiment: A, B-7 days; C, D-14 days; E, F- 21 days; G, H- 28 days. Different magnifications were used to highlight for cell distribution within the scaffolds and the cells morphology.

3.4. Real-Time PCR

Gene expression during the experiment was assessed by Real - Time PCR. Results were normalized against the reference gene expression, *GAPDH* (Figure 6.3). Expression of cartilage related genes was detected in the bioreactor and also in static culture conditions samples. The gene expression patterns were considerably different in both conditions. In the bioreactor samples, cartilage related genes were expressed, indicating the hBM-MSCs were differentiating into chondrocytes. *Aggrecan* expression is maintained along the experiment, as well as *Sox9* expression. *Collagen type II* expression increases at 28 days. *Collagen type I* is expressed in low levels, and shows a decreasing trend of expression until the last time point. Hypertrophy related genes – *Collagen type X* and *Runx2* – have low expression and also decrease with time. These results point towards chondrogenic differentiation of the cells and the production of proteins present in the ECM of cartilage, as expected.

A different pattern of expression was observed in static control conditions. Although *Aggrecan* expression is slightly higher than in bioreactor samples, cartilage related genes showed a lower expression for all time points. On the other hand, *Collagen type I* had a large increase from the 21st to the 28th day of experiment. Moreover, hypertrophy related genes showed an increase along the time, as well as a higher expression than the one found for bioreactor samples. In *Aggrecan* expression no significant differences between both conditions was found at 14 ($p=0.548$), 21 ($p=0.905$) or 28 days ($p=0.038$) of experiment. The same was observed for *Collagen type II* expression at 14 ($p=0.038$), 21 ($p=0.114$) or 28th days ($p=1$). *Collagen type I* expression showed no significant difference at 14 ($p=0.114$) and 21 days ($p=0.139$). However, at 28 days, static control conditions displayed a significantly higher expression of *Collagen type I* than the bioreactor samples ($p<0.01$). *Sox9* did not show significant differences, either at 14 ($p=0.277$), 21 ($p=0.190$) or 28 days ($p=0.222$). The same was found for *Collagen type X* at 14 ($p=0.100$), 21 ($p=0.200$) or 28 days ($p=0.100$), and *Runx2* at 14 ($p=0.100$), 21 ($p=0.100$) or 28 days ($p=0.100$).

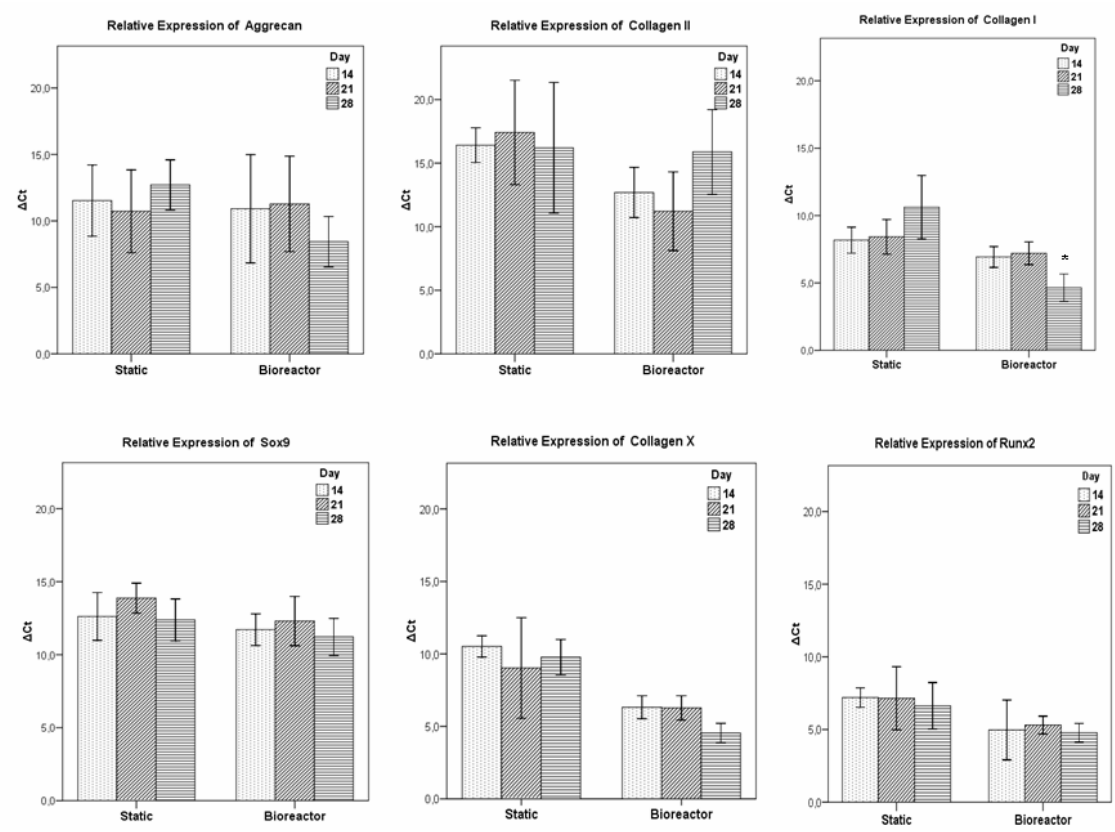

Figure 6.3 – Bar plots of chondrogenic markers of flow perfusion bioreactor samples and static control conditions normalized for the reference gene, *GAPDH*, after 14, 21 and 28 days of culture. Data were analyzed with a Mann–Whitney U test. In the bar plot for *Collagen type I*, data were analyzed with a Mann–Whitney U test (* $p<0.01$, vs static culture conditions).

3.5. Histological staining

Samples of CPBTA fiber meshes seeded with hBMSCs from both studied culture conditions were collected at the end of the experiment. Histological longitudinal sections were stained for cell localisation with hematoxylin-eosin. Toluidine blue and safranin O stain were used for detection of ECM proteoglycans (Figure 6.4). Cells from the bioreactor sections are more round-shaped (Figure 6.4A) when compared to the static cultures sections, that are stretched (Figure 6.4B). ECM production was detected in the bioreactor samples, either in the toluidine blue sections (Figure 6.4C) or in the safranin O sections (Figure 6.4E). The observation of the static culture sections revealed

that cells were able to produce ECM (Figures 6.4D and 6.4F), but in a smaller amount than in the bioreactor.

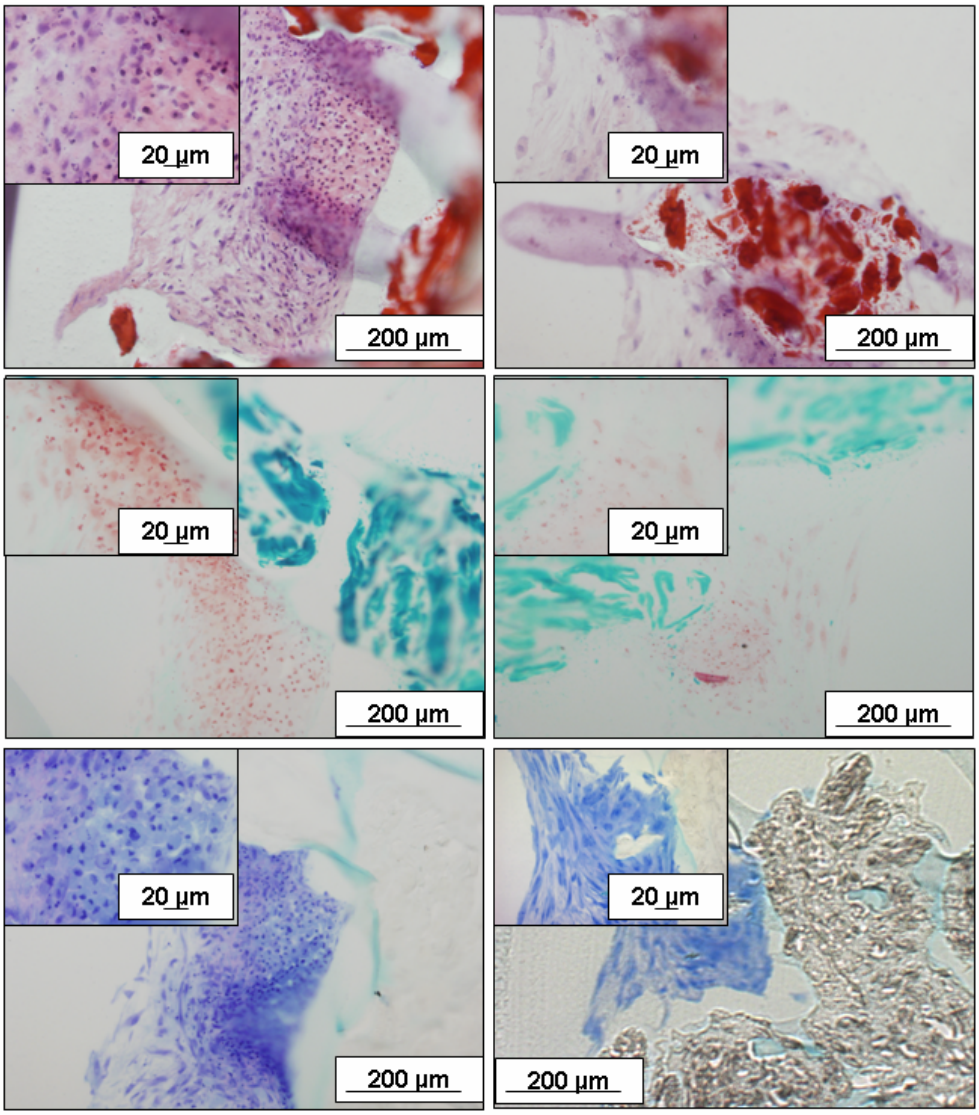

Figure 6.4 - Histological section of hBMSCs seeded onto CPBTA fiber meshes for chondrogenic differentiation, at 28 days of culture, in the flow perfusion bioreactor (A, C, E) and in the static control cultures (B, D, F). A, B – Hematoxylin-eosin stain for cell localisation; C, D – Safranin O stain for ECM localisation; E, F – Toluidine blue stain for proteoglycans.

3.6. Immunolocalisation of type I and type II collagens

Histological sections of CPBTA fiber meshes seeded with hBMSCs and of the static cultures were stained for imunolocalisation of collagens type I and II (Figure 6.5). In both culture conditions there was some level of unspecific staining, as showed in the control slides (Figures 6.5A and 6.5B). Collagen type I staining was not so clear in the bioreactor sections than in the static culture ones, as shown in Figures 6.5C and 6.5D, respectively. The contrary was observed for collagen type II: there is a darker staining in the bioreactor sections than in the static culture ones (Figures 6.5E and 6.5F).

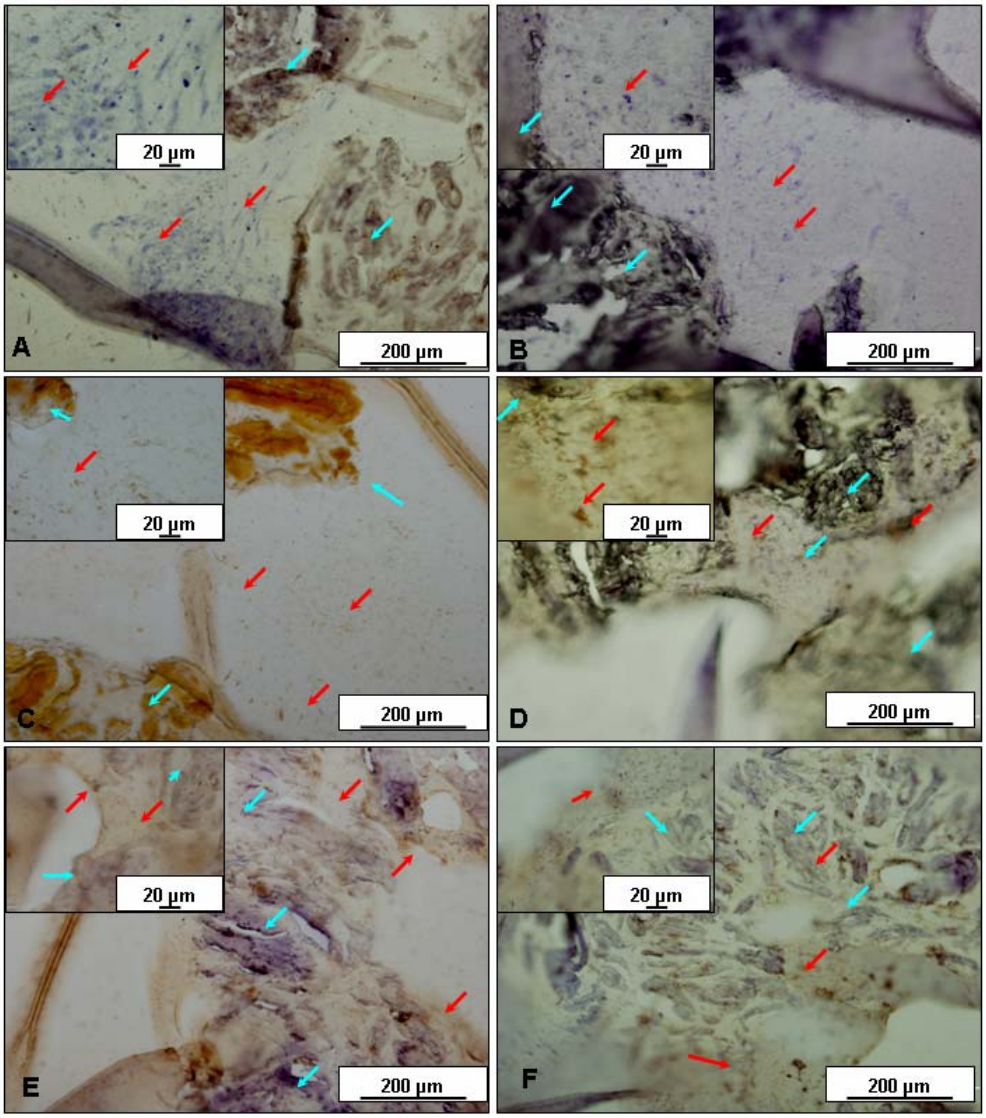

Figure 6.5 – Immunolocalisation of collagen type I (C, D) and type II (E, F) in CPBTA fiber meshes, after 28 days of culture in the bioreactor (A, C, E) and in static

cultures (B, D, F). Controls of the immunolocalisation assay (A, B) were performed with normal horse serum. Red arrows show areas with positive stain and cells nuclei. Blue arrows show CPBTA fiber meshes.

4. Discussion

Perfusion and shear stress can affect in a positive way the chondrogenic differentiation and ECM deposition [25, 27, 28, 32]. In the present work, our aim was to determine the outcome of a flow perfusion bioreactor strategy in the chondrogenic differentiation of hBM-MSCs. We intended to find out if the shear stress caused by the medium perfusion trough the constructs was capable of improving the differentiation process.

DNA quantification revealed a decrease of this nucleic acid in the bioreactor samples from the 21st day of culture. On the other hand, static cultures DNA increased along the time, indicating that cells are driven for proliferation rather than towards differentiation. No significant differences were found between both culture conditions at 14 days of experiment. However, static cultures displayed a significantly higher DNA quantification than the bioreactor, at 21 and 28 days. SEM observations are consistent with the DNA results. In the bioreactor samples, hBM-MSCs show evidence of early differentiation, as they present a round-shaped morphology in all time points. On the contrary, for static samples, SEM observations show that the morphology of the cells is fibroblastic, until the 21st day of experiment, evidencing low differentiation rate and high proliferation. These observations can also be sustained by the DNA concentration, which is higher in static cultures than in the bioreactor, for all time points.

Expression analysis of several cartilage related genes indicates a significant difference between the *Collagen type I* expression in the static cultures when compared to the bioreactor. This significant difference suggests the formation of fibrotic cartilage. Imunolocalisation results are consistent with this observation, as there is positive staining for collagen type I in the static culture samples, whereas there is a very weak staining in the bioreactor sections. Moreover, collagen type II was stained positive for both cultures and the expression of *Collagen type II* was detected. Histological stain results indicated the production of ECM in both bioreactor and static cultures. However, the stain is stronger in the bioreactor sections. Proteoglycans accumulation and the

expression of cartilage-related genes (*AGC, Col II, Sox9*) show that cells cultured in the bioreactor were able to differentiate into cartilagineous tissue. The cartilage-like tissue formed in the static cultures is more fibrotic, as there is an increased collagen type I production. ECM detection by histological methods revealed regions of deposit matrix, leading to the conclusion that, when hBM-MSCs are cultured statically, their chondrogenic differentiation ability decreases. There is a weaker tissue staining than the one formed when cells are cultured under flow perfusion. A similar result was found when bovine articular chondrocytes were seeded onto chitosan/PBS scaffolds and cultured under stirred conditions; ECM deposition was enhanced in scaffolds subjected to stirring, compared to static cultures [20]. This result suggests that not only the ECM components are produced in larger amounts in shear conditions, but also the GAGs are produced in a greater extent. Another work using ovine chondrocytes cultures on PGA scaffolds demonstrated the beneficial effect of perfusion on GAGs synthesis [33].

ECM stain is in agreement with the expression of *Aggrecan* and *Sox9*. At 28 days of culture, an expression decrease of both *Aggrecan* and *Sox9* in the bioreactor samples may suggest that cells are undergoing hypertrophy, since at the same time there is expression of *Collagen type X* and *Runx2*. The same behaviour was not observed for the static cultures. Mechanical stress is known to be necessary to produce and maintain healthy and fully functional articular cartilage. Some regulatory systems are believed to exist in the tissue, which influence GAGs and collagens metabolism [25, 27, 28, 32]. The size, concentration or linkage form of the tissues can be influenced by the mechanical culture conditions, being all these factors pivotal for the tissues functional behaviour. Therefore, it is difficult to state rules for the biochemistry of proteoglycans. In any case, it has been shown that GAGs content can be influenced by shear stress, whereas in its absence the GAGs concentration decreases [25, 32, 34]. Fitzgerald and colleagues (2006) showed that compressive and shear stress differently regulates chondrocyte transcription. Furthermore, they found that dynamic shear, in the absence of hydrostatic pressure gradients, induced matrix proteins transcription [27]. These findings are consistent with other works that also found that dynamic shear increased the transcription of matrix proteins to a greater extent than dynamic compression, and at the same time augmented matrix biosynthesis, preferentially collagen synthesis [28]. Our results are consistent with these observations. We found enhanced ECM deposition and collagen type II production in the bioreactor samples. Furthermore, it is possible to

observe that hBM-MSCs, in static cultures, take longer to differentiate, and that ECM accumulation in the sections is lower than in the bioreactor sections.

5. Conclusions

Herein our aim was to determine if the perfusion shear stress produced by a home-made flow perfusion bioreactor was able to enhance the chondrogenic differentiation of hBM-MSCs. Our observations showed improved ECM deposition and collagen type II production in the bioreactor samples, when compared to the static controls. Moreover, it was observed that hBM-MSCs chondrogenic differentiation in static cultures begins at later time points than in the bioreactor samples. ECM accumulation is likewise lower than in the bioreactor sections, and there is a significant difference in the expression of collagen type I. In the present work, we found evidences that confirm our hypothesis that shear stress has a beneficial effect on the chondrogenic differentiation of hBM-MSCs.

Acknowledgments

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References

1 - Kisiday JD, Kopesky PW, Evans CH, Grodzinsky AJ, McIlwraith CW, Frisbie DD. *Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures*. J Orthop Res. 2008. 26(3):322-31.

2 - Park J, Gelse K, Frank S, von der Mark K, Aigner T, Schneider H. *Transgene-activated mesenchymal cells for articular cartilage repair: A comparison of primary bone marrow-, perichondrium/periosteum- and fat-derived cells*. J Gene Med. 2006. 8(1):112-25.

3 - Yoshimura H, Muneta T, Nimura A, Yokoyama A, Koga H, Sekiya I. *Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle*. Cell Tissue Res. 2007. 327(3):449-62.

4 - Correlo VM, Boesel LM, Bhattacharya M, Mano JF, Neves NM, Reis RL. *Hydroxyapatite reinforced chitosan and polyester blends for biomedical applications*. Macromol Mater Eng. 2005. 290:1157–65.

5 - Correlo VM, Boesel LM, Bhattacharya M, Mano JF, Neves NM, Reis RL. *Properties of melt processed chitosan and aliphatic polyester blends*. Materials Science and Engineering A. 2005. 403:57–68.

6 - Ho M-H, Wang D-M, Hsieh H-J, Liu H-C, Hsien T-Y, Lai J-Y*, et al. Preparation and characterization of RGD-immobilized chitosan scaffolds*. Biomaterials. 2005. 26:3197–206.

7 - Medrado GCB, Machado CB, Valerio P, Sanches MD, Goes AM. *The effect of a chitosan–gelatin matrix and dexamethasone on the behavior of rabbit mesenchymal stem cells*. Biomedical Materials. 2006. 1:155-61.

8 - Zhao L, Chang J. *Preparation and characterization of macroporous chitosan/wollastonite composite scaffolds for tissue engineering*. J Mater Sci Mater Med. 2004. 15(5):625-9.

9 - Di Martino A, Sittinger M, Risbud MV. *Chitosan: A versatile biopolymer for orthopaedic tissue-engineering*. Biomaterials. 2005. 26:5983–90.

10 - VandeVord PJ, Matthew HW, DeSilva SP, Mayton L, Wu B, Wooley PH. *Evaluation of the biocompatibility of a chitosan scaffold in mice*. J Biomed Mater Res. 2002. 59(3):585-90.

11 - Hu Q, Li B, Wang M, Shen J. *Preparation and characterization of biodegradable chitosan/hydroxyapatite nanocomposite rods via in situ hybridization: A potential material as internal fixation of bone fracture*. Biomaterials. 2004. 25(5):779-85.

12 - Frenkel SR, Bradica G, Brekke JH, Goldman SM, Ieska K, Issack P*, et al. Regeneration of articular cartilage - evaluation of osteochondral defect repair in the rabbit using multiphasic implants*. Osteoarthritis and Cartilage. 2005. 13:798 - 807.

13 - Montembault A, Tahiri K, Korwin-Zmijowska C, Chevalier X, Corvol MT, Domard A. *A material decoy of biological media based on chitosan physical hydrogels: Application to cartilage tissue engineering*. Biochimie. 2006. 88(5):551-64.

14 - Hoemann CD, Sun J, Legare A, McKee MD, Buschmann MD. *Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle*. Osteoarthritis Cartilage. 2005. 13(4):318-29.

15 - Costa-Pinto AR, Salgado AJ, Correlo VM, Sol P, Bhattacharya M, Charbord P*, et al. Adhesion, proliferation, and osteogenic differentiation of a mouse mesenchymal stem cell line (BMC9) seeded on novel melt-based chitosan/polyester 3D porous scaffolds*. Tissue Eng Part A. 2008. 14(6):1049-57.

16 - Malafaya PB, Reis RL. *Bilayered chitosan-based scaffolds for osteochondral tissue engineering: Influence of hydroxyapatite on in vitro cytotoxicity and dynamic bioactivity studies in a specific double-chamber bioreactor*. Acta Biomater. 2009. 5(2):644-60.

17 - Martins AM, Santos MI, Azevedo HS, Malafaya PB, Reis RL. *Natural origin scaffolds with in situ pore forming capability for bone tissue engineering applications*. Acta Biomater. 2008. 4(6):1637-45.

18 - Muzzarelli RA. *Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone*. Carbohydrate Polymers. 2009. 76:167-82.

19 - Santos TC, Marques AP, Silva SS, Oliveira JM, Mano JF, Castro AG*, et al. Chitosan improves the biological performance of soy-based biomaterials*. Tissue Eng Part A. 2010.

20 - Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol P, Bhattacharya M*, et al. Chitosan/polyester-based scaffolds for cartilage tissue engineering: Assessment of extracellular matrix formation*. Acta Biomater. 2010. 6(3):1149-57.

21 - Correlo VM, Boesel LF, Pinho E, Costa-Pinto AR, Alves da Silva ML, Bhattacharya M*, et al. Melt-based compression-molded scaffolds from chitosan-polyester blends and composites: Morphology and mechanical properties*. J Biomed Mater Res A. 2009. 91(2):489-504.

22 - Oliveira J, Correlo VM, Sol P, Costa-Pinto A, Salgado A, Bhattacharya M*, et al. Assessment of the suitability of chitosan/polybutylene succinate scaffolds seeded with mouse mesenchymal progenitor cells for a cartilage tissue engineering approach* Tissue Eng. 2008. 14(10):1651-61.

23 - Oliveira JT, Crawford A, Mundy JL, Sol PC, Correlo VM, Bhattacharya M*, et al. Novel melt-processable chitosan-polybutylene succinate fibre scaffolds for cartilage tissue engineering*. J Biomater Sci Polym Ed. 2010.

24 - Ragetly GR, Griffon DJ, Lee HB, Fredericks LP, Gordon-Evans W, Chung YS. *Effect of chitosan scaffold microstructure on mesenchymal stem cell chondrogenesis*. Acta Biomater. 2010. 6(4):1430-6.

25 - Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD. *Biosynthetic response of cartilage explants to dynamic compression*. J Orthop Res. 1989. 7(5):619-36.

26 - Voronov R, Vangordon S, Sikavitsas VI, Papavassiliou DV. *Computational modeling of flow-induced shear stresses within 3D salt-leached porous scaffolds imaged via micro-CT*. J Biomech. 2010.

27 - Fitzgerald JB, Jin M, Grodzinsky AJ. *Shear and compression differentially regulate clusters of functionally related temporal transcription patterns in cartilage tissue*. J Biol Chem. 2006. 281(34):24095-103.

28 - Jin M, Frank EH, Quinn TM, Hunziker EB, Grodzinsky AJ. *Tissue shear deformation stimulates proteoglycan and protein biosynthesis in bovine cartilage explants*. Arch Biochem Biophys. 2001. 395(1):41-8.

29 - McBride SH, Falls T, Knothe Tate ML. *Modulation of stem cell shape and fate B: Mechanical modulation of cell shape and gene expression*. Tissue Eng Part A. 2008. 14(9):1573-80.

30 - Costa P, Martins A, Gomes M, Neves NM, Reis RL. *Multichamber bioreactor with bidirectional perfusion integrated in a culture system for tissue engineering strategies*. European Patent Office. 2010. EP 2151491 A2(09009863.3):11.

31 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, N.M. N, *et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromolecular Bioscience. 2010. accepted for publication.

32 - Sah RL, Grodzinsky AJ, Plaas AH, Sandy JD. *Effects of tissue compression on the hyaluronate-binding properties of newly synthesized proteoglycans in cartilage explants*. Biochem J. 1990. 267(3):803-8.

33 - Davisson T, Sah RL, Ratcliffe A. *Perfusion increases cell content and matrix synthesis in chondrocyte three-dimensional cultures*. Tissue Eng. 2002. 8(5):807-16.

34 - Grodzinsky AJ, Levenston ME, Jin M, Frank EH. *Cartilage tissue remodeling in response to mechanical forces*. Annu Rev Biomed Eng. 2000. 2:691-713.

SECTION V – CO-CULTURES OF ARTICULAR CHONDROCYTES AND MESENCHYMAL STEM CELLS USING MICROFIBER MESHES AS CELL SUPPORT

Chapter 7

Co-culture of human articular chondrocytes with two different sources of human mesenchymal stem cells as a new strategy for cartilage tissue engineering

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This chapter is based on the following publication: Alves da Silva ML, Costa-Pinto AR, Correlo VM, Sol P, Bhattacharya M, Faria S, Reis RL, Neves NM. *Co-culture of human articular chondrocytes with two different sources of human mesenchymal stem cells as a new strategy for cartilage tissue engineering*. Submitted. 2010.

Abstract

Chondrocytes are mainly responsible for maintaining the integrity of the extracellular matrix of articular cartilage and for the protection of the underlying subchondral bone. Mesenchymal Stem Cells (MSCs) are also believed to contribute to the maintenance of cartilage. Moreover, these cells have been recognized for their ability to differentiate into cells of different tissues such as bone, cartilage or adipose tissue, and therefore might be of great interest for developing cartilage therapeutic strategies. These cells are typically induced to differentiate by growth factors supplementation in the culture medium that will trigger differentiation into the desired cell phenotype.

One frequent problem that occurs in cartilage tissue engineering is that expanded chondrocytes undergo dedifferentiation when in 2D culture. This process leads frequently to the undesired production of fibrocartilage, and is associated with the loss of their phenotype in vitro. The use of an additional cell source to enhance cell numbers, not loosing the differentiation potential upon expansion in vitro may be an original choice. Moreover, soluble factors released by chondrocytes have been shown to influence stem cells differentiation onto the chondrogenic lineage. The use of conditioned medium obtained from chondrocytes for stimulating stem cells chondrogenic differentiation may be a very interesting alternative for moving into the clinical application of these cells.

In the present work, direct contact co-cultures and indirect co-cultures (using conditioned medium obtained from a culture of human articular chondrocytes) of human bone marrow-derived MSCs (hBMSCs) and human Wharton´s jelly MSCs (hWJSCs) were established. Cells were isolated from human samples collected at a local hospital, under donors' informed consent. The co-cultures were performed in previously produced 3D scaffolds, composed by a blend of 50/50 chitosan and poly (butylene succinate) – CPBS. Co-cultures were maintained during 28 days. Both types of stem cells were able to undergo chondrogenic differentiation, either in direct or indirect co-cultures. By the end of the experiment, the indirect co-cultures (using conditioned medium) showed significantly higher values in terms of DNA content, glycosaminoglycans (GAGs) accumulation and specific cartilage-related genes expression, when compared to direct co-cultures, for both types of adult MSCs tested. Human BMSCs resulted in fibrous cartilage, as it can be concluded by the significantly higher expression of *Collagen type I*. The hWJSCs showed higher chondrogenic

differentiation ability when compared to hBMSCs, as denoted by the higher values for GAGs accumulation and cartilage related genes expression.

Therefore, we were able to demonstrate the high chondrogenic potential of hWJSCs, and proved that co-cultures of articular chondrocytes with these cells are able to produce more cartilaginous ECM, when compared to co-cultures with hBMSCs. The use of conditioned medium obtained from articular chondrocytes induced MSCs chondrogenic differentiation and ECM formation. The obtained results showed that this new strategy is very interesting and should be further explored for clinical applications.

1. Introduction

Research concerning cartilage regeneration is of special importance, since this tissue lacks self-repairing capacity and causes a huge social and economic impact. Several cell types have been proposed for cartilage tissue engineering, as for example the direct use of autologous chondrocytes. However, this method is not very convenient, since it involves a biopsy used as cell source for primary chondrocytes that is always limited in size and consequently in cell numbers obtained [1]. Also, chondrocytes tend to dedifferentiate when expanded in 2D culture, in vitro [1]. The use of stem cells can be very useful to overcome these issues, as they can be expanded into clinically relevant numbers and further differentiate into the chondrogenic lineage. Applying a 3D milieu is pivotal when designing a cartilage TE strategy, because chondrocytes dedifferentiation is reversible, and when these cells are further cultured in a 3D environment, they may regain their phenotype [2-5].

