Universidade do Minho
Escola de Engenharia

José Rui Ribeiro da Cruz Pereira

Insights into the role of biochemical factors and extracellular matrix environment during chondrocyte culture: relevance on articular cartilage repair strategies

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I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, February 5th, 2015

Full name: José Rui Ribeiro da Cruz Pereira

Signature: _____

To my Parents and Sister

“É apenas o começo. Só depois dói,
e se lhe dá nome…”

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INSIGHTS INTO THE ROLE OF BIOCHEMICAL FACTORS AND EXTRACELLULAR MATRIX ENVIRONMENT DURING CHONDROCYTE CULTURE: RELEVANCE ON ARTICULAR CARTILAGE REPAIR STRATEGIES

Abstract

Considering the high multifaceted biology environment of cartilage connective tissue it is fairly clear that understanding the cellular interactions that regulate cartilaginous homeostasis under physiological and inflammatory conditions is a keystone necessary for a successful cartilage tissue engineering strategy approach. Total regeneration and repair of this structurally organized tissue after damage caused by trauma or/and inflammatory process is, in XXI century, still quite limited. This tissue, that covers almost all the terminal bone extremities of human body, presents a limited capacity of self-regeneration after injury mainly due to its avascular nature and, by consequence, poor access to lesion site of systemic growth factors and cytokines as well as host recruited cells that, together, could play an important role tissue repair.

Chondrocytes, the only cellular type present in articular cartilage, and the extracellular components of their dense and compact matrix, have become the focus of several investigations driven by tissue engineering, basic and clinical research fields seeking to improve the current clinical approaches to repair cartilage. Articular chondrocytes are the obvious cell source for cartilage regeneration and are nowadays the state of the art for several clinical procedures. The harvest of a sufficient number of autologous primary cells and consequent preservation of their somatic phenotype memoir are the first major issues concerning the actual clinical approaches to repair cartilage lesions. Due to the above-mentioned limitations of articular chondrocytes, stem cells from different sources can and have been used as an alternative cell source in cartilage tissue repair strategies. With their intrinsic plasticity, adult or embryonic stem cells can be a supporting tool for new ideas and strategies in cartilage tissue engineering. In this context, using a non-direct contact culture system composed of human umbilical wharton's jelly stem cells and human articular chondrocytes we demonstrated an *in vitro* cartilaginous strengthening of the uncommitted stem cells. Chondrocytes secreted soluble factors were able to enhance wharton's jelly gene expression of cartilage markers over time with contemporaneous augmentation of cartilage extracellular proteins deposition in pellet culture.

The use of blood derivates and biodegradable biomaterials in cartilage tissue engineering has been increased in the last years. The use of platelet lysate (PL) in human articular chondrocyte culture augmented cellular kinetic growth with the maintenance of their somatic cartilaginous phenotype memoir provided evidences by the performed *in vitro* and *in vivo* assays. The different action of platelet lysate on human chondrocytes depends on the environment. Physiological or inflammatory conditions

were also addressed in this first part of the thesis. Here it is demonstrated that PL plays a key role in inflammation resolution; initially causing a boost of inflammatory cytokines with subsequent, over time, diminish of COX2 expression and NF-kB activation values. Furthermore, PL has shown to have a strong chemotactic effect on human articular chondrocytes particularly under mimicked inflammatory conditions. A serious limitation of the paradigmatic use of biomaterials, with micro and/or nano dimensions, is their biocompatibility effect on the target cells. The development of new biomaterials for cartilage *in situ* delivery and consequent study of their effect on articular cartilage resident cells was aimed in the following part of the thesis. Novel Hyaluronic acid (HA) microparticles were made and characterized. The presence of HA microparticles, in direct and non-direct contact with chondrocytes did not interfere with cellular viability and growth over time. In the aforementioned conditions, cells retained their gene expression profile and their expression of cluster of differentiation 44 (CD 44) confirming the potential use of HA microparticles in a cartilage tissue engineering strategy.

The employ of polyelectrolyte polymer complexes for cartilage tissue engineering can be potentially advantageous. HA, polyanion polysaccharide and one of the main components of chondrocyte extracellular matrix, and poly-L-lysine (PLL), polycation polymer known for its biocompatibility and adhesion properties were used to form polyelectrolytes structures. The final size and structure stability of the formed nano complexes was demonstrated to be dependent of polymers combined ratios and solution salt molarity. The possibility to deliver cells within the cartilage lesion site has been focus of research in the last years as a new frontier in cell-base strategies. In the final part of this thesis, we developed a biodegradable injectable hydrogel to be use as a delivery vehicle for human articular chondrocytes. The novel hydrogel has able to support chondrocyte viability and proliferation. Encapsulated cells retained their capacity to form a neocartilaginous tissue positive for human type II collagen as demonstrated by both *in vitro* and *in vivo* evidences. In overall, the work exposed in this thesis presents valid insights into the role of biochemical factors and extracellular matrix environment during chondrocyte culture. Observations can potentially have high relevance on articular cartilage repair strategies.

EFEITO DE FACTORES BIOQUÍMICOS E COMPONENTES DA MATRIZ EXTRACELULAR NA CULTURA DE CONDRÓCITOS: IMPORTÂNCIA EM ESTRATÉGIAS DE REGENERAÇÃO DE CARTILAGEM ARTICULAR

Resumo

Tendo em consideração a complexa organização do tecido cartilagíneo, é de fundamental importância compreender as interacções célula – célula e célula- matriz que mantêm o equilíbrio homeostático em condições fisiológicas e de inflamação, para ter sucesso em estratégias de reparação deste tecido. Mesmo em pleno século XXI, a total cura e regeneração deste tecido conectivo com elevada organização estrutural, não é ainda satisfatória. Devido ao seu carácter avascular, que impede ou diminui fortemente a possibilidade de ter acesso, a nível sistémico e local, a factores de crescimento, a quemoquinas e a células com potencial regenerativo, a cartilagem articular tem um nível de auto regeneração muito baixo ou quase nulo. O único tipo celular que está presente neste tecido conectivo – condrócitos –, assim como os componentes da matriz extracelular que os rodeiam têm sido alvo, desde sempre, de investigação da ciência de base até à aplicação clínica na procura de novas abordagens para melhorar a regeneração e reparo deste tecido. Os condrócitos articulares são, deste modo, a fonte celular mais utilizada em ensaios clínicos, para reparar e regenerar defeitos articulares. Neste âmbito, um dos maiores aspectos limitantes do uso autólogo deste tipo celular é a dificuldade em obter um número suficiente de células mantendo, ao mesmo tempo, a sua memória fenotípica, ambos necessários para o sucesso final da sua utilização. Devido a este facto, a utilização de células estaminais adultas ou fetais tem vindo a ser considerada uma forte alternativa ao uso das células somáticas deste tecido conectivo. Assim sendo, modelar a plasticidade fenotípica estaminal e direccioná-la para um possível diferenciamento cartilagíneo, é de fundamental importância. Neste contexto, pretendeu-se, num sistema de co-cultura indirecta entre células Wharton Jelly de cordão umbilical e condrócitos articulares, ambos humanos, incrementar o potencial condrogénico das células estaminais. Factores de crescimento produzidos pelos condrócitos foram capazes de agir paracrinamente e aumentar a expressão génica de marcadores cartilagíneos nas células de Wharton Jelly. Pellets destas células cultivadas na presença de condrócitos apresentaram uma maior deposição de matriz extracelular comparada com as cultivadas na ausência de condrócitos. O uso de derivados de sangue, bem como de biomateriais em engenharia de cartilagem, tem aumentado nos últimos anos. Em particular, a utilização de lisados de plaquetas em substituição do soro bovino incrementou a capacidade replicativa dos condrócitos articulares humanos mantendo, ao mesmo tempo, a sua capacidade de diferenciação, como se demonstrou nos testes *in vitro* e *in vivo*. Na presença de estímulos inflamatórios, os lisados das plaquetas causam nos condrócitos um forte aumento inicial da secreção de proteínas inflamatórias que, com o decorrer do tempo, decresce acompanhado da

redução da expressão da ciclo-oxigenase2, assim como dos valores de activação de NF-Kb. Para além disto, os lisados das plaquetas têm um efeito atractivo nos condrócitos em particular em situações de inflamação. Um dos aspectos importantes da utilização de biomateriais é a sua biocompatibilidade com as células alvo da sua utilização. Uma parte desta tese foi focalizada no estudo e desenvolvimento de novos biomateriais para utilização *in situ* na regeneração cartilagínea e respectivo efeito nos condrócitos. Foram desenvolvidas e caracterizadas inovadoras micro partículas de ácido hialurónico. A viabilidade celular e a capacidade mitótica dos condrócitos articulares não foi significativamente alterada quando estes foram cultivados na presença directa ou indirecta das micro partículas. Os condrócitos mantiveram a expressão dos seus marcadores genéticos característicos, assim como a expressão do marcador de membrana CD44, o que indica que as micro partículas de ácido hialurónico poderão ter um bom potencial em estratégias de reparo e regeneração da cartilagem. O uso de complexos polieletrólitos em engenharia de tecidos da cartilagem pode trazer grandes benefícios. O ácido hialurónico é um dos componentes da matriz extracelular cartilagínea com carga negativa o que lhe confere a possibilidade de conjugar-se com polímeros de carga oposta. A poli-lisina é um polímero com carga positiva, altamente bio compatível, e com propriedades de adesão. A união destes dois polímeros originou a formação de complexos polieletrólitos. A formação destas estruturas, a sua carga e dimensões, estão fortemente dependentes dos ratios de polímeros utilizados. A possibilidade de injectar células no local da lesão cartilagínea tem sido, na última década, fonte de investigação no campo da engenharia de tecidos da cartilagem. Na parte final desta tese, foi desenvolvido um gel biodegradável e termo sensível, para injectar os condrócitos articulares humanos, no local lesionado da cartilagem. Neste contexto, foi utilizado este gel inovador no qual os condrócitos encapsulados mantiveram a sua vitalidade e capacidade proliferativa. As células encapsuladas mantiveram a sua memória fenotípica, ao longo do tempo, formando um novo tecido cartilagíneo positivo para a presença de colagénio de tipo II, em ambos os estudos *in vitro* e *in vivo*.

Em geral, o trabalho exposto nesta tese revela propostas inovadoras e funcionais na cultura de condrócitos. Estas observações são muito relevantes, em novas estratégias na engenharia de tecidos da cartilagem.

APPROCCI INNOVATIVI COINVOLTI NEL REPARO DELLA CARTILLAGINE: RUOLO DI FATTORI BIOCHIMICI E MATRICE EXTRACELLULARE IN CULTURE *IN VITRO* DI CONDROCITI UMANI.

Abstract

La cartilagine è un tessuto complesso, capire le interazioni cellulari che regolano l'omeostasi cartilaginea in condizioni fisiologiche e infiammatorie, è fondamentale per affrontare con successo la rigenerazione di tale tessuto.

La cartilagine articolare è un tessuto privo di vasi sanguigni, terminazioni nervose e cellule del sistema immunitario, da qui la scarsa capacità rigenerativa. L'unico elemento cellulare è costituito dai condrociti, che sono preposti alla produzione di matrice extracellulare in cui sono immersi.

I danni della cartilagine, sia traumi che progressiva degenerazione, portano a una perdita della funzione dell'articolazione a causa dell'incapacità del tessuto di rigenerarsi. Sebbene si siano sviluppate diverse tecniche chirurgiche, nessuna ha mai avuto un successo consistente, e le strategie terapeutiche attuali sono orientate verso l'impiego dell'ingegneria tessutale.

I condrociti articolari sono considerati la fonte cellulare ideale in diverse procedure cliniche ricostruttive del tessuto cartilagineo. Il loro limitato potere proliferativo e la difficoltà di recuperare tessuti sani, da cui prelevare ed isolare condrociti limitano l'impiego di queste cellule in medicina rigenerativa. L'utilizzo di cellule staminali fetali o adulte altamente plastiche e differenzianti, potrebbe essere una fonte alternativa per sviluppo di approcci per la rigenerazione di cartilagine.

In questo contesto, in uno degli studi riportati, abbiamo allestito co-culture di cellule staminali derivate dalla gelatina ombelicale di Wharton e chondrociti articolari. Le cellule non sono state coltivate in contatto diretto, i fattori solubili secreti dai condrociti hanno aumentato il potenziale differenziativo in senso condrogenico delle cellule di Whorton; è stato osservato inoltre nelle culture in vitro un aumento di geni specifici espressi in cartilagine e deposito di matrice extracellulare cartilaginea.

Negli ultimi anni l'uso dei derivati piastrinici e biomateriali biodegradabili è da considerarsi una pratica comune per la rigenerazione di danni cartilaginei. La somministrazione di lisato piastrinico a coltura *in vitro* di condrociti articolari umani determina un aumento della proliferazione cellulare e il mantenimento delle caratteristiche somatiche delle cellule cartilaginee.

Nella prima parte della tesi ho studiato l'effetto dei derivati piastrinici in colture di condrociti umani coltivati in vitro in condizioni fisiologiche e/o infiammatorie. E' stato dimostrato che il lisato piastrinico (PL) svolge un ruolo importante nella risoluzione del processo infiammatorio; il trattamento delle cellule con PL determina un iniziale repentino aumento di citochine infiammatorie seguito da una graduale diminuzione dell'espressione di classici stimoli infiammatori quali, COX2 e l'attivazione della via del

segnale regolata da NF-kB. Inoltre, è stato evidenziato che in colture di condrociti articolari coltivati in presenza di stimoli infiammatori, i derivati piastrinici determinano un effetto chemiotattico.

Un secondo aspetto che abbiamo voluto affrontare nella tesi riguarda l'uso di biomateriali nei processi di rigenerazione cartilaginea. I nuovi biomateriali devono essere biocompatibili, presentare micro e/o nano dimensioni ideali per veicolare nel sito di lesione cellule e molecole in grado di ricostituire un tessuto. Micro-particelle di acido ialuronico (HA) di nuova generazione sono state utilizzate per coltivare *in vitro* condrociti umani. Abbiamo dimostrato che la presenza del biomateriale non interferisce sulla vitalità e crescita cellulare, le cellule mantengono un profilo di espressione genica tipico cartilagineo, ciò conferma il potenziale utilizzo di micro-particelle di acido ialuronico per rigenerare cartilagine.

L'utilizzo di complessi polimerici polielettrolitici potrebbe essere molto vantaggioso per ottimizzare biomateriali da utilizzare in ambito cartilagineo. Il polisaccaride HA, uno degli elementi fondamentali della matrice extracellulare dei condrociti, insieme alla poly-L-lysina (PLL), polimero policationico conosciuto per le sue proprietà adesive, sono stati utilizzati per formare strutture polielettrolitiche. Nello studio abbiamo caratterizzato le dimensioni, la carica e la stabilità strutturale del nano complesso sviluppato.

In fine abbiamo voluto studiare possibili approcci per veicolare cellule nel sito di lesione e sviluppare nuovi approcci cellulari da utilizzare in campo clinico per il riparo della cartilagine. A tal fine, abbiamo sviluppato un'idrogel iniettabile biodegradabile in grado di trasportare condrociti articolari umani. Studi condotti *in vitro* ed *in vivo* hanno dimostrato che il nuovo idrogel non interferisce con la vitalità e la proliferazione delle cellule, anzi i condrociti incapsulati all'interno del gel crescono e sono in grado di depositare matrice extracellulare e formare nuovo tessuto cartilagineo.

In conclusione, in questo studio si evince che fattori biochimici, matrice extracellulare e nuovi biomateriali giocano un ruolo rilevante nel guidare i condrociti umani a rigenerare nel sito di lesione un nuovo tessuto.

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A

ACI Autologous chonrocyte implantation

ADH Adipic dihidrazide acid

C

Ca^{2+} Calcium ion

$CaCl_2$ Calcium Cloride

CD Cluster of differentiation

COMP Cartilage oligomeric matrix protein

COX-2 Cyclooxygenase 2

CFU-f Colony-forming unit-fibroblasts

Cytl1 Cytokyne-like 1

D

Da Dalton

DAPI 4',6-diamidino-2-phenylindole

Dh Hydrodynamic diameter

DLS Dynamic light backscattering

DMEM Dulbecco's modified eagle's medium

dsDNA double strand Dna

E

ECM Extracellular matrix

EDCI-HCl Ethyl-3-[3-dimethylamino] propyl carboddimide

ELISA Enzyme-linked immuno sorbent assay

ESC Embryonic stem cells

F

FCS Fetal calf serum

FDA Food and Drug Admisnistration

FELASA Federation of European animal science association

FGF Fibroblast growth factor

G

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

GAGs Glycosaminoglycans

GFs Growth factors

H

HA Hyaluronic Acid

HAC Human articular chondrocytes

HBMSCs Human bone marrow stromal cells

H&E Hematoxylin&Eosin

HUVEC Human umbilical vein endothelial cells

HWJSCs Human wharton jelly stem cells

I

IL-1 alpha Interleukin 1 alpha

IL-6 Interleukin 6

IL-8 Interleukin 8

ITS Insulin-transferin-selenium

K

K^+ Potassium ion

KCl Potassium Chloride

L

LDV Laser droppler velocimetry

M

MPa Megapascal

MACI Matrix Induced Chondrocyte Implantation

MAPK Mitogenic-activated protein kinase

mRNA messenger Ribonucleic acid

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NGAL Neutrophil gelatinase associated lipocalin

nm Nanometer

NIFNAHA Non-Inflammatory fractions sodium hyaluronan

NSAIDs Non-steroidal anti-inflammatory drugs

O

OA Osteoarthritis

P

P Cell passage

PBS Phosphate buffered solution

PCR Polymerase chain reaction

PGA Polyglycolic acid

PL Platelet lysate

PLL Poly-L-Lysine

PPP Poor platelet plasma

PRP Platelet rich plasma

R

RA Rheumatoid Arthritis

RT Room temperature

RT-PCR Reverse transcriptional polymerase chain reaction

RUNX2 Runt-related transcription factor 2

S

SEM Scanning electron microscope

SOX9	Sex determining region Y-box9
SF	Serum free
T	
TGF	Transforming growth factor
2D	two-dimensional
3D	thee-dimensional

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Chapter VII – Novel injectable gel (system) as a vehicle for human articular chondrocytes in cartilage tissue regeneration

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This thesis is divided in four sections containing eight chapters, which are proposed to provide a widespread report of the progress work achieved during the PhD time. Chapters are ordered in a way that explains the sequential progress obtained in the different works. Obtained results originated five scientific papers and one review manuscript. The contents of each chapter are summarized bellow.