The most widespread tissue source of adult mesenchymal stem cells (MSCs) is the bone marrow [6, 7]. Bone marrow MSCs (BMSCs) have been proposed and applied for various tissue engineering applications, including for cartilage repair [6, 8-11]. Yet, the number of available MSCs in bone marrow [12], and the possibility of donor site morbidity in the procedure to obtain bone marrow aspirates, leads to the need to identify other MSCs sources. Human umbilical cord Wharton´s jelly has been reported as a potential alternative tissue source [13, 14], since human umbilical cord MSCs share many properties with bone marrow mesenchymal stem cells, thus supporting their applicability for cell-based therapies [15]. Human WJSCs (hWJSCs) have a higher frequency of colony forming units (CFUs-F) than hBMSCs, thus a larger number of

MSCs may be obtained in the initial isolation from Wharton´s jelly [13]. Further details on Wharton´s jelly cells as a primitive stromal cell population can be found elsewhere [16, 17]. Human WJSCs have also shown a higher proliferative potential than hBMSCs [15], being able to undergo osteogenic, chondrogenic and adipogenic differentiation [14]. Thus, those cells are prospective candidates for cartilage tissue engineering, as they display a distinct chondrogenic potential. Moreover, the improved chondrogenic potential of cells derived from the umbilical cord was confirmed in a study comparing the differentiation potential of human BMSCs and human umbilical cord stem cells (UCSCs) in 2D cultures [18].

Several strategies have been proposed to direct chondrogenesis of stem cells, which can be applied either alone or in conjunction. The referred two strategies comprise the use of specific growth factors, employing 3D structures to act as supports for cell growth and differentiation, or to co-culture progenitor cells with conditioned medium obtained from cartilage explants [19]. Cartilage is a paracrine organ, which secretes humoral factors that influence the proliferation and differentiation of cells present in the surrounding tissues [20]. Chondrogenesis and osteogenesis of MSCs during in vitro micromass culture was affected by the use of conditioned medium obtained from cultures of chondrocytes [21]. It was observed that chondrocytes secrete factors that may affect the differentiation status of stem cells, and can promote chondrogenesis and osteogenesis [21]. In indirect co-cultures with chondrocytes and embryonic stem cells the interaction of paracrine factors secreted by chondrocytes with the referred cells was observed [22]. This interaction led to the enhancement of the embryonic stem cells chondrogenic commitment [22].

The use of a porous biodegradable scaffold to support cell growth is frequently proposed for tissue engineering approaches [23-26]. Chondrocytes tend to switch into a fibroblast-like phenotype when cultured in monolayer, in tissue culture flasks. The presence of a porous structure providing a 3D environment for their culture is pivotal for the phenotype development and stability in vitro [27]. In the case of adult stem cells, the influence of 3D porous structures is relevant when considering its chondrogenic differentiation. Both the proliferation and differentiation of MSCs can be positively influenced by the culture in 3D scaffolds. It was demonstrated in the literature that mesenchymal stem cells can be attracted into a cartilage defect by the guidance of a collagenous matrix after drilling a channel in the cartilage structure until reaching the subchondral bone [28].

Our group has been investigating scaffolds produced with several of chitosan/ poly(butylene succinate) (CPBS) for cartilage tissue engineering. Those scaffolds showed good performance in supporting cell proliferation and ECM deposition [29-31]. CPBS scaffolds were reported to sustain the chondrogenic differentiation of a mouse mesenchymal progenitor cell line [30] and to promote cartilaginous ECM deposition when cultured with bovine articular chondrocytes [29, 31]. Moreover, CPBS fiber meshes were also proposed for bone-related strategies using MSCs, with positive results [23, 32]. These fiber meshes present a highly connected 3D porous structure, as well as a large surface area for cell attachment and proliferation [33]. The versatility of these scaffolds makes them very promising candidates for future osteochondral applications. Nevertheless, their suitability for cartilage TE using MSCs has not been assessed previously. We herein hypothesize that CPBS scaffolds can also support and promote human MSCs chondrogenic differentiation. This is the main reason for the selection of these scaffolds for the present work.

Co-cultures aiming the regeneration of cartilage are the subject of recent scientific interest. Several studies in the literature propose their applicability for cartilage tissue engineering, either using articular chondrocytes from different sources [34] or passages [35], stem cells and articular chondrocytes [36], or embryonic stem cells and articular chondrocytes [37]. The co-culture of sheep BMSCs with synovial cells showed the expression of chondrocytic markers [38], while the use of rat BMSCs with allogenic cartilage explants revealed a sustained expression of Sox9 in an early stage of chondrogenesis and collagen type X at a later stage, which is an undesired outcome since it indicates hypertrophy [39]. Co-culture of synovial-derived stem cells with TGF-β3 transfected articular chondrocytes resulted in significantly improved chondrogenesis of the progenitor cells [40]. The chondrogenic effect of co-culturing chondrocytes with MSCs may be influenced by the tissue of origin of stem cells [41, 42]. For instance, in a study with human adipose-derived MSCs (ASCs) co-cultures with human articular chondrocytes [41], it was demonstrated that co-cultured ASCs and chondrocytes clearly differ in their chondrogenic potential, when compared to articular chondrocytes cultured alone. The co-culture of ASCs with chondrocytes resulted in decreased chondrogenesis [41]. Conversely, in a work comparing chondrogenesis of ASCs and BMSCs seeded onto hyaluronic acid scaffolds, it was shown that chondrogenesis was more efficient using BMSCs than ASCs or chondrocytes alone [42]. In the case of WJSCs, to our best knowledge, only one report can be found in the literature referring to co-cultures of

hWJSCs with articular chondrocytes for chondrogenic differentiation [43]. Thus, we herein aimed at determining whether their previously reported enhanced chondrogenic potential when compared to BMSCs would be maintained, of even improved, in co-cultures with articular chondrocytes using a 3D scaffold. Additionally, we aimed to verify if using conditioned medium from a separate articular chondrocyte culture would improve the induction of chondrogenic differentiation of stem cells when compared with direct contact between these cells in co-culture. The possibility of using conditioned medium opens interesting possibilities for obtaining larger numbers of differentiated cells prior to implantation, in the context of its clinical application.

2. Materials and Methods

2.1. CPBS fiber meshes

The porous fiber meshes used as 3D scaffolds in the present work have been produced and successfully tested earlier by our group for bone related applications using human BMSCs [44]. A detailed description of the production methods can be found elsewhere [45]. The chitosan used for scaffolds production was supplied by France Chitin (Orange, France), with a degree of deacetylation of 85%. Bionolle™ 1050, a polybutylene succinate copolymer (MFI~50) was obtained from Showa Highpolymer Co. Ltd., Tokyo, Japan. Briefly, the CPBS fibers were obtained using a prototype single screw micro-extruder coupled to a capillary die. The extruded fibers were chopped and further loaded into a mould, which was heated above the melting temperature of the thermoplastic blend during 10 minutes. Immediately after removing the moulds from the oven, the fibers were slightly compressed by a Teflon cylinder to pack and bond the fibers. Finally, standardized scaffolds in the form of 2 mm thick discs, and with a diameter of 8 mm were obtained by cutting the fiber bonded meshes with a circular punch tool.

2.2. Isolation and expansion of hBMSCs

Human BMSCs were isolated from bone-marrow aspirates collected after obtaining informed consent from patients undergoing knee arthroplasties. Those samples were

obtained under the scope of the cooperation agreement established between the 3B´s Research Group of the University of Minho and the Orthopaedics Department of the Hospital de São Marcos, Braga, Portugal. During the surgeries, bone marrow was collected into a container with α-MEM medium, supplemented with antibiotic/ antimycotic solution and 5000 units of heparin (Sigma, H3393) and maintained in ice until the isolation procedure. Aspirates were homogenised, diluted in phosphate saline buffer – PBS (Sigma, D8537) (1:1) and incubated for 5 minutes at room temperature. Then, bone marrow was diluted in lyses buffer (1:10) and left under agitation for 10 minutes. Lyses buffer was prepared with 10 mM of Tris-HCl (Sigma, T3253), 1.21 g of Tris Base (Sigma, T1503) and 8.3 g of NH_4Cl (Merck, 1011455000), in 1L of distilled water. Afterwards, the suspension was centrifuged at 1200 rpm, for 15 minutes at room temperature. Cells were ressuspended in α-MEM medium, supplemented with antibiotic/ antimycotic solution and 20 % FBS. Cell suspension was filtered for disposal of debris, using 100 μm and 70 μm Cell Strainer (BD Falcon™, 352360 and BD Falcon™, 352350). Cells were counted and plated at the density of 4.7x103 cells/cm^2. Cells were expanded in the referred culture medium until obtaining the cell numbers needed for this study. The stemness character of the isolated hBMSCs was analysed previously by flow cytometry, (CD34 and CD45 –negative; CD29, CD44, CD73, CD90, CD105 and CD106 - positive), using a flow cytometer, and differentiation studies into osteogenic, chondrogenic, and adipogenic lineage [32].

2.3. Isolation and expansion of hWJSCs

Human umbilical cords were obtained after obtaining informed consent from full-term caesarian sections donors, under the scope of the cooperation agreement of the 3B´s Research Group of the University of Minho and the Obstetrics Department of the Hospital de São Marcos, Braga, Portugal. Human WJSCs were isolated according to the procedure originally described by Sarugaser *et al*. [13]. Briefly, umbilical cords (UC) were cut into 3–5 cm segments. These segments were then dissected by separation of the UC section epithelium along its length, exposing the underlying Wharton's jelly. Each vessel, with its surrounding Wharton's jelly matrix, was pulled away, and the ends of each dissected vessel were tied together with a suture creating "loops". Following, the loops were digested using a 1 mg/ml collagenase type I solution (Sigma, C0130) prepared with PBS. After 18–24 hours, the loops were removed. The remaining

suspension was diluted with PBS to reduce its viscosity, followed by a centrifugation at 1200 rpm for 5 minutes. Cells were ressuspended in 10 ml culture medium consisting of α-MEM medium (Invitrogen, 12000-063), supplemented with antibiotic/ antimycotic solution (Gibco, 15240062), 10% fetal bovine serum (Baptista Marques, BSC0115/0943k) and counted in a hemocytometer. Finally, cells were plated and expanded until reaching 80–90% confluence. The stemness character of these cells was previously studied by flow cytometry for MSCs markers (CD34, CD45-negative and CD29, CD73, CD90, CD105, CD166-positive cells), using a flow cytometer (FACS Calibur (BD Biosciences)), and differentiation studies into osteogenic, chondrogenic, and adipogenic lineage [46].

2.4. Isolation and expansion of human articular chondrocytes

The isolation of human articular chondrocytes (hACs) was performed according to a method previously reported in the literature [47]. HACs were isolated from human cartilage samples collected under informed consent from patients undergoing knee arthroplasties in the Orthopaedics Department of the Hospital de São Marcos, Braga, Portugal, under the scope of the previously referred agreement. During surgeries, the knee was collected into a container with PBS and maintained in ice until the isolation procedure. Cartilage was dissected in small full-depth pieces and washed twice with PBS buffer. Then, it was digested with 0.25% (w/v) of trypsin solution (Sigma, E5134) for 30 minutes at 37ºC on a rotator. The solution was removed, cartilage was washed again in PBS buffer and then incubated in a 2 mg/ml collagenase type II solution (Sigma, E0130) overnight at 37ºC on a rotator. The following day, cells were washed twice with PBS, counted and platted at a density of $2x10^6$ cells per Petri dish. Cells were cultivated with expansion medium: Dulbecco's modified Eagle's medium (Sigma, D5671), containing 10 mM Hepes buffer (Sigma, H0887), L-alanyl-L-glutamine (Sigma, G8541), Non Essential Aminoacids (Sigma, M7145), antibiotic/ antimycotic solution, 10% fetal bovine serum and 10 ng/ml of basic Fibroblast Growth Factor (bFGF) (PeproTech, 100-18B).

2.5. Co-cultures

Cells were expanded until they reached the needed number. They were then split, counted and then seeded onto the CPBS fiber meshes. Two study groups were established: direct co-culture using one source of hMSCs (either hBMSCs or hWJSCs) and hACs; indirect co-culture using conditioned medium obtained from hACs cultures. The indirect co-culture method herein described is a variation of the transwell inserts technique [22, 48]. Instead of separating the two cultures with a transwell, we cultured separately the chondrocytes and the stem cells. As explained in the introduction, it is known that the 3D structure of the scaffolds can positively influence the chondrogenic commitment of MSCs. We hypothesised that by providing the microenvironment and the soluble factors present in the chondrocytes medium we would be able to influence the chondrogenic differentiation of both types of MSCs used in the present work. Thus, as the medium provided was previously consumed by the chondrocytes, we used conditioned medium mixed (50/50 in volume) with fresh medium. By means of diluting the factors secreted by the chondrocytes in fresh medium, it was our intention to provide the required nutrients to the MSCs indirect co-cultures, avoiding the risk of nutrient deprivation.

2.5.1. Direct co-cultures

A suspension of $1x10^6$ cells ($5x10^5$ hBMSCs and $5x10^5$ hACs) per scaffold was used for seeding the fiber meshes, in a rotator inside the incubator at 37ºC and 5% CO_2, during 24 hours. Basic medium was used: Dulbecco's modified Eagle's Medium containing 10 mM Hepes solution, L–alanyl–L–glutamine, Non Essential Aminoacids, antibiotic/ antimicotic and 10% fetal bovine serum. Afterwards, constructs were transferred to new culture plates containing differentiation medium. This medium is a modification of the expansion medium: instead of adding bFGF, 1mg/ml of L-ascorbic acid (Sigma, A8960) and 50 mg/ml of insulin (Sigma, I5500) were added. Constructs were placed inside the incubator under the same conditions and cultured for 28 days. Medium was changed every other day. The same procedure was used for the direct co-cultures of hWJSCs.

2.5.2. Indirect co-cultures

Indirect co-cultures were performed using conditioned medium obtained from the chondrocytes in culture. First, a cell suspension containing $1x10^6$ cells per scaffold was prepared for each type of cell (hBMSCs or hACs). Each cell type was seeded onto different CPBS scaffolds, in different culture plates. The seeding procedure was similar to the one described above for the direct co-cultures. Afterwards, cells were placed in new culture plates containing differentiation medium. After two days, the medium was changed. Human BMSCs medium was completely removed, and replaced by the mixture of conditioned and fresh medium. It consisted of a mixture of the medium which was removed from the chondrocytes culture, plus fresh differentiation medium (50/50). Medium of these cultures (hBMSCs) was changed every other day, using the referred mixture of conditioned medium. For the hACs cultures, fresh differentiation medium was used, and changed every other day. The two types of cultures were maintained for 28 days, in an incubator, at 37ºC and 5% CO_2. The same procedure was used for the indirect co-cultures of hWJSCs.

2.6. Proliferation assay (DNA quantification)

Evaluation of cells proliferation was performed using the PicoGreen dsDNA quantification Kit (Molecular Probes, P7589, Invitrogen), according to the manufacturer protocol. Samples from every culture were collected at each time point. Triplicates of each time point were produced. A standard curve was established. Fluorescence of both samples and standard curve was read with an excitation of 485 nm and an emission of 528 nm, in a microplate reader (Synergie HT). The DNA concentration was extrapolated directly from the standard curve.

2.7. Dimethylmethylene blue (DMB) assay for glycosaminoglycans quantification

Dimethylmethylene blue assay was performed according to the method described by Kafienah and Sims, 2004 [49]. Briefly, samples were collected at 1 and 4 weeks of culture, frozen overnight and then digested. This digestion allowed separating the formed ECM from the scaffold. The digestion solution was prepared by adding 1 mg/mL of proteinase K (Sigma, P8044) diluted in 50 mM Tris-HCl, at pH of 7.6. Tris-HCl solution was prepared using the appropriate amount of Tris-HCl, adding sodium

azide (Sigma, 13412) to a final concentration of 0.05% (w/v). The pH was corrected to obtain the final value of 7.6. Samples were placed in 1.5 mL tubes and incubated with 1 mL of the referred solution, overnight at 60ºC. Afterwards, samples were centrifuged in a bench centrifuge at 13000 rpm for 10 minutes and the supernatant was collected. Solutions for this assay were prepared as follows. Dimethymethylene Blue (DMB) stock solution was prepared dissolving 16 mg of DMB powder (Aldrich, 34088) in 900 ml of distilled water containing 3.04 g of glycine (Sigma, G8898) and 2.73 g of NaCl (Sigma, S3014). This was mixed for 2 hours, covered with aluminium foil. pH was adjusted to 3.0 with HCl (Panreac, 1310202424) and a final volume of 1L. The solution was stored at room temperature covered with aluminium foil. Chondroitin sulphate (Sigma, C8529) solution was prepared in water, in a 5 mg/ml stock solution and kept refrigerated. This solution was diluted with water resulting in 5 unit increments from 0 µg/ml to 50 µg/ml, in order to make a standard curve. 20 µl of water were added to a 96 well plate, as a blank. The same quantity of chondroitin sulphate diluted solutions was added, in triplicate, and the same was performed with all the samples. DMB solution was added, 250 µl to each well, the plates were incubated for 10 minutes and then the optical density was measured in a microplate reader, at 525 nm.

2.8. RNA isolation

Samples were collected from all controls at 7 and 28 days of experiment. Samples were washed in PBS, immersed in TRIzol reagent (Invitrogen, 15596-018) and kept at -80ºC for posterior RNA extraction. When the extraction was performed, samples were taken from the freezer, in order to thaw. Chloroform (Sigma, C2432) was added; samples were vigorously agitated for 15 seconds and then incubated in ice for 15 minutes. After that incubation, samples were centrifuged at 13000 rpm, for 15 minutes, at 4ºC. Afterwards, the supernatant was collected for a sterile 1.5 mL tube, and an equivalent volume of isopropanol (Sigma, I9516) was added. Samples were incubated at -20ºC overnight, to precipitate the RNA. The next day, samples were centrifuged at 13000 rpm, for 15 minutes, at 4ºC. Then, the supernatant was taken and 800 µl of ethanol 70% was added, in order to wash away the isopropanol. This ethanol 70% solution was prepared from absolute ethanol (Merck, 1.00983.2511) and ultra pure water. Tubes were agitated vigorously and centrifuged again, at 9000 rpm for 5 minutes, at 4ºC. The supernatant was again removed, and the pellet was left to air dry. Finally,

the pellet was ressuspended in 50 µl of distilled water DNase, RNase free (Gibco, 10977-015). The concentration and purity of the extracted RNA was evaluated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA).

2.9. Real-Time PCR

The Real-Time PCR procedure used in the present work consisted of a two step fluorogenic assay using the PerfeCta™ SYBR® Green system (Quanta Biosciences, 95055-100). All reagents used in this procedure were purchased from Quanta Biosciences, following the instructions of the manufacturer. Thermocycler reaction conditions used were also the ones mentioned in the kits. In the first step, RNA was reversed transcribed into cDNA, using the qScript cDNA Synthesis Kit (95047-500). A MasterCycler EP Gradient detection system (Eppendorf, USA) was used to perform the reaction. Afterwards, the obtained cDNA was used as template for the amplification of the targets genes sohwn in Table I, with the PerfeCta™ SYBR® Green FastMix™ kit (95072-05K). Tested genes were aggrecan, collagens type I and II and Sox9. GAPDH was used as house-keeping gene, and the expression of all target genes was normalized against the GAPDH of that sample for each time point of the study. All primer sequences were generated using Primer3 software [50] and acquired from MWG Biotech AG, Germany. Table I shows the primers sequences used.

Table 7.1 – Primer sequences used for RT-PCR procedures *.

Gene	Forward (5´-3´)	Reverse (5´-3´)
AGC	TGAGTCCTCAAGCCTCCTGT	TGGTCTGCAGCAGTTGATTC
COL II	CGGTGAGAAGGGAGAAGTTG	GACCGGTCACTCCAGTAGGA
COL I	AGCCAGCAGATCGAGAACAT	ACACAGGTCTCACCGGTTTC
Sox9	TTCATGAAGATGACCGACGC	GTCCAGTCGTAGCCCTTGAG
GAPDH	ACAGTCAGCCGCATCTTCTT	ACGACCAAATCCGTTGACTC

*AGC = Agreccan; COL II = Collagen type II; COL I = Collagen type I; Sox9 = Sry-type high mobility group box 9; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

2.10. Histological analysis

Samples were collected at the end of the experiment and processed for histology. Samples were then fixed in 10% neutral buffered formalin and then dehydrated through crescent concentrations of ethanol, immersed in infiltration solutions, embedded in Technovit glycol methacrylate (kit 8100, Heraeus Kulzer, EBSciences, CO, USA) and then polymerized. Specimens were cut to obtain longitudinal sections of 7 μm of thickness, using a modified microtome equipped with a tungsten blade (Leica RM 2155). Staining solution for toluidine blue assay was prepared by adding 1% of toluidine blue (Sigma, T0394) dissolved in distilled water containing 0.5 g of sodium borate, followed by filtering. One drop of this solution was added to each section for 1 minute. Then, the sections were rinsed with distilled water and let to air dry overnight. Sections were cleared in xylene substitute (Sigma, A5597) and mounted in Histo clear (Frilabo, HS200). Safranin O staining was performed by washing slides in tap water, then immersed in 0.02% fast green solution (Fluka, 44715) for 6 minutes. Then, they were immersed in 1% acetic acid (Panreac, 131008) solution for 1 minute. After, slides were immersed in 0.1% safranin O (Fluka, 84120) solution for 10 minutes. By the end, slides were washed in tap water and let to air dry. Sections were cleared in xylene substitute and mounted as previously described.

2.11. Statistical analysis

Statistical analysis was performed using the SPSS statistic software (Release 15.0.0 for Windows). Firstly, a Shapiro-Wilk test was used to ascertain about the data normality and variance equality. The normality condition was rejected and, consequently, nonparametric tests were used in further comparisons between direct and indirect co-cultures. A Kruskal-Wallis test followed by Tukey's HSD test was applied to compare differences in between the days in culture type. P values lower than 0.001 were considered statistically significant in the analysis of the results.

3. Results

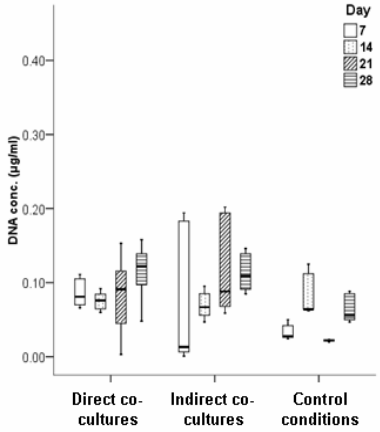
3.1. DNA quantification

The DNA concentration for each type of co-culture was assessed. Samples were collected at each time point of the experiment. We determined if there were any differences in between the days of culture, for each type of co-culture (direct or indirect), and also in the control conditions.

3.1.1. Co-cultures using hBMSCs

In terms of DNA concentration, both types of co-cultures using hBMSCs seem to follow the same trend of proliferation. DNA contents increased with time in both types of co-cultures, whereas the positive control shows a decrease at 21 days, increasing again towards the end of the experiment (Figure 7.1A). These results indicate that both types of co-cultures using hBMSCs were proliferating at a comparable rate, whereas in the control conditions (hACs) cell numbers did not increased between 14 and 21 days, recovering only in the last time point. Figure 7.1B shows the table of results for the statistical analysis concerning the differences between the various time points of the experiment, for each type of co-culture using hBMSCs. We found no significant differences between the various time points either in direct co-cultures ($p=0.172$) or indirect co-cultures ($p=0.062$), showing that the proliferation was not very strong during the experiment. The control conditions show a significant increase from the 7^{th} to the 28^{th} day of experiment, as observed in the statistical analysis results in Figure 7.1C. It is noteworthy that both the direct and indirect co-cultures show the highest levels of DNA concentration when compared to the controls in the last time point.

A



B

Days	Direct co-cultures	Indirect co-cultures	Control conditions
Day 7	0.081(0.069-0.108)	0.013 (0.005-0.188)	0.028 (0.025-0.046)
Day 14	0.076 (0.054-0.090)	0.067(0.054-0.090)	0.064(0.064-0.116)
Day 21	0.091(0.034-0.121)	0.088(0.067-0.194)	0.022(0.021-0.023)
Day 28	0.122(0.078-0.145)	0.109(0.090-0.140)	0.056(0.050-0.087)
P-value	0.172	0.062	<0.001

C

Control conditions	P-Value
Day 7 vs Day14	<0.001
Day 7 vs Day 21	<0.001
Day 7 vs Day 28	<0.001
Day 14 vs Day 21	0.046
Day 14 vs Day 28	<0.001
Day 21 vs Day 28	<0.001

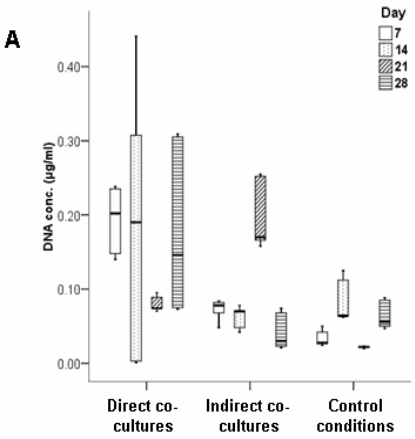
Figure 7.1 - Box plot of the DNA concentration in co-cultures using human bone marrow MSCs. A - Box plot of DNA concentration for direct and indirect co-cultures with hBMSCs and control conditions (hACs alone). B – Table with the statistical analysis results for the between days of culture differences (Kruskal-Wallis test, presented as median ± interquartile range (IQR)). C – Table with the statistical analysis results for between days differences in the control conditions (Kruskal-Wallis test followed by Tukey's HSD test.).

3.1.2. Co-cultures using hWJSCs

The trend of DNA concentration of hWJSCs co-cultures is rather different than the DNA concentration found for the co-cultures using hBMSCs. Both direct and indirect co-cultures show a decreasing tendency in the first days of culture. Then, the DNA concentration in direct co-cultures continues decreasing until the 21st day, increasing afterwards until the end of the experiment (Figure 7.2A). This result might indicate that the proliferation of the direct co-cultures with hWJSCs slowed down up to the 21st day. Conversely, indirect co-cultures with hWJSCs show an increase in DNA concentration after 14 days of culture, showing a marked decrease towards the last time point.

Comparing the various time points, we found no significant differences between the days in direct co-cultures (p=0.04) using hWJSCs (Figure 7.2B). At 21 days, indirect co-cultures using hWJSCs displayed a significantly higher DNA concentration than in the other time points (p<0.001). On the contrary, at 28 days these cultures displayed a significantly lower DNA concentration than all the other time points (p<0.001). This result seems to indicate that the proliferation is slowing down towards the end of the experiment. On the contrary, the control conditions showed a significant increase comparing the 7th and 28th day of the experiment (Figure 7.2C). The highest DNA concentration after 28 days of culture was obtained by direct co-cultures.

A



B

Days	Direct co-cultures	Indirect co-cultures	Control conditions
Day 7	0.202 (0.146-0.237)	0.078(0.075-0.083)	0.028(0.025-0.046)
Day 14	0.190(0.003-0.312)	0.070(0.047-0.720)	0.064(0.064-0.116)
Day 21	0.074(0.073-0.092)	0.170(0.165-0.252)	0.022(0.021-0.023)
Day 28	0.146(0.074-0.307)	0.030(0.023-0.069)	0.056(0.049-0.086)
P-value	0.04	<0.001	<0.001

C

Indirect co-cultures	P-Value	Control conditions	P-Value
Day 7 vs Day14	0.018	Day 7 vs Day14	<0.001
Day 7 vs Day 21	<0.001	Day 7 vs Day 21	<0.001
Day 7 vs Day 28	<0.001	Day 7 vs Day 28	<0.001
Day 14 vs Day 21	<0.001	Day 14 vs Day 21	0.046
Day 14 vs Day 28	0.033	Day 14 vs Day 28	<0.001
Day 21 vs Day 28	<0.001	Day 21 vs Day 28	<0.001

Figure 7.2 - Box plot of the DNA concentration in co-cultures using human WJSCs. A - Box plot of DNA concentration for direct and indirect co-cultures with hWJSCs and control conditions (hACs alone). B – Table with the statistical analysis results for the between days of culture differences (Kruskal-Wallis test, presented as median ± interquartile range (IQR)). C – Table with the statistical analysis results for between days differences in the indirect co-cultures and in control conditions (Kruskal-Wallis test followed by Tukey's HSD test.).

3.2. Glycosaminoglycans quantification

The GAGs content for each type of co-culture was assessed. Samples were collected at 7 and 28 days of the experiment. We compared GAGs content between the types of co-cultures (direct vs indirect). We also compared the differences between both types of co-cultures and our control condition, hACs to determine if the co-cultures were accumulating more GAGs than chondrocytes alone. Afterwards, we determined if any differences were observed between the different time points, for each type of co-culture (direct or indirect).

3.2.1. Co-cultures with hBMSCs

Both direct and indirect co-cultures with hBMSCs show a small decrease in the GAG content from 7 to 28 days of the experiment, whereas control conditions showed the expected significant increase in GAGs deposition over time (Figure 7.3A).

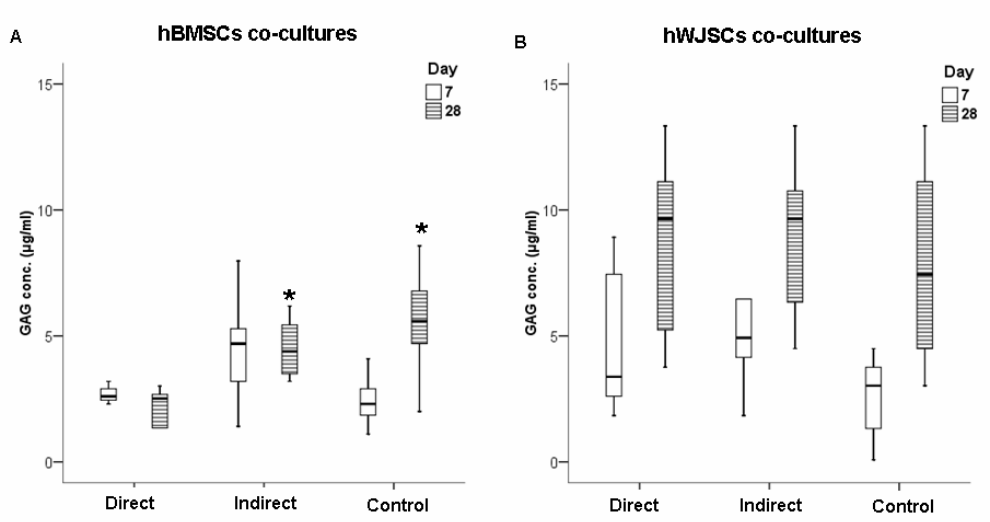


Figure 7.3 - Box plot of the GAGs concentration in both types of co-cultures, along the experiment. A - Box plot of GAGs concentration for direct and indirect co-cultures with hBMSCs and control conditions (hACs alone). B - Box plot of the GAGs concentration for direct and indirect co-cultures using hWJSCs and control conditions (hACs alone). Data were analyzed by nonparametric way of a Kruskal-Wallis test followed by Tukey's HSD test. * denotes significant differences compared to direct co-cultures.

No significant difference between direct and indirect co-cultures using hMSCs was found for 7 days of culture (p=0.061). At the end of the experiment, direct co-cultures displayed a significantly lower amount of GAGs than indirect co-cultures (p=0.006), as well as lower than the values for the control conditions (p=0.002). These results show that those co-cultures did not deposit more GAGs than the control condition. No significant differences between 7 and 28 days were observed in direct co-cultures (p=0.534) and in indirect co-cultures with hBMSCs (p=0.963).

3.2.2. Co-cultures using hWJSCs

In co-cultures using hWJSCs, a consistent trend was observed for all the conditions. Direct and indirect co-cultures, as well as the control conditions, show an increase in GAGs content along the time in culture (Figure 7.3B). For co-cultures using hWJSCs no significant difference between direct and indirect co-cultures was found after 7 days (p=0.138) nor after 28 days (p=0.733). Significant differences were found between 7 and 28 days of culture for both direct and indirect co-cultures. After 28 days, all the conditions displayed a significantly higher GAG concentration compared to 7 days ($p<0.01$), meaning that the GAGs accumulation is very alike to the control conditions, with native chondrocytes.

The GAGs concentration values obtained for all the time points for direct or indirect co-cultures with hBMSCs show consistently lower values when comparing to the ones obtained for the co-cultures with hWJSCs.

3.3. Real-Time PCR

Samples were collected for Real-Time PCR at 7 and 28 days of experiment. The aim was to determine the expression of several cartilage-related genes and to compare the differences between the expressions in both types of co-cultures, on both time points.

3.3.1. Co-cultures using hBMSCs

In both types of co-cultures using hBMSCs, no significant differences were found between the cultures for *Aggrecan* or *Sox9*, at 7 days (p=0.072 and p=0.868, respectively) or 28 days (p=0.183 and p=0.115, respectively), as shown in Figure 7.4. Direct co-cultures using hBMSCs expressed significantly higher values of Collagen *type I*, compared either to indirect co-cultures or to the control conditions ($p<0.001$),

indicating the formation of fibrocartilage in direct co-cultures. *Collagen type II* expression in indirect co-cultures of hBMSCs, displayed a significantly higher expression than direct co-cultures and control conditions ($p<0.001$) at 7 day of culture. After 28 days, direct co-cultures displayed a significantly higher *Collagen type II* expression than indirect co-cultures ($p=0.009$) and control conditions ($p=0.002$).

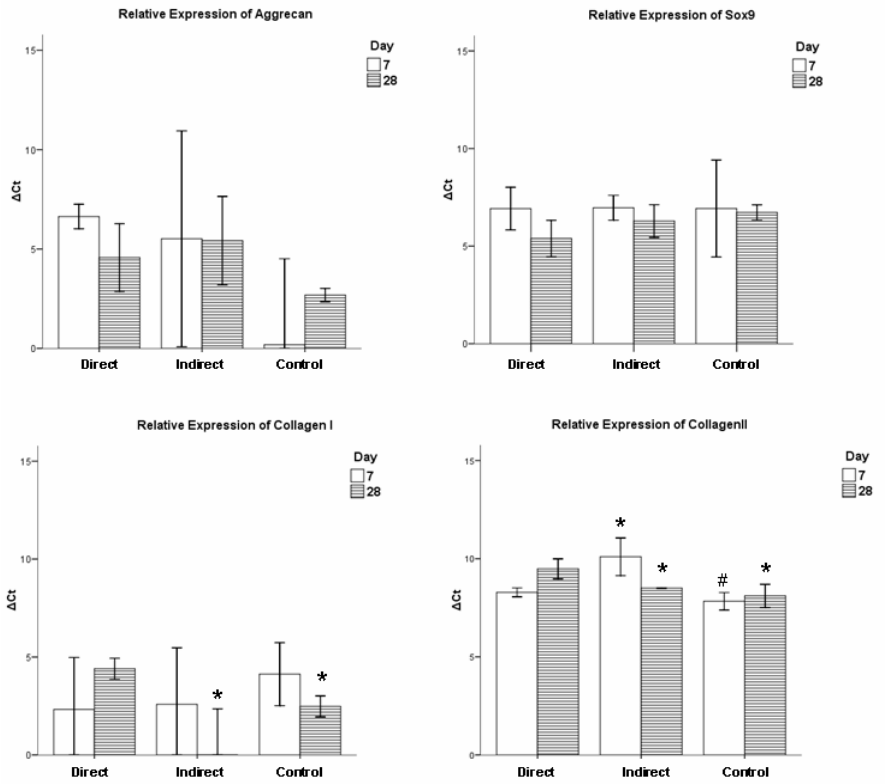


Figure 7.4 – Bar plots of chondrogenic markers present in direct and indirect co-cultures using hBMSCs, normalized for the reference gene *GAPDH*, after 7 and 28 days of culture. Data were analyzed by nonparametric way of a Kruskal-Wallis test for *Aggrecan* and *Sox9*. Data related with *Collagen type I* and *Collagen type II* were analyzed by nonparametric way of a Kruskal-Wallis test followed by Tukey's HSD test. * denotes significant differences compared to direct co-cultures, # denotes significant differences compared to indirect co-cultures.

3.3.2. Co-cultures using hWJSCs

Statistic analysis of gene expression in direct and indirect co-cultures using hWJSCs showed significant differences for almost all the genes (Figure 7.5). For *Aggrecan*, there was a significantly lower expression on the control conditions, when compared to direct and indirect co-cultures ($p<0.001$), at 7 days of culture. However, at 28 days of culture,

both types of co-cultures using hBMSCs showed a significantly higher expression of *Aggrecan* than control conditions ($p<0.001$). Additionally, indirect co-cultures also displayed a significantly higher value for *Aggrecan* expression when compared to direct co-cultures ($p=0.007$).

Indirect co-cultures also expressed significantly higher values of *Sox9* expression, at 28 days of culture, when compared to direct co-cultures and control conditions ($p<0.001$). For *Collagen type I*, no significant difference between cell type was found either at 7 ($p=0.044$) or 28 days of culture ($p=0.498$). However, the trend was to decrease its expression for the longer time periods.

Direct co-cultures using hWJSCs displayed a significantly higher expression of *Collagen type II* than indirect co-cultures, after 7 days in culture. In contrast, indirect co-cultures displayed a significantly higher *Collagen type II* expression than the control conditions ($p=0.006$), and a significant trend to increase the expression of this important gene, in contrast to other culture conditions.

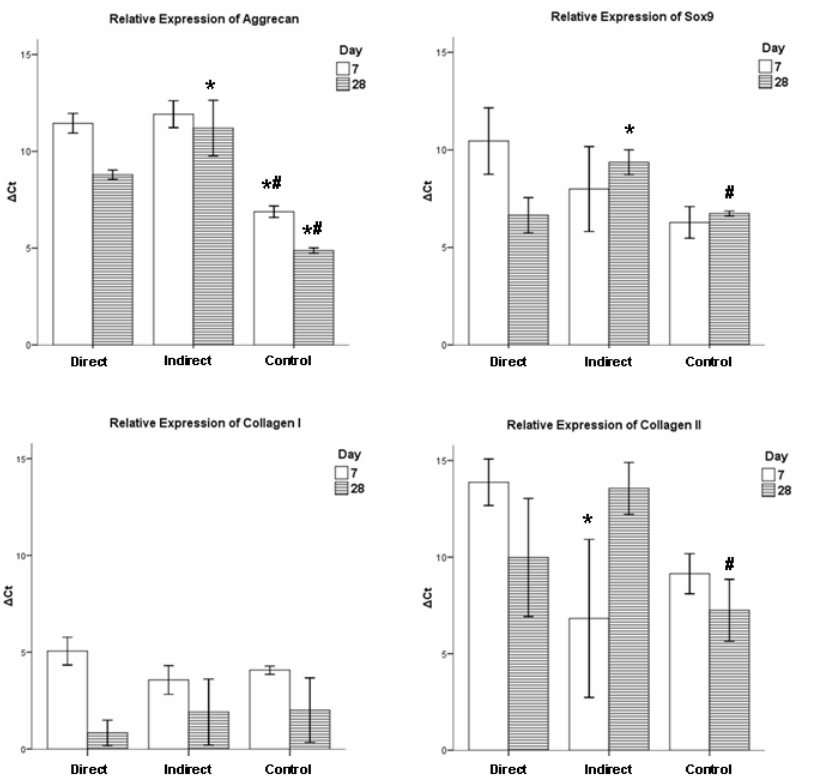


Figure 7.5 – Bar plots of chondrogenic markers present in direct and indirect co-cultures using hWJSCs, normalized for the reference gene *GAPDH*, after 7 and 28 days of culture. Data were analyzed by nonparametric way of a Kruskal-Wallis test for

Collagen type I. Data related to *Aggrecan*, *Sox9* and *Collagen type II* expressions were analyzed by nonparametric way of a Kruskal-Wallis test followed by Tukey's HSD test. * denotes significant differences compared to direct co-cultures, # denotes significant differences compared to indirect co-cultures.

3.4. Histological staining

By the end of the experiment, samples of every co-culture were collected for histological sectioning. Sections were stained for cartilagineous ECM using toluidine blue and safranin O assays (Figure 7.6). Both types of cells, either in direct or indirect co-culture, were able to produce ECM, as shown by the positive stain of toluidine blue and safranin O for ECM components. The cells were able to proliferate around the fiber meshes, and were able to grow in between the fibers inside the structure of the scaffolds. For co-cultures using hBMSCs no evident differences in terms of the quantity of cartilage ECM formation are observed in the histological staining results, either for direct or indirect co-cultures. Nevertheless, positive staining for proteoglycans can be observed either with toluidine blue or with safranin O stain. These observations are consistent with the previously obtained results for GAGs accumulation, as well as the RT-PCR results that showed the expression of cartilage related genes at the end of the experiment.

In the case of co-cultures using hWJSCs, the same observations were made. It is possible to state ECM formation both in direct and indirect co-cultures, stained with toluidine blue and safranin O. These observations are also consistent with the previously obtained results for GAGs accumulation and cartilage related genes expression at 28 days of culture.

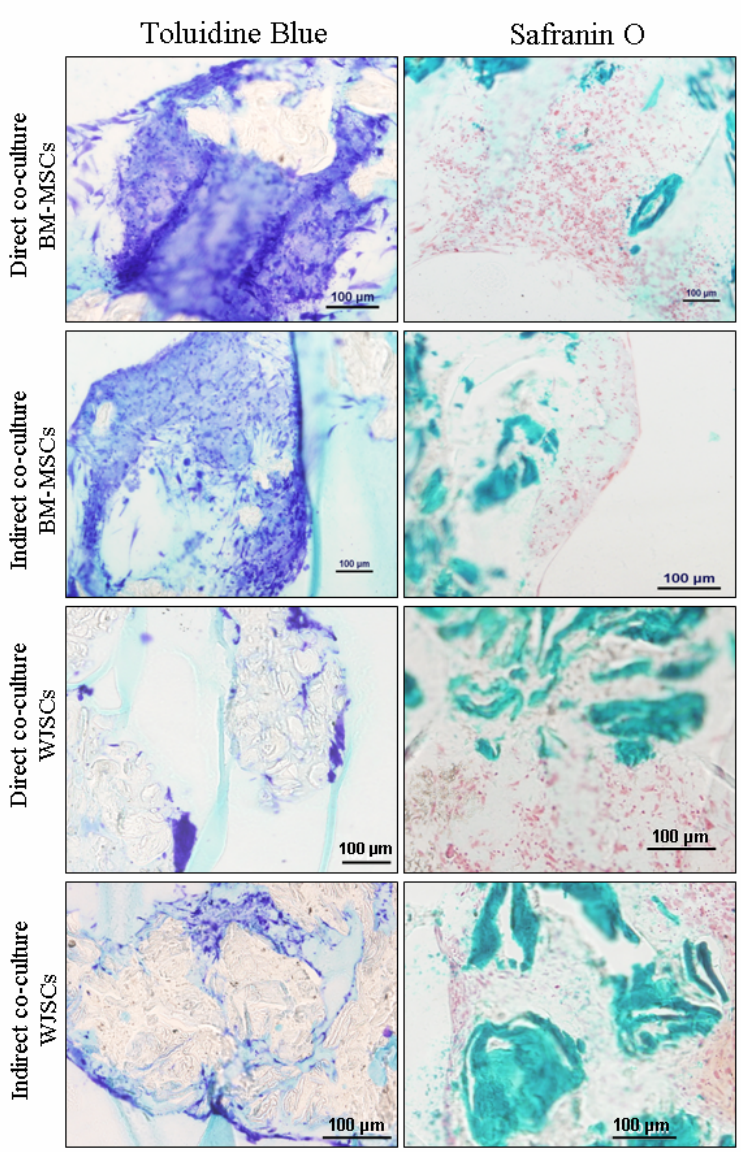


Figure 7.6 – Histological sections of direct and indirect co-cultures using human BMSCs and WJSCs, stained for ECM localisation.

4. Discussion

The use of biomaterial scaffolds to enhance ECM-cell interactions has been reported as a very important factor when using stem cells for TE [7, 42, 51]. For detailed overview on this matter, please refer to [52, 53]. The positive effect of 3D scaffolds in the chondrogenic differentiation of BMSCs [54-56] and UCSCs [32, 57] has been reported elsewhere. In the present work we showed that both hBMSCs and hWJSCs were able to undergo chondrogenic differentiation when seeded onto CPBS fiber

meshes. Polyglycolic acid (PGA) scaffolds were recently used for comparing the potential of hBMSCs and hUCSCs for cartilage tissue engineering. The authors observed enhanced chondrogenic differentiation of hUCSCs when compared to hBMSCs [7]. The chondrogenic differentiation of MSCs on chitosan fibrous scaffolds and chitosan sponges was compared, demonstrating that chondrogenesis on chitosan scaffolds is superior on microfibers, when compared to sponges [58].

Herein, chondrogenic differentiation of human BMSCs and WJSCs was observed both in direct and indirect co-cultures. The soluble signals released by articular chondrocytes, either by the direct contact or in the form of soluble factors released in the culture medium, did effectively promote chondrogenic differentiation of hBMSCs and of hWJSCs. A recent study reported similar conclusions for the chondrogenic differentiation of adipose stem cells when co-cultured with chondrocytes [59]. In that report, adipose stem cells were cultured in pellets either directly or indirectly with chondrocytes, and it was concluded that the chondrocytes secreted signals promoted chondrogenic differentiation of adipose stem cells. The co-culture systems used in the present work were able to provide MSCs with the signals and the stimulation needed for their chondrogenic differentiation. A similar result was found when using conditioned medium from chondrocytes in the chondrogenic differentiation of embryonic stem cells [48]. The authors could show the coordination of signals between the co-cultures, suggesting that chondrogenic differentiation of embryonic stem cells can be achieved by co-cultures with chondrocytes, without other exogenous growth factors being required.

No significant differences between the days in culture, in terms of proliferative ability, were found for the co-cultures using hBMSCs. Likewise, no significant differences were found for direct co-cultures using hWJSCs. However, when analysing the proliferative ability of hWJSCs in indirect co-cultures we observed significantly higher differences between the 21st day and all the other time points. Moreover, the values obtained for the DNA concentration of hWJSCs are higher than the ones obtained for the hBMSCs. The higher proliferative potential of hWJSCs compared to hBMSCs has been demonstrated earlier [15], as well as their enhanced chondrogenic potential [14]. Both types of co-cultures were able to produce and deposit GAGs. For the cultures with hBMSCs, a significantly lower GAG concentration value was found for the direct co-cultures, comparing to the indirect. For the cultures using hWJSCs, no significant differences were found in terms of GAGs concentration between direct and indirect co-cultures. These results show that indirect co-cultures may be more effective in ECM

components production, at least in terms of chondrogenic differentiation of hBMSCs, since no differences were found for hWJSCs. Our data with co-cultures of hBMSCs are similar to the results obtained in a study using passaged chondrocytes and primary bovine chondrocytes [19]. In that study, indirect co-cultures of primary and passaged chondrocytes were performed using filter inserts and it was found that the passaged cells accumulated ECM, when in contact with the conditioned medium produced by the primary chondrocytes. Although indirect co-cultures using hBMSCs seem to have an advantage in terms of GAGs accumulation, both types of co-cultures using these cells displayed a significantly lower value when compared to the chondrocytes culture (control condition). These results show that co-cultures with hBMSCs produced and accumulated less GAGs than the native chondrocytes cultures. In terms of GAGs, we can say that the hBMSCs co-cultures do not show a clear advantage over chondrocytes.

Interestingly, both direct and indirect co-cultures using hWJSCs displayed a significantly higher concentration of GAGs at 28 days of culture, compared to 7 days. The values for GAGs concentration were higher in the cultures using hWJSCs than in the cultures using hBMSCs, indicating more formation and accumulation of these important components of the cartilaginous ECM. Our results showed enhanced chondrogenesis in hWJSCs, when in indirect or direct co-cultures.

In the histological sections, it is noticeable that both cultures of hBMSCs and of hWJSCs were able to produce ECM components, namely proteoglycans. These components were stained both with toludine blue and with safranin O, showing ECM deposition. ECM accumulation was also observed in passaged human chondrocytes, when in co-cultures with bovine chondrocytes [34]. Co-cultures of equine BMSCs with equine articular chondrocytes improved the expression of cartilage related genes, and induced the production of a more homogeneous ECM within the neo-cartilage [36].

In terms of gene expression, for the cultures using hBMSCs, direct co-cultures showed significantly higher expression of *Collagen type I* than indirect co-cultures, at the end of the experiment. In terms of *Collagen type II* expression, direct co-cultures displayed a significantly higher expression at 7 days of culture than indirect co-cultures. On the other hand, towards the end of the experiment, indirect co-cultures showed a higher expression of collagen type II compared to the direct co-cultures. Again, the positive effect of using conditioned medium is evidenced by the results. For hWJSCs co-cultures, a significantly higher expression of *Aggrecan* was found for indirect co-cultures of hWJSCs at 28 days, compared to the direct ones. The same significantly

higher expression was observed for *Sox9* expression. For *Collagen type II*, there was a decrease in the last day of culture for the direct co-cultures. In this last time point, a significantly higher expression was found for the indirect co-cultures, when compared to the control conditions. Therefore, this system of co-culturing has a considerable clinic potential.

The use of conditioned medium to promote the differentiation of MSCs for further implantation is a valuable concept in terms of regenerative medicine, because it will allow obtained conditioned medium of unrelated chondrocytes to promote the differentiation of autologous stem cells. In the present work, we propose a new strategy for cartilage tissue engineering strategies based on the use of conditioned medium, and we were able to demonstrate its effect in MSCs chondrogenesis. The positive effect of the conditioned medium in osteogenesis and chondrogenesis has been recently demonstrated, using MSCs seeded onto polycaprolactone/hydroxyapatite scaffolds and on alginate, respectively [60]. In a different approach, using porcine cells for chondrogenic differentiation for intervertebral disc repair, notochordal cell conditioned medium promoted MSCs differentiation, induced significantly more collagen type II expression and GAGs production in MSCs [61]. In the present work, the soluble factors released by hACs in culture were able to induce chondrogenic differentiation of both hBMSCs and hWJSCs without the addition of growth factors. This is a very interesting result, since in a clinical perspective it is preferable to use conditioned medium for chondrogenic differentiation of MSCs than to induce it with growth factors or even with direct co-cultures. There are many cost associated with the use of growth factors, so this cost could be reduced when using conditioned medium. Indeed, this technique should be further explored, as some reports show that the use of conditioned medium is very versatile. For example, conditioned medium from a human hepatocarcinoma cell line (HepG2) enhanced in vitro chondrogenesis of murine embryonic stem cells [62]. Therefore, chondrocyte cell lines could be eventually established for conditioned medium production, to further induce chondrogenic differentiation of autologous MSCs in 3D scaffolds prior to its implantation. However, it is very important to identify, in the first place, the soluble factors that are being release into the hACs culture medium and that successfully induced the chondrogenic differentiation of hBMSCs and hWJSCs.

Indirect co-cultures of human MSCs using conditioned medium from hACs seem to be more suitable for engineered cartilage formation. The results of indirect co-cultures (either with BMSCs or with WJSCs) towards the end of the experiment were

significantly higher for all the tested parameters, namely GAGs accumulation and cartilage related-genes expression (*Collagen type II*, *Aggrecan* and *Sox9*). Concerning the type of human MSCs tested, we obtained strong evidence that hWJSCs are more promising than hBMSCs for cartilage related applications using this strategy. Additionally, more GAGs accumulation was measured again for both direct and indirect co-cultures using hWJSCs. These cultures also expressed *Aggrecan*, *Sox9* and *Collagen type II*, genes related to articular cartilage. *Collagen type I*, related to fibrotic cartilage, did not have a significant expression in these cultures with hWJSCs. This result may be indirectly related to a superior chondrogenic performance of hWJSCs when compared to hBMSCs [7].

5. Conclusions

Human adult MSCs were able to differentiate into the chondrogenic lineage, when co-cultured with hACs in CPBS scaffolds. In terms of type of culture, indirect co-cultures (using conditioned medium) seem more effective for chondrogenic differentiation. This is a very interesting result to be further explored for clinical translation. In this way, conditioned medium without the addition of other supplements or growth factors can be employed to enhance MSCs expansion and chondrogenic differentiation, prior to implantation. Indirect co-cultures using conditioned medium, using either BMSCs or WJSCs, showed significant higher values in terms of GAGs accumulation and cartilage related genes expression, when compared to direct co-cultures. In terms of cell source, hWJSCs showed higher proliferation rates and higher chondrogenic differentiation ability when compared to hBMSCs. Human WJSCs expressed significant higher values of DNA concentration, GAGs accumulation and cartilage related genes (*Aggrecan, Collagen type II* and *Sox9*). Human BMSCs chondrogenic differentiation produced fibrous cartilage, as stated by the significantly higher expression of *Collagen type I* when compared to hWJSCs.

In conclusion, indirect co-cultures of hWJSCs using conditioned medium obtained from hACs cultures are a new approach for cartilage tissue engineering that should be extensively studied for future clinical applications, as an alternative method for expanding autologous cells prior to implantation.

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References

1 - Schulze-Tanzil G. *Activation and dedifferentiation of chondrocytes: Implications in cartilage injury and repair*. Ann Anat. 2009. 191(4):325-38.

2 - Barbero A, Ploegert S, Heberer M, Martin I. *Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes*. Arthritis & Rheumatism. 2003. 48(5):1315 – 25.

3 - Brun P, Abatangelo G, Radice M, Zacchi V, Guidolin D, Gordini DD*, et al. Chondrocyte aggregation and reorganization into three-dimensional scaffolds*. J Biomed Mater Res. 1999. 46:337 – 46.

4 - Darling EM, Athanasiou KA. *Retaining zonal chondrocyte phenotype by means of novel growth environments*. Tissue Eng. 2005. 11(3-4):395-403.

5 - Miot S, Woodfield TB, Daniels AU, Suetterlin R, Peterschmitt I, Heberer M*, et al. Effects of scaffold composition and architecture on human nasal chondrocyte redifferentiation and cartilaginous matrix deposition*. Biomaterials. 2005. 26:2479 – 89.

6 - Sekiya I, Larson B, Smith JR, Pochampally R, Cui J-O, Prockop DJ. *Expansion of human adult stem cells from bone marrow stroma: Conditions that maximize the yields of early progenitors and evaluate their quality*. Stem Cells. 2002. 6:530 - 41.

7 - Wang L, Tran I, Seshareddy K, Weiss ML, Detamore MS. *A comparison of human bone marrow-derived mesenchymal stem cells and human umbilical cord-derived*

mesenchymal stromal cells for cartilage tissue engineering. Tissue Eng Part A. 2009. 15(8):2259-66.

8 - Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. *Isolation and multilineage differentiation of bovine bone marrow mesenchymal stem cells*. Cell Tissue Res. 2005. 319:243 – 53.

9 - Mauck RL, Yuan X, Tuan RS. *Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture*. Osteoarthritis Cartilage. 2006. 14(2):179-89.

10 - Hu J, Feng K, Liu X, Ma PX. *Chondrogenic and osteogenic differentiations of human bone marrow-derived mesenchymal stem cells on a nanofibrous scaffold with designed pore network*. Biomaterials. 2009. 30(28):5061-7.

11 - Markway BD, Tan GK, Brooke G, Hudson JE, Cooper-White JJ, Doran MR. *Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures*. Cell Transplant. 2010. 19(1):29-42.

12 - 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.

13 - Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. *Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors*. Stem Cells. 2005. 23(2):220-9.

14 - Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, *et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord*. Stem Cells. 2004. 22(7):1330-7.

15 - Baksh D, Yao R, Tuan RS. *Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow*. Stem Cells. 2007. 25(6):1384-92.

16 - Can A, Karahuseyinoglu S. *Concise review: Human umbilical cord stroma with regard to the source of fetus-derived stem cells*. Stem Cells. 2007. 25(11):2886-95.

17 - Troyer DL, Weiss ML. *Wharton's jelly-derived cells are a primitive stromal cell population*. Stem Cells. 2008. 26(3):591-9.

18 - Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, *et al. Biology of stem cells in human umbilical cord stroma: In situ and in vitro surveys*. Stem Cells. 2007. 25(2):319-31.

19 - Taylor DW, Ahmed N, Gan L, Gross AE, Kandel R. *Proteoglycan and collagen accumulation by passaged chondrocytes can be enhanced through side by side culture with primary chondrocytes*. Tissue Eng Part A. 2009.

20 - Bos PK, van Osch GJ, Frenz DA, Verhaar JA, Verwoerd-Verhoef HL. *Growth factor expression in cartilage wound healing: Temporal and spatial immunolocalization in a rabbit auricular cartilage wound model*. Osteoarthritis Cartilage. 2001. 9(4):382-9.

21 - Hwang NS, Varghese S, Puleo C, Zhang Z, Elisseeff J. *Morphogenetic signals from chondrocytes promote chondrogenic and osteogenic differentiation of mesenchymal stem cells*. J Cell Physiol. 2007. 212(2):281-4.

22 - Hwang NS, Varghese S, Elisseeff J. *Derivation of chondrogenically-committed cells from human embryonic cells for cartilage tissue regeneration*. PLoS ONE. 2008. 3(6):e2498.

23 - Costa-Pinto AR, Salgado AJ, Correlo VM, Sol P, Bhattacharya M, Charbord P*, et al. Adhesion, proliferation, and osteogenic differentiation of a mouse mesenchymal stem cell line (BMC9) seeded on novel melt-based chitosan/polyester 3D porous scaffolds*. Tissue Eng Part A. 2008. 14(6):1049-57.

24 - Cruz DM, Ivirico JL, Gomes MM, Ribelles JL, Sanchez MS, Reis RL*, et al. Chitosan microparticles as injectable scaffolds for tissue engineering*. J Tissue Eng Regen Med. 2008. 2(6):378-80.

25 - Gomes ME, Azevedo HS, Moreira AR, Ella V, Kellomaki M, Reis RL. *Starch-poly(epsilon-caprolactone) and starch-poly(lactic acid) fibre-mesh scaffolds for bone tissue engineering applications: Structure, mechanical properties and degradation behaviour*. J Tissue Eng Regen Med. 2008. 2(5):243-52.

26 - Pereira RC, Scaranari M, Castagnola P, Grandizio M, Azevedo HS, Reis RL*, et al. Novel injectable gel (system) as a vehicle for human articular chondrocytes in cartilage tissue regeneration*. J Tissue Eng Regen Med. 2009. 3(2):97-106.

27 - Wang Y, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL. *In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells*. Biomaterials. 2005. 26(34):7082-94.

28 - Kramer J, Bohrnsen F, Lindner U, Behrens P, Schlenke P, Rohwedel J. *In vivo matrix-guided human mesenchymal stem cells*. Cell Mol Life Sci. 2006. 63(5):616-26.

29 - Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol P, Bhattacharya M*, et al. Chitosan/polyester-based scaffolds for cartilage tissue engineering: Assessment of extracellular matrix formation*. Acta Biomater. 2010. 6(3):1149-57.

30 - Oliveira J, Correlo VM, Sol P, Costa-Pinto A, Salgado A, Bhattacharya M*, et al. Assessment of the suitability of chitosan/polybutylene succinate scaffolds seeded with mouse mesenchymal progenitor cells for a cartilage tissue engineering approach* Tissue Eng. 2008. 14(10):1651-61.

31 - Oliveira JT, Crawford A, Mundy JL, Sol PC, Correlo VM, Bhattacharya M*, et al. Novel melt-processable chitosan-polybutylene succinate fibre scaffolds for cartilage tissue engineering*. J Biomater Sci Polym Ed - In press. 2010.

32 - Costa-Pinto AR, Frias AM, Alves da Silva ML, Fernandes S, Barros A, Reis RL*, et al. Comparative study of the multidifferentiation potential of human Wharton´s jelly and amniotic fluid derived stem cells*. Tissue Eng Part A. 2008. 14:713.

33 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, Neves NM*, et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromol Biosci. 2010.

34 - Ahmed N, Taylor DW, Wunder J, Nagy A, Gross AE, Kandel RA. *Passaged human chondrocytes accumulate extracellular matrix when induced by bovine chondrocytes*. J Tissue Eng Regen Med. 2010. 4(3):233-41.

35 - Ahmed N, Gan L, Nagy A, Zheng J, Wang C, Kandel RA. *Cartilage tissue formation using redifferentiated passaged chondrocytes in vitro*. Tissue Eng Part A. 2009. 15(3):665-73.

36 - Lettry V, Hosoya K, Takagi S, Okumura M. *Coculture of equine mesenchymal stem cells and mature equine articular chondrocytes results in improved chondrogenic differentiation of the stem cells*. Jpn J Vet Res. 2010. 58(1):5-15.

37 - Bigdeli N, Karlsson C, Strehl R, Concaro S, Hyllner J, Lindahl A. *Coculture of human embryonic stem cells and human articular chondrocytes results in significantly altered phenotype and improved chondrogenic differentiation*. Stem Cells. 2009. 27(8):1812-21.