SECTION 1 (Chapter I)

Chapter I present an extensive literature review survey on the field of cartilage. It presents a comprehensive overview of cartilage structure, composition and properties that allows this connective tissue to be of extreme importance in the human body. It is also focused in discussing in detail the problems concerning cartilage current repair strategies presenting a widespread outline of those applied in clinics. This chapter should inspire and stimulate the debate in the perspective of works presented in this thesis.

SECTION 2 (Chapter II)

Chapter II provides a detailed description of the materials and methods used within the scope of the thesis. Although each chapter, witch describes the scientific work performed, is accompanied by its specific materials and methods sector, in this particular section, materials, methods and basic principles behind the methodologies used within the scope of this thesis are detailed exposed.

SECTION 3 (Chapters III to VII)

Chapters III to chapter VII describe the experimental work performed within the thesis.

Chapter III

This chapter includes a paper proposing a non-direct co-culture strategy between human articular chondrocytes and human wharton's jelly stem cells. Here are presented results sustaining a possible *in vitro* chondrogenic commitment of human wharton's jelly stem cells by co-culture system with human articular chondrocytes.

Chapter IV

The work within this chapter demonstrates the outcomes of the use of platelet lysate on human articular chondrocytes behaviour. It is verified an interrelated dual role of platelet lysate on human articular chondrocytes, i) maintenance of chondrogenic somatic characteristics, ii) transient pro-inflammatory burst activity follow-through an pro-resolving loop.

Chapter V

This chapter includes a study aimed to develop novel hyaluronic acid microparticles and to understand their effect on primary articular chondrocytes behaviour. In overall, in both culture conditions, direct and non-direct contact, hyaluronic acid microparticles did not affect adult articular chondrocytes mitogenesis and phenotype.

Chapter VI

The chapter VI reports the use of opposite charged polymers to develop novel nano-complexes to be applied in cartilage repair strategies. Complexes formed by hyaluronic acid (poly-anion) and poly-l-lysine (poly-cation) molecules in presence of sodium carbonate were analyzed. Influence of polymers ratios and salt solution in final size and charge of the complexes was the principal aim of the study.

Chapter VII

This chapter describes an innovative injectable biodegradable hydrogel to be use locally as a deliverable vehicle of human articular chondrocytes in cartilage tissue engineering repair strategies. On the whole, human articular chondrocytes within the novel carragenan/fibrin/Hyaluronic acid-based hydrogel demonstrated both *in vitro* and *in vivo* capacity to duplicate with the maintenance of their intrinsic differentiation potential and capability to deposit extracellular matrix over time.

SECTION 4 (Chapter VIII)

The final chapter of this thesis highlights the contribution and conclusions derived from the presented works. It includes general conclusions and final remarks regarding the overall work carried out under the scope of this thesis. It also aims to present a discussion on future perspectives and work to accomplish progress in articular cartilage regenerative medicine.

The six chapters presented in this thesis are based on the following articles:

Chapter I

R.C. Pereira, H.S. Azevedo, R.L.Reis, Cartilage structure and biological function: current therapeutic approaches, *submitted*.

Chapter III

R.C. Pereira, A.R. Pinto, A.M. Frias, N.M. Neves, H.S. Azevedo, R.L. Reis, *In vitro* chondrogenic commitment of human wharton's jelly stem cells by co-culture with human articular chondrocytes, *submitted*.

Chapter IV

R. C. Pereira, M. Scaranari, R. Benelli, P. Strada, R. L. Reis, R. Cancedda, and C. Gentili, Dual effect of platelet lysate on human articular cartilage: a maintenance of chondrocgenic potential and a transient pro-inflammatory activity followed by an inflammation resolution, *Tissue Eng Part A. 2013 Jun;19(11-12):1476-88*.

Chapter V

R.C. Pereira, R.L. Reis, H.S. Azevedo, Hyaluronic acid microparticles as intraarticular injectable drug carrier for cartilage repair: effect on human articular chondrocyte behavior, *submitted*.

Chapter VI

R.C. Pereira, da Silva RMP, H.S. Azevedo, R.L. Reis, Hyaluronic acid/Poly-Lysine nano complexes for cartilage repair strategies, *submitted*.

Chatper VII

R.C. Pereira, M Scaranari, P. Castagnola, M. Grandizio, H.S. Azevedo, R.L. Reis, R. Cancedda, 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.*

SECTION 1
BACKGROUND

CHAPTER I

CARTILAGE STRUCTURE AND BIOLOGICAL FUNCTION: CURRENT THERAPEUTIC APPROACHES

CHAPTER I

CARTILAGE STRUCTURE AND BIOLOGICAL FUNCTION: CURRENT THERAPEUTIC APPROACHES.*

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Abstract

Cartilage is a connective tissue responsible to cover and protect bone of diarthrodial joints from load bearing, impact, allowing frictionless motion between articular surfaces. There are three different kinds of cartilage: hyaline cartilage, elastic cartilage and fibrocartilage. In this review it is discussed the development or cartilage, its structure and composition, and its biochemistry and biomechanical properties. Also, we have review the state of the art of cartilage lesions and trauma, and current treatments for cartilage repair. Lastly, several examples of biomaterials, injections, based on hyaluronic acid in cartilage therapies have been proposed.

Existing clinical methods are normally limited in their ability to regenerate functional cartilage. For this reason, cartilage research has been focus on tissue engineering strategies. So far, no engineered long lasting hyaline cartilage meeting the functional demands of the tissue *in vivo* was developed. New directions are crucial to achieve new goals combining strategies of different areas such as chemistry, biology, engineering and medicine.

Keywords

Hyaluronic acid, cartilage structure, functionality, cartilage repair strategies

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*This chapter is based on the following publication:
R.C. Pereira, H.S. Azevedo and R.L.Reis, "*Chapter I. Cartilage structure and biological function: current therapeutic approaches*", submitted

1. Cartilage

Cartilage is a specialized form of connective tissue that covers and protects bone of diarthrodial joints from load bearing, impact associated forces, and allows nearly frictionless motion between articular surfaces [1]. Depending on their constitution and function, there are three distinct kinds of cartilage, hyaline cartilage, elastic cartilage and fibrocartilage [2]. Hyaline cartilage is the most abundant type of cartilage. Most of the skeleton of the fetus is laid down in cartilage before being replaced by bone. Hyaline cartilage in the adult is found in the nose, parts of the respiratory tract, at the ends of ribs and at the articular surfaces of bones.

1.1. Cartilage development/embryology

Cartilage has its origin in mesenchyme. During embryonic development, the peripheral cells of the bastema – aggregates of mesenchymal cells condense to form a bilaminar perichondrium where the outer layer becomes fibrous while the developed interior part, more packed, start to secrete cartilage matrix molecules giving origin to chondroblasts. These cells, individually separated and surrounded by continuous deposit of extracellular matrix, are now, *per se*, called chondrocytes [3].

Cellular condensations events are the initial structures responsible for proper formation of cartilage during which cells make surface contact with no other [4].

In chondrogenic condensation, process dependent of cell-cell and cell-matrix interactions, hyaluronic acid (HA), the predominant glycosaminoglycan of initial mesenchymal cells extracellular matrix (ECM), plays a fundamental role on the regulation of a embryogenic morphogenetic events [5, 6]. During cartilage oncogenesis, cells express specific binding sites for HA which influence cell behavior. Do to the molecule structure and hydrophilic nature, HA is responsible for ECM expansion, allowing cell proliferation, patterned migration and cell-cell communication, postponing cartilage differentiation [5, 7].

Embryonic cartilage is destined for one of several fates; remain as permanent cartilage, like the one present on articular surfaces of bones or provide a template for formation of long bones through the process of endochondral ossification.

Adult cartilage, connective tissue with origin in mesenchymal cells, exists in the entire articular surface of bones. Composed by a small number of a unique cellular type – chondrocytes – this tissue appears to be of high simplicity. This fake minimalism masks a convoluted balance between anabolic and catabolic processes needed to maintain metabolically active the tissue and its extracellular matrix. Adult articular cartilage has very important biomechanical functions directly related to its biochemical structure and location in the body: responsible for mechanisms of shock absorption and, supply lubrication to the articular zones with consequent spread of the applied loads to the underlying subchondral bone [8-11].

1.2. Cartilage structure and composition

It is known that in cartilage, the ECM is responsible for outcomes on chondrocyte cell morphology, metabolism and phenotype. Chondrocytes, occupying 5% of the total volume of the tissue, have as primary function the regulation and maintenance of the complex ECM homeostasis [12]. Chondrocyte structure, organization and function, as well as collagen, proteoglycans and non-collagenous extracellular matrix components are hierarchy organized from the external to the inner part of the connective tissue.

Healthy articular cartilage is an anisotropic tissue and its macroscopic structure can be divided into four morphologically distinct zones, as illustrated in Figure 1.

In contact with the synovial fluid, the superficial zone represents 10 up to 20% of the total cartilage full thickness. It is composed of flattened ellipsoid chondrocytes and high concentration of collagen fibers, both parallel oriented to the joint surface. This zone is responsible for providing high tensile and shear strength and protection from synovial fluid immune system. With low cellular density, the middle zone represents 40–60% of the connective tissue. Within this zone, rich in aggrecan and collagen fibers, randomly oriented chondrocytes are embedded in a

dense matrix assuming a spheroid shape. Immediately after, the deep zone occupies 30% of the distal end region of the cartilage. This zone is populated by larger spheroidal cells with perpendicular orientation to the surface having as bottom delimitation, just above the subchondral bone, a thin line of calcified cartilage. This forth-distal zone – calcified cartilage zone - is composed of a small amount of cells within a calcified matrix. Chondrocytes present a hypertrophic phenotype with synthesis of collagen type X. This zone is the transitional frontier with the subchondral bone [10, 13, 14].

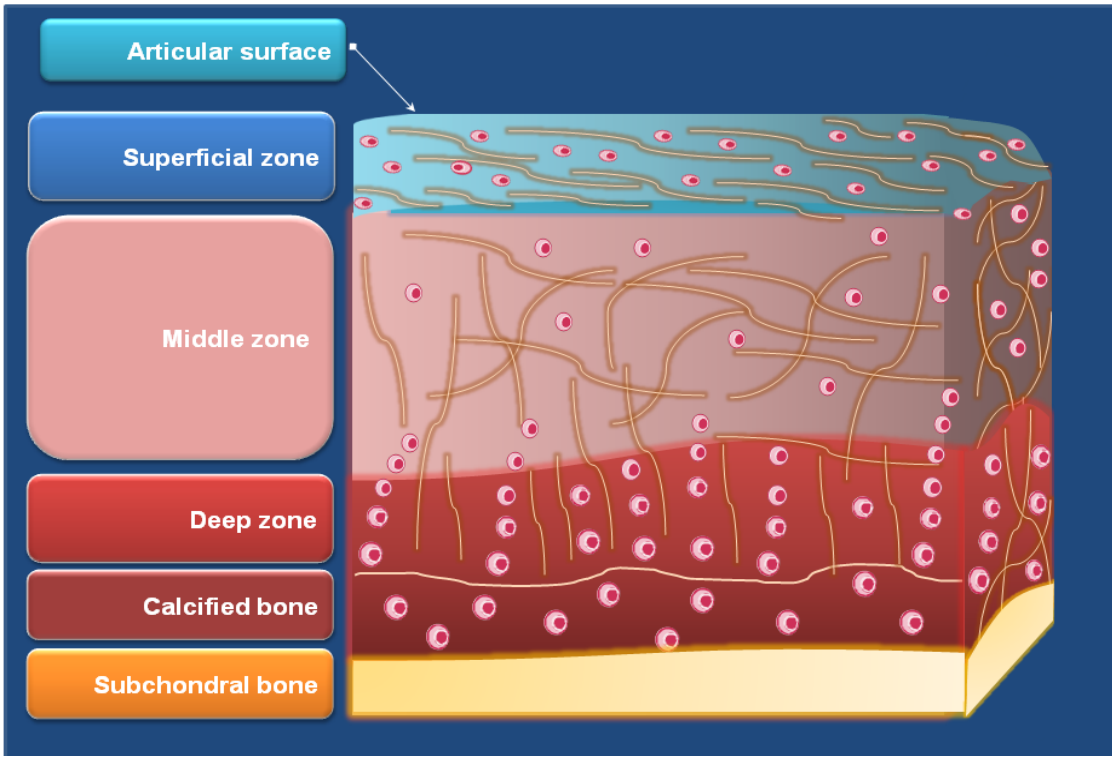

Figure 1 - Illustration showing the distinct zones of the anisotropic structure of mature articular cartilage (adapted from [15]). Articular cartilage is aneural, alymphatic and avascular tissue.

This entire anisotropic connective tissue has as main component water that occupies between 60 and 85% of its total constitution. Beyond this, the dry fraction is composed by two types of molecules: collagenous – 45% and non-collagenous – 35% having, depending of their nature, different functions. Collagen type II fibers are the most representative type of collagen in adult articular cartilage - almost 95% of this tissue portion. Other type of fibrils are also present such as collagen type V, VI, IX, X and XI [16-21]. Collagen type II has the ability to fold up with two other fibrils forming a triple helical structure. This biochemical phenomenum is product of the union of several alpha chains namely glycine, proline and hydroxyproline. This structure confers to collagen type II fibrils a

higher stability and resistance to compression forces [19, 22, 23]. Type XI collagen is covalently linked with collagen type II fibers conferring them more stability. Collagen type VI is present in the pericellular matrix surrounding each single chondrocyte [24]. Depending on the cartilage zone, these fibers can also encircle single column of cells, like it happens in the deep zone. All the fibrils network present in full cartilage structure act as a support for the proteoglycans that provide compressive strength to the tissue. Figure 2 shows the major constituents of cartilage matrix and their arrangement into large molecular assemblies. HA is strategic player in cartilage ECM, functioning as the central filament of the cartilage proteoglycan aggregate (aggrecan).

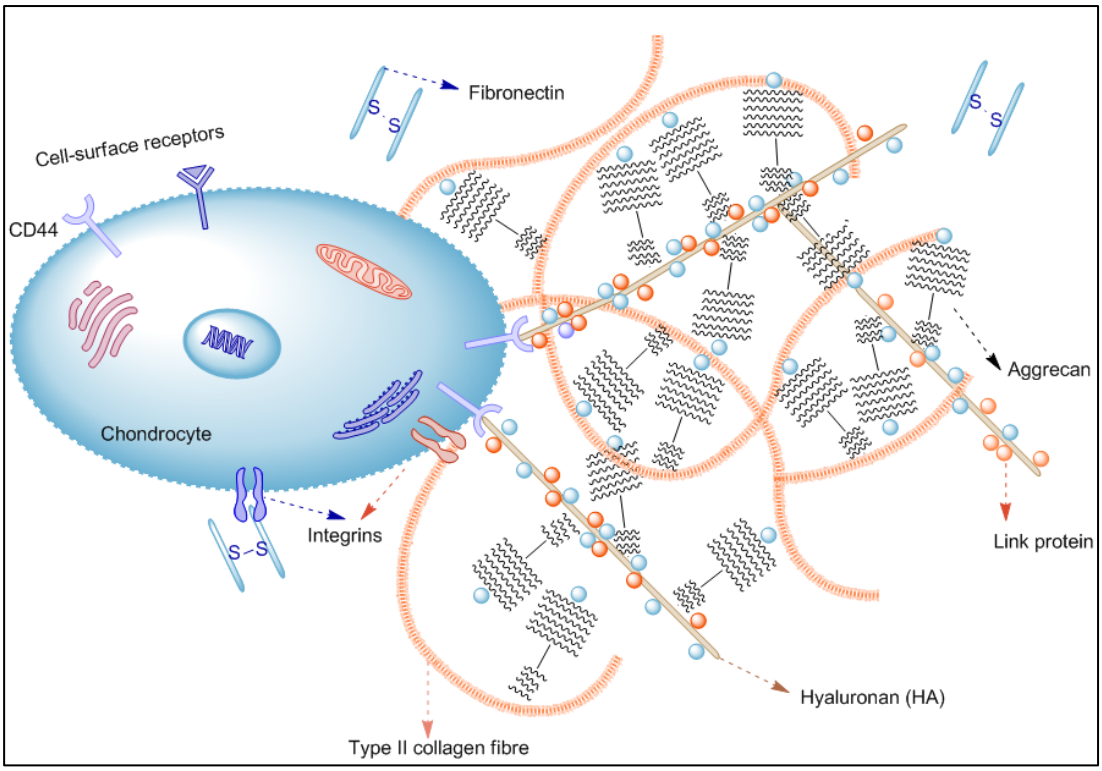

Figure 2 - Illustration of articular cartilage ECM. The matrix can be viewed as a composite with a network of type II collagen fibers, which is reinforced by crosslinks formed between chains of HA, proteoglycans, and other non-collagenous proteins. The load-bearing properties of cartilage are provided by the tensile properties of the collagen fiber network and the osmotic swelling pressure of the high concentration of aggrecan. The aggrecan is immobilized within the matrix by forming supramolecular aggregates with HA and link protein.

In cartilage there are two major classes of proteoglycans, those that are large aggregates of proteoglycan monomers – aggrecans and others smaller that include decorin, fibromodulinn and biglycan. Proteoglycans sub-units are called glycosaminoglycans (GAGs) [25]. These molecules are divided into chondroitin sulphate and keratin sulphate that are long chains of disaccharide units with the ability to attach to hyaluronic acid and other

link proteins. [26-28] The role of link protein is to stabilize the chain with a central hyaluronic acid backbone forming and intrinsic structure of the GAG macromolecule [27]. These events lead to the increase concentration of inorganic ions within the matrix and consequent osmolarity augment. By this manner, proteoglycans are the main responsible for the upholding of fluid and electrolyte balance in articular cartilage [29, 30].

1.3. Biochemistry and Biomechanical Properties of Articular Cartilage

With a unique architecture, single cells entrapped in dense extracellular matrix and location, isolated from systemic regulation with lack of blood supply, articular cartilage has a poor capacity of self-regeneration when damaged.

The combination of all distinct components and different zones in this avascular and alymphatic tissue makes cartilage highly specialized with low metabolic activity.

Chondrocytes are the only responsible cells for the growth and maintenance of the tissue. As so, they are often asked to perform multiple functions, including matrix synthesis and matrix degradation, that in other tissues are performed by more than one cellular type (e.g., bone deposition by osteoblasts and bone resorption by osteoclasts). The remodeling of cartilage tissue requires regulated cell proliferation, growth, synthesis of extracellular matrix proteins, production of matrix degrading enzymes, and in some cases, at the final, cell death and ECM calcification. With this, presenting a low mitotic activity, adult articular chondrocytes, play a fundamental role in maintaining the integrity of the cartilage through balanced orchestra of anabolic and catabolic activities. The functional role of cartilage is directly related with its ECM composition.