38 - Chen J, Wang C, Lü S, Wu J, Guo X, Duan C*, et al. In vivo chondrogenesis of adult bone-marrow-derived autologous mesenchymal stem cells*. Cell Tissue Res. 2005. 319:429 – 38.

39 - Ahmed N, Dreier R, Gopferich A, Grifka J, Grassel S. *Soluble signalling factors derived from differentiated cartilage tissue affect chondrogenic differentiation of rat adult marrow stromal cells*. Cell Physiol Biochem. 2007. 20(5):665-78.

40 - Varshney RR, Zhou R, Hao J, Yeo SS, Chooi WH, Fan J*, et al. Chondrogenesis of synovium-derived mesenchymal stem cells in gene-transferred co-culture system*. Biomaterials. 2010. 31(26):6876-91.

41 - Hildner F, Concaro S, Peterbauer A, Wolbank S, Danzer M, Lindahl A*, et al. Human adipose-derived stem cells contribute to chondrogenesis in coculture with human articular chondrocytes*. Tissue Eng Part A. 2009. 15(12):3961-9.

42 - Jakobsen RB, Shahdadfar A, Reinholt FP, Brinchmann JE. *Chondrogenesis in a hyaluronic acid scaffold: Comparison between chondrocytes and msc from bone marrow and adipose tissue*. Knee Surg Sports Traumatol Arthrosc. 2009.

43 - Pereira RC, Costa-Pinto AR, Frias AM, N.M. N, Azevedo HS, Reis RL. *Co-culture of human Wharton jelly stem cells with human articular chondrocytes provides a suitable environment for their chondrogenic commitment*. 5th International Annual Meeting of the Portuguese Society for Stem Cells and Cellular Therapy (SPCE-TC); 2010; Avepark, Caldas das Taipas , Guimarães; 2010. p. 19-20.

44 - Costa-Pinto AR, Correlo VM, Sol PC, Bhattacharya M, Charbord P, Delorme B*, et al. Osteogenic differentiation of human bone marrow mesenchymal stem cells seeded on melt based chitosan scaffolds for bone tissue engineering applications*. Biomacromolecules. 2009. 10(8):2067-73.

45 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, N.M. N*, et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromolecular Bioscience. 2010. accepted for publication.

46 - Costa-Pinto A. R., Frias AM, Alves da Silva ML, Barros A., Fernandes S., RL R*, et al. Comparative study of human Wharton's jelly and amniotic fluid derived stem cells differentiation potential*. Tissue Engineering: . 2008. 14:713.

47 - Crawford A, Dickinson S. *Chondrocyte isolation, expansion, and culture in polymer scaffolds.* In: Hollander AP, Hatton PV, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 147-57.

48 - Vats A, Bielby RC, Tolley N, Dickinson SC, Boccaccini AR, Hollander AP*, et al. Chondrogenic differentiation of human embryonic stem cells: The effect of the micro-environment*. Tissue Eng. 2006. 12(6):1687-97.

49 - Kafienah W, Sims TS. *Biochemical methods for the analysis of tissue engineered cartilage.* In: Hollander Ap, Hatton Pv, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 217 - 29.

50 - http://frodo.wi.mit.edu/.

51 - Mahmoudifar N, Doran PM. *Chondrogenic differentiation of human adipose-derived stem cells in polyglycolic acid mesh scaffolds under dynamic culture conditions.* Biomaterials. 2010. 31(14):3858-67.

52 - Chai C, Leong KW. *Biomaterials approach to expand and direct differentiation of stem cells*. Mol Ther. 2007. 15(3):467-80.

53 - Vinatier C, Mrugala D, Jorgensen C, Guicheux J, Noel D. *Cartilage engineering: A crucial combination of cells, biomaterials and biofactors*. Trends Biotechnol. 2009. 27(5):307-14.

54 - Kafienah W, Mistry S, Dickinson SC, Sims TJ, Learmonth I, Hollander AP. *Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients*. Arthritis Rheum. 2007. 56(1):177-87.

55 - Kim HJ, Lee JH, Im GI. *Chondrogenesis using mesenchymal stem cells and PCL scaffolds*. J Biomed Mater Res A. 2010. 92(2):659-66.

56 - Shao X, Hutmacher DW, Ho ST, Goh CH, Lee EH. *Evaluation of a hybrid scaffold/cell construct in repair of high-load-bearing osteochondral defects in rabbits.* Biomaterials. 2006. 27(7):1071 - 80.

57 - Wang L, Sheshareddy K, Weiss ML, Detamore MS. *Effect of initial seeding density on human umbilical cord mesenchymal stromal cells for fibrocartilage tissue engineering*. Tissue Eng Part A. 2009. 15(5):1009-17.

58 - Ragetly GR, Griffon DJ, Lee HB, Fredericks LP, Gordon-Evans W, Chung YS. *Effect of chitosan scaffold microstructure on mesenchymal stem cell chondrogenesis*. Acta Biomater. 2010. 6(4):1430-6.

59 - Lee JS, Im GI. *Influence of chondrocytes on the chondrogenic differentiation of adipose stem cells*. Tissue Eng Part A. 2010.

60 - Maxson S, Burg KJ. *Conditioned media enhance osteogenic differentiation on poly(l-lactide-co-epsilon-caprolactone)/hydroxyapatite scaffolds and chondrogenic differentiation in alginate*. J Biomater Sci Polym Ed. 2010. 21(11):1441-58.

61 - Korecki CL, Taboas JM, Tuan RS, Iatridis JC. *Notochordal cell conditioned medium stimulates mesenchymal stem cell differentiation toward a young nucleus pulposus phenotype*. Stem Cell Res Ther. 2010. 1(2):18.

62 - Hwang Y-S, Bishop AE, Polak JM, Mantalaris A. *Enhanced in vitro chondrogenesis of murine embryonic stem cells*. biotechnology and Bioprocesses Engineering. 2007. 12:696-706.

Chapter 7

Co-culture of human articular chondrocytes with two different sources of human mesenchymal stem cells as a new strategy for cartilage tissue engineering

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This chapter is based on the following publication: Alves da Silva ML, Costa-Pinto AR, Correlo VM, Sol P, Bhattacharya M, Faria S, Reis RL, Neves NM. *Co-culture of human articular chondrocytes with two different sources of human mesenchymal stem cells as a new strategy for cartilage tissue engineering*. Submitted. 2010.

Abstract

Chondrocytes are mainly responsible for maintaining the integrity of the extracellular matrix of articular cartilage and for the protection of the underlying subchondral bone. Mesenchymal Stem Cells (MSCs) are also believed to contribute to the maintenance of cartilage. Moreover, these cells have been recognized for their ability to differentiate into cells of different tissues such as bone, cartilage or adipose tissue, and therefore might be of great interest for developing cartilage therapeutic strategies. These cells are typically induced to differentiate by growth factors supplementation in the culture medium that will trigger differentiation into the desired cell phenotype.

One frequent problem that occurs in cartilage tissue engineering is that expanded chondrocytes undergo dedifferentiation when in 2D culture. This process leads frequently to the undesired production of fibrocartilage, and is associated with the loss of their phenotype in vitro. The use of an additional cell source to enhance cell numbers, not loosing the differentiation potential upon expansion in vitro may be an original choice. Moreover, soluble factors released by chondrocytes have been shown to influence stem cells differentiation onto the chondrogenic lineage. The use of conditioned medium obtained from chondrocytes for stimulating stem cells chondrogenic differentiation may be a very interesting alternative for moving into the clinical application of these cells.

In the present work, direct contact co-cultures and indirect co-cultures (using conditioned medium obtained from a culture of human articular chondrocytes) of human bone marrow-derived MSCs (hBMSCs) and human Wharton´s jelly MSCs (hWJSCs) were established. Cells were isolated from human samples collected at a local hospital, under donors' informed consent. The co-cultures were performed in previously produced 3D scaffolds, composed by a blend of 50/50 chitosan and poly (butylene succinate) – CPBS. Co-cultures were maintained during 28 days. Both types of stem cells were able to undergo chondrogenic differentiation, either in direct or indirect co-cultures. By the end of the experiment, the indirect co-cultures (using conditioned medium) showed significantly higher values in terms of DNA content, glycosaminoglycans (GAGs) accumulation and specific cartilage-related genes expression, when compared to direct co-cultures, for both types of adult MSCs tested. Human BMSCs resulted in fibrous cartilage, as it can be concluded by the significantly higher expression of *Collagen type I*. The hWJSCs showed higher chondrogenic

differentiation ability when compared to hBMSCs, as denoted by the higher values for GAGs accumulation and cartilage related genes expression.

Therefore, we were able to demonstrate the high chondrogenic potential of hWJSCs, and proved that co-cultures of articular chondrocytes with these cells are able to produce more cartilaginous ECM, when compared to co-cultures with hBMSCs. The use of conditioned medium obtained from articular chondrocytes induced MSCs chondrogenic differentiation and ECM formation. The obtained results showed that this new strategy is very interesting and should be further explored for clinical applications.

1. Introduction

Research concerning cartilage regeneration is of special importance, since this tissue lacks self-repairing capacity and causes a huge social and economic impact. Several cell types have been proposed for cartilage tissue engineering, as for example the direct use of autologous chondrocytes. However, this method is not very convenient, since it involves a biopsy used as cell source for primary chondrocytes that is always limited in size and consequently in cell numbers obtained [1]. Also, chondrocytes tend to dedifferentiate when expanded in 2D culture, in vitro [1]. The use of stem cells can be very useful to overcome these issues, as they can be expanded into clinically relevant numbers and further differentiate into the chondrogenic lineage. Applying a 3D milieu is pivotal when designing a cartilage TE strategy, because chondrocytes dedifferentiation is reversible, and when these cells are further cultured in a 3D environment, they may regain their phenotype [2-5].

The most widespread tissue source of adult mesenchymal stem cells (MSCs) is the bone marrow [6, 7]. Bone marrow MSCs (BMSCs) have been proposed and applied for various tissue engineering applications, including for cartilage repair [6, 8-11]. Yet, the number of available MSCs in bone marrow [12], and the possibility of donor site morbidity in the procedure to obtain bone marrow aspirates, leads to the need to identify other MSCs sources. Human umbilical cord Wharton´s jelly has been reported as a potential alternative tissue source [13, 14], since human umbilical cord MSCs share many properties with bone marrow mesenchymal stem cells, thus supporting their applicability for cell-based therapies [15]. Human WJSCs (hWJSCs) have a higher frequency of colony forming units (CFUs-F) than hBMSCs, thus a larger number of

MSCs may be obtained in the initial isolation from Wharton´s jelly [13]. Further details on Wharton´s jelly cells as a primitive stromal cell population can be found elsewhere [16, 17]. Human WJSCs have also shown a higher proliferative potential than hBMSCs [15], being able to undergo osteogenic, chondrogenic and adipogenic differentiation [14]. Thus, those cells are prospective candidates for cartilage tissue engineering, as they display a distinct chondrogenic potential. Moreover, the improved chondrogenic potential of cells derived from the umbilical cord was confirmed in a study comparing the differentiation potential of human BMSCs and human umbilical cord stem cells (UCSCs) in 2D cultures [18].

Several strategies have been proposed to direct chondrogenesis of stem cells, which can be applied either alone or in conjunction. The referred two strategies comprise the use of specific growth factors, employing 3D structures to act as supports for cell growth and differentiation, or to co-culture progenitor cells with conditioned medium obtained from cartilage explants [19]. Cartilage is a paracrine organ, which secretes humoral factors that influence the proliferation and differentiation of cells present in the surrounding tissues [20]. Chondrogenesis and osteogenesis of MSCs during in vitro micromass culture was affected by the use of conditioned medium obtained from cultures of chondrocytes [21]. It was observed that chondrocytes secrete factors that may affect the differentiation status of stem cells, and can promote chondrogenesis and osteogenesis [21]. In indirect co-cultures with chondrocytes and embryonic stem cells the interaction of paracrine factors secreted by chondrocytes with the referred cells was observed [22]. This interaction led to the enhancement of the embryonic stem cells chondrogenic commitment [22].

The use of a porous biodegradable scaffold to support cell growth is frequently proposed for tissue engineering approaches [23-26]. Chondrocytes tend to switch into a fibroblast-like phenotype when cultured in monolayer, in tissue culture flasks. The presence of a porous structure providing a 3D environment for their culture is pivotal for the phenotype development and stability in vitro [27]. In the case of adult stem cells, the influence of 3D porous structures is relevant when considering its chondrogenic differentiation. Both the proliferation and differentiation of MSCs can be positively influenced by the culture in 3D scaffolds. It was demonstrated in the literature that mesenchymal stem cells can be attracted into a cartilage defect by the guidance of a collagenous matrix after drilling a channel in the cartilage structure until reaching the subchondral bone [28].

Our group has been investigating scaffolds produced with several of chitosan/ poly(butylene succinate) (CPBS) for cartilage tissue engineering. Those scaffolds showed good performance in supporting cell proliferation and ECM deposition [29-31]. CPBS scaffolds were reported to sustain the chondrogenic differentiation of a mouse mesenchymal progenitor cell line [30] and to promote cartilaginous ECM deposition when cultured with bovine articular chondrocytes [29, 31]. Moreover, CPBS fiber meshes were also proposed for bone-related strategies using MSCs, with positive results [23, 32]. These fiber meshes present a highly connected 3D porous structure, as well as a large surface area for cell attachment and proliferation [33]. The versatility of these scaffolds makes them very promising candidates for future osteochondral applications. Nevertheless, their suitability for cartilage TE using MSCs has not been assessed previously. We herein hypothesize that CPBS scaffolds can also support and promote human MSCs chondrogenic differentiation. This is the main reason for the selection of these scaffolds for the present work.

Co-cultures aiming the regeneration of cartilage are the subject of recent scientific interest. Several studies in the literature propose their applicability for cartilage tissue engineering, either using articular chondrocytes from different sources [34] or passages [35], stem cells and articular chondrocytes [36], or embryonic stem cells and articular chondrocytes [37]. The co-culture of sheep BMSCs with synovial cells showed the expression of chondrocytic markers [38], while the use of rat BMSCs with allogenic cartilage explants revealed a sustained expression of Sox9 in an early stage of chondrogenesis and collagen type X at a later stage, which is an undesired outcome since it indicates hypertrophy [39]. Co-culture of synovial-derived stem cells with TGF-β3 transfected articular chondrocytes resulted in significantly improved chondrogenesis of the progenitor cells [40]. The chondrogenic effect of co-culturing chondrocytes with MSCs may be influenced by the tissue of origin of stem cells [41, 42]. For instance, in a study with human adipose-derived MSCs (ASCs) co-cultures with human articular chondrocytes [41], it was demonstrated that co-cultured ASCs and chondrocytes clearly differ in their chondrogenic potential, when compared to articular chondrocytes cultured alone. The co-culture of ASCs with chondrocytes resulted in decreased chondrogenesis [41]. Conversely, in a work comparing chondrogenesis of ASCs and BMSCs seeded onto hyaluronic acid scaffolds, it was shown that chondrogenesis was more efficient using BMSCs than ASCs or chondrocytes alone [42]. In the case of WJSCs, to our best knowledge, only one report can be found in the literature referring to co-cultures of

hWJSCs with articular chondrocytes for chondrogenic differentiation [43]. Thus, we herein aimed at determining whether their previously reported enhanced chondrogenic potential when compared to BMSCs would be maintained, of even improved, in co-cultures with articular chondrocytes using a 3D scaffold. Additionally, we aimed to verify if using conditioned medium from a separate articular chondrocyte culture would improve the induction of chondrogenic differentiation of stem cells when compared with direct contact between these cells in co-culture. The possibility of using conditioned medium opens interesting possibilities for obtaining larger numbers of differentiated cells prior to implantation, in the context of its clinical application.

2. Materials and Methods

2.1. CPBS fiber meshes

The porous fiber meshes used as 3D scaffolds in the present work have been produced and successfully tested earlier by our group for bone related applications using human BMSCs [44]. A detailed description of the production methods can be found elsewhere [45]. The chitosan used for scaffolds production was supplied by France Chitin (Orange, France), with a degree of deacetylation of 85%. Bionolle™ 1050, a polybutylene succinate copolymer (MFI~50) was obtained from Showa Highpolymer Co. Ltd., Tokyo, Japan. Briefly, the CPBS fibers were obtained using a prototype single screw micro-extruder coupled to a capillary die. The extruded fibers were chopped and further loaded into a mould, which was heated above the melting temperature of the thermoplastic blend during 10 minutes. Immediately after removing the moulds from the oven, the fibers were slightly compressed by a Teflon cylinder to pack and bond the fibers. Finally, standardized scaffolds in the form of 2 mm thick discs, and with a diameter of 8 mm were obtained by cutting the fiber bonded meshes with a circular punch tool.

2.2. Isolation and expansion of hBMSCs

Human BMSCs were isolated from bone-marrow aspirates collected after obtaining informed consent from patients undergoing knee arthroplasties. Those samples were

obtained under the scope of the cooperation agreement established between the 3B´s Research Group of the University of Minho and the Orthopaedics Department of the Hospital de São Marcos, Braga, Portugal. During the surgeries, bone marrow was collected into a container with α-MEM medium, supplemented with antibiotic/ antimycotic solution and 5000 units of heparin (Sigma, H3393) and maintained in ice until the isolation procedure. Aspirates were homogenised, diluted in phosphate saline buffer – PBS (Sigma, D8537) (1:1) and incubated for 5 minutes at room temperature. Then, bone marrow was diluted in lyses buffer (1:10) and left under agitation for 10 minutes. Lyses buffer was prepared with 10 mM of Tris-HCl (Sigma, T3253), 1.21 g of Tris Base (Sigma, T1503) and 8.3 g of NH_4Cl (Merck, 1011455000), in 1L of distilled water. Afterwards, the suspension was centrifuged at 1200 rpm, for 15 minutes at room temperature. Cells were ressuspended in α-MEM medium, supplemented with antibiotic/ antimycotic solution and 20 % FBS. Cell suspension was filtered for disposal of debris, using 100 μm and 70 μm Cell Strainer (BD Falcon™, 352360 and BD Falcon™, 352350). Cells were counted and plated at the density of 4.7x103 cells/cm^2. Cells were expanded in the referred culture medium until obtaining the cell numbers needed for this study. The stemness character of the isolated hBMSCs was analysed previously by flow cytometry, (CD34 and CD45 –negative; CD29, CD44, CD73, CD90, CD105 and CD106 - positive), using a flow cytometer, and differentiation studies into osteogenic, chondrogenic, and adipogenic lineage [32].

2.3. Isolation and expansion of hWJSCs

Human umbilical cords were obtained after obtaining informed consent from full-term caesarian sections donors, under the scope of the cooperation agreement of the 3B´s Research Group of the University of Minho and the Obstetrics Department of the Hospital de São Marcos, Braga, Portugal. Human WJSCs were isolated according to the procedure originally described by Sarugaser *et al*. [13]. Briefly, umbilical cords (UC) were cut into 3–5 cm segments. These segments were then dissected by separation of the UC section epithelium along its length, exposing the underlying Wharton's jelly. Each vessel, with its surrounding Wharton's jelly matrix, was pulled away, and the ends of each dissected vessel were tied together with a suture creating "loops". Following, the loops were digested using a 1 mg/ml collagenase type I solution (Sigma, C0130) prepared with PBS. After 18–24 hours, the loops were removed. The remaining

suspension was diluted with PBS to reduce its viscosity, followed by a centrifugation at 1200 rpm for 5 minutes. Cells were ressuspended in 10 ml culture medium consisting of α-MEM medium (Invitrogen, 12000-063), supplemented with antibiotic/ antimycotic solution (Gibco, 15240062), 10% fetal bovine serum (Baptista Marques, BSC0115/0943k) and counted in a hemocytometer. Finally, cells were plated and expanded until reaching 80–90% confluence. The stemness character of these cells was previously studied by flow cytometry for MSCs markers (CD34, CD45-negative and CD29, CD73, CD90, CD105, CD166-positive cells), using a flow cytometer (FACS Calibur (BD Biosciences)), and differentiation studies into osteogenic, chondrogenic, and adipogenic lineage [46].

2.4. Isolation and expansion of human articular chondrocytes

The isolation of human articular chondrocytes (hACs) was performed according to a method previously reported in the literature [47]. HACs were isolated from human cartilage samples collected under informed consent from patients undergoing knee arthroplasties in the Orthopaedics Department of the Hospital de São Marcos, Braga, Portugal, under the scope of the previously referred agreement. During surgeries, the knee was collected into a container with PBS and maintained in ice until the isolation procedure. Cartilage was dissected in small full-depth pieces and washed twice with PBS buffer. Then, it was digested with 0.25% (w/v) of trypsin solution (Sigma, E5134) for 30 minutes at 37ºC on a rotator. The solution was removed, cartilage was washed again in PBS buffer and then incubated in a 2 mg/ml collagenase type II solution (Sigma, E0130) overnight at 37ºC on a rotator. The following day, cells were washed twice with PBS, counted and platted at a density of $2x10^6$ cells per Petri dish. Cells were cultivated with expansion medium: Dulbecco's modified Eagle's medium (Sigma, D5671), containing 10 mM Hepes buffer (Sigma, H0887), L-alanyl-L-glutamine (Sigma, G8541), Non Essential Aminoacids (Sigma, M7145), antibiotic/ antimycotic solution, 10% fetal bovine serum and 10 ng/ml of basic Fibroblast Growth Factor (bFGF) (PeproTech, 100-18B).

2.5. Co-cultures

Cells were expanded until they reached the needed number. They were then split, counted and then seeded onto the CPBS fiber meshes. Two study groups were established: direct co-culture using one source of hMSCs (either hBMSCs or hWJSCs) and hACs; indirect co-culture using conditioned medium obtained from hACs cultures. The indirect co-culture method herein described is a variation of the transwell inserts technique [22, 48]. Instead of separating the two cultures with a transwell, we cultured separately the chondrocytes and the stem cells. As explained in the introduction, it is known that the 3D structure of the scaffolds can positively influence the chondrogenic commitment of MSCs. We hypothesised that by providing the microenvironment and the soluble factors present in the chondrocytes medium we would be able to influence the chondrogenic differentiation of both types of MSCs used in the present work. Thus, as the medium provided was previously consumed by the chondrocytes, we used conditioned medium mixed (50/50 in volume) with fresh medium. By means of diluting the factors secreted by the chondrocytes in fresh medium, it was our intention to provide the required nutrients to the MSCs indirect co-cultures, avoiding the risk of nutrient deprivation.

2.5.1. Direct co-cultures

A suspension of $1x10^6$ cells ($5x10^5$ hBMSCs and $5x10^5$ hACs) per scaffold was used for seeding the fiber meshes, in a rotator inside the incubator at 37ºC and 5% CO_2, during 24 hours. Basic medium was used: Dulbecco's modified Eagle's Medium containing 10 mM Hepes solution, L–alanyl–L–glutamine, Non Essential Aminoacids, antibiotic/ antimicotic and 10% fetal bovine serum. Afterwards, constructs were transferred to new culture plates containing differentiation medium. This medium is a modification of the expansion medium: instead of adding bFGF, 1mg/ml of L-ascorbic acid (Sigma, A8960) and 50 mg/ml of insulin (Sigma, I5500) were added. Constructs were placed inside the incubator under the same conditions and cultured for 28 days. Medium was changed every other day. The same procedure was used for the direct co-cultures of hWJSCs.

2.5.2. Indirect co-cultures

Indirect co-cultures were performed using conditioned medium obtained from the chondrocytes in culture. First, a cell suspension containing $1x10^6$ cells per scaffold was prepared for each type of cell (hBMSCs or hACs). Each cell type was seeded onto different CPBS scaffolds, in different culture plates. The seeding procedure was similar to the one described above for the direct co-cultures. Afterwards, cells were placed in new culture plates containing differentiation medium. After two days, the medium was changed. Human BMSCs medium was completely removed, and replaced by the mixture of conditioned and fresh medium. It consisted of a mixture of the medium which was removed from the chondrocytes culture, plus fresh differentiation medium (50/50). Medium of these cultures (hBMSCs) was changed every other day, using the referred mixture of conditioned medium. For the hACs cultures, fresh differentiation medium was used, and changed every other day. The two types of cultures were maintained for 28 days, in an incubator, at 37ºC and 5% CO_2. The same procedure was used for the indirect co-cultures of hWJSCs.

2.6. Proliferation assay (DNA quantification)

Evaluation of cells proliferation was performed using the PicoGreen dsDNA quantification Kit (Molecular Probes, P7589, Invitrogen), according to the manufacturer protocol. Samples from every culture were collected at each time point. Triplicates of each time point were produced. A standard curve was established. Fluorescence of both samples and standard curve was read with an excitation of 485 nm and an emission of 528 nm, in a microplate reader (Synergie HT). The DNA concentration was extrapolated directly from the standard curve.

2.7. Dimethylmethylene blue (DMB) assay for glycosaminoglycans quantification

Dimethylmethylene blue assay was performed according to the method described by Kafienah and Sims, 2004 [49]. Briefly, samples were collected at 1 and 4 weeks of culture, frozen overnight and then digested. This digestion allowed separating the formed ECM from the scaffold. The digestion solution was prepared by adding 1 mg/mL of proteinase K (Sigma, P8044) diluted in 50 mM Tris-HCl, at pH of 7.6. Tris-HCl solution was prepared using the appropriate amount of Tris-HCl, adding sodium

azide (Sigma, 13412) to a final concentration of 0.05% (w/v). The pH was corrected to obtain the final value of 7.6. Samples were placed in 1.5 mL tubes and incubated with 1 mL of the referred solution, overnight at 60ºC. Afterwards, samples were centrifuged in a bench centrifuge at 13000 rpm for 10 minutes and the supernatant was collected. Solutions for this assay were prepared as follows. Dimethymethylene Blue (DMB) stock solution was prepared dissolving 16 mg of DMB powder (Aldrich, 34088) in 900 ml of distilled water containing 3.04 g of glycine (Sigma, G8898) and 2.73 g of NaCl (Sigma, S3014). This was mixed for 2 hours, covered with aluminium foil. pH was adjusted to 3.0 with HCl (Panreac, 1310202424) and a final volume of 1L. The solution was stored at room temperature covered with aluminium foil. Chondroitin sulphate (Sigma, C8529) solution was prepared in water, in a 5 mg/ml stock solution and kept refrigerated. This solution was diluted with water resulting in 5 unit increments from 0 µg/ml to 50 µg/ml, in order to make a standard curve. 20 µl of water were added to a 96 well plate, as a blank. The same quantity of chondroitin sulphate diluted solutions was added, in triplicate, and the same was performed with all the samples. DMB solution was added, 250 µl to each well, the plates were incubated for 10 minutes and then the optical density was measured in a microplate reader, at 525 nm.

2.8. RNA isolation

Samples were collected from all controls at 7 and 28 days of experiment. Samples were washed in PBS, immersed in TRIzol reagent (Invitrogen, 15596-018) and kept at -80ºC for posterior RNA extraction. When the extraction was performed, samples were taken from the freezer, in order to thaw. Chloroform (Sigma, C2432) was added; samples were vigorously agitated for 15 seconds and then incubated in ice for 15 minutes. After that incubation, samples were centrifuged at 13000 rpm, for 15 minutes, at 4ºC. Afterwards, the supernatant was collected for a sterile 1.5 mL tube, and an equivalent volume of isopropanol (Sigma, I9516) was added. Samples were incubated at -20ºC overnight, to precipitate the RNA. The next day, samples were centrifuged at 13000 rpm, for 15 minutes, at 4ºC. Then, the supernatant was taken and 800 µl of ethanol 70% was added, in order to wash away the isopropanol. This ethanol 70% solution was prepared from absolute ethanol (Merck, 1.00983.2511) and ultra pure water. Tubes were agitated vigorously and centrifuged again, at 9000 rpm for 5 minutes, at 4ºC. The supernatant was again removed, and the pellet was left to air dry. Finally,

the pellet was ressuspended in 50 µl of distilled water DNase, RNase free (Gibco, 10977-015). The concentration and purity of the extracted RNA was evaluated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA).

2.9. Real-Time PCR

The Real-Time PCR procedure used in the present work consisted of a two step fluorogenic assay using the PerfeCta™ SYBR® Green system (Quanta Biosciences, 95055-100). All reagents used in this procedure were purchased from Quanta Biosciences, following the instructions of the manufacturer. Thermocycler reaction conditions used were also the ones mentioned in the kits. In the first step, RNA was reversed transcribed into cDNA, using the qScript cDNA Synthesis Kit (95047-500). A MasterCycler EP Gradient detection system (Eppendorf, USA) was used to perform the reaction. Afterwards, the obtained cDNA was used as template for the amplification of the targets genes sohwn in Table I, with the PerfeCta™ SYBR® Green FastMix™ kit (95072-05K). Tested genes were aggrecan, collagens type I and II and Sox9. GAPDH was used as house-keeping gene, and the expression of all target genes was normalized against the GAPDH of that sample for each time point of the study. All primer sequences were generated using Primer3 software [50] and acquired from MWG Biotech AG, Germany. Table I shows the primers sequences used.

Table 7.1 – Primer sequences used for RT-PCR procedures *.

Gene	Forward (5´-3´)	Reverse (5´-3´)
AGC	TGAGTCCTCAAGCCTCCTGT	TGGTCTGCAGCAGTTGATTC
COL II	CGGTGAGAAGGGAGAAGTTG	GACCGGTCACTCCAGTAGGA
COL I	AGCCAGCAGATCGAGAACAT	ACACAGGTCTCACCGGTTTC
Sox9	TTCATGAAGATGACCGACGC	GTCCAGTCGTAGCCCTTGAG
GAPDH	ACAGTCAGCCGCATCTTCTT	ACGACCAAATCCGTTGACTC

*AGC = Agreccan; COL II = Collagen type II; COL I = Collagen type I; Sox9 = Sry-type high mobility group box 9; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

2.10. Histological analysis

Samples were collected at the end of the experiment and processed for histology. Samples were then fixed in 10% neutral buffered formalin and then dehydrated through crescent concentrations of ethanol, immersed in infiltration solutions, embedded in Technovit glycol methacrylate (kit 8100, Heraeus Kulzer, EBSciences, CO, USA) and then polymerized. Specimens were cut to obtain longitudinal sections of 7 μm of thickness, using a modified microtome equipped with a tungsten blade (Leica RM 2155). Staining solution for toluidine blue assay was prepared by adding 1% of toluidine blue (Sigma, T0394) dissolved in distilled water containing 0.5 g of sodium borate, followed by filtering. One drop of this solution was added to each section for 1 minute. Then, the sections were rinsed with distilled water and let to air dry overnight. Sections were cleared in xylene substitute (Sigma, A5597) and mounted in Histo clear (Frilabo, HS200). Safranin O staining was performed by washing slides in tap water, then immersed in 0.02% fast green solution (Fluka, 44715) for 6 minutes. Then, they were immersed in 1% acetic acid (Panreac, 131008) solution for 1 minute. After, slides were immersed in 0.1% safranin O (Fluka, 84120) solution for 10 minutes. By the end, slides were washed in tap water and let to air dry. Sections were cleared in xylene substitute and mounted as previously described.