During lifetime, cartilage is subjected to loads exceeding five times the body weight that corresponds approximately to pressure values near 5 megapascal (MPa) [31]. Above this physiological level, pressure can cause cartilage fissures with further ruptures in calcified cartilage zone. Cartilage ability to act as weight bearing specialized tissue is directly related to collagen and negatively charge aggrecan ECM components [32, 33]. Stratified into zones with distinct morphological appearance, biochemical composition and structure, cartilage

mechanical properties of each layer vary to suit the functional role of them. These zonal biomechanics makes, for instance, the superficial layer more stiffness in tension then the other zones because of the parallel arrangement of the collagens fibrils [33]. Compression modulus for the superficial zone are significantly lower than those presented in deeper zones [34]. The remarkable mechanical and load-bearing properties of cartilage tissue are given by the combination of type II collagen fibers, responsible for tensile strength, and GAGs, responsible for load distribution and compressive stiffness. Healthy human adult articular cartilage present values of tensile modulus between 5 and 25 MPa and compressive modulus of approximately 800 KPa, depending on the joint depth [32].

Like in other tissues, cartilage permeability depends on pore size and interconnectivity [35]. Cartilage present approximately 70% of porosity with an average pore diameter between 3 and 6 nanometer (nm) [36]. Due to this fact and because compression causes further reduction in the average pore size, cartilage shows low values of permeability.

Physiological durability of articular cartilage, in light of the constant mechanical loads, is attributed to cell-matrix interdependence. In the cell-matrix crosstalk phenomenon, chondrocytes, through a balance between synthesis and degradation, are responsible for matrix maintenance receiving in the other hand, from ECM components, mechanical protection and stimuli to maintain their intrinsic phenotype. With so, cartilage properties depend in major on the ability of the resident cells to maintain the composition and structure of their inner matrix. Although, chondrocytes can, in some extent play that role, they present limited capacity to repair damaged or degenerated tissue.

2. Cartilage injuries/lesions and current treatments for cartilage repair

Cartilage lesions caused by trauma or disease has been recognized as a cause of significant morbidity for more than 200 years, affecting a significant part of the worldwide population [37, 38].

Articular cartilage has a relatively high incident of damage and deterioration, commonly caused by trauma such as sports injury and diseases like osteoarthritis (OA) and rheumatoid arthritis (RA). Consequently, different joint injuries lead to different possible repair responses.

Adult hyaline superficial lesions damage the flattened chondrocytes highly packed within paralleled oriented collagen type II fibers causing the diminish of proteoglycans content. Consequently, increase of tissue permeability and augment of direct biomechanical response during compression. Deepest lesions of articular cartilage lead to chondrocyte apoptosis and disturbance of cartilage matrix turnover. As a consequence, this effect results ultimately as long-term absence of the tissue homeostasis and disruption of structure and biomechanical functions. Due to demographic and life style changes, degenerative diseases are an enormous socio-economic and medical challenge in industrialized countries. Musculoskeletal disease such as OA is one of the most prevalent.

Osteoarthritis is a chronic disease that causes serious pain and disability. This disease is not totally understood, and causes alterations, not only in cartilage, but also beneath it, at the subchondral level. OA is characterized by progressive loss of articular cartilage, subchondral bone sclerosis, osteophyte formation, changes in the synovial membrane and increase volume of synovial fluid with reduced viscosity [39, 40]. Currently, there is no gold standard for the repair or prevention of onset of this disease and neither preventive measures nor disease-modified drugs are available. There are three major challenges: reducing inflammation, cartilage repair and subchondral bone repair.

OA, as described above, is a chronic disease that causes serious pain and limitations in activities. OA is the most prevalent joint disease, but neither preventive measures nor disease-modifying OA drugs are available in patients with established or advanced disease state.

Several authors demonstrated that different intraarticular corticosteroid injections provided symptomatic and functional pain relief on patients with knee OA. Intraarticular corticostereoid injections, combined with pharmacological treatment and physiotherapy, have been also used to control the local inflammation and relieve

pain in patients with OA. Attempts of cartilage and subchondral bone repair can be made either using only cells or cells within different biomaterials.

Clinical consequences of articular cartilage defects, caused by trauma or degenerative joint diseases, result in severe knee pain, swelling, mechanical symptoms, functional disability. These clinical problems result in disability for millions of people worldwide causing a social impact with high costs in terms of therapeutic treatments. The challenge to heal articular cartilage full thickness defects is a multidisciplinary task faced by both scientists and orthopedics/surgeons/physicians.

The technique of autologous cartilage implantation (ACI) described initially by Peterson *et al.* [41] was the first cell based therapy in cartilage orthopaedic field. Such modus operandi was developed to overcome the limitations of the already existing approaches in the early 90's. The initial results regarding the follow up after 39 months of 23 patients were published in 1994 by Brittgerg *et al.* [42] demonstrating good and even excellent results in approximately 70% of the cases. Involving the implantation of a suspension of healthy autologous chondrocytes into the lesion site and further covered with autologous periosteum membrane, ACI, has been used in a vast variety of patients and pathologies maintaining good outcomes as demonstrated by diverse studies [43-47]. Nevertheless, ACI has associated limitations, like high complexity of the surgical procedure, and generation of fibro and hypertrophic cartilage [48]. In addition, due to the low cell retention caused by the direct transplantation of cellular suspensions in the lesion site, in addition to some donor site morbility and elevate cost, new porous three-dimensional (3D) scaffolds start to be used aiming to a superior cell attachment to the scaffold/lesion site and consequently, improve cartilage repair.

Several alternatives to autologous periosteal cover procedure used in the first cell therapy, has been proposed in the past years. Three dimensional (3D) scaffolds have been seeded with chondrocytes and used in clinical applications in the last years showing good signs of acquisition and maintenance of chondrogenic phenotype. The application by physicians of the second generation of cell grafts started in Europe more than a decade ago. From there until now, scaffolds clinically applied can be divided by the nature of their matrix, being mainly made of collagen [49] and HA [50]. Both cases of tissue-engineered implants require the isolation of chondrocytes, with

subsequent cell seeding onto the scaffolds (with or without cell expansion period) and further final implantation within the lesion site.

The first 3D matrix induced chondrocyte implantation (MACI) was of a porcine collagen type I/III membrane (Chondro-Gide, Switzerland) using the matrix-induced technology was performed in the 1998 and since then, in Europe and Australia, more them 3000 patients have been treated [45, 49]. This method comprehends two distinct surgery steps: i) initial harvesting of articular cartilage from non-weight bearing area and subsequent chondrocyte isolation, expansion and seeding into the 3D biomaterial; ii) implantation of the graft into the damaged tissue.

HYAFF is a wide class of biopolymers resulted from the improvement of HA stability by sterification [51]. The use of these biomaterials associated with cells led to a new concept in cartilage repair strategies named Hyalograft C®. Introduced clinically in european countries in 1999 for the treatment of full-thickness cartilage defects, Hyalograft C® has already been applied in almost 5000 patients demonstrating good results [50, 52, 53]. The application of this technique requires also chondrocyte isolation from a non-weight loaded cartilage zone, expansion and finally, the cellular seeding within the HA scaffold. Globally, patients treated with Hyalograft C® demonstrated to have an increase mobility with reduction of pain and other symptoms of cartilage injuries. Repaired cartilage assumes high quality and good integration with the healthy surrounding tissue representing, in this manner, a valid option to treat knee cartilage injuries.

In all the cases involving the application of a 3D scaffolds, clinical data sustains a significant improvement regarding the ACI approach. Outcomes demonstrate improvement of graft fixation and significantly reduced operating time. During the past years, the features of these biomaterials have also allowed the development of new surgical *modus operandi* like arthroscopy. The application of this technique provided not only the reduced surgical time, but also, better recovery, and less patient morbility when compared to open surgery procedures.

2.1. Applications of HA biomaterials in cartilage therapies

In the past years cell-based tissue-engineered implants technology has been used in Europe and Australia with promising outcomes. However, this approach in cartilage repair is not usually available in the United States of America due to Food and Drug Administration (FDA) restrictions. FDA did not approve the application of these techniques in human beings due to problems that can be raised from the *ex vivo* chondrocyte isolation, culture and expansion before their seeding within the scaffold. In a world where dwindle frontiers are a well establish reality, we do believe that rules should be instituted to regulate the existence of a sort of worldwide "standard operation proceedings".

HA is an important ECM component that affects cellular behavior such as cell-cell adhesion, migration and differentiation as well as to play a critical role in the maintenance of ECM structure. Several studies have shown that hyaluronic acid is able to modulate the phenotype and behavior of articular chondrocytes. Schmitz *et al.*, [54] demonstrated that HA oligosaccharides displace HA from the cell surface inducing different bovine articular chondrocyte signaling events. They showed that these changes influences chondrocyte – matrix interactions by inducing altered matrix metabolism caused by the activation of at least two distinct signaling pathways. Abe *et al.*, [55] using different molecular weights of hyaluronic acid within a type I collagen matrix showed a direct relation between high hyaluronic acid molecular weights and matrix maturation. Due to its intrinsic protective physiochemical functions, hyaluronic acid has also been proposed as supplementation factor during chondrocyte culture. Aiming to determine the *in vitro* effects of HA supplementation on articular chondrocytes metabolism cultured within an alginate hydrogel culture system, Akmal *et al.*, demonstrated that phenotype maintenance, cellular proliferation and ECM synthesis within the construct is HA dose dependent [56]. At low doses HA had a significant stimulatory effect on metabolic activity of chondrocytes that could provide an explanation for the longer term clinical benefits of this glycosaminoglycan. It has also been demonstrated that hyaluronic acid present in other type of hydrogels is responsible for increasing cartilage ECM expression and cartilaginous phenotype maintenance [57].

The signaling pathways associated with known biological effects of HA on articular chondrocytes changes (e.g. cell migration and proliferation, cellular homeostasis and ECM deposition), have not been clearly defined. It has been shown that hyaluronan oligosaccharides are incorporated in articular chondrocytes through interactions with HA CD44 receptor [4, 58]. Knudson *et al.* [58] showed that HA oligosaccharides internalized on articular chondrocytes induced, not only an up regulation of matrix repair genes (i.e. collagen type II and aggrecan), but also the stimulation of catabolic metalloproteinases involved in cartilage matrix degeneration. The metabolic changes induced by hyaluronic oligosaccharides on chondrocytes were activated by two different signaling pathways [59, 60]. These data support the emerging paradigm that CD44 mediated intra-cellular signaling involves both articular chondrocyte anabolic and catabolic pathways.

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2.1.1. HA intra-articular injections for treating osteoarthritis

Due to its rheological and functional properties, HA does play a crucial role in joint homeostasis. The use of viscous solutions of HA or crosslinked derivatives as intra-articular injections to treat OA is nominated as viscosupplementation. Intraarticular injection of HA appears as an alternative method to nonsteroidal anti-inflammatory drugs (NSAIDs) in reducing pain and inflammation in OA. The use of several NSAIDs present severe side effects at gastrointestinal level (e.g. intestinal bleeds and ulcers) and increase risk of cardiovascular events. Viscosupplemetation has been used mainly to restore joint rheological properties, to promote a normalization of HA synthesis and consequent pain reduction. The first HA preparation ever made was described by Balazs *et al.* in the sixth decade of the last century [61]. Named as non inflammatory fraction sodium hyaluronan (NIFNAHA), this HA sterile preparation, depleted of inflammatory content and with sufficient purity levels, was initially applied in cartilage medical field. Nowadays, it is called Healon® and has applications in ocular surgery. Currently, several viscosupplementation products are used for treatment of knee OA. These clinical products are listed by increasing molecular weight, ARTZ® and Hyalgan®; Orthovisc® and Synvisc®. The use of these products in animal and clinical trials was initially supported by *in vitro* studies of the distinct molecular weight solutions that demonstrated the regulatory and protective effects on different cell types and tissues. It was proved the existence of a direct relationship between concentration, dosage and molecular weight of HA solution and cellular protective effect [62, 63].

ARTZ® is a 1% solution of HA with a molecular weight of an average of 6 to 800 000 Dalton (Da). Several authors, using diverse animal models, showed through histological analysis that ARTZ® injections leaded to a significant decrease of friction of the joint and partial preservation of the cartilage thickness [64, 65]. Williams *et al.* [66] described distinct results using a rabbit model. They have demonstrated that initial good effects of ARTZ® on reduction of severe cartilage changes after 1 week of induced OA were not maintained over time. Other authors have shown that reduce of pain and cartilage tissue maintenance depends on the intraarticular injections frequency [67, 68]. Clinical trial studies indicate that the satisfactory results of ARTZ® injection depends not only on the number of serial injections, but also on the population age and also on the clinicians [68, 69].

Other example of intraarticular preparation is Hyalan®, a solution composed of HA with a molecular weight between 5 and 730 000 Da. One clinical study showed that less deterioration was found in the structural parameters analyzed in patients that were injected with Hyalan® injection compared to the placebo group [70]. Several others clinical trials proved the efficacy of Hyalan® versus placebo administration by reduced pain and increase cartilage quality corroborated by arthroscopic results [70-72]. However, some contradictory results were reported by Henderson *et al.* showed no statistical differences on the final cartilage achievements between this injectable HA formulation and placebo [73].

Orthovisc® has a concentration slightly higher than both solutions aforementioned. Busconi *et al*. [74] evaluated the safety and the efficacy of Orthovisc® injection at the same arthroscopic debridement in a osteoarthritic joint. These authors concluded that the synergetic effect of Orthovisc® delivery with arthroscopic debridement might be responsible for the decrease joint infection and pain. However, it is therefore difficult to conclude more about this study due to the lack of placebo controls and blind trials.

Other HA injectable preparation is Synvisc®. This composition developed to treat OA by viscosupplementation is a mixture of hyaluronic polymers, 80% (V/V) of hylan A and 20% of hylan B gel presenting the highest molecular weight, (6000000 Da) of all the products clinically tested until now [75]. Clinical trials [75-78] revealed the improvement of cartilage quality baseline, as well as reduction of pain when compared to placebo and NSAIDs injections, indicating that this intraarticular HA formulation acts successfully reducing OA symptoms.

These findings should help to improve successful alternatives and to better evaluate/understand the safety and efficacy of marked products, as well as future ones seeking better solutions to treat OA. In general, all the intraarticular products present an excellent safety profile with almost absence of systemic adverse effects. It can only be pointed as significant adverse effect transient reactions in the injected knee site, that are benign, and the short half-life of the product. The reestablish of the homeostatic elastoviscosity conditions within the knee joint cavity and pain reduction as consequence of intraarticular injections of HA demonstrate their valid application on OA treatment.

2.1.2. HA scaffolds and hydrogels for cartilage tissue engineering

Cartilage was one of the first connective tissues under study to be regenerated/repaired for commercial targets. Even after efforts made in the last 20 years, clinically matching the native properties and functionality of cartilage remains a desirable and elusive aim.

The combination of few types of hyaluronic acid products and preparations with different approaches is commercially available and clinically tested [79-82].

Used since 1999, Hyalograf C®, a HA-based scaffold is one of the best alternatives to autologous chondrocyte transplantation [79]. The clinical approach consist in using this scaffold, composed of benzylic ester of HA with a 20 μm tick fiber network, to seed previously expanded autologous cells. Delivery in the lesion site by arthroscopic surgical technique, Hyalograf C® showed to have similar results to those obtained with autologous chondrocyte implantation [79].

Other authors proved with one year clinical follow-up outcome that autologous articular chondroyctes combined with HA and fibrin were able to induce cartilage regeneration [80].

Due to costs retention, health markets are driving their attention to products not involving cell usage commonly call “on-the-shelf biomaterials”. These ideal biomaterials from a surgical and commercial standpoint are in the last years been proposed for cartilage repair. Recently, a clinical trial using a cell-free scaffold made of HA and polyglycolic acid (PGA) with platelet rich plasma (PRP) showed good repair and improvement of cartilage functionality after one year of trial [82]. The celerity gain of cartilage regeneration using this cell-free scaffold must be followed in the incoming years. Other study proved that articular hyaline cartilage regeneration is possible combining HA with autologous peripheral blood progenitor cells [81].

Lately, a different approach has been used with HA hydrogels. These products were launched by sereval companies (e.g. Fidia S.p.a, LifeCore and Glycosan Biosystems). Hydrogels have as best properties the tunable mechanical strength and gelation rate that allow them to deliver into the cartilaginous lesion site easily then those already pre-molded.

Conclusions and future directions

Despite promising experimental advances, none of the current cartilage repair strategy has until now fulfilled the demanded needs of the field. Current clinical methods are generally limited in their ability to regenerate functional cartilage. For this reason, research has been focus on tissue engineering strategies. No one has engineered long lasting hyaline cartilage that meets the functional demands of the tissue *in vivo*. Perhaps at the present, the breakthroughs in cartilage repair and regeneration strategies do not rely totally in the achievement of other findings, but more, in looking to the existing problems with a keener way. In an age of dwindling frontiers, seek new directions is absolutely crucial to achieve innovative goals. It is of extreme importance to improve the exchange of knowledge between different areas such as chemistry, biology, engineering, medicine/surgery. Only the combination of biomaterials, cells and signals in cartilage tissue engineering can carry additional benefits when compared to the separate implementation of these individual fractions. On the spur of the moment, it is strictly necessary to use the known results to encourage the development of new and more rational strategies in cartilage field. The accomplishment of better results bypasses by understanding and controlling mutually, local microenvironment (paracrine regulation, extracellular matrix and additional extrinsic factors); cell-released signs (autocrine regulation); and cellular genomic potential and materials with detailed structure and composition.

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SECTION 2

EXPERIMENTAL

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

The objective of this chapter is to describe the materials and procedures used in the experimental work exposed in this thesis. This section is divided in sub titles depending on the raw materials used to process scaffolds/biomaterials and cellular culture conditions employed in the different chapters of this thesis. Each chapter contains the materials and methods used, but herein we intended to explain and describe in more detail the experiments that were done as well as the techniques applied.

1. Materials

1.1. Platelet –Rich Plasma (PRP) and Platelet Lysate (PL)

Due to its high spectrum content of growth factors (GFs), Platelet-Rich Plasma (PRP), an natural concentrate of autologous blood platelet GFs has been proposed as a cell culture medium supplement (1). Obtained with a simple, low cost and minimally invasive method, PRP was introduced into clinical therapies in 1998 to augment bone grafts (2). Since then, PRP or Patelet Lysate (PL), one derivate of PRP, has been applied in distinct fields of medicine aiming the improvement of tissue regeneration.