2.11. Statistical analysis

Statistical analysis was performed using the SPSS statistic software (Release 15.0.0 for Windows). Firstly, a Shapiro-Wilk test was used to ascertain about the data normality and variance equality. The normality condition was rejected and, consequently, nonparametric tests were used in further comparisons between direct and indirect co-cultures. A Kruskal-Wallis test followed by Tukey's HSD test was applied to compare differences in between the days in culture type. P values lower than 0.001 were considered statistically significant in the analysis of the results.

3. Results

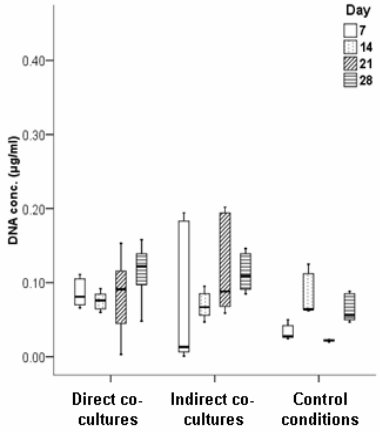
3.1. DNA quantification

The DNA concentration for each type of co-culture was assessed. Samples were collected at each time point of the experiment. We determined if there were any differences in between the days of culture, for each type of co-culture (direct or indirect), and also in the control conditions.

3.1.1. Co-cultures using hBMSCs

In terms of DNA concentration, both types of co-cultures using hBMSCs seem to follow the same trend of proliferation. DNA contents increased with time in both types of co-cultures, whereas the positive control shows a decrease at 21 days, increasing again towards the end of the experiment (Figure 7.1A). These results indicate that both types of co-cultures using hBMSCs were proliferating at a comparable rate, whereas in the control conditions (hACs) cell numbers did not increased between 14 and 21 days, recovering only in the last time point. Figure 7.1B shows the table of results for the statistical analysis concerning the differences between the various time points of the experiment, for each type of co-culture using hBMSCs. We found no significant differences between the various time points either in direct co-cultures ($p=0.172$) or indirect co-cultures ($p=0.062$), showing that the proliferation was not very strong during the experiment. The control conditions show a significant increase from the 7^{th} to the 28^{th} day of experiment, as observed in the statistical analysis results in Figure 7.1C. It is noteworthy that both the direct and indirect co-cultures show the highest levels of DNA concentration when compared to the controls in the last time point.

A



B

Days	Direct co-cultures	Indirect co-cultures	Control conditions
Day 7	0.081(0.069-0.108)	0.013 (0.005-0.188)	0.028 (0.025-0.046)
Day 14	0.076 (0.054-0.090)	0.067(0.054-0.090)	0.064(0.064-0.116)
Day 21	0.091(0.034-0.121)	0.088(0.067-0.194)	0.022(0.021-0.023)
Day 28	0.122(0.078-0.145)	0.109(0.090-0.140)	0.056(0.050-0.087)
P-value	0.172	0.062	<0.001

C

Control conditions	P-Value
Day 7 vs Day14	<0.001
Day 7 vs Day 21	<0.001
Day 7 vs Day 28	<0.001
Day 14 vs Day 21	0.046
Day 14 vs Day 28	<0.001
Day 21 vs Day 28	<0.001

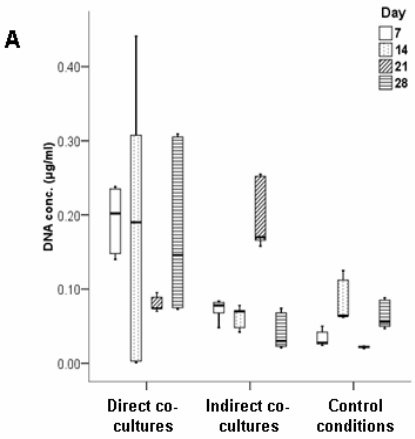
Figure 7.1 - Box plot of the DNA concentration in co-cultures using human bone marrow MSCs. A - Box plot of DNA concentration for direct and indirect co-cultures with hBMSCs and control conditions (hACs alone). B – Table with the statistical analysis results for the between days of culture differences (Kruskal-Wallis test, presented as median ± interquartile range (IQR)). C – Table with the statistical analysis results for between days differences in the control conditions (Kruskal-Wallis test followed by Tukey's HSD test.).

3.1.2. Co-cultures using hWJSCs

The trend of DNA concentration of hWJSCs co-cultures is rather different than the DNA concentration found for the co-cultures using hBMSCs. Both direct and indirect co-cultures show a decreasing tendency in the first days of culture. Then, the DNA concentration in direct co-cultures continues decreasing until the 21st day, increasing afterwards until the end of the experiment (Figure 7.2A). This result might indicate that the proliferation of the direct co-cultures with hWJSCs slowed down up to the 21st day. Conversely, indirect co-cultures with hWJSCs show an increase in DNA concentration after 14 days of culture, showing a marked decrease towards the last time point.

Comparing the various time points, we found no significant differences between the days in direct co-cultures (p=0.04) using hWJSCs (Figure 7.2B). At 21 days, indirect co-cultures using hWJSCs displayed a significantly higher DNA concentration than in the other time points (p<0.001). On the contrary, at 28 days these cultures displayed a significantly lower DNA concentration than all the other time points (p<0.001). This result seems to indicate that the proliferation is slowing down towards the end of the experiment. On the contrary, the control conditions showed a significant increase comparing the 7th and 28th day of the experiment (Figure 7.2C). The highest DNA concentration after 28 days of culture was obtained by direct co-cultures.

A



B

Days	Direct co-cultures	Indirect co-cultures	Control conditions
Day 7	0.202 (0.146-0.237)	0.078(0.075-0.083)	0.028(0.025-0.046)
Day 14	0.190(0.003-0.312)	0.070(0.047-0.720)	0.064(0.064-0.116)
Day 21	0.074(0.073-0.092)	0.170(0.165-0.252)	0.022(0.021-0.023)
Day 28	0.146(0.074-0.307)	0.030(0.023-0.069)	0.056(0.049-0.086)
P-value	0.04	<0.001	<0.001

C

Indirect co-cultures	P-Value	Control conditions	P-Value
Day 7 vs Day14	0.018	Day 7 vs Day14	<0.001
Day 7 vs Day 21	<0.001	Day 7 vs Day 21	<0.001
Day 7 vs Day 28	<0.001	Day 7 vs Day 28	<0.001
Day 14 vs Day 21	<0.001	Day 14 vs Day 21	0.046
Day 14 vs Day 28	0.033	Day 14 vs Day 28	<0.001
Day 21 vs Day 28	<0.001	Day 21 vs Day 28	<0.001

Figure 7.2 - Box plot of the DNA concentration in co-cultures using human WJSCs. A - Box plot of DNA concentration for direct and indirect co-cultures with hWJSCs and control conditions (hACs alone). B – Table with the statistical analysis results for the between days of culture differences (Kruskal-Wallis test, presented as median ± interquartile range (IQR)). C – Table with the statistical analysis results for between days differences in the indirect co-cultures and in control conditions (Kruskal-Wallis test followed by Tukey's HSD test.).

3.2. Glycosaminoglycans quantification

The GAGs content for each type of co-culture was assessed. Samples were collected at 7 and 28 days of the experiment. We compared GAGs content between the types of co-cultures (direct vs indirect). We also compared the differences between both types of co-cultures and our control condition, hACs to determine if the co-cultures were accumulating more GAGs than chondrocytes alone. Afterwards, we determined if any differences were observed between the different time points, for each type of co-culture (direct or indirect).

3.2.1. Co-cultures with hBMSCs

Both direct and indirect co-cultures with hBMSCs show a small decrease in the GAG content from 7 to 28 days of the experiment, whereas control conditions showed the expected significant increase in GAGs deposition over time (Figure 7.3A).

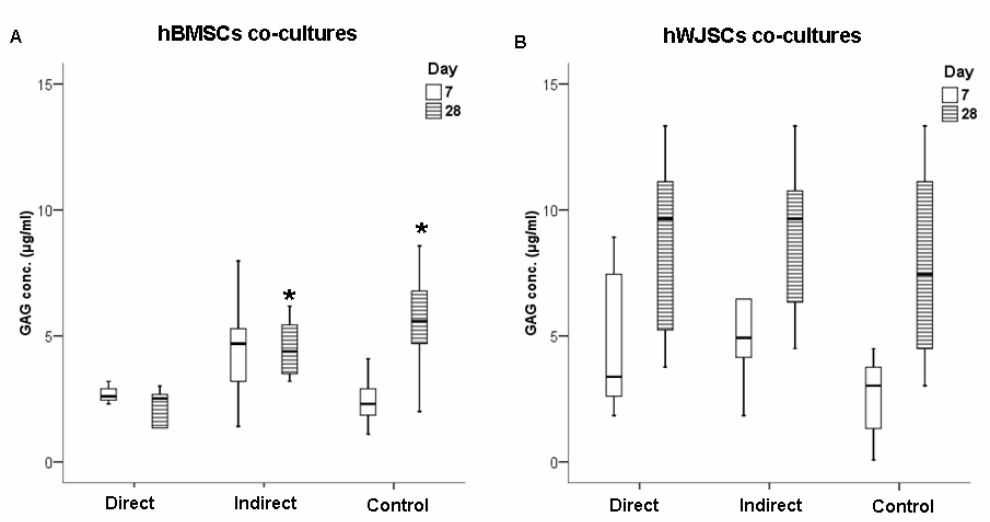


Figure 7.3 - Box plot of the GAGs concentration in both types of co-cultures, along the experiment. A - Box plot of GAGs concentration for direct and indirect co-cultures with hBMSCs and control conditions (hACs alone). B - Box plot of the GAGs concentration for direct and indirect co-cultures using hWJSCs and control conditions (hACs alone). Data were analyzed by nonparametric way of a Kruskal-Wallis test followed by Tukey's HSD test. * denotes significant differences compared to direct co-cultures.

No significant difference between direct and indirect co-cultures using hMSCs was found for 7 days of culture (p=0.061). At the end of the experiment, direct co-cultures displayed a significantly lower amount of GAGs than indirect co-cultures (p=0.006), as well as lower than the values for the control conditions (p=0.002). These results show that those co-cultures did not deposit more GAGs than the control condition. No significant differences between 7 and 28 days were observed in direct co-cultures (p=0.534) and in indirect co-cultures with hBMSCs (p=0.963).

3.2.2. Co-cultures using hWJSCs

In co-cultures using hWJSCs, a consistent trend was observed for all the conditions. Direct and indirect co-cultures, as well as the control conditions, show an increase in GAGs content along the time in culture (Figure 7.3B). For co-cultures using hWJSCs no significant difference between direct and indirect co-cultures was found after 7 days (p=0.138) nor after 28 days (p=0.733). Significant differences were found between 7 and 28 days of culture for both direct and indirect co-cultures. After 28 days, all the conditions displayed a significantly higher GAG concentration compared to 7 days ($p<0.01$), meaning that the GAGs accumulation is very alike to the control conditions, with native chondrocytes.

The GAGs concentration values obtained for all the time points for direct or indirect co-cultures with hBMSCs show consistently lower values when comparing to the ones obtained for the co-cultures with hWJSCs.

3.3. Real-Time PCR

Samples were collected for Real-Time PCR at 7 and 28 days of experiment. The aim was to determine the expression of several cartilage-related genes and to compare the differences between the expressions in both types of co-cultures, on both time points.

3.3.1. Co-cultures using hBMSCs

In both types of co-cultures using hBMSCs, no significant differences were found between the cultures for *Aggrecan* or *Sox9*, at 7 days (p=0.072 and p=0.868, respectively) or 28 days (p=0.183 and p=0.115, respectively), as shown in Figure 7.4. Direct co-cultures using hBMSCs expressed significantly higher values of Collagen *type I*, compared either to indirect co-cultures or to the control conditions ($p<0.001$),

indicating the formation of fibrocartilage in direct co-cultures. *Collagen type II* expression in indirect co-cultures of hBMSCs, displayed a significantly higher expression than direct co-cultures and control conditions ($p<0.001$) at 7 day of culture. After 28 days, direct co-cultures displayed a significantly higher *Collagen type II* expression than indirect co-cultures ($p=0.009$) and control conditions ($p=0.002$).

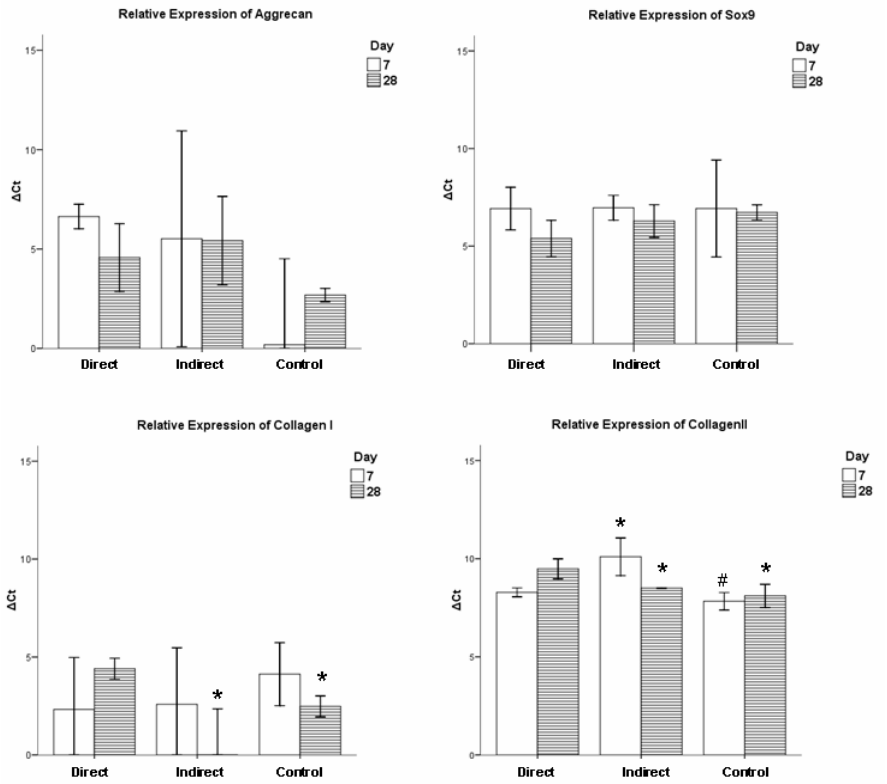


Figure 7.4 – Bar plots of chondrogenic markers present in direct and indirect co-cultures using hBMSCs, normalized for the reference gene *GAPDH*, after 7 and 28 days of culture. Data were analyzed by nonparametric way of a Kruskal-Wallis test for *Aggrecan* and *Sox9*. Data related with *Collagen type I* and *Collagen type II* were analyzed by nonparametric way of a Kruskal-Wallis test followed by Tukey's HSD test. * denotes significant differences compared to direct co-cultures, # denotes significant differences compared to indirect co-cultures.

3.3.2. Co-cultures using hWJSCs

Statistic analysis of gene expression in direct and indirect co-cultures using hWJSCs showed significant differences for almost all the genes (Figure 7.5). For *Aggrecan*, there was a significantly lower expression on the control conditions, when compared to direct and indirect co-cultures ($p<0.001$), at 7 days of culture. However, at 28 days of culture,

both types of co-cultures using hBMSCs showed a significantly higher expression of *Aggrecan* than control conditions ($p<0.001$). Additionally, indirect co-cultures also displayed a significantly higher value for *Aggrecan* expression when compared to direct co-cultures ($p=0.007$).

Indirect co-cultures also expressed significantly higher values of *Sox9* expression, at 28 days of culture, when compared to direct co-cultures and control conditions ($p<0.001$). For *Collagen type I*, no significant difference between cell type was found either at 7 ($p=0.044$) or 28 days of culture ($p=0.498$). However, the trend was to decrease its expression for the longer time periods.

Direct co-cultures using hWJSCs displayed a significantly higher expression of *Collagen type II* than indirect co-cultures, after 7 days in culture. In contrast, indirect co-cultures displayed a significantly higher *Collagen type II* expression than the control conditions ($p=0.006$), and a significant trend to increase the expression of this important gene, in contrast to other culture conditions.

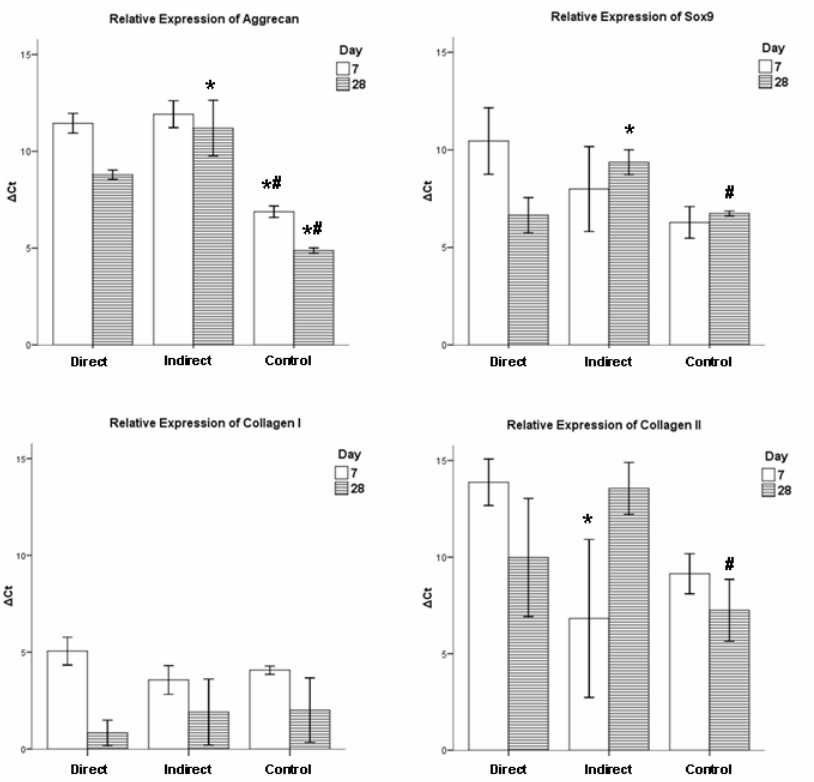


Figure 7.5 – Bar plots of chondrogenic markers present in direct and indirect co-cultures using hWJSCs, normalized for the reference gene *GAPDH*, after 7 and 28 days of culture. Data were analyzed by nonparametric way of a Kruskal-Wallis test for

Collagen type I. Data related to *Aggrecan*, *Sox9* and *Collagen type II* expressions were analyzed by nonparametric way of a Kruskal-Wallis test followed by Tukey's HSD test. * denotes significant differences compared to direct co-cultures, # denotes significant differences compared to indirect co-cultures.

3.4. Histological staining

By the end of the experiment, samples of every co-culture were collected for histological sectioning. Sections were stained for cartilagineous ECM using toluidine blue and safranin O assays (Figure 7.6). Both types of cells, either in direct or indirect co-culture, were able to produce ECM, as shown by the positive stain of toluidine blue and safranin O for ECM components. The cells were able to proliferate around the fiber meshes, and were able to grow in between the fibers inside the structure of the scaffolds. For co-cultures using hBMSCs no evident differences in terms of the quantity of cartilage ECM formation are observed in the histological staining results, either for direct or indirect co-cultures. Nevertheless, positive staining for proteoglycans can be observed either with toluidine blue or with safranin O stain. These observations are consistent with the previously obtained results for GAGs accumulation, as well as the RT-PCR results that showed the expression of cartilage related genes at the end of the experiment.

In the case of co-cultures using hWJSCs, the same observations were made. It is possible to state ECM formation both in direct and indirect co-cultures, stained with toluidine blue and safranin O. These observations are also consistent with the previously obtained results for GAGs accumulation and cartilage related genes expression at 28 days of culture.

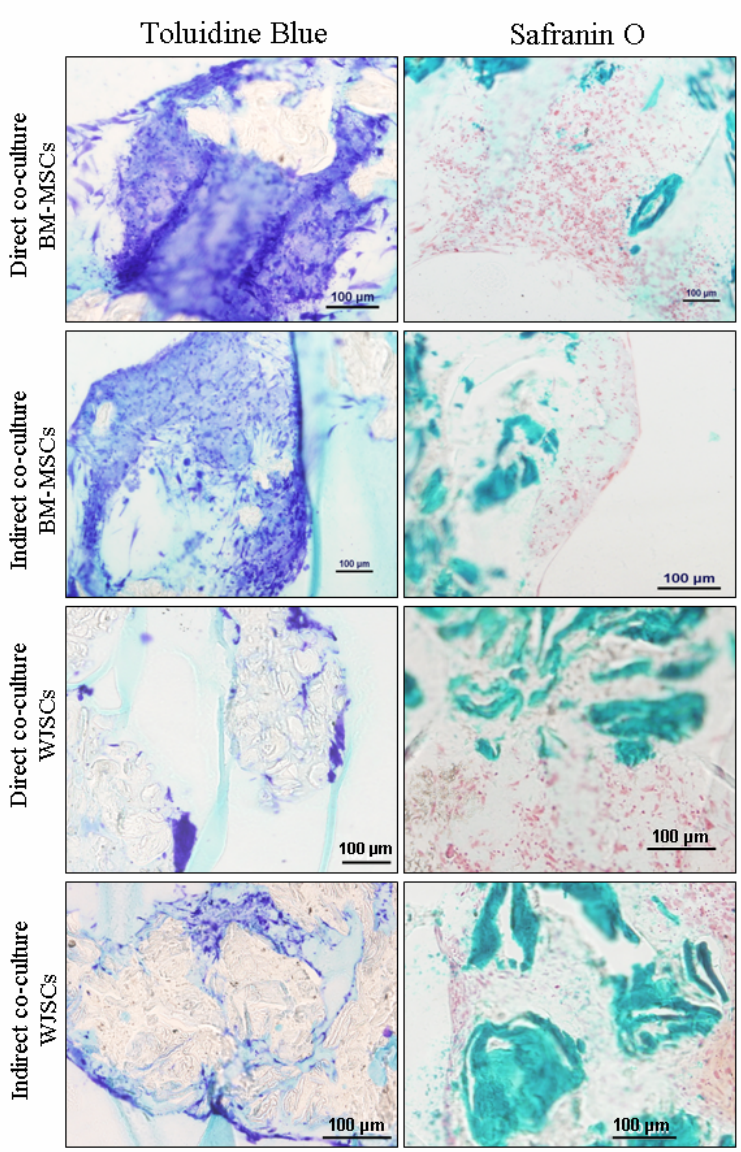


Figure 7.6 – Histological sections of direct and indirect co-cultures using human BMSCs and WJSCs, stained for ECM localisation.

4. Discussion

The use of biomaterial scaffolds to enhance ECM-cell interactions has been reported as a very important factor when using stem cells for TE [7, 42, 51]. For detailed overview on this matter, please refer to [52, 53]. The positive effect of 3D scaffolds in the chondrogenic differentiation of BMSCs [54-56] and UCSCs [32, 57] has been reported elsewhere. In the present work we showed that both hBMSCs and hWJSCs were able to undergo chondrogenic differentiation when seeded onto CPBS fiber

meshes. Polyglycolic acid (PGA) scaffolds were recently used for comparing the potential of hBMSCs and hUCSCs for cartilage tissue engineering. The authors observed enhanced chondrogenic differentiation of hUCSCs when compared to hBMSCs [7]. The chondrogenic differentiation of MSCs on chitosan fibrous scaffolds and chitosan sponges was compared, demonstrating that chondrogenesis on chitosan scaffolds is superior on microfibers, when compared to sponges [58].

Herein, chondrogenic differentiation of human BMSCs and WJSCs was observed both in direct and indirect co-cultures. The soluble signals released by articular chondrocytes, either by the direct contact or in the form of soluble factors released in the culture medium, did effectively promote chondrogenic differentiation of hBMSCs and of hWJSCs. A recent study reported similar conclusions for the chondrogenic differentiation of adipose stem cells when co-cultured with chondrocytes [59]. In that report, adipose stem cells were cultured in pellets either directly or indirectly with chondrocytes, and it was concluded that the chondrocytes secreted signals promoted chondrogenic differentiation of adipose stem cells. The co-culture systems used in the present work were able to provide MSCs with the signals and the stimulation needed for their chondrogenic differentiation. A similar result was found when using conditioned medium from chondrocytes in the chondrogenic differentiation of embryonic stem cells [48]. The authors could show the coordination of signals between the co-cultures, suggesting that chondrogenic differentiation of embryonic stem cells can be achieved by co-cultures with chondrocytes, without other exogenous growth factors being required.

No significant differences between the days in culture, in terms of proliferative ability, were found for the co-cultures using hBMSCs. Likewise, no significant differences were found for direct co-cultures using hWJSCs. However, when analysing the proliferative ability of hWJSCs in indirect co-cultures we observed significantly higher differences between the 21st day and all the other time points. Moreover, the values obtained for the DNA concentration of hWJSCs are higher than the ones obtained for the hBMSCs. The higher proliferative potential of hWJSCs compared to hBMSCs has been demonstrated earlier [15], as well as their enhanced chondrogenic potential [14]. Both types of co-cultures were able to produce and deposit GAGs. For the cultures with hBMSCs, a significantly lower GAG concentration value was found for the direct co-cultures, comparing to the indirect. For the cultures using hWJSCs, no significant differences were found in terms of GAGs concentration between direct and indirect co-cultures. These results show that indirect co-cultures may be more effective in ECM

components production, at least in terms of chondrogenic differentiation of hBMSCs, since no differences were found for hWJSCs. Our data with co-cultures of hBMSCs are similar to the results obtained in a study using passaged chondrocytes and primary bovine chondrocytes [19]. In that study, indirect co-cultures of primary and passaged chondrocytes were performed using filter inserts and it was found that the passaged cells accumulated ECM, when in contact with the conditioned medium produced by the primary chondrocytes. Although indirect co-cultures using hBMSCs seem to have an advantage in terms of GAGs accumulation, both types of co-cultures using these cells displayed a significantly lower value when compared to the chondrocytes culture (control condition). These results show that co-cultures with hBMSCs produced and accumulated less GAGs than the native chondrocytes cultures. In terms of GAGs, we can say that the hBMSCs co-cultures do not show a clear advantage over chondrocytes.

Interestingly, both direct and indirect co-cultures using hWJSCs displayed a significantly higher concentration of GAGs at 28 days of culture, compared to 7 days. The values for GAGs concentration were higher in the cultures using hWJSCs than in the cultures using hBMSCs, indicating more formation and accumulation of these important components of the cartilaginous ECM. Our results showed enhanced chondrogenesis in hWJSCs, when in indirect or direct co-cultures.

In the histological sections, it is noticeable that both cultures of hBMSCs and of hWJSCs were able to produce ECM components, namely proteoglycans. These components were stained both with toludine blue and with safranin O, showing ECM deposition. ECM accumulation was also observed in passaged human chondrocytes, when in co-cultures with bovine chondrocytes [34]. Co-cultures of equine BMSCs with equine articular chondrocytes improved the expression of cartilage related genes, and induced the production of a more homogeneous ECM within the neo-cartilage [36].

In terms of gene expression, for the cultures using hBMSCs, direct co-cultures showed significantly higher expression of *Collagen type I* than indirect co-cultures, at the end of the experiment. In terms of *Collagen type II* expression, direct co-cultures displayed a significantly higher expression at 7 days of culture than indirect co-cultures. On the other hand, towards the end of the experiment, indirect co-cultures showed a higher expression of collagen type II compared to the direct co-cultures. Again, the positive effect of using conditioned medium is evidenced by the results. For hWJSCs co-cultures, a significantly higher expression of *Aggrecan* was found for indirect co-cultures of hWJSCs at 28 days, compared to the direct ones. The same significantly

higher expression was observed for *Sox9* expression. For *Collagen type II*, there was a decrease in the last day of culture for the direct co-cultures. In this last time point, a significantly higher expression was found for the indirect co-cultures, when compared to the control conditions. Therefore, this system of co-culturing has a considerable clinic potential.

The use of conditioned medium to promote the differentiation of MSCs for further implantation is a valuable concept in terms of regenerative medicine, because it will allow obtained conditioned medium of unrelated chondrocytes to promote the differentiation of autologous stem cells. In the present work, we propose a new strategy for cartilage tissue engineering strategies based on the use of conditioned medium, and we were able to demonstrate its effect in MSCs chondrogenesis. The positive effect of the conditioned medium in osteogenesis and chondrogenesis has been recently demonstrated, using MSCs seeded onto polycaprolactone/hydroxyapatite scaffolds and on alginate, respectively [60]. In a different approach, using porcine cells for chondrogenic differentiation for intervertebral disc repair, notochordal cell conditioned medium promoted MSCs differentiation, induced significantly more collagen type II expression and GAGs production in MSCs [61]. In the present work, the soluble factors released by hACs in culture were able to induce chondrogenic differentiation of both hBMSCs and hWJSCs without the addition of growth factors. This is a very interesting result, since in a clinical perspective it is preferable to use conditioned medium for chondrogenic differentiation of MSCs than to induce it with growth factors or even with direct co-cultures. There are many cost associated with the use of growth factors, so this cost could be reduced when using conditioned medium. Indeed, this technique should be further explored, as some reports show that the use of conditioned medium is very versatile. For example, conditioned medium from a human hepatocarcinoma cell line (HepG2) enhanced in vitro chondrogenesis of murine embryonic stem cells [62]. Therefore, chondrocyte cell lines could be eventually established for conditioned medium production, to further induce chondrogenic differentiation of autologous MSCs in 3D scaffolds prior to its implantation. However, it is very important to identify, in the first place, the soluble factors that are being release into the hACs culture medium and that successfully induced the chondrogenic differentiation of hBMSCs and hWJSCs.

Indirect co-cultures of human MSCs using conditioned medium from hACs seem to be more suitable for engineered cartilage formation. The results of indirect co-cultures (either with BMSCs or with WJSCs) towards the end of the experiment were

significantly higher for all the tested parameters, namely GAGs accumulation and cartilage related-genes expression (*Collagen type II*, *Aggrecan* and *Sox9*). Concerning the type of human MSCs tested, we obtained strong evidence that hWJSCs are more promising than hBMSCs for cartilage related applications using this strategy. Additionally, more GAGs accumulation was measured again for both direct and indirect co-cultures using hWJSCs. These cultures also expressed *Aggrecan*, *Sox9* and *Collagen type II*, genes related to articular cartilage. *Collagen type I*, related to fibrotic cartilage, did not have a significant expression in these cultures with hWJSCs. This result may be indirectly related to a superior chondrogenic performance of hWJSCs when compared to hBMSCs [7].

5. Conclusions

Human adult MSCs were able to differentiate into the chondrogenic lineage, when co-cultured with hACs in CPBS scaffolds. In terms of type of culture, indirect co-cultures (using conditioned medium) seem more effective for chondrogenic differentiation. This is a very interesting result to be further explored for clinical translation. In this way, conditioned medium without the addition of other supplements or growth factors can be employed to enhance MSCs expansion and chondrogenic differentiation, prior to implantation. Indirect co-cultures using conditioned medium, using either BMSCs or WJSCs, showed significant higher values in terms of GAGs accumulation and cartilage related genes expression, when compared to direct co-cultures. In terms of cell source, hWJSCs showed higher proliferation rates and higher chondrogenic differentiation ability when compared to hBMSCs. Human WJSCs expressed significant higher values of DNA concentration, GAGs accumulation and cartilage related genes (*Aggrecan, Collagen type II* and *Sox9*). Human BMSCs chondrogenic differentiation produced fibrous cartilage, as stated by the significantly higher expression of *Collagen type I* when compared to hWJSCs.

In conclusion, indirect co-cultures of hWJSCs using conditioned medium obtained from hACs cultures are a new approach for cartilage tissue engineering that should be extensively studied for future clinical applications, as an alternative method for expanding autologous cells prior to implantation.

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References

1 - Schulze-Tanzil G. *Activation and dedifferentiation of chondrocytes: Implications in cartilage injury and repair*. Ann Anat. 2009. 191(4):325-38.

2 - Barbero A, Ploegert S, Heberer M, Martin I. *Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes*. Arthritis & Rheumatism. 2003. 48(5):1315 – 25.

3 - Brun P, Abatangelo G, Radice M, Zacchi V, Guidolin D, Gordini DD*, et al. Chondrocyte aggregation and reorganization into three-dimensional scaffolds*. J Biomed Mater Res. 1999. 46:337 – 46.

4 - Darling EM, Athanasiou KA. *Retaining zonal chondrocyte phenotype by means of novel growth environments*. Tissue Eng. 2005. 11(3-4):395-403.

5 - Miot S, Woodfield TB, Daniels AU, Suetterlin R, Peterschmitt I, Heberer M*, et al. Effects of scaffold composition and architecture on human nasal chondrocyte redifferentiation and cartilaginous matrix deposition*. Biomaterials. 2005. 26:2479 – 89.

6 - Sekiya I, Larson B, Smith JR, Pochampally R, Cui J-O, Prockop DJ. *Expansion of human adult stem cells from bone marrow stroma: Conditions that maximize the yields of early progenitors and evaluate their quality*. Stem Cells. 2002. 6:530 - 41.

7 - Wang L, Tran I, Seshareddy K, Weiss ML, Detamore MS. *A comparison of human bone marrow-derived mesenchymal stem cells and human umbilical cord-derived*

mesenchymal stromal cells for cartilage tissue engineering. Tissue Eng Part A. 2009. 15(8):2259-66.

8 - Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. *Isolation and multilineage differentiation of bovine bone marrow mesenchymal stem cells*. Cell Tissue Res. 2005. 319:243 – 53.

9 - Mauck RL, Yuan X, Tuan RS. *Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture*. Osteoarthritis Cartilage. 2006. 14(2):179-89.

10 - Hu J, Feng K, Liu X, Ma PX. *Chondrogenic and osteogenic differentiations of human bone marrow-derived mesenchymal stem cells on a nanofibrous scaffold with designed pore network*. Biomaterials. 2009. 30(28):5061-7.

11 - Markway BD, Tan GK, Brooke G, Hudson JE, Cooper-White JJ, Doran MR. *Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures*. Cell Transplant. 2010. 19(1):29-42.

12 - 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.

13 - Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. *Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors*. Stem Cells. 2005. 23(2):220-9.

14 - Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, *et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord*. Stem Cells. 2004. 22(7):1330-7.

15 - Baksh D, Yao R, Tuan RS. *Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow*. Stem Cells. 2007. 25(6):1384-92.

16 - Can A, Karahuseyinoglu S. *Concise review: Human umbilical cord stroma with regard to the source of fetus-derived stem cells*. Stem Cells. 2007. 25(11):2886-95.

17 - Troyer DL, Weiss ML. *Wharton's jelly-derived cells are a primitive stromal cell population*. Stem Cells. 2008. 26(3):591-9.

18 - Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, *et al. Biology of stem cells in human umbilical cord stroma: In situ and in vitro surveys*. Stem Cells. 2007. 25(2):319-31.

19 - Taylor DW, Ahmed N, Gan L, Gross AE, Kandel R. *Proteoglycan and collagen accumulation by passaged chondrocytes can be enhanced through side by side culture with primary chondrocytes*. Tissue Eng Part A. 2009.

20 - Bos PK, van Osch GJ, Frenz DA, Verhaar JA, Verwoerd-Verhoef HL. *Growth factor expression in cartilage wound healing: Temporal and spatial immunolocalization in a rabbit auricular cartilage wound model*. Osteoarthritis Cartilage. 2001. 9(4):382-9.

21 - Hwang NS, Varghese S, Puleo C, Zhang Z, Elisseeff J. *Morphogenetic signals from chondrocytes promote chondrogenic and osteogenic differentiation of mesenchymal stem cells*. J Cell Physiol. 2007. 212(2):281-4.

22 - Hwang NS, Varghese S, Elisseeff J. *Derivation of chondrogenically-committed cells from human embryonic cells for cartilage tissue regeneration*. PLoS ONE. 2008. 3(6):e2498.

23 - Costa-Pinto AR, Salgado AJ, Correlo VM, Sol P, Bhattacharya M, Charbord P*, et al. Adhesion, proliferation, and osteogenic differentiation of a mouse mesenchymal stem cell line (BMC9) seeded on novel melt-based chitosan/polyester 3D porous scaffolds*. Tissue Eng Part A. 2008. 14(6):1049-57.

24 - Cruz DM, Ivirico JL, Gomes MM, Ribelles JL, Sanchez MS, Reis RL*, et al. Chitosan microparticles as injectable scaffolds for tissue engineering*. J Tissue Eng Regen Med. 2008. 2(6):378-80.

25 - Gomes ME, Azevedo HS, Moreira AR, Ella V, Kellomaki M, Reis RL. *Starch-poly(epsilon-caprolactone) and starch-poly(lactic acid) fibre-mesh scaffolds for bone tissue engineering applications: Structure, mechanical properties and degradation behaviour*. J Tissue Eng Regen Med. 2008. 2(5):243-52.

26 - Pereira RC, Scaranari M, Castagnola P, Grandizio M, Azevedo HS, Reis RL*, et al. Novel injectable gel (system) as a vehicle for human articular chondrocytes in cartilage tissue regeneration*. J Tissue Eng Regen Med. 2009. 3(2):97-106.

27 - Wang Y, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL. *In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells*. Biomaterials. 2005. 26(34):7082-94.

28 - Kramer J, Bohrnsen F, Lindner U, Behrens P, Schlenke P, Rohwedel J. *In vivo matrix-guided human mesenchymal stem cells*. Cell Mol Life Sci. 2006. 63(5):616-26.

29 - Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol P, Bhattacharya M*, et al. Chitosan/polyester-based scaffolds for cartilage tissue engineering: Assessment of extracellular matrix formation*. Acta Biomater. 2010. 6(3):1149-57.

30 - Oliveira J, Correlo VM, Sol P, Costa-Pinto A, Salgado A, Bhattacharya M*, et al. Assessment of the suitability of chitosan/polybutylene succinate scaffolds seeded with mouse mesenchymal progenitor cells for a cartilage tissue engineering approach* Tissue Eng. 2008. 14(10):1651-61.

31 - Oliveira JT, Crawford A, Mundy JL, Sol PC, Correlo VM, Bhattacharya M*, et al. Novel melt-processable chitosan-polybutylene succinate fibre scaffolds for cartilage tissue engineering*. J Biomater Sci Polym Ed - In press. 2010.

32 - Costa-Pinto AR, Frias AM, Alves da Silva ML, Fernandes S, Barros A, Reis RL*, et al. Comparative study of the multidifferentiation potential of human Wharton´s jelly and amniotic fluid derived stem cells*. Tissue Eng Part A. 2008. 14:713.

33 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, Neves NM*, et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromol Biosci. 2010.

34 - Ahmed N, Taylor DW, Wunder J, Nagy A, Gross AE, Kandel RA. *Passaged human chondrocytes accumulate extracellular matrix when induced by bovine chondrocytes*. J Tissue Eng Regen Med. 2010. 4(3):233-41.

35 - Ahmed N, Gan L, Nagy A, Zheng J, Wang C, Kandel RA. *Cartilage tissue formation using redifferentiated passaged chondrocytes in vitro*. Tissue Eng Part A. 2009. 15(3):665-73.

36 - Lettry V, Hosoya K, Takagi S, Okumura M. *Coculture of equine mesenchymal stem cells and mature equine articular chondrocytes results in improved chondrogenic differentiation of the stem cells*. Jpn J Vet Res. 2010. 58(1):5-15.

37 - Bigdeli N, Karlsson C, Strehl R, Concaro S, Hyllner J, Lindahl A. *Coculture of human embryonic stem cells and human articular chondrocytes results in significantly altered phenotype and improved chondrogenic differentiation*. Stem Cells. 2009. 27(8):1812-21.

38 - Chen J, Wang C, Lü S, Wu J, Guo X, Duan C*, et al. In vivo chondrogenesis of adult bone-marrow-derived autologous mesenchymal stem cells*. Cell Tissue Res. 2005. 319:429 – 38.

39 - Ahmed N, Dreier R, Gopferich A, Grifka J, Grassel S. *Soluble signalling factors derived from differentiated cartilage tissue affect chondrogenic differentiation of rat adult marrow stromal cells*. Cell Physiol Biochem. 2007. 20(5):665-78.

40 - Varshney RR, Zhou R, Hao J, Yeo SS, Chooi WH, Fan J*, et al. Chondrogenesis of synovium-derived mesenchymal stem cells in gene-transferred co-culture system*. Biomaterials. 2010. 31(26):6876-91.

41 - Hildner F, Concaro S, Peterbauer A, Wolbank S, Danzer M, Lindahl A*, et al. Human adipose-derived stem cells contribute to chondrogenesis in coculture with human articular chondrocytes*. Tissue Eng Part A. 2009. 15(12):3961-9.

42 - Jakobsen RB, Shahdadfar A, Reinholt FP, Brinchmann JE. *Chondrogenesis in a hyaluronic acid scaffold: Comparison between chondrocytes and msc from bone marrow and adipose tissue*. Knee Surg Sports Traumatol Arthrosc. 2009.

43 - Pereira RC, Costa-Pinto AR, Frias AM, N.M. N, Azevedo HS, Reis RL. *Co-culture of human Wharton jelly stem cells with human articular chondrocytes provides a suitable environment for their chondrogenic commitment*. 5th International Annual Meeting of the Portuguese Society for Stem Cells and Cellular Therapy (SPCE-TC); 2010; Avepark, Caldas das Taipas , Guimarães; 2010. p. 19-20.

44 - Costa-Pinto AR, Correlo VM, Sol PC, Bhattacharya M, Charbord P, Delorme B*, et al. Osteogenic differentiation of human bone marrow mesenchymal stem cells seeded on melt based chitosan scaffolds for bone tissue engineering applications*. Biomacromolecules. 2009. 10(8):2067-73.

45 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, N.M. N*, et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromolecular Bioscience. 2010. accepted for publication.

46 - Costa-Pinto A. R., Frias AM, Alves da Silva ML, Barros A., Fernandes S., RL R*, et al. Comparative study of human Wharton's jelly and amniotic fluid derived stem cells differentiation potential*. Tissue Engineering: . 2008. 14:713.

47 - Crawford A, Dickinson S. *Chondrocyte isolation, expansion, and culture in polymer scaffolds.* In: Hollander AP, Hatton PV, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 147-57.

48 - Vats A, Bielby RC, Tolley N, Dickinson SC, Boccaccini AR, Hollander AP*, et al. Chondrogenic differentiation of human embryonic stem cells: The effect of the micro-environment*. Tissue Eng. 2006. 12(6):1687-97.

49 - Kafienah W, Sims TS. *Biochemical methods for the analysis of tissue engineered cartilage.* In: Hollander Ap, Hatton Pv, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 217 - 29.

50 - http://frodo.wi.mit.edu/.

51 - Mahmoudifar N, Doran PM. *Chondrogenic differentiation of human adipose-derived stem cells in polyglycolic acid mesh scaffolds under dynamic culture conditions.* Biomaterials. 2010. 31(14):3858-67.

52 - Chai C, Leong KW. *Biomaterials approach to expand and direct differentiation of stem cells*. Mol Ther. 2007. 15(3):467-80.

53 - Vinatier C, Mrugala D, Jorgensen C, Guicheux J, Noel D. *Cartilage engineering: A crucial combination of cells, biomaterials and biofactors*. Trends Biotechnol. 2009. 27(5):307-14.

54 - Kafienah W, Mistry S, Dickinson SC, Sims TJ, Learmonth I, Hollander AP. *Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients*. Arthritis Rheum. 2007. 56(1):177-87.

55 - Kim HJ, Lee JH, Im GI. *Chondrogenesis using mesenchymal stem cells and PCL scaffolds*. J Biomed Mater Res A. 2010. 92(2):659-66.

56 - Shao X, Hutmacher DW, Ho ST, Goh CH, Lee EH. *Evaluation of a hybrid scaffold/cell construct in repair of high-load-bearing osteochondral defects in rabbits.* Biomaterials. 2006. 27(7):1071 - 80.

57 - Wang L, Sheshareddy K, Weiss ML, Detamore MS. *Effect of initial seeding density on human umbilical cord mesenchymal stromal cells for fibrocartilage tissue engineering*. Tissue Eng Part A. 2009. 15(5):1009-17.

58 - Ragetly GR, Griffon DJ, Lee HB, Fredericks LP, Gordon-Evans W, Chung YS. *Effect of chitosan scaffold microstructure on mesenchymal stem cell chondrogenesis*. Acta Biomater. 2010. 6(4):1430-6.

59 - Lee JS, Im GI. *Influence of chondrocytes on the chondrogenic differentiation of adipose stem cells*. Tissue Eng Part A. 2010.

60 - Maxson S, Burg KJ. *Conditioned media enhance osteogenic differentiation on poly(l-lactide-co-epsilon-caprolactone)/hydroxyapatite scaffolds and chondrogenic differentiation in alginate*. J Biomater Sci Polym Ed. 2010. 21(11):1441-58.

61 - Korecki CL, Taboas JM, Tuan RS, Iatridis JC. *Notochordal cell conditioned medium stimulates mesenchymal stem cell differentiation toward a young nucleus pulposus phenotype*. Stem Cell Res Ther. 2010. 1(2):18.

62 - Hwang Y-S, Bishop AE, Polak JM, Mantalaris A. *Enhanced in vitro chondrogenesis of murine embryonic stem cells*. biotechnology and Bioprocesses Engineering. 2007. 12:696-706.

Chapter 7

Co-culture of human articular chondrocytes with two different sources of human mesenchymal stem cells as a new strategy for cartilage tissue engineering

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Abstract

Chondrocytes are mainly responsible for maintaining the integrity of the extracellular matrix of articular cartilage and for the protection of the underlying subchondral bone. Mesenchymal Stem Cells (MSCs) are also believed to contribute to the maintenance of cartilage. Moreover, these cells have been recognized for their ability to differentiate into cells of different tissues such as bone, cartilage or adipose tissue, and therefore might be of great interest for developing cartilage therapeutic strategies. These cells are typically induced to differentiate by growth factors supplementation in the culture medium that will trigger differentiation into the desired cell phenotype.

One frequent problem that occurs in cartilage tissue engineering is that expanded chondrocytes undergo dedifferentiation when in 2D culture. This process leads frequently to the undesired production of fibrocartilage, and is associated with the loss of their phenotype in vitro. The use of an additional cell source to enhance cell numbers, not loosing the differentiation potential upon expansion in vitro may be an original choice. Moreover, soluble factors released by chondrocytes have been shown to influence stem cells differentiation onto the chondrogenic lineage. The use of conditioned medium obtained from chondrocytes for stimulating stem cells chondrogenic differentiation may be a very interesting alternative for moving into the clinical application of these cells.

In the present work, direct contact co-cultures and indirect co-cultures (using conditioned medium obtained from a culture of human articular chondrocytes) of human bone marrow-derived MSCs (hBMSCs) and human Wharton´s jelly MSCs (hWJSCs) were established. Cells were isolated from human samples collected at a local hospital, under donors' informed consent. The co-cultures were performed in previously produced 3D scaffolds, composed by a blend of 50/50 chitosan and poly (butylene succinate) – CPBS. Co-cultures were maintained during 28 days. Both types of stem cells were able to undergo chondrogenic differentiation, either in direct or indirect co-cultures. By the end of the experiment, the indirect co-cultures (using conditioned medium) showed significantly higher values in terms of DNA content, glycosaminoglycans (GAGs) accumulation and specific cartilage-related genes expression, when compared to direct co-cultures, for both types of adult MSCs tested. Human BMSCs resulted in fibrous cartilage, as it can be concluded by the significantly higher expression of *Collagen type I*. The hWJSCs showed higher chondrogenic

differentiation ability when compared to hBMSCs, as denoted by the higher values for GAGs accumulation and cartilage related genes expression.

Therefore, we were able to demonstrate the high chondrogenic potential of hWJSCs, and proved that co-cultures of articular chondrocytes with these cells are able to produce more cartilaginous ECM, when compared to co-cultures with hBMSCs. The use of conditioned medium obtained from articular chondrocytes induced MSCs chondrogenic differentiation and ECM formation. The obtained results showed that this new strategy is very interesting and should be further explored for clinical applications.

1. Introduction

Research concerning cartilage regeneration is of special importance, since this tissue lacks self-repairing capacity and causes a huge social and economic impact. Several cell types have been proposed for cartilage tissue engineering, as for example the direct use of autologous chondrocytes. However, this method is not very convenient, since it involves a biopsy used as cell source for primary chondrocytes that is always limited in size and consequently in cell numbers obtained [1]. Also, chondrocytes tend to dedifferentiate when expanded in 2D culture, in vitro [1]. The use of stem cells can be very useful to overcome these issues, as they can be expanded into clinically relevant numbers and further differentiate into the chondrogenic lineage. Applying a 3D milieu is pivotal when designing a cartilage TE strategy, because chondrocytes dedifferentiation is reversible, and when these cells are further cultured in a 3D environment, they may regain their phenotype [2-5].

The most widespread tissue source of adult mesenchymal stem cells (MSCs) is the bone marrow [6, 7]. Bone marrow MSCs (BMSCs) have been proposed and applied for various tissue engineering applications, including for cartilage repair [6, 8-11]. Yet, the number of available MSCs in bone marrow [12], and the possibility of donor site morbidity in the procedure to obtain bone marrow aspirates, leads to the need to identify other MSCs sources. Human umbilical cord Wharton´s jelly has been reported as a potential alternative tissue source [13, 14], since human umbilical cord MSCs share many properties with bone marrow mesenchymal stem cells, thus supporting their applicability for cell-based therapies [15]. Human WJSCs (hWJSCs) have a higher frequency of colony forming units (CFUs-F) than hBMSCs, thus a larger number of

MSCs may be obtained in the initial isolation from Wharton´s jelly [13]. Further details on Wharton´s jelly cells as a primitive stromal cell population can be found elsewhere [16, 17]. Human WJSCs have also shown a higher proliferative potential than hBMSCs [15], being able to undergo osteogenic, chondrogenic and adipogenic differentiation [14]. Thus, those cells are prospective candidates for cartilage tissue engineering, as they display a distinct chondrogenic potential. Moreover, the improved chondrogenic potential of cells derived from the umbilical cord was confirmed in a study comparing the differentiation potential of human BMSCs and human umbilical cord stem cells (UCSCs) in 2D cultures [18].

Several strategies have been proposed to direct chondrogenesis of stem cells, which can be applied either alone or in conjunction. The referred two strategies comprise the use of specific growth factors, employing 3D structures to act as supports for cell growth and differentiation, or to co-culture progenitor cells with conditioned medium obtained from cartilage explants [19]. Cartilage is a paracrine organ, which secretes humoral factors that influence the proliferation and differentiation of cells present in the surrounding tissues [20]. Chondrogenesis and osteogenesis of MSCs during in vitro micromass culture was affected by the use of conditioned medium obtained from cultures of chondrocytes [21]. It was observed that chondrocytes secrete factors that may affect the differentiation status of stem cells, and can promote chondrogenesis and osteogenesis [21]. In indirect co-cultures with chondrocytes and embryonic stem cells the interaction of paracrine factors secreted by chondrocytes with the referred cells was observed [22]. This interaction led to the enhancement of the embryonic stem cells chondrogenic commitment [22].

The use of a porous biodegradable scaffold to support cell growth is frequently proposed for tissue engineering approaches [23-26]. Chondrocytes tend to switch into a fibroblast-like phenotype when cultured in monolayer, in tissue culture flasks. The presence of a porous structure providing a 3D environment for their culture is pivotal for the phenotype development and stability in vitro [27]. In the case of adult stem cells, the influence of 3D porous structures is relevant when considering its chondrogenic differentiation. Both the proliferation and differentiation of MSCs can be positively influenced by the culture in 3D scaffolds. It was demonstrated in the literature that mesenchymal stem cells can be attracted into a cartilage defect by the guidance of a collagenous matrix after drilling a channel in the cartilage structure until reaching the subchondral bone [28].

Our group has been investigating scaffolds produced with several of chitosan/ poly(butylene succinate) (CPBS) for cartilage tissue engineering. Those scaffolds showed good performance in supporting cell proliferation and ECM deposition [29-31]. CPBS scaffolds were reported to sustain the chondrogenic differentiation of a mouse mesenchymal progenitor cell line [30] and to promote cartilaginous ECM deposition when cultured with bovine articular chondrocytes [29, 31]. Moreover, CPBS fiber meshes were also proposed for bone-related strategies using MSCs, with positive results [23, 32]. These fiber meshes present a highly connected 3D porous structure, as well as a large surface area for cell attachment and proliferation [33]. The versatility of these scaffolds makes them very promising candidates for future osteochondral applications. Nevertheless, their suitability for cartilage TE using MSCs has not been assessed previously. We herein hypothesize that CPBS scaffolds can also support and promote human MSCs chondrogenic differentiation. This is the main reason for the selection of these scaffolds for the present work.

Co-cultures aiming the regeneration of cartilage are the subject of recent scientific interest. Several studies in the literature propose their applicability for cartilage tissue engineering, either using articular chondrocytes from different sources [34] or passages [35], stem cells and articular chondrocytes [36], or embryonic stem cells and articular chondrocytes [37]. The co-culture of sheep BMSCs with synovial cells showed the expression of chondrocytic markers [38], while the use of rat BMSCs with allogenic cartilage explants revealed a sustained expression of Sox9 in an early stage of chondrogenesis and collagen type X at a later stage, which is an undesired outcome since it indicates hypertrophy [39]. Co-culture of synovial-derived stem cells with TGF-β3 transfected articular chondrocytes resulted in significantly improved chondrogenesis of the progenitor cells [40]. The chondrogenic effect of co-culturing chondrocytes with MSCs may be influenced by the tissue of origin of stem cells [41, 42]. For instance, in a study with human adipose-derived MSCs (ASCs) co-cultures with human articular chondrocytes [41], it was demonstrated that co-cultured ASCs and chondrocytes clearly differ in their chondrogenic potential, when compared to articular chondrocytes cultured alone. The co-culture of ASCs with chondrocytes resulted in decreased chondrogenesis [41]. Conversely, in a work comparing chondrogenesis of ASCs and BMSCs seeded onto hyaluronic acid scaffolds, it was shown that chondrogenesis was more efficient using BMSCs than ASCs or chondrocytes alone [42]. In the case of WJSCs, to our best knowledge, only one report can be found in the literature referring to co-cultures of

hWJSCs with articular chondrocytes for chondrogenic differentiation [43]. Thus, we herein aimed at determining whether their previously reported enhanced chondrogenic potential when compared to BMSCs would be maintained, of even improved, in co-cultures with articular chondrocytes using a 3D scaffold. Additionally, we aimed to verify if using conditioned medium from a separate articular chondrocyte culture would improve the induction of chondrogenic differentiation of stem cells when compared with direct contact between these cells in co-culture. The possibility of using conditioned medium opens interesting possibilities for obtaining larger numbers of differentiated cells prior to implantation, in the context of its clinical application.

2. Materials and Methods

2.1. CPBS fiber meshes

The porous fiber meshes used as 3D scaffolds in the present work have been produced and successfully tested earlier by our group for bone related applications using human BMSCs [44]. A detailed description of the production methods can be found elsewhere [45]. The chitosan used for scaffolds production was supplied by France Chitin (Orange, France), with a degree of deacetylation of 85%. Bionolle™ 1050, a polybutylene succinate copolymer (MFI~50) was obtained from Showa Highpolymer Co. Ltd., Tokyo, Japan. Briefly, the CPBS fibers were obtained using a prototype single screw micro-extruder coupled to a capillary die. The extruded fibers were chopped and further loaded into a mould, which was heated above the melting temperature of the thermoplastic blend during 10 minutes. Immediately after removing the moulds from the oven, the fibers were slightly compressed by a Teflon cylinder to pack and bond the fibers. Finally, standardized scaffolds in the form of 2 mm thick discs, and with a diameter of 8 mm were obtained by cutting the fiber bonded meshes with a circular punch tool.

2.2. Isolation and expansion of hBMSCs

Human BMSCs were isolated from bone-marrow aspirates collected after obtaining informed consent from patients undergoing knee arthroplasties. Those samples were

obtained under the scope of the cooperation agreement established between the 3B´s Research Group of the University of Minho and the Orthopaedics Department of the Hospital de São Marcos, Braga, Portugal. During the surgeries, bone marrow was collected into a container with α-MEM medium, supplemented with antibiotic/ antimycotic solution and 5000 units of heparin (Sigma, H3393) and maintained in ice until the isolation procedure. Aspirates were homogenised, diluted in phosphate saline buffer – PBS (Sigma, D8537) (1:1) and incubated for 5 minutes at room temperature. Then, bone marrow was diluted in lyses buffer (1:10) and left under agitation for 10 minutes. Lyses buffer was prepared with 10 mM of Tris-HCl (Sigma, T3253), 1.21 g of Tris Base (Sigma, T1503) and 8.3 g of NH_4Cl (Merck, 1011455000), in 1L of distilled water. Afterwards, the suspension was centrifuged at 1200 rpm, for 15 minutes at room temperature. Cells were ressuspended in α-MEM medium, supplemented with antibiotic/ antimycotic solution and 20 % FBS. Cell suspension was filtered for disposal of debris, using 100 μm and 70 μm Cell Strainer (BD Falcon™, 352360 and BD Falcon™, 352350). Cells were counted and plated at the density of 4.7x103 cells/cm^2. Cells were expanded in the referred culture medium until obtaining the cell numbers needed for this study. The stemness character of the isolated hBMSCs was analysed previously by flow cytometry, (CD34 and CD45 –negative; CD29, CD44, CD73, CD90, CD105 and CD106 - positive), using a flow cytometer, and differentiation studies into osteogenic, chondrogenic, and adipogenic lineage [32].

2.3. Isolation and expansion of hWJSCs

Human umbilical cords were obtained after obtaining informed consent from full-term caesarian sections donors, under the scope of the cooperation agreement of the 3B´s Research Group of the University of Minho and the Obstetrics Department of the Hospital de São Marcos, Braga, Portugal. Human WJSCs were isolated according to the procedure originally described by Sarugaser *et al*. [13]. Briefly, umbilical cords (UC) were cut into 3–5 cm segments. These segments were then dissected by separation of the UC section epithelium along its length, exposing the underlying Wharton's jelly. Each vessel, with its surrounding Wharton's jelly matrix, was pulled away, and the ends of each dissected vessel were tied together with a suture creating "loops". Following, the loops were digested using a 1 mg/ml collagenase type I solution (Sigma, C0130) prepared with PBS. After 18–24 hours, the loops were removed. The remaining

suspension was diluted with PBS to reduce its viscosity, followed by a centrifugation at 1200 rpm for 5 minutes. Cells were ressuspended in 10 ml culture medium consisting of α-MEM medium (Invitrogen, 12000-063), supplemented with antibiotic/ antimycotic solution (Gibco, 15240062), 10% fetal bovine serum (Baptista Marques, BSC0115/0943k) and counted in a hemocytometer. Finally, cells were plated and expanded until reaching 80–90% confluence. The stemness character of these cells was previously studied by flow cytometry for MSCs markers (CD34, CD45-negative and CD29, CD73, CD90, CD105, CD166-positive cells), using a flow cytometer (FACS Calibur (BD Biosciences)), and differentiation studies into osteogenic, chondrogenic, and adipogenic lineage [46].

2.4. Isolation and expansion of human articular chondrocytes

The isolation of human articular chondrocytes (hACs) was performed according to a method previously reported in the literature [47]. HACs were isolated from human cartilage samples collected under informed consent from patients undergoing knee arthroplasties in the Orthopaedics Department of the Hospital de São Marcos, Braga, Portugal, under the scope of the previously referred agreement. During surgeries, the knee was collected into a container with PBS and maintained in ice until the isolation procedure. Cartilage was dissected in small full-depth pieces and washed twice with PBS buffer. Then, it was digested with 0.25% (w/v) of trypsin solution (Sigma, E5134) for 30 minutes at 37ºC on a rotator. The solution was removed, cartilage was washed again in PBS buffer and then incubated in a 2 mg/ml collagenase type II solution (Sigma, E0130) overnight at 37ºC on a rotator. The following day, cells were washed twice with PBS, counted and platted at a density of $2x10^6$ cells per Petri dish. Cells were cultivated with expansion medium: Dulbecco's modified Eagle's medium (Sigma, D5671), containing 10 mM Hepes buffer (Sigma, H0887), L-alanyl-L-glutamine (Sigma, G8541), Non Essential Aminoacids (Sigma, M7145), antibiotic/ antimycotic solution, 10% fetal bovine serum and 10 ng/ml of basic Fibroblast Growth Factor (bFGF) (PeproTech, 100-18B).