1.1.1. Preparation of purified Platelet Lysate (PL)

Platelet lysate was prepared according to Zaky and El Backly et al (3, 4) with slight modifications. Human whole blood samples were collected from six healthy donors (30 to 70 year old) from the Blood Transfusion Center of the San Martino Hospital, Genoa, Italy in consensus with the guidelines of the institutional ethics committee. To remove lymphocytes from PRP, buffy coats were centrifuged for five cycles at 1500 rpm for 15 minutes at room temperature as represented in Figure 1. After the final washing, platelet final pellet was resuspended in poor platelet plasma (PPP) arriving at a final concentration of 1x 10^7platelets/ml. To obtain platelet membrane rupture

and consequent GFs release, PRP preparations were immersed in liquid nitrogen for 1 minute before transferring at 37°C for 6 minutes. Three consecutive freeze-thaw cycles were repeated and platelet membranes removed by centrifugation. Supernatants (PL) were harvested, divided in aliquots and stored at –80°C until use. Platelet lysate was used in work described in chapter IV.

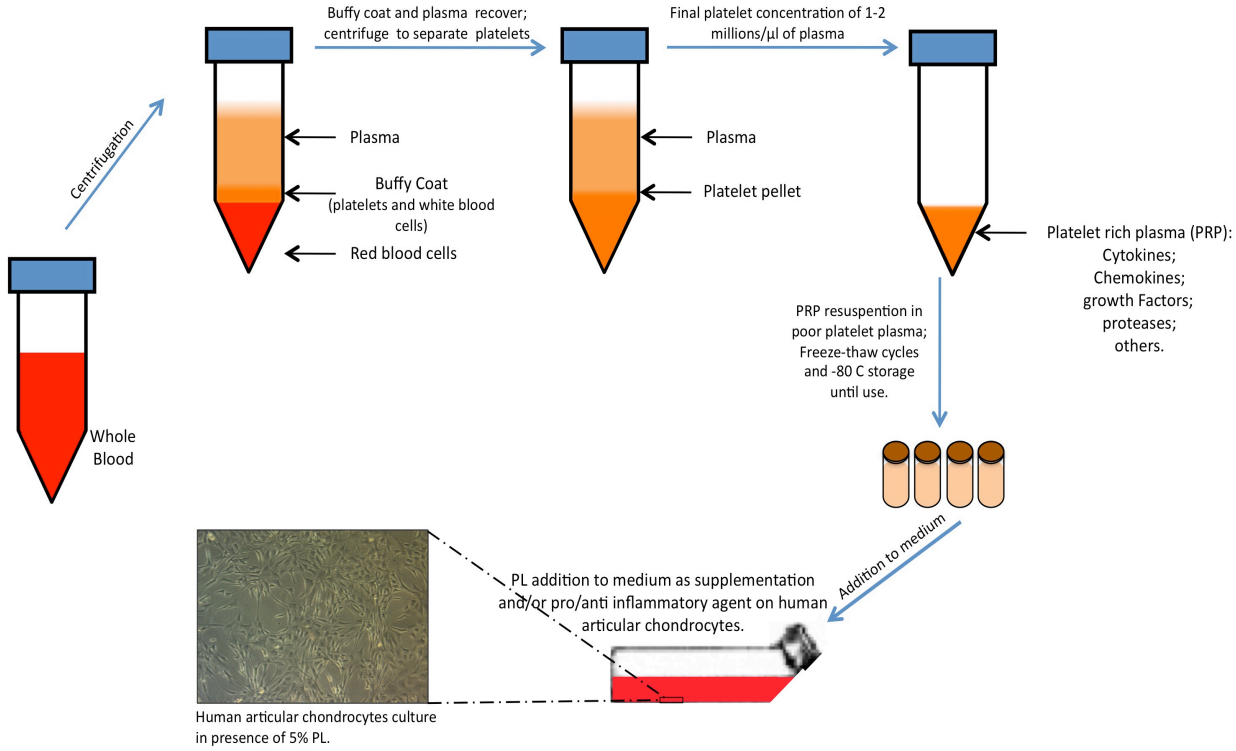

Figure 1 - Ilustration showing the preparation and use of platelet lysate on *in vitro* culture of human articular chondrocytes.

1.2. Hyaluronic Acid (HA)

HA is a naturally occurring glycosaminoglycan distributed in cellular extracellular matrix, connective tissues and animal organs. The structure of HA consists of repeating disaccharide units of D-glucuronic acid and 1to 3 beta N - acetyl-D-glucosamine (5-7). It presents a big range of molecular weight that can go from $1x10^5$ to $5x10^6$ Daltons (Da). The importance of this polysaccharide relies on the fact that it has key roles in organization and structure extracellular matrix, regulation of cellular adhesion, morphogenesis, nutrient transportation and modulation of inflammation (7-11). This vast panoply of properties regarding the human body and in particular cartilage

connective tissue makes HA an ideal biomaterial for tissue engineering. In this manner, the abovementioned polymer was used in works described in chapters V and VI.

1.3. Poly-L- lysine (PLL)

Poly-L- lysine is a polycation that presents high levels of biocompatibility witch has been used for promoting cell adhesion to solid surfaces. It offers the possibility to be easily conjugated with bioactive molecules and with opposite charged polysaccharides. Used as a polycationic drug carrier its known to increase the uptake efficiency by endocytosis (12). Poly(L-lysine) hydrobromide with two different molecular weights, $4.2x10^6$ and $26x10^6$ Da were used in the work described in chapter VI.

1.4. Carrageenans

Carrageenans are high molecular weight sulfated polygalactans derived from several species of red seaweeds (*Rhodophyceae*). The most common forms of carrageenan are lambda (λ), kappa (κ) and iota (ι). Carrageenan has alternating disaccharide units composed of D-galactose-2-sulfate and D-galactose- 2,6-disulfate, being the galactose residues joined by -1,3 and -1,4 linkages. Carrageenans resemble to some extent the naturally occurring glycosaminoglycans owing to their backbone composition of sulfated disaccharides. The different composition and conformation of carrageenans results in a wide range of rheological and functional properties. Carrageeenans form thermoreversible gels. The polysaccharide is water soluble when heated and gels upon cooling the solution in presence of electrolytes (Ca^{2+} and K^+). κ-carrageenan gels in the presence of K^+ ions to form strong crisp gels, whereas ι–carrageenan gels in the presence of Ca^{2+} ions to form elastic gels (13). Carrageenans have been used extensively in the food industry. In addition to incorporation in foods, carrageenan has been used as an ingredient in pharmaceuticals and personal care products, such as toothpaste and cosmetics (14).

The use of carrageenan has not been fully explored as potential biomaterial and we believe that the structural diversity of carrageenans can provide very interesting rheological and functional properties in cell encapsulation technology. Due to its widespread use in food and pharmaceutical industry, purified carrageenan materials are

readily available with reliable, predictable and chemically defined composition. This material was used in chapter VII.

1.5. Fibrinogen & Thrombin (TISSEL®)

The TISSEL®, fibrin sealant product is a trademark of Baxterhealthcare company. Briefly, this product is composed of a double-chamber syringe with Thrombin and Sealer Protein Solution (both from human origin) solutions. The sealer protein solution contains fibrinogen as main ingredient, that work as the active factor of the thrombin causing a reaction that mimic the key features of the physiological coagulation process. When both components of the two solutions come into contact, polymerization occurs given origin to a white and elastic clot. This vehicle was used to aggregate micromass pellets prior to mice subcutaneous implantation. Fibrinogen and thrombin were used in the work explained in chapter VII.

2. Biomaterials Production and Characterization

2.1. Particles production

HA microparticles were produced using a water-in-oil-emulsion technique.

An emulsion composed of 95 ml of mineral oil and 5 ml of Span 85 (both from Sigma-Aldrich, St. Louis, MO) was prepared in a thermal bath at 35 °C adapted from Yan *et al.* (15). Then, 1% (w/v) of HA (235 KDa, LifeCore, USA) was dissolved at 4 °C overnight under smooth rotation. The homogeneous polymer solution was then dropped with a syringe with a 23 Gauge needle into the water-in-oil emulsion. The obtained HA microparticles were kept under mechanical agitation at 600 rpm for 10 hours. After that, the microparticles were centrifuged at 1800 rpm for 10 minutes. Mineral phase was removed and the microparticles deposited in the bottom of the tubes were washed with n-hexane and centrifuged at 1500 rpm for 8 minutes. This procedure was repeated tree times to assure total absence of the mineral phase.

After the final wash, HA microparticles were resuspended in a solution of 50 mg of adipic dihydrazide acid (ADH, Sigma-Aldrich, St. Louis, MO) and 25 mg of ethyl-3-[3-dimethylamino] propyl carbodiimide (EDCI-HCl) in 90%

(v/v) of isopropanol (Sigma-Aldrich, St. Louis. MO). This solution was kept under slow magnetic agitation for 4 hours.

After this time, HA microparticles were centrifuged and washed with isopropanol 90% (v/v). At the end, HA microparticles were sterilized by ethanol 70%, for 1 hour and stored under sterile conditions for further analysis and cellular culture.

2.2. Polyelectrolyte assessment

The hydrodynamic diameter was determined by Dynamic Light Scattering (DLS) using a Zetasizer NanoZS Instrument (ZEN3600, Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He-Ne laser (λ_0 = 633 nm) and with non-invasive backscattering (NIBS) detection at a scattering angle of 173°.

The electrophoretic mobility was obtained by Laser Doppler Velocimetry (LDV) using a Zetasizer NanoZS Instrument (ZEN3600, Malvern Instruments, Worcestershire, UK) at a scattering angle of 17° and capillary folded cells (DTS1060, Malvern, Worcestershire, UK).

The apparent hydrodynamic diameters (D_h) were taken as the mean position of the peak in volume-D_h distributions.

Measurements were performed in the temperature range 20-30 °C with a temperature interval of 2°C and an equilibration time of 2 minutes. Solutions with varying salt concentration were prepared in ultrapure water and filtered using a 0.20 μm disposable PES membrane filter (TPP, Trasadingen, Switzerland).

2.3. Hydrogel formation

K and iota carrageenan powders were sterilized by autoclave procedure. Afterwards, under sterile conditions, cell culture laminar flow cabinet polymers (Sigma-Aldrich, Germany) were dissolved in bi-distillate water (0.8 and 1.2 % w/v) and solution was heated at 65°C under constant stirring to obtain a final homogeneous preparation. Both polymers have the ability to form gels in the presence of potassium (K^+) and calcium (Ca^{2+}) ions. Polymer gelation occurs in different ways in presence of the abovementioned mono and divalent ions. With K^+ ions,

k-carrageenan forms strong crisp gels, whereas iota–carrageenan in the presence of Ca^{2+} ions forms elastic gels (16, 17).

K and i-carrageenans (Sigma-Aldrich, Germany) were dissolved in bi-distillate water (0.8 and 1.2 % w/v) and the solution was heated at 65°C under constant stirring to obtain a final homogeneous preparation. Carrageenans are high molecular weight sulfated polygalactans derived from several species of red seaweeds (Rhodophyceae). The most common forms of carrageenan are lambda (l), kappa (k) and iota (i). Carrageenan has alternating disaccharide units composed of D-galactose-2-sulfate and D-galactose- 2,6-disulfate, being the galactose residues joined by -1,3 and -1,4 linkages. These polysaccharides form gels upon cooling in presence of specific ions due to the development of three-dimensional networks as the result of the formation of helical chains complexes. In the presence of K^{+} ions, k-carrageenan forms strong crisp gels, whereas i–carrageenan in the presence of Ca^{2+} ions forms elastic gels (16, 17).

The other component of the biodegradable hydrogel is composed of a mixture of fibrinogen and thrombin. The addition of these components was made to increase elastic properties gellation time of the hydrogel. Fibrinogen and thrombin were dissolved in an aprotinin solution (10,000 U/ml) and in $CaCl_2$ solution (40mM) respectively to achieve a final concentration of 3,000 UIK/ml 500U/ml. Hyaluronic acid was only added during the cell encapsulation process.

2.4. Cell encapsulation

Cell encapsulation occurred only when the total number of human articular chondrocytes expanded *in vitro* (Figure 2 A) reached the desirable cell number necessary to perform the experiments described in chapter VII.

At the correct time, human articular chondrocytes were resuspended at a concentration of $2x10^6$ cells/ml in culture medium (Biochrom A.G. Berlin, Germany) containing extra concentration of calcium chloride ($CaCl_2$) - 0.265 g/L and potassium chloride (KCL) - 0.4 g/L in order to trigger gel formation when mixing with the carrageenan polymeric solution.

At that time, cell suspension was supplemented with the thrombin solution (5,9% v/v) and with 0,75% (v/v) of hyaluronic acid before loading in syringe A of the injection system (Figure 2 B). Syringe B (Figure 2 B) was loaded with the carrageenan solution supplemented with the fibrinogen solution at 35% (v/v). The carrageenan/fibrinogen solution was mixed with the cells/thrombin/hyaluronic acid solution by activating the two syringes at the same time. The gel formed almost instantaneously. Macroscopic morphology of the hydrogel with encapsulated cells within is shown in Figure 2 B.

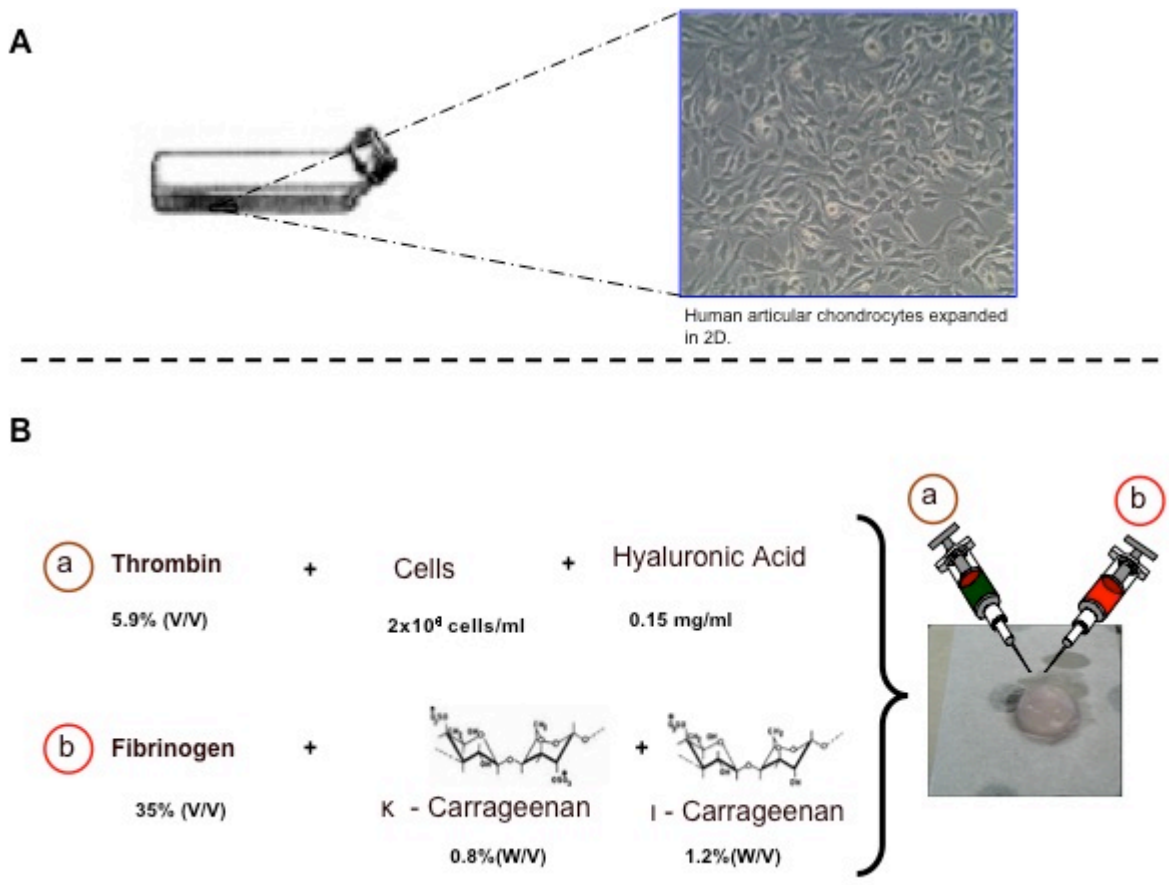

Figure 2 – Illustrative scheme of the procedure followed to encapsulated human articular chondrocytes within the biodegradable hydrogel A) Light microscopy of a primary human articular chondrocytes monolayer cultured in serum free (SF) medium before their encapsulation occur; B) Biodegradable hydrogel system: content composition in "a" and "b" syringes and gross morphology of the formed gel.

3. Biomaterials Characterization

3.1. Scanning Electron Microscopy (SEM)

The shape and surface characteristics of the microparticles were studied by scanning electron microscopy (SEM). First of all, hyaluronic acid microparticles were collected by centrifugation (1000 r.p.m. for 10 minutes). Afterwards, samples were washed several times with PBS to remove mineral oil or/and culture medium proteins presence and kept to dry at room temperature for 24 hours. After, samples were mounted on aluminium stubs and coated with Pt/Pd and then observed with a scanning electron microscope (FEGSEM, FEI Nova 200 NanoSEM).

3.2. Dynamic Light Scattering (DLS)

Polyelectrolyte complexes formed with the association of HA (anion) and Poly (L-lysine) (cation) were analysed by dynamic light scattering (DLS).

DLS was performed using a Zetasizer NanoZS Instrument (ZEN3600, Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He-Ne laser (λ_0=633 nm) and with non-invasive backscattering (NIBS) detection at a scattering angle of 173°. Owing to this configuration, the equipment can decrease the scattered light path length through the sample by adjusting automatically the measuring position, hence reducing multiple scattering for larger particle size, i.e. opaque samples. This is especially useful in colloidal aggregation experiments, where scattered light intensity can rapidly increase several orders of magnitude, because it reduces the need of sample dilution. Both measuring position and attenuator were adjusted automatically before each measurement. The autocorrelation function was converted in a volume weighted particle size distribution with Dispersion Technology Software 5.06 from Malvern Instruments.

The apparent hydrodynamic diameters (D_h) were taken as the mean position of the peak in volume-D_h distributions. The measurements were performed in the temperature range 20-30 °C with a temperature interval of 2°C and an equilibration time of 2 minutes. Solutions with varying salt concentration were prepared in ultrapure water and filtered using a 0.20 μm disposable PES membrane filter (TPP, Trasadingen, Switzerland).