2.5. Co-cultures

Cells were expanded until they reached the needed number. They were then split, counted and then seeded onto the CPBS fiber meshes. Two study groups were established: direct co-culture using one source of hMSCs (either hBMSCs or hWJSCs) and hACs; indirect co-culture using conditioned medium obtained from hACs cultures. The indirect co-culture method herein described is a variation of the transwell inserts technique [22, 48]. Instead of separating the two cultures with a transwell, we cultured separately the chondrocytes and the stem cells. As explained in the introduction, it is known that the 3D structure of the scaffolds can positively influence the chondrogenic commitment of MSCs. We hypothesised that by providing the microenvironment and the soluble factors present in the chondrocytes medium we would be able to influence the chondrogenic differentiation of both types of MSCs used in the present work. Thus, as the medium provided was previously consumed by the chondrocytes, we used conditioned medium mixed (50/50 in volume) with fresh medium. By means of diluting the factors secreted by the chondrocytes in fresh medium, it was our intention to provide the required nutrients to the MSCs indirect co-cultures, avoiding the risk of nutrient deprivation.

2.5.1. Direct co-cultures

A suspension of $1x10^6$ cells ($5x10^5$ hBMSCs and $5x10^5$ hACs) per scaffold was used for seeding the fiber meshes, in a rotator inside the incubator at 37ºC and 5% CO_2, during 24 hours. Basic medium was used: Dulbecco's modified Eagle's Medium containing 10 mM Hepes solution, L–alanyl–L–glutamine, Non Essential Aminoacids, antibiotic/ antimicotic and 10% fetal bovine serum. Afterwards, constructs were transferred to new culture plates containing differentiation medium. This medium is a modification of the expansion medium: instead of adding bFGF, 1mg/ml of L-ascorbic acid (Sigma, A8960) and 50 mg/ml of insulin (Sigma, I5500) were added. Constructs were placed inside the incubator under the same conditions and cultured for 28 days. Medium was changed every other day. The same procedure was used for the direct co-cultures of hWJSCs.

2.5.2. Indirect co-cultures

Indirect co-cultures were performed using conditioned medium obtained from the chondrocytes in culture. First, a cell suspension containing $1x10^6$ cells per scaffold was prepared for each type of cell (hBMSCs or hACs). Each cell type was seeded onto different CPBS scaffolds, in different culture plates. The seeding procedure was similar to the one described above for the direct co-cultures. Afterwards, cells were placed in new culture plates containing differentiation medium. After two days, the medium was changed. Human BMSCs medium was completely removed, and replaced by the mixture of conditioned and fresh medium. It consisted of a mixture of the medium which was removed from the chondrocytes culture, plus fresh differentiation medium (50/50). Medium of these cultures (hBMSCs) was changed every other day, using the referred mixture of conditioned medium. For the hACs cultures, fresh differentiation medium was used, and changed every other day. The two types of cultures were maintained for 28 days, in an incubator, at 37ºC and 5% CO_2. The same procedure was used for the indirect co-cultures of hWJSCs.

2.6. Proliferation assay (DNA quantification)

Evaluation of cells proliferation was performed using the PicoGreen dsDNA quantification Kit (Molecular Probes, P7589, Invitrogen), according to the manufacturer protocol. Samples from every culture were collected at each time point. Triplicates of each time point were produced. A standard curve was established. Fluorescence of both samples and standard curve was read with an excitation of 485 nm and an emission of 528 nm, in a microplate reader (Synergie HT). The DNA concentration was extrapolated directly from the standard curve.

2.7. Dimethylmethylene blue (DMB) assay for glycosaminoglycans quantification

Dimethylmethylene blue assay was performed according to the method described by Kafienah and Sims, 2004 [49]. Briefly, samples were collected at 1 and 4 weeks of culture, frozen overnight and then digested. This digestion allowed separating the formed ECM from the scaffold. The digestion solution was prepared by adding 1 mg/mL of proteinase K (Sigma, P8044) diluted in 50 mM Tris-HCl, at pH of 7.6. Tris-HCl solution was prepared using the appropriate amount of Tris-HCl, adding sodium

azide (Sigma, 13412) to a final concentration of 0.05% (w/v). The pH was corrected to obtain the final value of 7.6. Samples were placed in 1.5 mL tubes and incubated with 1 mL of the referred solution, overnight at 60ºC. Afterwards, samples were centrifuged in a bench centrifuge at 13000 rpm for 10 minutes and the supernatant was collected. Solutions for this assay were prepared as follows. Dimethymethylene Blue (DMB) stock solution was prepared dissolving 16 mg of DMB powder (Aldrich, 34088) in 900 ml of distilled water containing 3.04 g of glycine (Sigma, G8898) and 2.73 g of NaCl (Sigma, S3014). This was mixed for 2 hours, covered with aluminium foil. pH was adjusted to 3.0 with HCl (Panreac, 1310202424) and a final volume of 1L. The solution was stored at room temperature covered with aluminium foil. Chondroitin sulphate (Sigma, C8529) solution was prepared in water, in a 5 mg/ml stock solution and kept refrigerated. This solution was diluted with water resulting in 5 unit increments from 0 µg/ml to 50 µg/ml, in order to make a standard curve. 20 µl of water were added to a 96 well plate, as a blank. The same quantity of chondroitin sulphate diluted solutions was added, in triplicate, and the same was performed with all the samples. DMB solution was added, 250 µl to each well, the plates were incubated for 10 minutes and then the optical density was measured in a microplate reader, at 525 nm.

2.8. RNA isolation

Samples were collected from all controls at 7 and 28 days of experiment. Samples were washed in PBS, immersed in TRIzol reagent (Invitrogen, 15596-018) and kept at -80ºC for posterior RNA extraction. When the extraction was performed, samples were taken from the freezer, in order to thaw. Chloroform (Sigma, C2432) was added; samples were vigorously agitated for 15 seconds and then incubated in ice for 15 minutes. After that incubation, samples were centrifuged at 13000 rpm, for 15 minutes, at 4ºC. Afterwards, the supernatant was collected for a sterile 1.5 mL tube, and an equivalent volume of isopropanol (Sigma, I9516) was added. Samples were incubated at -20ºC overnight, to precipitate the RNA. The next day, samples were centrifuged at 13000 rpm, for 15 minutes, at 4ºC. Then, the supernatant was taken and 800 µl of ethanol 70% was added, in order to wash away the isopropanol. This ethanol 70% solution was prepared from absolute ethanol (Merck, 1.00983.2511) and ultra pure water. Tubes were agitated vigorously and centrifuged again, at 9000 rpm for 5 minutes, at 4ºC. The supernatant was again removed, and the pellet was left to air dry. Finally,

the pellet was ressuspended in 50 µl of distilled water DNase, RNase free (Gibco, 10977-015). The concentration and purity of the extracted RNA was evaluated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA).

2.9. Real-Time PCR

The Real-Time PCR procedure used in the present work consisted of a two step fluorogenic assay using the PerfeCta™ SYBR® Green system (Quanta Biosciences, 95055-100). All reagents used in this procedure were purchased from Quanta Biosciences, following the instructions of the manufacturer. Thermocycler reaction conditions used were also the ones mentioned in the kits. In the first step, RNA was reversed transcribed into cDNA, using the qScript cDNA Synthesis Kit (95047-500). A MasterCycler EP Gradient detection system (Eppendorf, USA) was used to perform the reaction. Afterwards, the obtained cDNA was used as template for the amplification of the targets genes sohwn in Table I, with the PerfeCta™ SYBR® Green FastMix™ kit (95072-05K). Tested genes were aggrecan, collagens type I and II and Sox9. GAPDH was used as house-keeping gene, and the expression of all target genes was normalized against the GAPDH of that sample for each time point of the study. All primer sequences were generated using Primer3 software [50] and acquired from MWG Biotech AG, Germany. Table I shows the primers sequences used.

Table 7.1 – Primer sequences used for RT-PCR procedures *.

Gene	Forward (5´-3´)	Reverse (5´-3´)
AGC	TGAGTCCTCAAGCCTCCTGT	TGGTCTGCAGCAGTTGATTC
COL II	CGGTGAGAAGGGAGAAGTTG	GACCGGTCACTCCAGTAGGA
COL I	AGCCAGCAGATCGAGAACAT	ACACAGGTCTCACCGGTTTC
Sox9	TTCATGAAGATGACCGACGC	GTCCAGTCGTAGCCCTTGAG
GAPDH	ACAGTCAGCCGCATCTTCTT	ACGACCAAATCCGTTGACTC

*AGC = Agreccan; COL II = Collagen type II; COL I = Collagen type I; Sox9 = Sry-type high mobility group box 9; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase

2.10. Histological analysis

Samples were collected at the end of the experiment and processed for histology. Samples were then fixed in 10% neutral buffered formalin and then dehydrated through crescent concentrations of ethanol, immersed in infiltration solutions, embedded in Technovit glycol methacrylate (kit 8100, Heraeus Kulzer, EBSciences, CO, USA) and then polymerized. Specimens were cut to obtain longitudinal sections of 7 μm of thickness, using a modified microtome equipped with a tungsten blade (Leica RM 2155). Staining solution for toluidine blue assay was prepared by adding 1% of toluidine blue (Sigma, T0394) dissolved in distilled water containing 0.5 g of sodium borate, followed by filtering. One drop of this solution was added to each section for 1 minute. Then, the sections were rinsed with distilled water and let to air dry overnight. Sections were cleared in xylene substitute (Sigma, A5597) and mounted in Histo clear (Frilabo, HS200). Safranin O staining was performed by washing slides in tap water, then immersed in 0.02% fast green solution (Fluka, 44715) for 6 minutes. Then, they were immersed in 1% acetic acid (Panreac, 131008) solution for 1 minute. After, slides were immersed in 0.1% safranin O (Fluka, 84120) solution for 10 minutes. By the end, slides were washed in tap water and let to air dry. Sections were cleared in xylene substitute and mounted as previously described.

2.11. Statistical analysis

Statistical analysis was performed using the SPSS statistic software (Release 15.0.0 for Windows). Firstly, a Shapiro-Wilk test was used to ascertain about the data normality and variance equality. The normality condition was rejected and, consequently, nonparametric tests were used in further comparisons between direct and indirect co-cultures. A Kruskal-Wallis test followed by Tukey's HSD test was applied to compare differences in between the days in culture type. P values lower than 0.001 were considered statistically significant in the analysis of the results.

3. Results

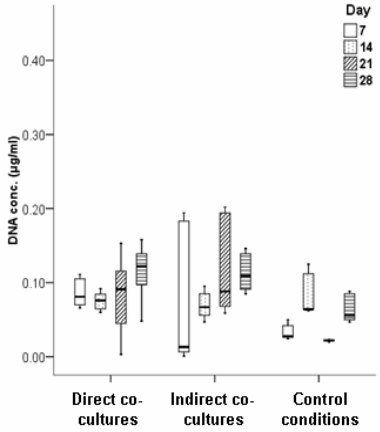
3.1. DNA quantification

The DNA concentration for each type of co-culture was assessed. Samples were collected at each time point of the experiment. We determined if there were any differences in between the days of culture, for each type of co-culture (direct or indirect), and also in the control conditions.

3.1.1. Co-cultures using hBMSCs

In terms of DNA concentration, both types of co-cultures using hBMSCs seem to follow the same trend of proliferation. DNA contents increased with time in both types of co-cultures, whereas the positive control shows a decrease at 21 days, increasing again towards the end of the experiment (Figure 7.1A). These results indicate that both types of co-cultures using hBMSCs were proliferating at a comparable rate, whereas in the control conditions (hACs) cell numbers did not increased between 14 and 21 days, recovering only in the last time point. Figure 7.1B shows the table of results for the statistical analysis concerning the differences between the various time points of the experiment, for each type of co-culture using hBMSCs. We found no significant differences between the various time points either in direct co-cultures ($p=0.172$) or indirect co-cultures ($p=0.062$), showing that the proliferation was not very strong during the experiment. The control conditions show a significant increase from the 7^{th} to the 28^{th} day of experiment, as observed in the statistical analysis results in Figure 7.1C. It is noteworthy that both the direct and indirect co-cultures show the highest levels of DNA concentration when compared to the controls in the last time point.

A



B

Days	Direct co-cultures	Indirect co-cultures	Control conditions
Day 7	0.081(0.069-0.108)	0.013 (0.005-0.188)	0.028 (0.025-0.046)
Day 14	0.076 (0.054-0.090)	0.067(0.054-0.090)	0.064(0.064-0.116)
Day 21	0.091(0.034-0.121)	0.088(0.067-0.194)	0.022(0.021-0.023)
Day 28	0.122(0.078-0.145)	0.109(0.090-0.140)	0.056(0.050-0.087)
P-value	0.172	0.062	<0.001

C

Control conditions	P-Value
Day 7 vs Day14	<0.001
Day 7 vs Day 21	<0.001
Day 7 vs Day 28	<0.001
Day 14 vs Day 21	0.046
Day 14 vs Day 28	<0.001
Day 21 vs Day 28	<0.001

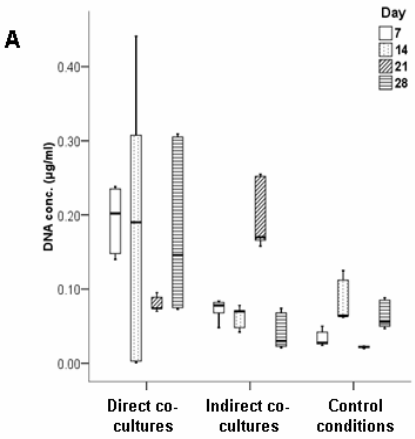
Figure 7.1 - Box plot of the DNA concentration in co-cultures using human bone marrow MSCs. A - Box plot of DNA concentration for direct and indirect co-cultures with hBMSCs and control conditions (hACs alone). B – Table with the statistical analysis results for the between days of culture differences (Kruskal-Wallis test, presented as median ± interquartile range (IQR)). C – Table with the statistical analysis results for between days differences in the control conditions (Kruskal-Wallis test followed by Tukey's HSD test.).

3.1.2. Co-cultures using hWJSCs

The trend of DNA concentration of hWJSCs co-cultures is rather different than the DNA concentration found for the co-cultures using hBMSCs. Both direct and indirect co-cultures show a decreasing tendency in the first days of culture. Then, the DNA concentration in direct co-cultures continues decreasing until the 21st day, increasing afterwards until the end of the experiment (Figure 7.2A). This result might indicate that the proliferation of the direct co-cultures with hWJSCs slowed down up to the 21st day. Conversely, indirect co-cultures with hWJSCs show an increase in DNA concentration after 14 days of culture, showing a marked decrease towards the last time point.

Comparing the various time points, we found no significant differences between the days in direct co-cultures (p=0.04) using hWJSCs (Figure 7.2B). At 21 days, indirect co-cultures using hWJSCs displayed a significantly higher DNA concentration than in the other time points (p<0.001). On the contrary, at 28 days these cultures displayed a significantly lower DNA concentration than all the other time points (p<0.001). This result seems to indicate that the proliferation is slowing down towards the end of the experiment. On the contrary, the control conditions showed a significant increase comparing the 7th and 28th day of the experiment (Figure 7.2C). The highest DNA concentration after 28 days of culture was obtained by direct co-cultures.

A



B

Days	Direct co-cultures	Indirect co-cultures	Control conditions
Day 7	0.202 (0.146-0.237)	0.078(0.075-0.083)	0.028(0.025-0.046)
Day 14	0.190(0.003-0.312)	0.070(0.047-0.720)	0.064(0.064-0.116)
Day 21	0.074(0.073-0.092)	0.170(0.165-0.252)	0.022(0.021-0.023)
Day 28	0.146(0.074-0.307)	0.030(0.023-0.069)	0.056(0.049-0.086)
P-value	0.04	<0.001	<0.001

C

Indirect co-cultures	P-Value	Control conditions	P-Value
Day 7 vs Day14	0.018	Day 7 vs Day14	<0.001
Day 7 vs Day 21	<0.001	Day 7 vs Day 21	<0.001
Day 7 vs Day 28	<0.001	Day 7 vs Day 28	<0.001
Day 14 vs Day 21	<0.001	Day 14 vs Day 21	0.046
Day 14 vs Day 28	0.033	Day 14 vs Day 28	<0.001
Day 21 vs Day 28	<0.001	Day 21 vs Day 28	<0.001

Figure 7.2 - Box plot of the DNA concentration in co-cultures using human WJSCs. A - Box plot of DNA concentration for direct and indirect co-cultures with hWJSCs and control conditions (hACs alone). B – Table with the statistical analysis results for the between days of culture differences (Kruskal-Wallis test, presented as median ± interquartile range (IQR)). C – Table with the statistical analysis results for between days differences in the indirect co-cultures and in control conditions (Kruskal-Wallis test followed by Tukey's HSD test.).

3.2. Glycosaminoglycans quantification

The GAGs content for each type of co-culture was assessed. Samples were collected at 7 and 28 days of the experiment. We compared GAGs content between the types of co-cultures (direct vs indirect). We also compared the differences between both types of co-cultures and our control condition, hACs to determine if the co-cultures were accumulating more GAGs than chondrocytes alone. Afterwards, we determined if any differences were observed between the different time points, for each type of co-culture (direct or indirect).

3.2.1. Co-cultures with hBMSCs

Both direct and indirect co-cultures with hBMSCs show a small decrease in the GAG content from 7 to 28 days of the experiment, whereas control conditions showed the expected significant increase in GAGs deposition over time (Figure 7.3A).

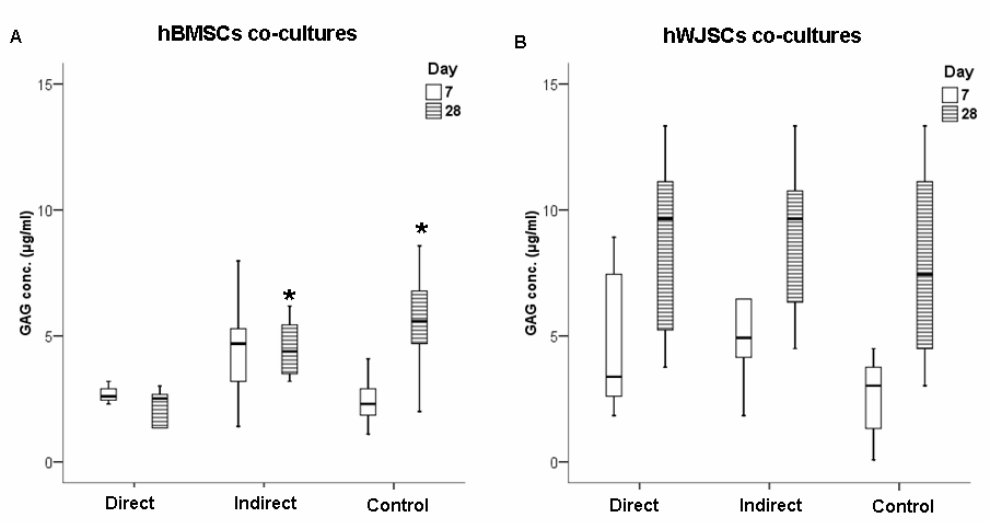


Figure 7.3 - Box plot of the GAGs concentration in both types of co-cultures, along the experiment. A - Box plot of GAGs concentration for direct and indirect co-cultures with hBMSCs and control conditions (hACs alone). B - Box plot of the GAGs concentration for direct and indirect co-cultures using hWJSCs and control conditions (hACs alone). Data were analyzed by nonparametric way of a Kruskal-Wallis test followed by Tukey's HSD test. * denotes significant differences compared to direct co-cultures.

No significant difference between direct and indirect co-cultures using hMSCs was found for 7 days of culture (p=0.061). At the end of the experiment, direct co-cultures displayed a significantly lower amount of GAGs than indirect co-cultures (p=0.006), as well as lower than the values for the control conditions (p=0.002). These results show that those co-cultures did not deposit more GAGs than the control condition. No significant differences between 7 and 28 days were observed in direct co-cultures (p=0.534) and in indirect co-cultures with hBMSCs (p=0.963).

3.2.2. Co-cultures using hWJSCs

In co-cultures using hWJSCs, a consistent trend was observed for all the conditions. Direct and indirect co-cultures, as well as the control conditions, show an increase in GAGs content along the time in culture (Figure 7.3B). For co-cultures using hWJSCs no significant difference between direct and indirect co-cultures was found after 7 days (p=0.138) nor after 28 days (p=0.733). Significant differences were found between 7 and 28 days of culture for both direct and indirect co-cultures. After 28 days, all the conditions displayed a significantly higher GAG concentration compared to 7 days ($p<0.01$), meaning that the GAGs accumulation is very alike to the control conditions, with native chondrocytes.

The GAGs concentration values obtained for all the time points for direct or indirect co-cultures with hBMSCs show consistently lower values when comparing to the ones obtained for the co-cultures with hWJSCs.

3.3. Real-Time PCR

Samples were collected for Real-Time PCR at 7 and 28 days of experiment. The aim was to determine the expression of several cartilage-related genes and to compare the differences between the expressions in both types of co-cultures, on both time points.

3.3.1. Co-cultures using hBMSCs

In both types of co-cultures using hBMSCs, no significant differences were found between the cultures for *Aggrecan* or *Sox9*, at 7 days (p=0.072 and p=0.868, respectively) or 28 days (p=0.183 and p=0.115, respectively), as shown in Figure 7.4. Direct co-cultures using hBMSCs expressed significantly higher values of Collagen *type I*, compared either to indirect co-cultures or to the control conditions ($p<0.001$),

indicating the formation of fibrocartilage in direct co-cultures. *Collagen type II* expression in indirect co-cultures of hBMSCs, displayed a significantly higher expression than direct co-cultures and control conditions ($p<0.001$) at 7 day of culture. After 28 days, direct co-cultures displayed a significantly higher *Collagen type II* expression than indirect co-cultures ($p=0.009$) and control conditions ($p=0.002$).

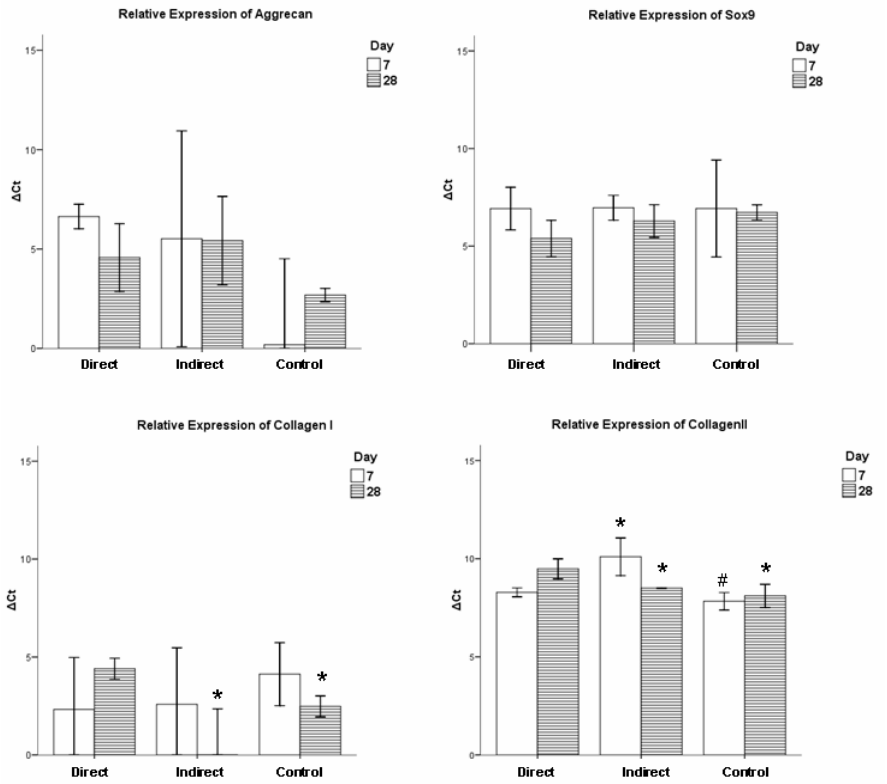


Figure 7.4 – Bar plots of chondrogenic markers present in direct and indirect co-cultures using hBMSCs, normalized for the reference gene *GAPDH*, after 7 and 28 days of culture. Data were analyzed by nonparametric way of a Kruskal-Wallis test for *Aggrecan* and *Sox9*. Data related with *Collagen type I* and *Collagen type II* were analyzed by nonparametric way of a Kruskal-Wallis test followed by Tukey's HSD test. * denotes significant differences compared to direct co-cultures, # denotes significant differences compared to indirect co-cultures.

3.3.2. Co-cultures using hWJSCs

Statistic analysis of gene expression in direct and indirect co-cultures using hWJSCs showed significant differences for almost all the genes (Figure 7.5). For *Aggrecan*, there was a significantly lower expression on the control conditions, when compared to direct and indirect co-cultures ($p<0.001$), at 7 days of culture. However, at 28 days of culture,

both types of co-cultures using hBMSCs showed a significantly higher expression of *Aggrecan* than control conditions ($p<0.001$). Additionally, indirect co-cultures also displayed a significantly higher value for *Aggrecan* expression when compared to direct co-cultures ($p=0.007$).

Indirect co-cultures also expressed significantly higher values of *Sox9* expression, at 28 days of culture, when compared to direct co-cultures and control conditions ($p<0.001$). For *Collagen type I*, no significant difference between cell type was found either at 7 ($p=0.044$) or 28 days of culture ($p=0.498$). However, the trend was to decrease its expression for the longer time periods.

Direct co-cultures using hWJSCs displayed a significantly higher expression of *Collagen type II* than indirect co-cultures, after 7 days in culture. In contrast, indirect co-cultures displayed a significantly higher *Collagen type II* expression than the control conditions ($p=0.006$), and a significant trend to increase the expression of this important gene, in contrast to other culture conditions.

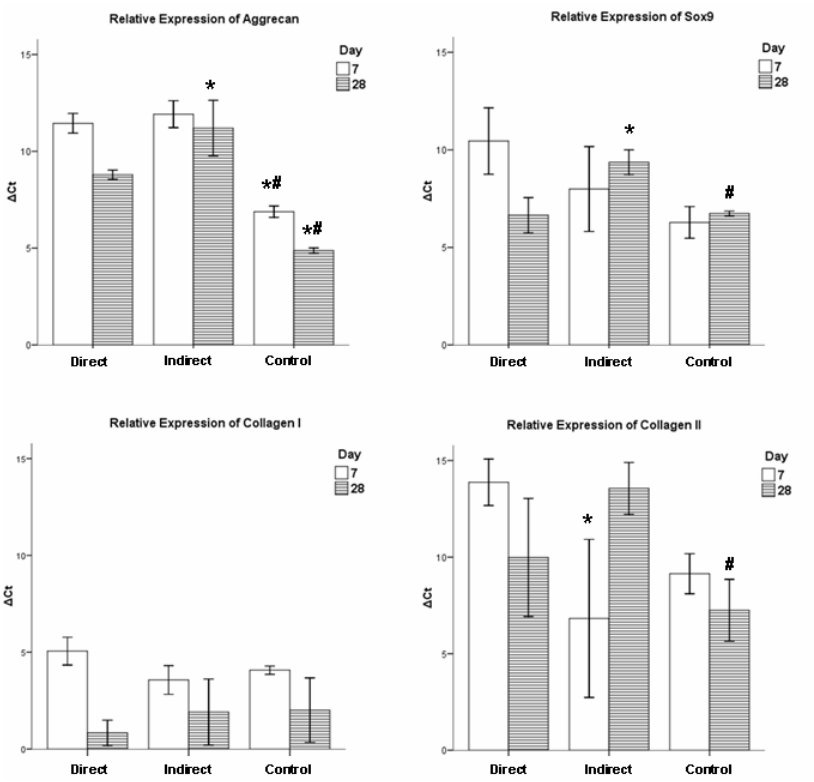


Figure 7.5 – Bar plots of chondrogenic markers present in direct and indirect co-cultures using hWJSCs, normalized for the reference gene *GAPDH*, after 7 and 28 days of culture. Data were analyzed by nonparametric way of a Kruskal-Wallis test for

Collagen type I. Data related to *Aggrecan*, *Sox9* and *Collagen type II* expressions were analyzed by nonparametric way of a Kruskal-Wallis test followed by Tukey's HSD test. * denotes significant differences compared to direct co-cultures, # denotes significant differences compared to indirect co-cultures.

3.4. Histological staining

By the end of the experiment, samples of every co-culture were collected for histological sectioning. Sections were stained for cartilagineous ECM using toluidine blue and safranin O assays (Figure 7.6). Both types of cells, either in direct or indirect co-culture, were able to produce ECM, as shown by the positive stain of toluidine blue and safranin O for ECM components. The cells were able to proliferate around the fiber meshes, and were able to grow in between the fibers inside the structure of the scaffolds. For co-cultures using hBMSCs no evident differences in terms of the quantity of cartilage ECM formation are observed in the histological staining results, either for direct or indirect co-cultures. Nevertheless, positive staining for proteoglycans can be observed either with toluidine blue or with safranin O stain. These observations are consistent with the previously obtained results for GAGs accumulation, as well as the RT-PCR results that showed the expression of cartilage related genes at the end of the experiment.

In the case of co-cultures using hWJSCs, the same observations were made. It is possible to state ECM formation both in direct and indirect co-cultures, stained with toluidine blue and safranin O. These observations are also consistent with the previously obtained results for GAGs accumulation and cartilage related genes expression at 28 days of culture.

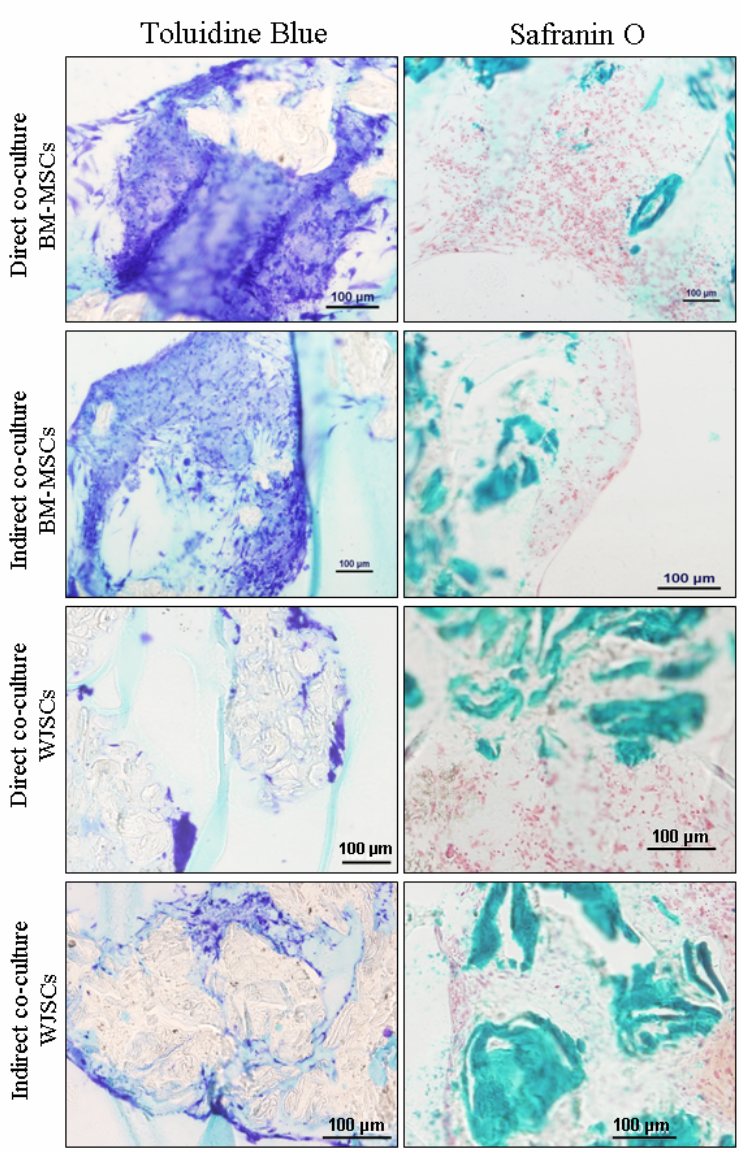


Figure 7.6 – Histological sections of direct and indirect co-cultures using human BMSCs and WJSCs, stained for ECM localisation.