4. CELL SOURCES – Isolation And Expansion

In the overall of the work preformed and exposed in this thesis, one main cell source was used – human articular chondrocytes. Besides that, as described in the next sections, other human cell types were employed in the different works, such as Wharton's Jelly Stem Cells (HWJSCs), Bone Marrow Stromal Cells (HBMSCS) and Umbilical Vein Endothelial Cells (HUVEC). In this part of the chapter II we intended to provide a summary of the different cell sources as well as the usage methodologies to isolate, culture and expand the distinct primarily obtained cells.

4.1. Tissue samples protocol

In this thesis, distinct tissues were used to obtain primary human cells. For all of them, in both laboratories, 3B's Research Group, Braga, Portugal and Laboratory of Regenerative Medicine, Genova, Italy, when isolation was necessary, a strict protocol was followed as agreed between the clinical institutions; Hospital de Sao Marcos, Braga, Portugal and Ospedale S. Antonio Recco, ASL 3 Genovese, Genova, Italy. Signed protocols required the approval of the respective ethical committees. The *modus operandi* was based in rigorous rules to assure patients agreement for bioptic material gathering as well as patient's full anonymity.

4.2. Human Articular Chondocytes (HACs)

Adult chondrocytes are the unique cellular type present in cartilage. This connective tissue with mesenchymal origin covers and protects bone of diarthrodial joints from load bearing impact-associated forces, and allows nearly frictionless motion between articular surfaces (18). Depending on their constitution and function, there are three distinct kinds of cartilage, hyaline cartilage, elastic cartilage and fibrocartilage (19, 20). HACs occupy approximately 5% of the total volume of the tissue and have as main function, thought anabolic and catabolic processes, regulate and maintain the homeostasis of the ECM where they are embedded in (18, 21).

4.2.1. Human articular chondrocytes isolation

Isolation of human articular chondrocytes was done using biological material of patients undergoing partial and total hip replacement and knee arthroplasty (chapters III, IV, V and VII).

The method applied to isolate HACs was performed according to the methodology previously reported by Malpeli *et al.*, (22). The importance of protocol design in yielding cells of adequate quantity and quality is paramount for the success of this technology. Therefore, several variables should be considered when performing any type of primary culture (i.e., the total final number of cells enzymatically obtained during the tissue digestion and its biological quality). During the first step, it is very important to clean the biopsy sample from all adherent tissues (muscular, connective, and bone) to avoid possible contamination of cartilage cells. The biological quality of the primary culture will depend on the biological functionality of the tissue (cartilage in this case) and also on the degree of tissue purity.

Cartilage was cleaned of connective tissue and/or subchondral bone by serials washing with phosphate buffer solution (PBS) and dissected into small fragments. Individual HAC were released by repeated enzymatic digestions with collagenase I (400 U/mL); collagenase II (1,000 U/mL) (Worthing Biochemical, Lakewood, NJ); hyaluronidase (1 mg/ml) (Sigma, St. Louis, MO) and 0.25% trypsin (Invitrogen Life Technologies, Carlsbad, CA). Solutions with minced cartilage pieces were kept in a thermostatic bath at 37°C under constant mild agitation. The sequential enzymatic digestions were repeated until no biopsy material was available. At the final, cells were pooled, counted and seeded for culture in Petri dishes (100mm x 20mm) using F-12 culture medium (Biochrom A.G. Berlin, Germany) with 10% fetal calf serum (FCS). After 48 hours from seeding, the chondrocytes were washed with phosphate buffer solution (PBS), enzymatically detached by trypsin, collected, counted and re-plated in petri dishes for further use.

4.2.2. Human articular chondrocyte standard 2D monolayer expansion

Primarily obtained human articular chondrocytes were cultured in 2D using F-12 Coon's medium supplemented with 10% FCS, 1% antibiotics and 1% L-Glutamine. Cells were kept until 80-90% of confluence at each passage. Media were changed every two days.

4.2.3. Human articular chondrocytes Serum Free (SF) 2D monolayer expansion

An alternative methodology to standard use of fetal calf serum supplementing culture media is the use of SF medium (22). In chapter VII, cells were expanded in this specific medium before encapsulation within the injectable hydrogel.

At a certain time point, 'standard' culture medium was then replaced with the serum-free (SF) medium described by Malpeli *et al*. (22). Cells were expanded *in vitro* in this culture conditions until they were used for encapsulation; Rna extraction and/or micromass pellet assay. Medium growth factor composition is described bellow in Table 1.

Table 1. Serum free medium composition (adapted from (23)).

Component (Protein/Growth Factor)	Final Concentration
Apo-Transferrin	25 µg/mL
Ascorbic Acid	250 µM
Biotin	33 µM
Cholesterol	13 µM
Dexamethasone	10 nM
Epidermal Growth Factor	5 ng/mL
Fibroblast Growth Factor 2	5 ng/mL
Holo-Transferrin	25 µg/mL
Human Serum Albumin	1% (w/v)
Insulin	10 µg/mL
Linoleic Acid	4.5 µM
N-acetylcysteine	50 µM
Platelet Derived Growth Factor	5ng/mL
Sodium Pantothenate	17 µM
Sodium Selenite	30 nM

4.2.1.4 Human articular chondrocytes 2D monolayer expansion with Platelet Lysate (PL)

Human articular chondrocytes at passage 0 were counted, washed several times with PBS to avoid presence of serum components and seeded in culture dishes at a density of $10x10^3$ cells/cm^2 with F-12 supplemented with 5% PL. Media were changed every two days. At each cell passage, 80-90% cell confluence achieved, cells were washed with PBS and detached with trypsin. The following seeding was performed previous cellular density.

4.3. Human Wharton Jelly Stem Cells (HWJSCs)

Recently, fetal stem cells have attracted increased interest due to their unique features, such as high competence to proliferate maintaining the self-renewal potential over time and the ability to differentiate toward almost all cell types (24, 25). Being an extra-embryonic tissue with a large volume of mass, these tissues are potential sources of stem cells for isolation. In particular, stem cells derived from the human umbilical cord outer region, known as wharton's jelly stem cells (WJSCs), have been isolated from the mucoid connective tissue that surrounds the two arteries and the single vein of the umbilical cord. Harvested from umbilical cords, hWJSCs are easily accessible, presenting high levels of proliferation during *in vitro* expansion making possible to obtain a plentiful amount of cells in a short period of time (26, 27). Controlling cell differentiation is one of the most challenging aspects of cell-based regenerative therapies since the mechanisms by which stem cells differentiate can be difficult to recapitulate with current technologies. By this manner hWJSCs could be a useful cellular source to seek cartilage regeneration.

4.3.1. Human Wharton jelly stem cells isolation and expansion

Human umbilical cords were collected from mothers' who underwent full-term pregnancy after their consent and São Marcos Hospital (Braga, Portugal) Ethics Committee approval (described in 4.1 - Tissue samples procurement)

Human WJSCs were isolated adapting the procedure originally described by Sarugaser *et al.*, (19). In brief, the cord was sectioned in 3 to 5 centimeter (cm) individual segments and the blood vessels were separated from the wharton's jelly. After, segments were dissected by the separation of the umbilical cord section epithelium along its length, exposing the underlying Wharton jelly. Individual vessels surrounding the matrix were removed from the cord. At the end of each dissection, collected vessels were washed in PBS and tied with a suture creating loops. The vessels were incubated in a 0.50 mg/ml collagenase type I (Sigma St. Louis, MO) solution for 18 hours at 37 °C under slow agitation (60 rpm).

After incubation, the digested vessels were collected, discarded and the remaining viscous solution diluted with PBS. The solutions were centrifuged for 10 minutes at 1150 rpm and this step was repeated until the solution viscosity was reduced. After that, the cell suspension was incubated for 5 minutes at room temperature (RT) with erythrocyte lyses buffer to lyse the erythrocytes. Finally, cell suspension was centrifuged and the obtained cell pellet re-suspended in culture medium, counted and plated into culture flasks.

4.4. Human Bone Marrow Stromal Cells (HBMSCs)

In part of the work presented in chapter IV, human bone marrow stromal cells (hBMSCs) were used as one cell source to test a possible chemotatic effect of hACs medium.

In the early's 70's, Friedenstein and colleagues were the first to report the existence of "fibroblastic" cells that could be flushed out from the inner of adult bone marrow, presenting after that the ability to form colonies on plastic (28). After, when transplanted subcutaneously using a proper carrier, cells could conduct to cartilage and bone formation in a host new microenvironment (28). Over time it has become gradually clear that hBMSCs could be a potential effective agent in a large variety of experimental models of injured tissues (29, 30).

4.4.1. Human bone marrow stromal stem cells isolation and expansion

In this thesis HBMSCs were used from cryopreserved cells previously isolated from human source. Nevertheless, it is important to briefly describe how the primary culture was performed. The employed procedure was based on methodology previously described by *Gallotto et al.* (31). With so, HBMSCs were obtained from iliac crest marrow

aspirates from healthy donors for bone marrow transplant procedures. Afterwards, collected aspirates were homogenized, diluted in PBS (1:1) and incubated for 5 minutes at room temperature. Bone marrow fraction was diluted in lyses buffer and left under agitation for 10 minutes. Then, the suspension was centrifuged at 1200 rpm for 15 minutes. Collected cells were ressuspended in Coon's modified Ham's F12 (Biochrom A.G. Berlin, Germany) supplemented with 1% of antibiotic solution and L-glutamine, 5ug/500ml FGF-b (Peprotech), as well as 10% of FBS and nucleated cells were counted and plated at high density: 3.2 – 5.1 x 10^4 cells/cm^2. Cells used in the experimental assays were shortly expanded with change of medium occurring twice a week. All the different cellular populations obtained from distinct primary cultures were characterized by flow cytometry for stemness markers (cell membrane clusters of differentiation – CDs) such as CD29, 44, 73, 90, 105, 133, 146 and as well as the hematopoietic exclusion markers CDs 34 and 45. Adherent cells were as well characterized at low passages for its multipotential differentiation ability; osteogenic, chondrogenic and adipogenic *in vitro* assays; as well as for their colony forming unit (CFU) faculty.

4.5. Human Umbilical vein endothelial cells (HUVEC)

Human umbilical vein endothelial cells (HUVECs) play a pivot role as a model system for the study of the regulation of endothelial cell function and the function of the endothelium in response of the blood vessel wall to mechanical forces, and the increase of angiogenesis (32). For their high mobility capacity, these cells are commonly used for *in vitro* and *in vivo* physiological and pharmacological investigations as "positive controls".

4.5.1. Human umbilical vein endothelial cells expansion

In chapter IV, HUVEC were used to perform chemotatic *in vitro* assays. We used cryopreserved cells previously isolated from human source. Human umbilical vein endothelial cells were plated in plastic dishes and cultured in M199 medium supplemented with 10% FBS; 1% penicillin/streptomycin; 5mg/ml heparin; 50μg/ml hydrocortisone; 10^{-3}μg/ml FGF-α; 10^{-3}μg/ml FGF-β; 10^{-3}/ml EGF. Media were changed twice a week until 80% of confluence was achieved. Cells were detached, counted and used in Boyden chamber assay.

5. Cell and/or Materials culture

5.1. Co-culture of human wharton's jelly stem cells with human articular chondrocytes

In Chapter III, co-culture of HWJSCs with HACs, was performed using a transwell system. Chondrocytes were plated in the transwell chambers at cell density of 18000 cells/cm^2 and HWJSCs seeded in the above layer at a density of 15000 cells/cm^2. Both cells shared the same medium but no direct cell-cell contact was possible due to the physical separation of by a polycarbonate membrane with pore size of 0.4 μm, which does not allow cell migration through the membrane.

Cells were cultured in alpha medium (Sigma, USA) supplemented with 10% fetal bovine serum (Biochrom AG, Germany), 5 mM L-Glutamine (Sigma, St. Louis, MO) and 1% of antibiotic-antimicotic mixture (Invitrogen, St. Louis, MO). Human articular chondrocytes plated in the inserts were replenished at each wharton's jelly enzymatic detachment at P1. Chondrocytes in transwell inserts were trypsinized and stored for subsequent molecular biology analysis. Both cell types were kept in culture at 37C with 5% CO_2.

5.2. Chondrogenic differentiation – 3D micromass pellets

For chondrogenic differentiation, *in vitro* micromass pellet culture system was used as described by Johnstone et al. (33). In Chapter III, chondrocyte differentiation capacity of hWJSCs, previously exposed to soluble factors secreted by human articular chondrocytes during expansion, was investigated by means of micromass culture pellet. In brief, cells were trypsinized and $2.5x10^5$ cells aliquots were collected in 15 ml conical tubes (Sarstedt, Numbrecht, Germany). Cells were suspended in chondrogenic medium consisting in serum free DMEM supplemented with ITS + 1 (insulin–transferrin–selenium; Sigma, St. Louis, MO); 10 ng/ml transforming growth factor (TGF)-β1; 100 nM dexamathasone; 50 μg/ml ascorbic acid; 2 mM L-glutanine; 40 μg/ml L-proline and 1 mM sodium pyruvate. Suspended cells were centrifuged for 2 minutes at 1400 rpm to allow the formation of spherical pellets. Pellets were cultured in previously described medium that was changed every two days during 3 weeks.

Within the works described in Chapters IV and VII, micromass pellets culture system was again used to assess chondrogenic phenotype of human articular chondrocytes over time. In this case, $2.5x10^5$ cells were pelleted in 15 ml conical tubes and cultured for 21 days in chondrogenic medium according to Johnstone et al. (33), without the usage of ITS +1. Cells were suspended in Coon's F-12 Modified Ham's medium (Biochrom A.G. Berlin, Germany) supplemented with 6.25 μg/ml bovine insulin; 6.25 μg/ml human apotransferin (both from Austral Biologicals, San ramon, CA, USA); 1.25 μg/ml linoleic acid (Peprotech Inc., London, UK); 5.35 μg/ml bovine serum albumin (Sigma-Aldrich, Steinheim, Germany); 1mM sodium piruvate (Sigma-Aldrich, Steinheim, Germany); 50 μg/ml ascorbic acid (Sigma-Aldrich, Steinheim, Germany); 10ng/ml transforming growth factor (TGF)-β1 (Peprotech Inc., London, UK) and 10^{-7} M dexamethasone (Sigma-Aldrich, Steinheim, Germany). In all the chapters cells were maintained in culture at 37 C with 5%CO_2. Medium was changed every two days.

5.3. HAC culture with PL

Human articular chondrocytes were culture in F-12 Coon's medium supplemented with 5% of PL. Cell expansion was performed following the same criteria as in presence of foetal calf serum, i.e., level of confluence, trypsinization and cell seeding density.

5.4. HAC under inflammatory conditions

To mimic an inflammatory environment, chondrocytes were stimulated with interleukin 1alpha (IL-1alpha). For this, before adding the stimuli, to remove the possible residual presence of FCS proteins in the media that could create artefacts in the studied conditions, cells were washed several times with PBS. Subsequently, IL-1alpha (100U/ml) was added to media with and without presence of platelet lysate.

5.5. HAC with HA particles Direct / Non direct contact culture

To study the effect of HA microparticles on the cellular behavior of human articular chondrocytes over *in vitro* time of culture (chapter V) different culture conditions were made; cells in direct contact with HA particles and in

non-direct contact. Control of the experiments was made using "standard" human articular chondrocytes 2D plastic culture.

Primary human articular chondrocytes were plated at density of 80000 cells/cm^2 and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) with phenol red at 37 °C supplemented with 10% fetal bovine serum (Biochrom AG, Germany), 5 mM L-Glutamine (Sigma, St. Louis, MO) and 1% of antibiotic-antimicotic mixture (Invitrogen, St. Louis, MO).

For culturing HAC in non-direct contact with HA microparticles, it was used a transwell system (Corning incorporated, Transwell®) where cells, were plated in the bottom of the well with the transwell chambers with HA micropartilces above. Cells shared the same medium but no direct cell-material contact due to the physical separation of the cells by a polycarbonate membrane with pore size of 0.4 μm.

Culture of HAC in direct contact with HA microparticles was made using the same number of cell seeding as aforementioned. After 1 day, time that allows cells to adhere to plastic surface, HA microparticles were added to culture. The same amount (mg/ml) of microparticles used for the no-direct contact condition was of used for this case.

5.6. Cell encapsulation

Human articular chondrocytes encapsulation within the hydrogel is extensively explain in section 2.4 – cell encapsulation present in the section Biomaterials Production and Characterization. Please see that point.

5.7. Organ Culture

Bovine articular cartilage in the case of the cartilage engineering, one of the major limit of this method is the lack of an appropriate articular cartilage surrounding the newly formed tissue and therefore the impossibility to investigate the integration of the newly formed cartilage with the pre-existing one. We used a organ culture model

recently described (34, 35) in which the hydrogel with and without encapsulated HAC was directly injected into the defect created on the bovine articular cartilage layer.

6. *In vitro* biological assays

6.1. Cell Imaging

The morphological aspect of cultured HACs, HWJSCs and BMSCs was observed by optical microscopy. Cells in different tissue culture flasks/ petrid dishes were observed using a bright field microscope (AXIOVERT 40 CFL, Germany) equipped with a digital camera (Canon Power Shot G8, Japan). Images at different passages were acquired at 5x and 10x magnification.

6.2. Proliferation assessment

Cellular proliferation can be consistently assessed by the calculation of cell number at each cellular passage – cell-doubling number and by dsDNA quantification. Using the first method, counted cells were re-plated and consequently, kept in culture for posterior analysis, which allowed the continuous use of the cells for further characterization techniques. Picogreen quantification of dsDNA from cells cultured in monolayer involved the use of a certain number of cells only for that purpose.

6.2.1 Growth kinetics by calculation of doubling number

The number of cell doublings performed by human articular chondrocytes and human Wharton jelly stem cells expanded *in vitro* was calculated considering the number of cells recovered and plated at each passage (Chapter III). Cells were detached from dishes by digestion with trypsin/EDTA (Invitrogen Life Technologies, Carlsbad, CA) before they reached confluence, counted and always re-plated at $2.5x10^4$ cells/cm^2 in a 60 mm x 15 mm cell culture dish. The number of duplications at each cellular passage was calculated according to the formula (3, 36):

Log_2 of cells obtained/cells plated

Obtained data were plotted against time of culture.

The growth kinetics of HACs cultured in presence of PL and in F-12 complete media (Chapter IV) and HACs expanded in Serum Free Medium before encapsulation within the injectable hydrogel (Chapter VII) was assessed by calculation of cellular doubling number as well according the same mathematical formula.

6.2.2. Proliferation rates by PicoGreen Quantification Assay

Proliferation of cells cultured in direct, non-direct presence and in total absence of of HA microparticles (Chapter V) over time of *in vitro* culture was assessed by dsDNA quantification. Picogreen is a fluorescent molecule that binds specifically to dsDNA and as a result allows the measurement of the proportional fluorescence augmentation (37). For this, PicoGreen Quantification Kit (Invitrogen, USA) was used according to the manufacturer instructions. To this propose, for each time point, cells were washed several times to remove HA micropaticles as well as proteins from medium that could interfere with dsDNA fluorescent labelling. Prior to dsDNA quantification, cellular samples collected over time of culture were lysated by consecutive osmotic and thermal shocks. Standard samples were prepared prepared from 20μg/mL standard solutions of dsDNA by successive dilutions. Samples and standards were mixed with picogreen on a 1:1 ratio in a 96 multiwell white opaque pate and incubated for 10 minutes protected from light. Fluorescence was measured using an excitation wavelength of 480 nm and emission of 528 nm in a Synergy microplate reader (Bio-Tek, USA). The obtained standard curve was used to extrapolate the dsDNA concentration values.

6.3. Viability by MTT assay

It was necessary for each time point to evaluate the viability of human articular chondrocytes in presence of HA microparticles (Chapter V) and encapsulated within the hydrogel (Chapter VII). In both cases 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide - MTT analysis was performed. This is a safe, sensitive, *in*

vitro colorimetric method that allows the measurement of cell viability, which determines in indirect manner potential cytotoxicity effects of materials on cells. This assay is based on MTT compound reduction by metabolically active cells to insoluble formazan dye crystals that are possible to detect spectrophotometrically after solubilization (38).

The viability of HACs was evaluated at different time intervals using the Thiazolyl Blue staining (MTT; Sigma-Aldrich). In brief, in triplicate for each time point, medium was removed from the analyzed samples and replaced with 0.5 ml of fresh serum-free medium supplemented with 25 µl of MTT stock solution (5mg/ml). After 3 hours of incubation, the medium was collected and the converted dye was solubilized with 1 ml absolute ethanol. Dye absorbance was measured at 570 nm with background subtraction at 670 nm (38).

6.4. Viability and cell spatial distribution by APotome Analysis

This is an epifluorescent illumination technique (39, 40), which allows the acquisition of images throughout tick biological samples. APotome analysis was used to obtain images of the embedded HACs within the hydrogel (chapter VII) without the need of any manipulation procedures such as the dehydration required for histology that can introduce artefacts. This technique is a powerful tool for *in vitro* cellular characterization - distribution and viability of encapsulated cells.

In brief, culture medium was carefully aspirate from the wells where the samples to be analyzed were and incubation (37 °C, 5% CO_2, protect from light) with a serum free medium containing Hoescht 33342 (Sigma-Aldrich, Germany) at the final concentration of 5 µg/mL was done for 30 minutes to assess live cells distribution. Afterwards, the hydrogel was transferred to a small Petri dish (3 ml) and optical sections along the Z axis were then acquired by structured epifluorescent illumination using an Axiovert 200M microscope equipped with the Apotome module with filter set 49 and the AxiocamHR camera (Carl Zeiss, Jena, Germany). Images acquisition, stacks of different optical sections and 3D sample reconstruction were performed by the Axiovision Software (Carl Zeiss, Jena, Germany) (39, 40).

6.5. Histological Analysis

At the different time points, samples were processed for histological analysis. One main histological technique was done within the course of the distinct works described in this thesis. In all of them, samples were processed for paraffin inclusion and subsequently analyzed.

6.5.1. Inclusion

Samples of the work performed *in vitro* within Chapters III, IV and VII were removed from their culture media, and washed in PBS. In Chapters III and IV samples were fixed in 4% formaldehyde in PBS for 15 minutes. In Chapter VII, this step of the inclusion procedure, occurred for 6 hours to allow the cells embedded within the hydrogel to be fixated. After the fixation, in all cases, samples were dehydrated by serial immersion in ethanol solutions (70%, 90%, 95%, and 100%) and xylene. Specimens were then embedded in paraffin and 5 μm sections were cut using a Microtome (Leica RM 2165).

6.5.2. Staining

Following the acquisition of 5 μm slices, sections were dewaxed and stained. Hematoxylin-eosin (H&E) staining is a widely used method for the analysis of cells overall distribution and general morphology. This staining was used before any other staining mentioned above to verify the overall morphological observation of the samples.

Chapter III

Cross-sections (4 μm thickness) were cut, dewaxed and stained with Safranin O for detection of sulfated glycosaminoglycan (sGAG). Briefly, slides were first washed in running tap water, quick destained with fresh acid ethanol (1% hydrochloric acid in 70%ethanol), re-washed in running tap water and immersed in 1:500 fast green (Fluka/44715) for 3 minutes. After that, they were immersed in 1% acetic acid (Panreac/131008) solution for 30 seconds and immersed in 0.1% Safranin O (Fluka/84120) for 4 minutes. Slides were washed in running tap water, counterstained with hematoxylin, cleared in xylene and mounted. Inserts containing human articular

chondrocytes were also processed for Safranin O staining. They were washed twice with PBS, fixed in formalin for 30 minutes and stained for GAG content. Pictures were acquired.

Chapter IV

Micromass pellet sections were paraffin embedded. Samples were cut in 5 µm thickness slices dewaxed and stained with Toluidine Blue (E. Merck, Darmstadt, Germany) to detect acidic proteoglycans present in cartilage tissue. In brief, slides were dewaxed, washed in running water and immersed in Toluidine Blue solution for 5 minutes. After, samples were dyed at 37C for two hours. Microscope pictures were acquired.

Chapter VII

Sections were then dewaxed and stained with Toluidine Blue (E. Merck, Darmstadt, Germany), and/or Alcian Blue (Sigma-Aldrich, Chemical, St. Louis, USA) to reveal cartilage matrix components. Histology samples were rinsed three times in PBS and then fixed in 4% formaldehyde in PBS for 6 hours. After fixation, the samples were dehydrated by serial immersion in ethanol solutions (70%, 90%, 95%, and 100%) and xylene. Specimens were then embedded in paraffin and 5 mm sections were cut using a Microtome (Leica RM 2165). Sections were then dewaxed and stained with Toluidine Blue (E. Merck, Darmstadt, Germany), or Alcian Blue (Sigma-Aldrich, Chemical, St. Louis, USA) to reveal cartilage matrix components.

6.5.3. Immunolocalization of collagen type I and II

For immunohistochemistry analysis, serial sections of paraffin embedded samples were dewaxed and treated with methanol/H_2O_2 (49/1) for 30 minutes to inhibit endogenous peroxidases. Sections were then treated with 1 mg/ml hyaluronidase in PBS pH 6.0 for 20 minutes at 37°C to smooth cartilaginous matrix and hence a better antigen/antibody recognition. After this fundamental step in the immunlocalization of matrix proteins, samples were washed carefully several times with PBS to remove hyaluronidase presence from the slices. After, samples were incubated with goat serum for 1 hour to reduce non-specific binding; the specific antibody was added and

incubated for 1 hour at room temperature. Sections were washed several times in PBS and challenged with biotinylated anti mouse IgG (Jackson Laboratory Inc., West Grove, PA, USA) and peroxidase-conjugated egg-white avidin (Jackson Laboratory Inc. West Grove, PA, USA). After additional washing of the sections with PBS and 50 mM Na Acetate pH 5.0, the peroxidase activity was visualized by enzymatic modification of the 3-amino-9-ethylcarbazole substratum (3-amino-9-ethylcarbazole 0.4% in dimethylformamide: 50 mM Na Acetate, pH 5: 30% hydrogen peroxide (H_2O_2); 100:900:1) performed in the dark at room temperature for 15 min. Sections were counterstained with Harri`s hematoxylin and mounted with Gel mount from Biomeda Corp. (Foster City, CA, USA). Slides were observed and images acquired with the Axiovert 200M microscope (Carl Zeiss).

6.5.4. Immunolocalization of tenascin

To better access the potential of human cells to generate a specific tissue, several *in vivo* models are used. In our case, to study the real possibility of the encapsulated cells to form a cartilaginous tissue after injection in a cartilage made defect we used nude mice model. In this way, the use of immunosuppressed mice avoids a possible inflammatory response of the recipient animal. Bovine cartilage made defects were fulfil with encapsulated human articular chondrocytes.

To distinguish the origin of the neoformed tissue within the defect, we performed immunoperoxidase against human tenascin.

Tenascin is an extracellular matrix (ECM) glycoprotein constitutively present in cartilage that has been suggested to have an important structural role in embryonic and differentiated cartilage (41, 42).

In brief, samples were dewaxed and treated with methanol/H_2O_2 (49/1) for 30 minutes to inhibit endogenous peroxidases. After, Hyaluronidase (1mg/ml in PBS pH 6) was used Sections were then treated with 1 mg/ml hyaluronidase in PBS pH 6.0 for 20 minutes at 37°C to smooth cartilaginous matrix and hence a better antigen/antibody recognition. After this fundamental step in the immunlocalization of matrix proteins, samples were washed carefully several times with PBS to remove Hyaluronidase presence from the slices.

6.6. Flow Cytometry Analysis

The phenotype of HWJSCs (chapter III) and HACs (chapter V) was screened by flow cytometry using different clusters of differentiation (CDs) markers as showed in the abovementioned chapters.

The phenotype of HWJS cells (chapter III) cultured in polystyrene tissue culture plates (monoculture) and in co-culture (non-direct contact) with human chondrocytes in multiwell inserts was assessed by flow cytometry.

In brief, harvested cells at each passage were incubated with fluorescent mice against human monoclonal antibodies against CD105 (AbD Serotec, UK), CD44, and CD90 (BD Biosciences Pharmingen, USA) for 15 minutes at room temperature. Cells were then washed in PBS with 1% sodium azide (Sigma) and fixed with 2% formaldehyde. Unlabeled controls were included in every experiment to evaluate the unspecific binding. Samples were analyzed using a FACScalibur (Becton-Dickinson, USA) with CellQuest analysis software (BectonDickinson, USA).

In chapter V, human articular chondrocytes cultured in direct contact and in non-direct contact with HA microparticles were characterized by flow cytometry. Changes of expression in CD44, the membrane receptor of Hyaluronic acid polysaccharide was verified. For this, HAC at the points were detached using trypsin. Cells were counted and marked with an anti-human mouse monoclonal antibody against CD 44. In brief, like in the procedure aforementioned, HAC were incubated the CD antibody for 15 minutes at room temperature in dark conditions. After, cells were washed with PBS and analyzed using a FACScalibur instrument. Data were treated using Summit analysis software.

6.7. Chemotatic assay

Boyden chamber chemotaxis assay of HUVEC, BMSCs and HAC was made as described by Benelli R. et al. (43). Previously to chemotactic assay execution, human chondrocyte culture media were supplemented with 5% PL, or 200 U/ml IL-1 a, or 5% PL + 200 U/ml IL-1a or without any supplement. After 16 hours, media were removed and cells transferred to serum-free culture medium. After 24 hours, 3 ml of conditioned culture media was collected and concentrated to 1 ml using Centricon Centrifugal Filter devices (Millipore Corporation, Bredforf, MA, USA).

The two compartments of the Boyden chamber were separated by 12mm pore-size polycarbonate filters previously coated with 2.5 mg/ml of type IV collagen solution (in H_2O, 0.1% CH_3COOH). Filters were air dried and stored at 4°C until used.

The analyzed human cells were extensively washed with PBS and resuspended in Coon's modified Ham's-F-12 serum-free media to the final dilution of 1.2 x 10^6 cell/ml before the seeding in the upper compartment of chemotaxis chamber. The concentrated conditioned media used as chemoattractant were placed in the lower chamber. After 6 hours of incubation at 37°C in a humidified incubator, the filters were recovered, fixed in ETOH 70% for 30 minutes and stained with toluidine blue. Non-migrated cells were removed previously from the upper surface by serial washes with PBS.

Chemotaxis was quantified counting five to ten random fields of migrated cells, for each filter, under a microscope (Carl Zeiss) at a 160x magnification. Each experimental point was made in triplicate. Means and standard deviations were calculated from three different experiments.

6.8. Western blot analysis

Confluent human articular chondrocytes were treated for 16 hours with medium supplemented with 5% PL, or 200U/ml IL-1a, or 5% PL + 200U/ml IL-1a or without any supplement. Media were then removed, and cells extensively washed with PBS in order to remove all possible contaminating PL proteins before being transferred to serum-free standard medium (no FCS) for 24 hours. Conditioned serum-free culture media and cells were collected for subsequent western blot analysis. Protein concentrations in the media and in the cell extracts were quantified using the Bradford assay (44). For each culture medium sample, 50 µg of proteins were loaded on 4-12% NuPAGE Bis-Tris gel (Invitrogen) and electrophoresis performed in reducing conditions.

For cell lysates, 100-150 µg of proteins were boiled in Laemmli buffer, and separated on 10% SDS-polyacrylamide gel. After electrophoresis, gels were blotted to a 0.2 µm Protran BA83 nitrocellulose membrane (Whatman GmbH, Dassel, Germany). Blots were saturated overnight under constant motion with 5% non-fat cow

milk in TTBS buffer (20 mM Tris HCl pH 7.5, 500 mM NaCl, 0.05% Tween 20), washed several times with TTBS before being probed with specific primary antibodies against IL-6, IL-8, COX-2 (1:200; Santa Cruz Biotechnology, CA, USA) and NGAL (1:1000; R&D Systems, Abingdon, UK). Blot membranes were washed and detection was performed by conjugated horseradish peroxidase (HRP) IgGs (Amersham, Biosciences, UK) for 1 hour at room temperature. Blots were then washed, incubated with an enhanced chemo-luminescence substrate mixture (GE healthcare, UK) and exposed to an x-ray film (GE healthcare, UK) to capture the image.

Western Blot analysis for IL-6, IL-8 and NGAL, secreted by cells cultured in the presence of the p38 inhibitor SB203580 was also performed (Calbiochem, Germany). To this purpose, cells were incubated in the presence of 5mM SB203580 for 2 hours under the same conditions as described above. Images were scanned using the Epson perfection 1260 scanner and band densities were quantified using the image J software. Different pixels mean densities were analyzed.

Means and standard deviations were obtained for statistical analysis.

6.9. NF-kB activity assay

NF-kB pathway regulates an extensive family of transcriptional regulators that promote expression of almost 100 target genes involved in host immune and inflammatory response. The implicated proteins include a multitude of cytokines, chemokines, immune recognition receptors, proteins implicated in antigen presentation and receptors needed for adhesion and migration during pro/anti inflammatory processes (45, 46). Because of this, understand if the activation levels of NF-kB were changed during human articular chondrocytes treatment with PL under physiological and inflammatory conditions is crucial.

To assess NF-kB activity the binding of the NF-kB, p65 subunit to the NF-kB binding consensus sequence 5'-GGGACTTTCC-3' was measured with the ELISA-based Trans-Am NF-kB kit (Active Motif, CA) using human articular chondrocyte lysates. Cell extracts from primary articular chondrocytes expanded in the presence of the different medium supplements, were prepared as recommended by the manufacturer. The Trans-Am kit employs 96-well plate coated with an oligonucleotide containing the NF-kB binding consensus sequence. The active form of the p65 subunit present in cell lysates was detected using Abs specific for an epitope accessible only in the

activated subunit bound to its target DNA. Specificity was checked by measuring the ability of soluble wild type or mutated oligonucleotides to inhibit binding. Results were expressed as specific binding, i.e., as the absorbance values observed in the presence of the mutated oligonucleotide minus absorbance values observed in the presence of the wild-type oligonucleotide. The NF-kB activity was measured in duplicate samples.

6.10. Gene expression analyses

The experimental work within the Chapters III, IV, V and VII implicated the use of molecular approaches to study the transduction levels of cartilage specific markers for the different aims of the aforementioned studies.

6.10.1. RNA isolation

The extraction of messenger ribonucleic acid mRNA from cells in different culture conditions and within biomaterials used in the experimental work described in chapters III, IV, V and VII was the first step employed to assess levels of gene expression by the use of reverse transcription polymerase chain reaction (RT-PCR).

The method herein used for RNA extraction an isolation was based on Trizol® reagent (Invitrogen, Carlsbad CA, USA). A basic Trizol® RNA extraction procedure includes tissue digestion, the separation of a RNA containing aqueous phase from organic phases and subsequent RNA precipitation.

Cells in 2D culture, in contact or within the biomaterials, were washed in PBS, immersed in Trizol® reagent (Invitrogen) and kept at -80 °C for subsequent RNA extraction. During this quick procedure samples were always kept in ice. Afterwards, total RNA was extracted as previously described (47). In brief, RNA samples immersed in Trizol® were incubated at 4 °C for 10 minutes with chloroform (Sigma) and after that centrifuged at 13000 rpm for 15 minutes. 700 µL of the supernatant were collected and an equivalent volume of isopropanol (Sigma/I-9516) was added. After RNA precipitation (occurred within 1 hour) the samples were centrifuged at 13000 rpm and 4 °C for 15 minutes. Supernatant was removed and 700 µl of 70% ethanol was added. Eppendorfs were again centrifuged at 13000 rpm and at 4 °C for 5 minutes, and the supernatant was removed. Pellet was left to air dry at room temperature and at the end was ressuspended in 50 µl of DNase/RNase-free distilled water

(Gibco/10977-015). The amount of isolated RNA and A260/A280 ratio was determined using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA).

6.10.2. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Real-Time PCR is a quantitative technique based in the Polymerase Chain Reaction (PCR) modus operandi. Widely used technique, PCR allows the amplification of a DNA fragment copied million of times to detectable quantities. This technique relies on the thermal cycling and enzyme (DNA polymerase) replication of DNA fragments. In the present thesis, we used a reverse transciptase RT-PCR consisting of a two steps fluorimetric assay.

Firstly, isolated RNA was reversed transcribed into complementary DNA (cDNA), followed by RT-PCR for the detection of gene of interest.

In Chapter III and V samples were studied using SyberGreen system (Bio-Rad Laboratories, CA, USA). All reagents employed in this procedure and thermocycler reaction conditions were those proposed in the kits from the manufacture. Relative gene expression quantification was performed using a BioRad CFX96 real-time PCR detection system (BioRad Laboratories, USA). Primer Express software was used to generate forward and reverse oligonucleotides. cDNA (2 µl for total volume of 25 ml per reaction) was analyzed for the gene of interest and for the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression level of each target gene was calculated using the -2^{DDCt} method as described Livak *et al.* (48). The number of amplification cycles used for every reaction was of 40.