4. Discussion

The use of biomaterial scaffolds to enhance ECM-cell interactions has been reported as a very important factor when using stem cells for TE [7, 42, 51]. For detailed overview on this matter, please refer to [52, 53]. The positive effect of 3D scaffolds in the chondrogenic differentiation of BMSCs [54-56] and UCSCs [32, 57] has been reported elsewhere. In the present work we showed that both hBMSCs and hWJSCs were able to undergo chondrogenic differentiation when seeded onto CPBS fiber

meshes. Polyglycolic acid (PGA) scaffolds were recently used for comparing the potential of hBMSCs and hUCSCs for cartilage tissue engineering. The authors observed enhanced chondrogenic differentiation of hUCSCs when compared to hBMSCs [7]. The chondrogenic differentiation of MSCs on chitosan fibrous scaffolds and chitosan sponges was compared, demonstrating that chondrogenesis on chitosan scaffolds is superior on microfibers, when compared to sponges [58].

Herein, chondrogenic differentiation of human BMSCs and WJSCs was observed both in direct and indirect co-cultures. The soluble signals released by articular chondrocytes, either by the direct contact or in the form of soluble factors released in the culture medium, did effectively promote chondrogenic differentiation of hBMSCs and of hWJSCs. A recent study reported similar conclusions for the chondrogenic differentiation of adipose stem cells when co-cultured with chondrocytes [59]. In that report, adipose stem cells were cultured in pellets either directly or indirectly with chondrocytes, and it was concluded that the chondrocytes secreted signals promoted chondrogenic differentiation of adipose stem cells. The co-culture systems used in the present work were able to provide MSCs with the signals and the stimulation needed for their chondrogenic differentiation. A similar result was found when using conditioned medium from chondrocytes in the chondrogenic differentiation of embryonic stem cells [48]. The authors could show the coordination of signals between the co-cultures, suggesting that chondrogenic differentiation of embryonic stem cells can be achieved by co-cultures with chondrocytes, without other exogenous growth factors being required.

No significant differences between the days in culture, in terms of proliferative ability, were found for the co-cultures using hBMSCs. Likewise, no significant differences were found for direct co-cultures using hWJSCs. However, when analysing the proliferative ability of hWJSCs in indirect co-cultures we observed significantly higher differences between the 21st day and all the other time points. Moreover, the values obtained for the DNA concentration of hWJSCs are higher than the ones obtained for the hBMSCs. The higher proliferative potential of hWJSCs compared to hBMSCs has been demonstrated earlier [15], as well as their enhanced chondrogenic potential [14]. Both types of co-cultures were able to produce and deposit GAGs. For the cultures with hBMSCs, a significantly lower GAG concentration value was found for the direct co-cultures, comparing to the indirect. For the cultures using hWJSCs, no significant differences were found in terms of GAGs concentration between direct and indirect co-cultures. These results show that indirect co-cultures may be more effective in ECM

components production, at least in terms of chondrogenic differentiation of hBMSCs, since no differences were found for hWJSCs. Our data with co-cultures of hBMSCs are similar to the results obtained in a study using passaged chondrocytes and primary bovine chondrocytes [19]. In that study, indirect co-cultures of primary and passaged chondrocytes were performed using filter inserts and it was found that the passaged cells accumulated ECM, when in contact with the conditioned medium produced by the primary chondrocytes. Although indirect co-cultures using hBMSCs seem to have an advantage in terms of GAGs accumulation, both types of co-cultures using these cells displayed a significantly lower value when compared to the chondrocytes culture (control condition). These results show that co-cultures with hBMSCs produced and accumulated less GAGs than the native chondrocytes cultures. In terms of GAGs, we can say that the hBMSCs co-cultures do not show a clear advantage over chondrocytes.

Interestingly, both direct and indirect co-cultures using hWJSCs displayed a significantly higher concentration of GAGs at 28 days of culture, compared to 7 days. The values for GAGs concentration were higher in the cultures using hWJSCs than in the cultures using hBMSCs, indicating more formation and accumulation of these important components of the cartilaginous ECM. Our results showed enhanced chondrogenesis in hWJSCs, when in indirect or direct co-cultures.

In the histological sections, it is noticeable that both cultures of hBMSCs and of hWJSCs were able to produce ECM components, namely proteoglycans. These components were stained both with toludine blue and with safranin O, showing ECM deposition. ECM accumulation was also observed in passaged human chondrocytes, when in co-cultures with bovine chondrocytes [34]. Co-cultures of equine BMSCs with equine articular chondrocytes improved the expression of cartilage related genes, and induced the production of a more homogeneous ECM within the neo-cartilage [36].

In terms of gene expression, for the cultures using hBMSCs, direct co-cultures showed significantly higher expression of *Collagen type I* than indirect co-cultures, at the end of the experiment. In terms of *Collagen type II* expression, direct co-cultures displayed a significantly higher expression at 7 days of culture than indirect co-cultures. On the other hand, towards the end of the experiment, indirect co-cultures showed a higher expression of collagen type II compared to the direct co-cultures. Again, the positive effect of using conditioned medium is evidenced by the results. For hWJSCs co-cultures, a significantly higher expression of *Aggrecan* was found for indirect co-cultures of hWJSCs at 28 days, compared to the direct ones. The same significantly

higher expression was observed for *Sox9* expression. For *Collagen type II*, there was a decrease in the last day of culture for the direct co-cultures. In this last time point, a significantly higher expression was found for the indirect co-cultures, when compared to the control conditions. Therefore, this system of co-culturing has a considerable clinic potential.

The use of conditioned medium to promote the differentiation of MSCs for further implantation is a valuable concept in terms of regenerative medicine, because it will allow obtained conditioned medium of unrelated chondrocytes to promote the differentiation of autologous stem cells. In the present work, we propose a new strategy for cartilage tissue engineering strategies based on the use of conditioned medium, and we were able to demonstrate its effect in MSCs chondrogenesis. The positive effect of the conditioned medium in osteogenesis and chondrogenesis has been recently demonstrated, using MSCs seeded onto polycaprolactone/hydroxyapatite scaffolds and on alginate, respectively [60]. In a different approach, using porcine cells for chondrogenic differentiation for intervertebral disc repair, notochordal cell conditioned medium promoted MSCs differentiation, induced significantly more collagen type II expression and GAGs production in MSCs [61]. In the present work, the soluble factors released by hACs in culture were able to induce chondrogenic differentiation of both hBMSCs and hWJSCs without the addition of growth factors. This is a very interesting result, since in a clinical perspective it is preferable to use conditioned medium for chondrogenic differentiation of MSCs than to induce it with growth factors or even with direct co-cultures. There are many cost associated with the use of growth factors, so this cost could be reduced when using conditioned medium. Indeed, this technique should be further explored, as some reports show that the use of conditioned medium is very versatile. For example, conditioned medium from a human hepatocarcinoma cell line (HepG2) enhanced in vitro chondrogenesis of murine embryonic stem cells [62]. Therefore, chondrocyte cell lines could be eventually established for conditioned medium production, to further induce chondrogenic differentiation of autologous MSCs in 3D scaffolds prior to its implantation. However, it is very important to identify, in the first place, the soluble factors that are being release into the hACs culture medium and that successfully induced the chondrogenic differentiation of hBMSCs and hWJSCs.

Indirect co-cultures of human MSCs using conditioned medium from hACs seem to be more suitable for engineered cartilage formation. The results of indirect co-cultures (either with BMSCs or with WJSCs) towards the end of the experiment were

significantly higher for all the tested parameters, namely GAGs accumulation and cartilage related-genes expression (*Collagen type II*, *Aggrecan* and *Sox9*). Concerning the type of human MSCs tested, we obtained strong evidence that hWJSCs are more promising than hBMSCs for cartilage related applications using this strategy. Additionally, more GAGs accumulation was measured again for both direct and indirect co-cultures using hWJSCs. These cultures also expressed *Aggrecan*, *Sox9* and *Collagen type II*, genes related to articular cartilage. *Collagen type I*, related to fibrotic cartilage, did not have a significant expression in these cultures with hWJSCs. This result may be indirectly related to a superior chondrogenic performance of hWJSCs when compared to hBMSCs [7].

5. Conclusions

Human adult MSCs were able to differentiate into the chondrogenic lineage, when co-cultured with hACs in CPBS scaffolds. In terms of type of culture, indirect co-cultures (using conditioned medium) seem more effective for chondrogenic differentiation. This is a very interesting result to be further explored for clinical translation. In this way, conditioned medium without the addition of other supplements or growth factors can be employed to enhance MSCs expansion and chondrogenic differentiation, prior to implantation. Indirect co-cultures using conditioned medium, using either BMSCs or WJSCs, showed significant higher values in terms of GAGs accumulation and cartilage related genes expression, when compared to direct co-cultures. In terms of cell source, hWJSCs showed higher proliferation rates and higher chondrogenic differentiation ability when compared to hBMSCs. Human WJSCs expressed significant higher values of DNA concentration, GAGs accumulation and cartilage related genes (*Aggrecan, Collagen type II* and *Sox9*). Human BMSCs chondrogenic differentiation produced fibrous cartilage, as stated by the significantly higher expression of *Collagen type I* when compared to hWJSCs.

In conclusion, indirect co-cultures of hWJSCs using conditioned medium obtained from hACs cultures are a new approach for cartilage tissue engineering that should be extensively studied for future clinical applications, as an alternative method for expanding autologous cells prior to implantation.

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References

1 - Schulze-Tanzil G. *Activation and dedifferentiation of chondrocytes: Implications in cartilage injury and repair*. Ann Anat. 2009. 191(4):325-38.

2 - Barbero A, Ploegert S, Heberer M, Martin I. *Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes*. Arthritis & Rheumatism. 2003. 48(5):1315 – 25.

3 - Brun P, Abatangelo G, Radice M, Zacchi V, Guidolin D, Gordini DD*, et al. Chondrocyte aggregation and reorganization into three-dimensional scaffolds*. J Biomed Mater Res. 1999. 46:337 – 46.

4 - Darling EM, Athanasiou KA. *Retaining zonal chondrocyte phenotype by means of novel growth environments*. Tissue Eng. 2005. 11(3-4):395-403.

5 - Miot S, Woodfield TB, Daniels AU, Suetterlin R, Peterschmitt I, Heberer M*, et al. Effects of scaffold composition and architecture on human nasal chondrocyte redifferentiation and cartilaginous matrix deposition*. Biomaterials. 2005. 26:2479 – 89.

6 - Sekiya I, Larson B, Smith JR, Pochampally R, Cui J-O, Prockop DJ. *Expansion of human adult stem cells from bone marrow stroma: Conditions that maximize the yields of early progenitors and evaluate their quality*. Stem Cells. 2002. 6:530 - 41.

7 - Wang L, Tran I, Seshareddy K, Weiss ML, Detamore MS. *A comparison of human bone marrow-derived mesenchymal stem cells and human umbilical cord-derived*

mesenchymal stromal cells for cartilage tissue engineering. Tissue Eng Part A. 2009. 15(8):2259-66.

8 - Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. *Isolation and multilineage differentiation of bovine bone marrow mesenchymal stem cells*. Cell Tissue Res. 2005. 319:243 – 53.

9 - Mauck RL, Yuan X, Tuan RS. *Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture*. Osteoarthritis Cartilage. 2006. 14(2):179-89.

10 - Hu J, Feng K, Liu X, Ma PX. *Chondrogenic and osteogenic differentiations of human bone marrow-derived mesenchymal stem cells on a nanofibrous scaffold with designed pore network*. Biomaterials. 2009. 30(28):5061-7.

11 - Markway BD, Tan GK, Brooke G, Hudson JE, Cooper-White JJ, Doran MR. *Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures*. Cell Transplant. 2010. 19(1):29-42.

12 - 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.

13 - Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. *Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors*. Stem Cells. 2005. 23(2):220-9.

14 - Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, *et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord*. Stem Cells. 2004. 22(7):1330-7.

15 - Baksh D, Yao R, Tuan RS. *Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow*. Stem Cells. 2007. 25(6):1384-92.

16 - Can A, Karahuseyinoglu S. *Concise review: Human umbilical cord stroma with regard to the source of fetus-derived stem cells*. Stem Cells. 2007. 25(11):2886-95.

17 - Troyer DL, Weiss ML. *Wharton's jelly-derived cells are a primitive stromal cell population*. Stem Cells. 2008. 26(3):591-9.

18 - Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, *et al. Biology of stem cells in human umbilical cord stroma: In situ and in vitro surveys*. Stem Cells. 2007. 25(2):319-31.

19 - Taylor DW, Ahmed N, Gan L, Gross AE, Kandel R. *Proteoglycan and collagen accumulation by passaged chondrocytes can be enhanced through side by side culture with primary chondrocytes*. Tissue Eng Part A. 2009.

20 - Bos PK, van Osch GJ, Frenz DA, Verhaar JA, Verwoerd-Verhoef HL. *Growth factor expression in cartilage wound healing: Temporal and spatial immunolocalization in a rabbit auricular cartilage wound model*. Osteoarthritis Cartilage. 2001. 9(4):382-9.

21 - Hwang NS, Varghese S, Puleo C, Zhang Z, Elisseeff J. *Morphogenetic signals from chondrocytes promote chondrogenic and osteogenic differentiation of mesenchymal stem cells*. J Cell Physiol. 2007. 212(2):281-4.

22 - Hwang NS, Varghese S, Elisseeff J. *Derivation of chondrogenically-committed cells from human embryonic cells for cartilage tissue regeneration*. PLoS ONE. 2008. 3(6):e2498.

23 - Costa-Pinto AR, Salgado AJ, Correlo VM, Sol P, Bhattacharya M, Charbord P*, et al. Adhesion, proliferation, and osteogenic differentiation of a mouse mesenchymal stem cell line (BMC9) seeded on novel melt-based chitosan/polyester 3D porous scaffolds*. Tissue Eng Part A. 2008. 14(6):1049-57.

24 - Cruz DM, Ivirico JL, Gomes MM, Ribelles JL, Sanchez MS, Reis RL*, et al. Chitosan microparticles as injectable scaffolds for tissue engineering*. J Tissue Eng Regen Med. 2008. 2(6):378-80.

25 - Gomes ME, Azevedo HS, Moreira AR, Ella V, Kellomaki M, Reis RL. *Starch-poly(epsilon-caprolactone) and starch-poly(lactic acid) fibre-mesh scaffolds for bone tissue engineering applications: Structure, mechanical properties and degradation behaviour*. J Tissue Eng Regen Med. 2008. 2(5):243-52.

26 - Pereira RC, Scaranari M, Castagnola P, Grandizio M, Azevedo HS, Reis RL*, et al. Novel injectable gel (system) as a vehicle for human articular chondrocytes in cartilage tissue regeneration*. J Tissue Eng Regen Med. 2009. 3(2):97-106.

27 - Wang Y, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL. *In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells*. Biomaterials. 2005. 26(34):7082-94.

28 - Kramer J, Bohrnsen F, Lindner U, Behrens P, Schlenke P, Rohwedel J. *In vivo matrix-guided human mesenchymal stem cells*. Cell Mol Life Sci. 2006. 63(5):616-26.

29 - Alves da Silva ML, Crawford A, Mundy JM, Correlo VM, Sol P, Bhattacharya M*, et al. Chitosan/polyester-based scaffolds for cartilage tissue engineering: Assessment of extracellular matrix formation*. Acta Biomater. 2010. 6(3):1149-57.

30 - Oliveira J, Correlo VM, Sol P, Costa-Pinto A, Salgado A, Bhattacharya M*, et al. Assessment of the suitability of chitosan/polybutylene succinate scaffolds seeded with mouse mesenchymal progenitor cells for a cartilage tissue engineering approach* Tissue Eng. 2008. 14(10):1651-61.

31 - Oliveira JT, Crawford A, Mundy JL, Sol PC, Correlo VM, Bhattacharya M*, et al. Novel melt-processable chitosan-polybutylene succinate fibre scaffolds for cartilage tissue engineering*. J Biomater Sci Polym Ed - In press. 2010.

32 - Costa-Pinto AR, Frias AM, Alves da Silva ML, Fernandes S, Barros A, Reis RL*, et al. Comparative study of the multidifferentiation potential of human Wharton´s jelly and amniotic fluid derived stem cells*. Tissue Eng Part A. 2008. 14:713.

33 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, Neves NM*, et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromol Biosci. 2010.

34 - Ahmed N, Taylor DW, Wunder J, Nagy A, Gross AE, Kandel RA. *Passaged human chondrocytes accumulate extracellular matrix when induced by bovine chondrocytes*. J Tissue Eng Regen Med. 2010. 4(3):233-41.

35 - Ahmed N, Gan L, Nagy A, Zheng J, Wang C, Kandel RA. *Cartilage tissue formation using redifferentiated passaged chondrocytes in vitro*. Tissue Eng Part A. 2009. 15(3):665-73.

36 - Lettry V, Hosoya K, Takagi S, Okumura M. *Coculture of equine mesenchymal stem cells and mature equine articular chondrocytes results in improved chondrogenic differentiation of the stem cells*. Jpn J Vet Res. 2010. 58(1):5-15.

37 - Bigdeli N, Karlsson C, Strehl R, Concaro S, Hyllner J, Lindahl A. *Coculture of human embryonic stem cells and human articular chondrocytes results in significantly altered phenotype and improved chondrogenic differentiation*. Stem Cells. 2009. 27(8):1812-21.

38 - Chen J, Wang C, Lü S, Wu J, Guo X, Duan C*, et al. In vivo chondrogenesis of adult bone-marrow-derived autologous mesenchymal stem cells*. Cell Tissue Res. 2005. 319:429 – 38.

39 - Ahmed N, Dreier R, Gopferich A, Grifka J, Grassel S. *Soluble signalling factors derived from differentiated cartilage tissue affect chondrogenic differentiation of rat adult marrow stromal cells*. Cell Physiol Biochem. 2007. 20(5):665-78.

40 - Varshney RR, Zhou R, Hao J, Yeo SS, Chooi WH, Fan J*, et al. Chondrogenesis of synovium-derived mesenchymal stem cells in gene-transferred co-culture system*. Biomaterials. 2010. 31(26):6876-91.

41 - Hildner F, Concaro S, Peterbauer A, Wolbank S, Danzer M, Lindahl A*, et al. Human adipose-derived stem cells contribute to chondrogenesis in coculture with human articular chondrocytes*. Tissue Eng Part A. 2009. 15(12):3961-9.

42 - Jakobsen RB, Shahdadfar A, Reinholt FP, Brinchmann JE. *Chondrogenesis in a hyaluronic acid scaffold: Comparison between chondrocytes and msc from bone marrow and adipose tissue*. Knee Surg Sports Traumatol Arthrosc. 2009.

43 - Pereira RC, Costa-Pinto AR, Frias AM, N.M. N, Azevedo HS, Reis RL. *Co-culture of human Wharton jelly stem cells with human articular chondrocytes provides a suitable environment for their chondrogenic commitment*. 5th International Annual Meeting of the Portuguese Society for Stem Cells and Cellular Therapy (SPCE-TC); 2010; Avepark, Caldas das Taipas , Guimarães; 2010. p. 19-20.

44 - Costa-Pinto AR, Correlo VM, Sol PC, Bhattacharya M, Charbord P, Delorme B*, et al. Osteogenic differentiation of human bone marrow mesenchymal stem cells seeded on melt based chitosan scaffolds for bone tissue engineering applications*. Biomacromolecules. 2009. 10(8):2067-73.

45 - Correlo VM, Costa-Pinto AR, Sol P, Covas JA, Bhattacharya M, N.M. N*, et al. Melt processing of chitosan-based fibers and fiber-mesh scaffolds for the engineering of connective tissues*. Macromolecular Bioscience. 2010. accepted for publication.

46 - Costa-Pinto A. R., Frias AM, Alves da Silva ML, Barros A., Fernandes S., RL R*, et al. Comparative study of human Wharton's jelly and amniotic fluid derived stem cells differentiation potential*. Tissue Engineering: . 2008. 14:713.

47 - Crawford A, Dickinson S. *Chondrocyte isolation, expansion, and culture in polymer scaffolds.* In: Hollander AP, Hatton PV, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 147-57.

48 - Vats A, Bielby RC, Tolley N, Dickinson SC, Boccaccini AR, Hollander AP*, et al. Chondrogenic differentiation of human embryonic stem cells: The effect of the micro-environment*. Tissue Eng. 2006. 12(6):1687-97.

49 - Kafienah W, Sims TS. *Biochemical methods for the analysis of tissue engineered cartilage.* In: Hollander Ap, Hatton Pv, editors. Methods in molecular biology. Totowa, NJ: Humana Press Inc.; 2004. p. 217 - 29.

50 - http://frodo.wi.mit.edu/.

51 - Mahmoudifar N, Doran PM. *Chondrogenic differentiation of human adipose-derived stem cells in polyglycolic acid mesh scaffolds under dynamic culture conditions.* Biomaterials. 2010. 31(14):3858-67.

52 - Chai C, Leong KW. *Biomaterials approach to expand and direct differentiation of stem cells*. Mol Ther. 2007. 15(3):467-80.

53 - Vinatier C, Mrugala D, Jorgensen C, Guicheux J, Noel D. *Cartilage engineering: A crucial combination of cells, biomaterials and biofactors*. Trends Biotechnol. 2009. 27(5):307-14.

54 - Kafienah W, Mistry S, Dickinson SC, Sims TJ, Learmonth I, Hollander AP. *Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients*. Arthritis Rheum. 2007. 56(1):177-87.

55 - Kim HJ, Lee JH, Im GI. *Chondrogenesis using mesenchymal stem cells and PCL scaffolds*. J Biomed Mater Res A. 2010. 92(2):659-66.

56 - Shao X, Hutmacher DW, Ho ST, Goh CH, Lee EH. *Evaluation of a hybrid scaffold/cell construct in repair of high-load-bearing osteochondral defects in rabbits.* Biomaterials. 2006. 27(7):1071 - 80.

57 - Wang L, Sheshareddy K, Weiss ML, Detamore MS. *Effect of initial seeding density on human umbilical cord mesenchymal stromal cells for fibrocartilage tissue engineering*. Tissue Eng Part A. 2009. 15(5):1009-17.

58 - Ragetly GR, Griffon DJ, Lee HB, Fredericks LP, Gordon-Evans W, Chung YS. *Effect of chitosan scaffold microstructure on mesenchymal stem cell chondrogenesis*. Acta Biomater. 2010. 6(4):1430-6.

59 - Lee JS, Im GI. *Influence of chondrocytes on the chondrogenic differentiation of adipose stem cells*. Tissue Eng Part A. 2010.

60 - Maxson S, Burg KJ. *Conditioned media enhance osteogenic differentiation on poly(l-lactide-co-epsilon-caprolactone)/hydroxyapatite scaffolds and chondrogenic differentiation in alginate*. J Biomater Sci Polym Ed. 2010. 21(11):1441-58.

61 - Korecki CL, Taboas JM, Tuan RS, Iatridis JC. *Notochordal cell conditioned medium stimulates mesenchymal stem cell differentiation toward a young nucleus pulposus phenotype*. Stem Cell Res Ther. 2010. 1(2):18.

62 - Hwang Y-S, Bishop AE, Polak JM, Mantalaris A. *Enhanced in vitro chondrogenesis of murine embryonic stem cells*. biotechnology and Bioprocesses Engineering. 2007. 12:696-706.

SECTION VI – CONCLUDING REMARKS AND FUTURE WORK

Chapter 8

Concluding remarks and future work

Concluding remarks and future work

In the present thesis, three distinct TE strategies were tested, based on different in vitro models. The rationale of the developed works relied on testing various primary cell cultures derived from different sources, combined with nano or macro scaffolds as supports for cell chondrogenic differentiation (in the case of adult MSCs) or for chondrocytes phenotype maintenance.

1. Concluding remarks

In section III of the present thesis, bovine articular chondrocytes (BAC) were cultured in vitro, using different scaffolds structures and compositions. In chapter 3 we used this model to determine if starch-polycaprolactone (SPCL) and polycaprolactone (PCL) electrospun nanofiber meshes would sustain BAC growth and ECM production. In this chapter, we confirmed the literature evidences which sustain that PCL nanofiber meshes are a valid scaffold for cartilage tissue engineering strategies. Moreover, we propose a new electrospun scaffold for this approach: SPCL nanofiber meshes.

In chapter 4, the BAC in vitro model was applied to microporous scaffolds with different pore sizes and geometries. Microporous 80 CPBS scaffolds facilitate the formation of cartilage like tissue in a greater extent than in the 60 CPBS scaffolds. The larger pores present in microporous 80 CPBS scaffolds may contribute to the enhanced ECM deposition.

From section III we concluded that BAC model is a valuable tool for cartilage tissue engineering. A massive colonization by BAC of all the tested structures was observed, producing cartilage ECM. We also were able to identify some advantages concerning the choice of scaffolds for further studies. Electrospun nanofiber meshes supported cartilage formation; therefore it was decided to continue the investigation of these structures using other cell types. Microporous scaffolds also supported ECM deposition, but we considered that other scaffold morphologies should be used in further studies, such as microfiber meshes. Dynamic culture conditions were considered as very

interesting for our strategies, as they stimulated ECM formation when using microporous scaffolds.

Section IV of the present thesis discussed the influence of flow perfusion in the chondrogenic differentiation of hBMSCs using nano and micro fiber meshes. In chapter 5 we described the culture of hBMSCs seeded onto electrospun PCL nanofiber meshes. We consider that this strategy is very useful in the pre-clinical context, as it can be employed in a MACI modified technique, using a bioreactor to expand and differentiate cells in the membrane that will be further applied for covering the cartilage defect.

In chapter 6 we explored chitosan-based microfiber meshes cultures in the referred bioreactor. Improved ECM deposition and collagen type II production was found in bioreactor samples when compared to static controls. The structure of the microfiber meshes seems to be adequate as cells were able to grow and differentiate into the CPBTA scaffolds, both in the bioreactor and in static controls. We showed that flow perfusion can influence hBMSCs chondrogenic differentiation, inducing cells differentiation at early time points than in the static conditions.

From section IV, we can conclude that perfusion conditions influence hBMSCs chondrogenic differentiation, independently of the scaffold formulation, shape or structure used for the culture. Indeed, the chondrogenic differentiation started at earlier stages of the experiment for cells seeded onto both nanofiber meshes and microfiber meshes in dynamic cultures.

Section V is composed by one chapter (chapter 7) referring to the last studied in vitro model: co-cultures. In this chapter, adult hMSCs were able to differentiate into chondrogenic lineage when co-cultured with hACs, and using CPBS microfiber meshes as 3D supports. Indirect co-cultures using conditioned medium obtained from the hACs cultures was more effective for chondrogenic differentiation. Human WJSCs showed enhanced chondrogenic differentiation ability when compared to hBMSCs.

All the referred results found during this PhD work allowed us to draw some key conclusions that will be very valuable for the future work. In terms of electrospun nanofiber meshes, we showed that both PCL and SPCL structures supported both BAC and hBMSCs models could be used for cartilage TE strategies. In all tested scaffolds, it was possible to produce cartilagineous ECM in vitro, therefore validating our proposed models. The use of chitosan-based scaffolds in different morphologies proved to

support the referred ECM formation in a greater extent than the one obtained with the other scaffolds tested. Our results evidenced the advantages of using chitosan blends for the models proposed herein.

Static and dynamic culture conditions showed different effects in our models, depending on the structure used as scaffold or on the type of cell used. We can state that nanofiber meshes cultures may be negatively affected by the stirring. On the other hand, the cultures using the flow perfusion bioreactor were positively affected, as enhanced chondrogenic differentiation was observed. Dynamic culture conditions effect in cartilage production in vitro was more pronounced in the cultures using microporous structures or microfiber meshes. Stirred conditions significantly enhanced ECM deposition by BAC. When CPBTA microfibrous meshes were cultured in the flow perfusion bioreactor, enhanced chondrogenic differentiation of hBMSCs was also observed. Results of these experiments revealed the important effect of the flow perfusion in our cultures. In the overall, we consider that dynamic stimulus is beneficial for the strategies proposed herein.

The final work describes a new approach that puts together the best features of all the previous works. Indirect co-cultures using conditioned medium from hACs cultures showed more ECM accumulation when compared to direct contact co-cultures. This strategy is a promising alternative method of expanding cells and mature constructs in vitro prior to implantation. The use of conditioned medium to enhance mesenchymal stem cells chondrogenic differentiation is, in our perspective, a very interesting tool to explore. This method could be applied in the clinic, avoiding the use of growth factors to induce chondrogenic differentiation, which is economically advantageous. Further studies and optimization of the method will be conducted in the future, having in mind its translation into the clinic.

2. Future work

After compiling all the results obtained in the 5 years of this PhD we have a perspective of what features could be further explored to optimize our models. In our future experiments, we will study some modifications of electrospun nanofiber meshes

to enhance chondrogenic differentiation, such as surface plasma modifications, combining nanofiber meshes with micro and macrostructures or incorporating bioactive agents. Moreover, in vivo studies are planned in order to study the nanofiber meshes in a large animal model.

Concerning the flow perfusion bioreactor herein described, it is our belief that further experiments will allow optimizing the dynamic conditions. A detailed study using several flow velocities and their effects either in electrospun nanofiber meshes or fiber meshes cultures, using the models herein described is required.

Further work will be pivotal to consolidate our last in vitro strategy, the co-cultures. We believe this is a very promising model in terms of its clinical translation potential. Therefore, extensive studies will be conducted for identification of the mechanisms underlying the conditioned medium potential to induce chondrogenic differentiation without the use of growth factors.