Biological samples in Chapter IV were analyzed using a different termocyler and different reaction conditions. In brief, after mRNA extraction and quantification, cDNA was synthesized starting from 1 µg of total RNA and using an Oligo(dT)20, random hexamers mix and a Superscript III first-strand synthesis system supermix for RT-PCR (Invitrogen). cDNAs were diluted 5 to 20 times, then subjected to PCR analysis. Gene expression levels were quantified by real-time quantitative RT-PCR (qPCR), using Taqman Probe and the ABI Prism 7700 Sequence Detector (Applied Biosystems, Branchburg, New Jersey, USA), according to the manufacturer instruction. Primers

and probes used for the reference gene (GAPDH) and the genes of interest were generated using Primer Express Software 3.0 version. The level of expression of the genes of interest was calculated as 2^{DCt} according to (48).

6.10.3. Semi quantitative reverse transcriptase polymerase chain reaction

In Chapter IV, we used semiquantitative PCR reactions to assess the influence of FCS and PL in chondrocyte phenotype over time of expansion. Once the mRNA was extracted and quantified using Trizol® reagent we analysed by a semiquantitative reverse transcriptase polymerase chain reaction the expression of some gene of interest (49).

In Chapter VII, to test the capability of the hydrogel embedded cells to preserve their native characteristics and form cartilage, the expression of chondrogenic master gene markers was analyzed by semiquantitative PCR.

After RNA extraction by Trizol® reagent (Invitrogen, Carlsbad CA, USA) and its purity quantification, semiquantitative reverse transcription – polymerase chain reaction (RT-PCR) was performed as described by Banfi et al. (49). The mRNAs analyzed were aggrecan, α1 (I) collagen (Collagen Type I), α 1 (II) collagen (Collagen Type II) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In brief, following a 4 minute denaturation step at 95°C, the adopted reaction profiles were: aggrecan, collagen type I and GAPDH: 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 25 cycles; Collagen type II: 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 30 cycles. All PCRs ended with 8 minutes of incubation at 72°C.

Values of the cycles used were in the linear range of amplification were those determined previously in the titration curve.

Afterwards, PCR products were run on 1% agarose gels and bands visualized by ethidium bromide.

7. *In vivo* Assays

7.1. Subcutaneous implantation of Micromass pellets

Human articular chondrocytes were centrifuged and maintained for 4 days *in vitro* in chondrogenic medium before implantation occurred. Pre-induced micromass pellets were then collected, aggregated in groups of 3 units per condition and afterwards implanted in nude mice (CD-1 nu/nu; Charles River Italia, Calco, Italy). Groups of 3 pellets for each culture condition were implanted in 10 different mice.

All animals were maintained in accordance with the standards of the Federation of European Laboratory Animal Science Associations (FELASA), as required by the Italian Ministry of Health. Animals were sacrificed, and implants were harvested after 4 weeks for histological analysis to assess cartilage formation.

Explants were collected at the time points mentioned in chapter V. For the histological full characterization the procedures made were already extensively described in section 6.5 – Histological analysis of this chapter.

7.2. Subcutaneous implantation of Bovine organ culture with the hydrogel

Defects were made in bovine articular cartilage tissues. Induced defects were fulfilled with the injectable hydrogel with $2x10^6$/ml of human articular chondrocytes. Positive controls, bovine articular cartilage lesion filled with the hydrogel without cells, and not filled with the hydrogel were also made. All the studied conditions were implanted subcutaneously in nude mice (CD-1 nu/nu; Charles River Italia, Calco, Italy). Animals were sacrificed after 4, 6 and 8 weeks. Final time point is shown in Figure 3. Six samples per condition were made for each time point. Histological analysis was performed

All animals were treated and maintained in accordance with the standards of the Federation of European Laboratory Animal Science Associations (FELASA), as required by the Italian Ministry of Health.

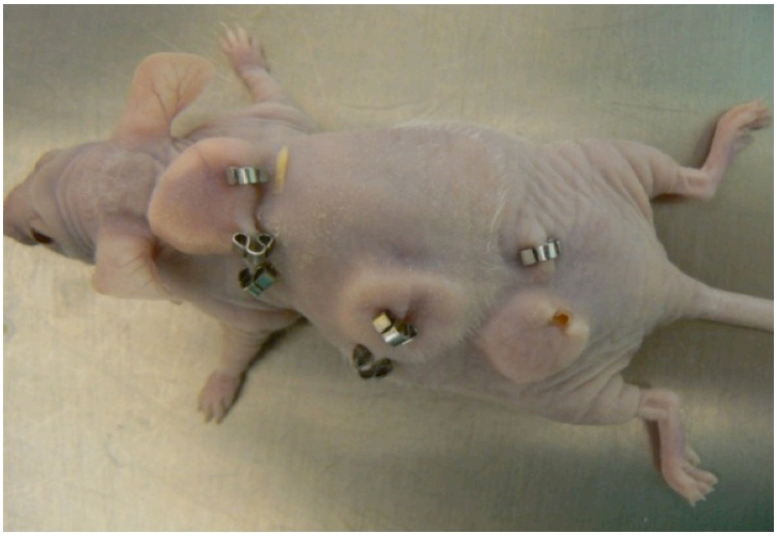

Figure 3 - Representative photograph of one sacrificed animal before sample explantation.

Explants were collected at time points described in chapter VII and fixated with PFA solution for 24 hours at 4C. Afterwards, samples were washed several times with PBS to assure the total absence of PFA residues that could interfere with immunodetection results. For the histological and immudetection assays, a detailed explanation was already made in this chapter in section 6.5. – Histological analysis.

8. Statistical analysis

Statistical analyses were performed in the majority of the works presented in this PhD thesis. In chapter III, during cell culture, cellular duplication number was expressed as mean ± standard deviation with number of cases equal to 3 for each group of interest. Still within this chapter, gene expression values quantified by real time polymerase chain reaction were subjected to statistical analysis as well. Significance of differences was determined using t-test multiple comparison with a confidence level of 95% ($p < 0.05$). Graphpad was used. Statistical analysis for chapter IV was performed using also Graphpad website.

Numbers of cell doublings and Western Blot were expressed as a mean ± standard deviation with $n = 6$ for each culture condition. Results of semi-quantitative PCR, Real Time-PCR and NF-κB and Western Blot for p38 analysis are expressed as mean ± standard deviation with $n = 3$ for each group of interest. Single factor analysis of variance (ANOVA) was used to determine statistical significance within a data set. All tests were conducted with a confidence interval of 95% ($p < 0.05$).

The same occurred in chapter V for real time polymerase chain reaction values. Single factor analysis of variance (ANOVA) was used to determine statistical significance within a data set. If ANOVA detected a significant difference within the data set, Tukey's honestly significantly different (HSD) multiple comparison tests was used to determine significant differences between groups and conditions. All tests were conducted with a confidence interval of 95% ($p < 0.05$) with Graphpad.

Statistical analysis for chapter VII was made for the MTT values regarding the encapsulated human articular chondrocytes within the hydrogel over time of culture. ANOVA with $p<0.05$ was used as reference.

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SECTION 3

RELEVANCE OF SEVERAL STRATEGIES ON ARTICULAR CARTILAGE REPAIR – THE ROLE OF BIOCHEMICAL FACTORS AND EXTRACELLULAR MATRIZ COMPONENTS

CHAPTER III

IN VITRO CHONDROGENIC COMMITMENT OF HUMAN WHARTON'S JELLY STEM CELLS BY CO-CULTURE WITH HUMAN ARTICULAR CHONDROCYTES

CHAPTER III

IN VITRO CHONDROGENIC COMMITMENT OF HUMAN WHARTON'S JELLY STEM CELLS BY CO-CULTURE WITH HUMAN ARTICULAR CHONDROCYTES*

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Abstract

Wharton's jelly stem cells (WJSCs) are a potential source of transplantable stem cells in cartilage regenerative applications due to their highly proliferative and multilineage differentiation capacity. With the current work, we hypothesized that a non-direct co-culture system with human articular chondrocytes (hACs) could enhance a potential chondrogenic phenotype of hWJSCs during expansion phase when compared to those expanded in monoculture conditions. Primary hWJSCs were plated in the bottom of a multiwell plate separated by a porous transwell membrane insert seeded with hACs. No statistically significant differences on hWJSCs duplication number were observed in both culture conditions during expansion. Relative expression quantification for chondrogenic genes revealed that hWJSCs in co-culture conditions showed an up-regulation of collagen type I and II, COMP, TGF-beta1 and aggrecan, as well as of main cartilage transcription factor SOX9, when compared to those cultured in absence of chondrocytes. The chondrogenic differentiation of hWJSCs, previously expanded in co-culture and monoculture conditions, was evaluated by *in vitro* micromass culture for each cellular passage. Cells expanded in co-culture showed higher accumulation of glycosaminoglycans (GAGs) compared to cells in monoculture, as indicated by Safranin O staining. Immunohistochemistry of collagen type I revealed a strong detection of this extracellular matrix protein when hWJSCs were expanded in monoculture conditions, while type II collagen was significantly detected when cells were expanded in co-culture conditions where numerous round-shaped cell clusters were observed. In conclusion, hWJSCs, exposed to soluble factors secreted by human chondrocytes, were able to express higher levels of chondrogenic genes as well as to differentiate along chondrogenic phenotype, as it was demonstrated by deposition of cartilage extracellular matrix components in micromass culture.

Keywords

Human stem cells; Human articular chondrocytes; Co-culture; Chondrogenic differentiation; Cartilage

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R.C. Pereira, A.R.Pinto, A.M. Frias, N.M. Neves, H.S. Azevedo, R.L.Reis, "*In vitro* chondrogenic commitment of human wharton's jelly stem cells by co-culture with human articular chondrocytes", submitted

1. INTRODUCTION

Cartilage is an avascular musculoskeletal tissue with a low capacity of self-repair. Damage, by trauma or disease, results in the loss of partial or complete tissue functionality. During the past 15 years, several clinical approaches, such as micro fracture, arthroscopy and laser abrasion, have been performed to restore pathologically altered cartilage architecture and function [1-4]. The autologous chondrocyte implantation (ACI) technique, proposed by Britteberg in 1994 [5] is a clear example of a cell-based therapy with excellent clinical results. The use of ACI is associated with a number of limitations such as morbidity of the surgical procedure, frequent occurrence of periosteal hypertrophy and inefficient cell retention. In addition, it involves lengthy and costly cell isolation and expansion steps of human articular chondrocytes (hACs), which have low cellular mitosis and are prone to dedifferentiation when expanded in 2D. A potential improvement in the ACI procedure is the application of mesenchymal stem cells (MSCs) [6] instead of hACs, in order to minimize additional donor site morbidity. MSCs are attractive candidates for cartilage cell-based therapeutics because they have the potential to differentiate into the chondrogenic lineage when supplied with proper differentiation signals [6-9]. Adult stem cells present a limited degree of proliferation with maintenance of their multipotency capacity of differentiation. Recently, fetal stem cells have attracted increased interest due to their unique features, such as high competence to proliferate maintaining the self-renewal potential over time and the ability to differentiate toward almost all cell types [10, 11]. These cells are derived from the fetus proper or from supportive extra-embryonic structures from fetal origin, such as umbilical cord blood, wharton's jelly, amniotic fluid, amniotic membrane and placenta. These cell sources are routinely discarded after birth. Being an extra- embryonic tissue with a large volume of mass, these tissues are potential sources of stem cells for isolation. In particular, stem cells derived from the human umbilical cord outer region, known as wharton's jelly stem cells (WJSCs), have been isolated from the mucoid connective tissue that surrounds the two arteries and the single vein of the umbilical cord. In the report on WJSCs, these cells are described as myofibroblasts [12]. These cells retain a combination of many embryonic stem cells (ESC) and MSC markers in primary culture at early passages and can be expanded without significant loss of stemness for at least 50 passages [10]. Recently, various authors have shown that WJSCs

present several advantages compared to adult MSCs, such as higher frequency of colony-forming-unit fibroblasts (CFU-Fs) and shorter population doubling time, both critical to scale up the cell numbers [13]. WJSCs can be differentiated into adipogenic, osteogenic, chondrogenic and cardiomyogenic lineages [14] and dopaminergic neurons [15, 16]. These cells also express some pluripotent stem cell markers including Oct- 4, Sox-2, and Nanog in cells from porcine origin [17], Oct-4, SSEA-4, and c-Kit in cells from equine sources [18], and SSEA-1, SSEA-4, Tra-1–60, and Tra-1–81 in cells from human sources [10], suggesting a more immature state than adult MSCs.

Controlling cell differentiation is one of the most challenging aspects of cell-based regenerative therapies since the mechanisms by which stem cells differentiate can be difficult to recapitulate with current technologies. Co-culture systems with relevant cells can be used as valuable tools for probing and manipulating the molecular mechanisms by which stem cells differentiate and could offer therapeutic possibilities in cartilage regeneration strategies. Previously, chondrocytes have been shown to secrete various soluble morphogenic factors that promoted the chondrogenic differentiation of MSCs [8]. In this study, we hypothesized that factors secreted by chondrocytes possess chondrogenic-differentiating effects and could enhance chondrogenesis of human wharton's jelly stem cells (hWJSCs) when co-cultured with human articular chondrocytes (hACs). Particularly, we intend to inquire: (i) if chondrocytes cultured in transwell system could influence the proliferation ratio of hWJSCs during expansion (as illustrated in Figure 1); (ii) compare the relative gene expression of human Wharton jelly stem cells in presence or absence of hACs; (iii) assess the effect of non direct co-culture on the hWJSCs capability to differentiate into chondrogenic lineage evaluated by micromass culture model.

To the best of our knowledge, the use of non-direct co-culture systems of hWJSCs with hACs was not reported before. We believe that considerable efforts will be directed to this area of research since wharton's jelly stem cells are a potential cell source for musculoskeletal tissue engineering.

2. MATERIALS AND METHODS

2.1. Human wharton jelly stem cells isolation and culture

Human umbilical cords were collected from mothers' who underwent full-term pregnancy after their consent and Sao Marcos Hospital (Braga, Portugal) Ethics Committee approval.

Briefly, the cord was sectioned in 4 pieces and the blood vessels were separated from the wharton's jelly. The collected vessels were washed in phosphate buffered saline solution (PBS, pH 7.4, Invitrogen, UK) and loops were created with sutures. The vessels were incubated in a 0.50 mg/ml collagenase type I (Sigma St. Louis, MO) solution for 18 hours at 37 °C under slow agitation. After incubation, the digested vessels were diluted with PBS. The solution was collected and centrifuged for 10 minutes at 1150 rpm. This step was repeated until the solution viscosity was reduced. After, vessels were discarded and the cell suspension was incubated with eritrocyte lysis buffer and incubated 5 minutes at room temperature (RT). The cell suspension was centrifuged and the obtained cell pellet dissolved in culture medium. The cells were counted and plated onto a culture flask.

2.2. Chondrocyte isolation and co-culture

Bioptic material to perform articular chondrocyte isolation was collected from femoral condyles of patients undergoing partial knee arthroplasty (3 female patients with median age 73 years). All patients had signed an informed consent approved by the Ethical Committee of São Marcos Hospital.

Articular cartilage was cleaned of connective tissue as well as of subchondral bone and minced in small fragments and rinsed in fresh phosphate buffered saline solution (PBS, pH 7.4) according to previously published procedures (19). Chondrocytes were individually released by consecutive enzymatic digestions; 400 U/mL collagenase I, 1000 U/mL collagenase II (Worthing Biochemical, Lakewood, NJ), 0.25% trypsin (Invitrogen, UK), 1 mg/mL hyaluronidase (Sigma, USA) in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Germany) with phenol red at 37 °C. For co-culturing hWJSCs with hACs, we have used a transwell system (Corning

incorporated, Transwell®) where hACs, at cell density of 18000 cells/cm^2 were plated in the transwell chambers and placed above the hWJSCs layer, plated at a density of 15000 cells/cm^2. Cells share the same medium but no direct cell-cell contact are possible due to the physical separation of the cells by a polycarbonate membrane with pore size of 0.4 μm which does not allow cell migration through the membrane (Figure 1-B). Cells were cultured in alpha medium (Sigma, USA) supplemented with 10% fetal bovine serum (Biochrom AG, Germany), 5 mM L-Glutamine (Sigma, St. Louis, MO) and 1% of antibiotic-antimicotic mixture (Invitrogen, St. Louis, MO). Human articular chondrocytes plated in the inserts were replenished at each wharton's jelly enzymatic detachment at P1. Chondrocytes in transwell inserts were trypsinized and stored for subsequent molecular biology analysis.

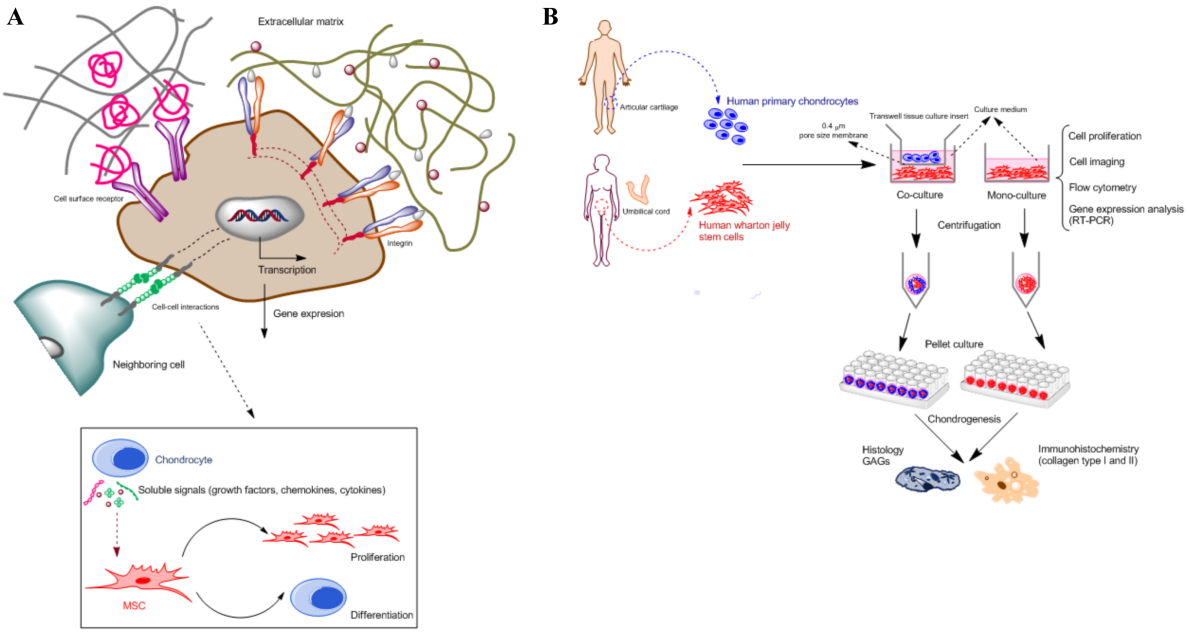

Figure 1 - (A) Environmental factors affecting cell behavior (adapted from [46]). Cells receive multiple signals from their surroundings including neighboring cells. In this study we investigated whether chondrocyte-secreted morphogenetic factors can stimulate the chondrogenic commitment of human wharton's jelly stem cells (hWJSCs). (B) Experimental design and schematic representation of the culture conditions used in this study.

2.3. Characterization of chondrogenic-stimulated hWJSCs

2.3.1. Growth kinetics

Cell doublings number of Wharton jelly stem cells, cultured in presence or absence of chondrocytes, was calculated respecting to starting cell number plated at each passage, $1.5x10^4$ cells/cm^2 in the 6 multi well tissue culture plates. Number of duplications was plotted versus time to show the effect of culture conditions on cell proliferation. Cells were successively passaged using trypsin/EDTA (Invitrogen Life Technologies, Carlsbad, CA), counted with trypan blue (Invitrogen, UK) staining using a Newbauer chamber. Each time point was assessed with n=3.

2.3.2. Cell Imaging

Over the course of culture, cells in 6 multi well tissue culture plates were observed using a bright field microscope (AXIOVERT 40 CFL, Germany) equipped with a digital camera (Canon Power Shot G8, Japan). Images at different passages were acquired at 5x and 10x magnification.

2.3.3. Flow cytometry

The phenotype of WJS cells cultured in polystyrene tissue culture plates (monoculture) and in co-culture with human chondrocytes in multiwell inserts was assessed by flow cytometry. Briefly, harvested cells were incubated with fluorescent monoclonal antibodies against CD105 (AbD Serotec, UK), CD44, and CD90 (BD Biosciences Pharmingen, USA) for 15 minutes at room temperature. Cells were then washed in phosphate-buffered saline with 1% sodium azide (Sigma) and fixed with 2% formaldehyde. Unlabeled controls were included in every experiment to evaluate the unspecific binding. Samples were analyzed using a FACScalibur (Becton-Dickinson, USA) with CellQuest analysis software (BectonDickinson, USA).

2.3.4. RNA extraction and quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Wharton's jelly stem cells co-cultured with hACs and in monoculture conditions were collected at each passage, washed in PBS, immersed in Trizol® reagent (Invitrogen) and kept at -80 °C for subsequent RNA extraction. Human articular chondrocytes were collected in the same manner for further molecular analysis. Afterwards, total RNA was extracted as previously described (19). Briefly, samples were incubated at 4 °C for 10 minutes with chloroform (Sigma) and after that centrifuged at 13000 rpm for 15 minutes. 700 µL of the supernatant were collected and an equivalent volume of isopropanol (Sigma/I-9516) was added. After RNA precipitation (occurred within 1 hour) the samples were centrifuged at 13000 rpm and 4 °C for 15 minutes. Supernatant was removed and 700 µl of 70% ethanol was added. Eppendorfs were again centrifuged at 13000 rpm and at 4 °C for 5 minutes, and the supernatant was removed. Pellet was left to air dry at room temperature and at the end was ressuspended in 50 µl of DNase/RNase-free distilled water (Gibco/10977-015). RNA content and integrity was assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA). Isolated RNA was transcribed into cDNA using the iScript cDNA synthesis kit (1708891). Relative gene expression quantification was performed by real-time quantitative RT-PCR (qPCR) using a BioRad CFX96 real-time PCR detection system (BioRad Laboratories, USA) and SYBR Green IQ Supermix (Bio-Rad Laboratories, CA, USA). Primer Express software was used to generate forward and reverse oligonucleotides listed in Table 1. cDNA (2 µl for total volume of 25 ml per reaction) was analyzed for the gene of interest and for the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression level of each target gene was calculated using the -2^{DDCt} method as described Livak et al (20). Each sample was repeated three times for the gene of interest.

Table 1 - Primers used for evaluating the gene expression of human wharton's jelly stem cells by quantitative real-time polymerase chain reaction.

Gene	Forward	Reserve
GAPDH	5'-ACAGTCAGCCGCATCTTCTT-3'	5'-ACGACCCAAATCCGTTGACTC-3'
Collagen type I	5'-CATCTCCCCTTCGTTTTTGA-3	5'-CCAAATCCGATGTTTCTGCT-3'
Collagen Type II	5'-GACAATCTGGCTCCCAAC-3'	5'-ACAGTCTTGCCCCACTTAC-3'
Collagen type X	5'-CCAGGTCTCGATGGTCCTAA-3'	5'-GTCCTCCAACTCCAGGATCA-3'
SOX9	5'-TACGACTACACCGACCACCA-3'	5'-TTAGGATCATCTCGGCCATC-3'
COMP	5'-CCCACAGACCCTTCCAAGTA-3'	5'-GGGGACAACTGGAGTGAAAA-3'
TGF-beta	5'-CTCCTCGGAAGACACTCTG-3'	5'-AGACTGCGCCTGGTAGTTG-3'
Aggrecan	5'-TGAGTCCtTCAAGCCTCCTGT-3'	5'-TGGTCTGCAGCAGTTGATTC-3'
Runx2	5'-TTCCAGACCAGCAGCACTC-3'	5'-CAGCGTCAACACCATCATTC-3'

2.4. Micromass culture

Chondrogenic differentiation capacity of hWJSCs, previously exposed to soluble factors secreted by chondrocytes during cellular expansion, was investigated by means of an *in vitro* assay of high dense cell micromass culture, as described by Johnstone et al. (7). Briefly, at each passage, cells were trypsinized and $2.5x10^5$ cells aliquots were collected in 15 ml conical tubes (Sarstedt, Numbrecht, Germany). Cells were suspended in chondrogenic medium consisting in serum free DMEM supplemented with ITS + 1 (insulin–transferrin–selenium; Sigma, St. Louis, MO), 10 ng/ml transforming growth factor (TGF)-β1, 100 nM dexamathasone, 50 µg/ml ascorbic acid, 2 mM L-glutanine, 40 µg/ml L-proline and 1 mM sodium pyruvate. Suspended cells were centrifuged for 2 minutes at 1400 rpm to allow the formation of spherical pellets. Pellets (n=3) were cultured in chondrogenic medium and medium was changed every two days during 3 weeks and subsequently processed for histological and immunohistochemical analysis.

2.4.1. Histology and immunohistochemistry

For histological and immunohistochemical analyses, pellets were fixed in 10% neutral buffered formalin (formalin, Sigma), washed twice in PBS for 15 minutes, dehydrated in graded ethanol series, and then paraffin-embedded. Cross-sections (4 µm thickness) were cut, dewaxed and stained with Safranin O for detection of sulfated glycosaminoglycan (sGAG). Briefly, slides were first washed in running tap water, quick destained with fresh acid ethanol (1% hydrochloric acid in 70%ethanol), re-washed in running tap water and immersed in 1:500 fast green (Fluka/44715) for 3 minutes. After that, they were immersed in 1% acetic acid (Panreac/131008) solution for 30 seconds and immersed in 0.1% Safranin O (Fluka/84120) for 4 minutes. Slides were washed in running tap water, counterstained with hematoxylin, cleared in xylene and mounted. Inserts containing human articular chondrocytes were also processed for Safranin O staining. They were washed twice with PBS, fixed in formalin and stained for GAG content.

For immunohistochemistry examination, slides were dewaxed, washed with PBS solution and the exogenous peroxidase activity quenched with 0.6% hydrogen peroxide in methanol for 10 minutes and blocked with R.T.U. Vectastain® normal horse serum (Vectastain® Universal Elite ABC Kit (Vector/ VCPK-7200)) for 20 minutes. Slides were then incubated with primary antibodies rabbit anti-collagen type I and II (Abcam Inc., Cambridge, MA)

overnight at 4 °C in a humidified atmosphere at 1:200 and 1:75 respectively in 3% BSA in PBS respectively. Proper secondary antibody (Vectastain ABC kit) was applied for 1 hour at room temperature in humidified atmosphere, followed by the avidin biotinylated enzyme complex (Vectastain ABC kit) and DAB reagent (Vector Labs, VCSK-4100). Slides were counterstained with hematoxylin and mounted. Slides stained without the addition of a primary antibody served as negative control. Native human articular cartilage was used as positive controls for collagens type I and II.

2.5. Statistic analysis

The number of cell duplications and the relative gene expression quantification are expressed as a mean ± standard deviation with n=3 for each group of culture. Statistical significance of differences was determined using student's t-test multiple comparison procedure at a confidence level of 95 % (p<0.05).

3. RESULTS

3.1. Characterization of human articular chondrocytes (hACs)

During the *in vitro* cell expansion, chondrocytes seeded on transwell inserts, were stained at each cellular passage with Alcian and Toluidine Blue, as well as with Safranin O to stain the cartilaginous proteoglycan content (Figure 2A). Multiwell inserts without chondrocytes were also stained and used as controls, to confirm the absence of possible staining artifacts. Membrane inserts did not color when cells were not present (data not shown). Morphology of human articular chondrocytes was monitored during *in vitro* culture. Cells maintained their characteristic polygonal shape with full colonization of the surface (Figure 2B). The levels of several transcripts of chondrocytes were assessed by means of quantitative polymerase chain reaction (qPCR) analysis. Amplified products were resolved on 1% agarose gels, stained with ethidium bromide and photographed under UV light, as shown in Figure 2C. Collagen type I and II are present in small amounts, as well as aggrecan. Cartilage-specific transcription factor Sex determining region Y-box9 (SOX9), cartilage oligomeric matrix protein (COMP) and transforming growth factor beta (TGF-beta1) genes were well detected. These results demonstrated that hACs were able to maintain the expected inherent chondrogenic phenotype.

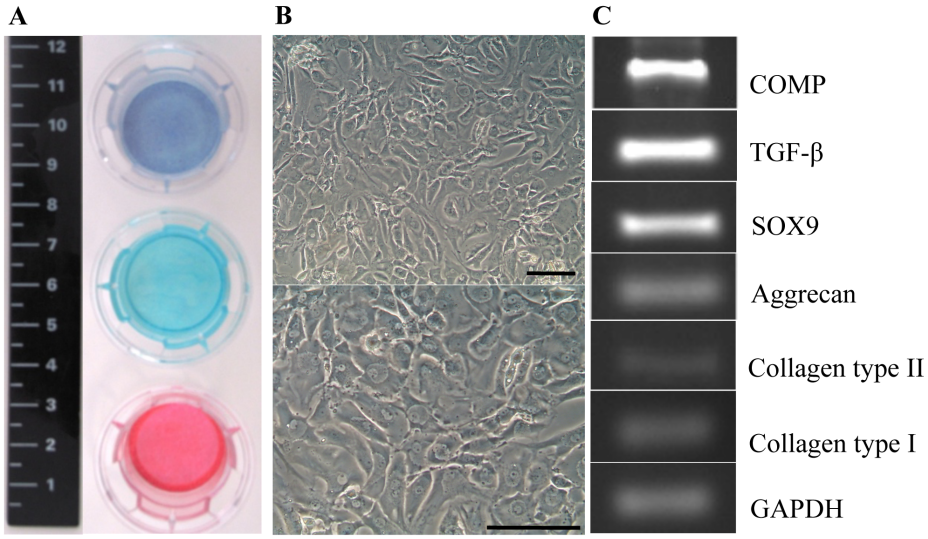

Figure 2 - Characterization of human articular chondrocytes (hACs) after 1 week in culture. (A) Toluidine, Alcian blue and Safranin O staining (from the top to bottom) of hACs cultured on multiwell inserts; (B) Light microscopy images showing chondrocyte morphology in 2D culture; (C) Real time RT-PCR analysis of chondrogenic marker genes confirming that chondrocytes maintain their phenotype during the culture time. DNA fragments, products of real time RT-PCR, for each analyzed gene were set in agarose gel. Scale bar = 100 μm.

3.2. Characterization of chondrogenic-stimulated hWJSCs

Calculating the duplication number versus time during the total period of culture allowed assessing the proliferation capacity of hWJSCs either in co-culture with chondrocytes or in monoculture (Figure 3A). Cellular duplication was constant for the whole culture time. Similar duplications number values were observed for both culture conditions with no statistically significant differences. Possible changes in cell morphology in the two culture systems were monitored during the *in vitro* culture (Figure 3B). Either in monoculture or in co-culture conditions, hWJSCs maintained their characteristic long shape and colonized the entire plastic surface over the time of culture. Co-cultured hWJSCs with hACs did show any changes in their morphology. Cluster of differentiation (CD) antigen of hWJSCs during the expansion phase in both culture conditions were assessed by flow cytometry (Figure 3C). Cultures were tested for the presence of mesenchymal stem cell markers (CD44, CD73 and CD105). Expression of CD44, CD73 and CD105 was higher than 95% No differences were observed for the different cell passages.

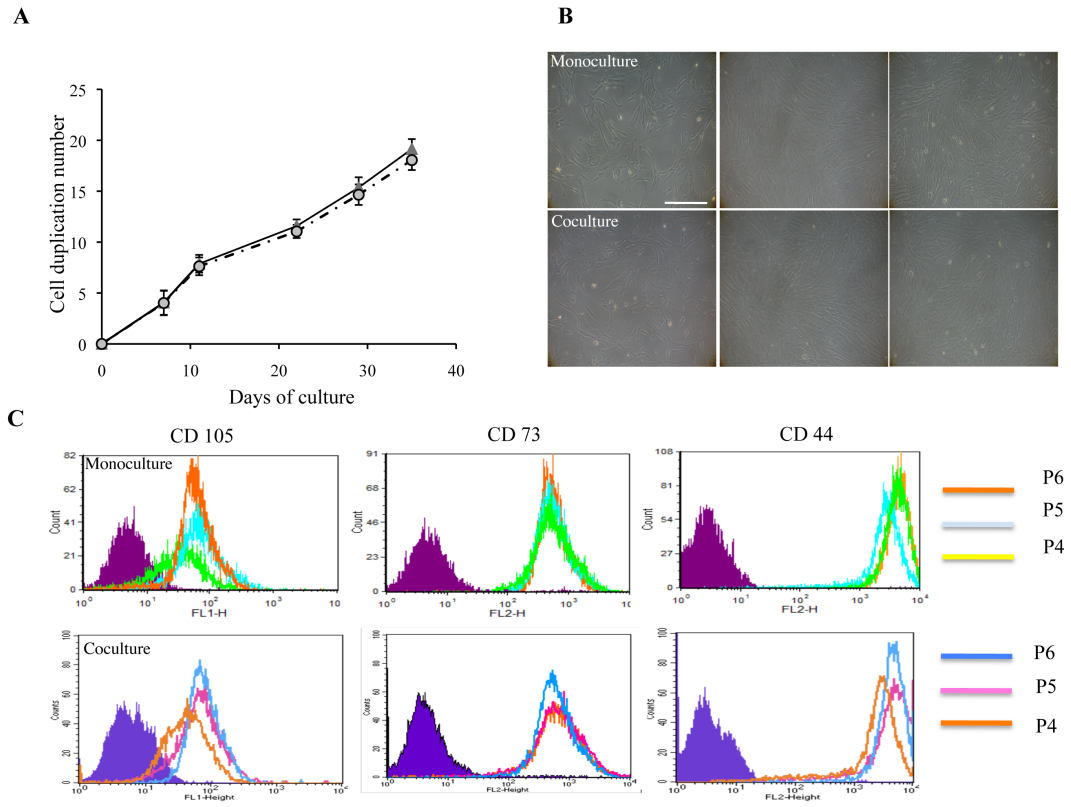

Figure 3 - Characterization of chondrogenic-stimulated hWJSCs. (A) Doubling numbers during the expansion phase. Growth kinetics plotted as number of cell duplication versus time of culture (proliferation). hWJSCs co-cultured with hACs (solid line); hWJSCs in monoculture (dashed line). (B) Optical microscopy images of hWJSCs cultured in both conditions during expansion phase. (C) Flow cytometry analysis of the expression of CD 105, CD 73 and CD44 surface markers on hWJSCs in co-culture with hACs and in monoculture at different passages.

3.3. hWJSCs expression profile of chondrocyte-specific genes

Real-time polymerase chain reaction is usually employed to simultaneously amplify and quantify specific sequences of DNA fragments. In order to quantify variations in gene expression by hWJSCs, either in co-culture or in monoculture, qPCR was performed to assess the expression profile of several genes by hWJSCs during the expansion phase. GAPDH was chosen as reference housekeeping gene. The threshold cycle (Ct) value for each sample was determined in the exponential phase of amplification. In each analyzed condition, for each cDNA sample, the Ct value of GAPDH was subtracted from the Ct value of the target gene, to derive Delta Ct. Relative gene expression quantification was performed having as normalization gene values those expressed by hWJSCs in monoculture.

Relative gene expression values of hWJSCs in co-culture with chondrocytes are always normalized with the values of gene expression relative to hWJSCs cultured alone (Figure 4). We observed an up-regulation of gene expression for all analyzed genes when hWJSCs were co-cultured with chondrocytes. Collagen type I presents the highest relative expression, a 4 fold at passage 3. With further passages during cell expansion, the level of collagen type I expression presents a continuous decrease of fold induction. Opposite tendency of expression fold may be observed for Type II collagen. This gene expression fold augments with cell expansion. A drastic reduction of aggrecan expression level was observed from passage 3 to passage 4, but then increases until P6. A slightly reduction of Sox9 relative expression was observed with cellular passage. An up-regulation of TGF-beta1 was observed for hWJSCs co-cultured with chondrocytes compared to those expanded alone. Relative induction fold values of this gene increase gradually during culture.

Cartilage oligomeric matrix protein (COMP) relative expression values tend to increase, especially for the latest stages of cell expansion. This propensity is particularly observed in the first 3 cellular passages. Type X collagen (a hypertrophic chondrocyte marker) and Runx2 (pre osteogenic gene marker) relative expression values showed an analogous behavior. A decrease in the first two passages is observed with further low expression until the sixth passage.

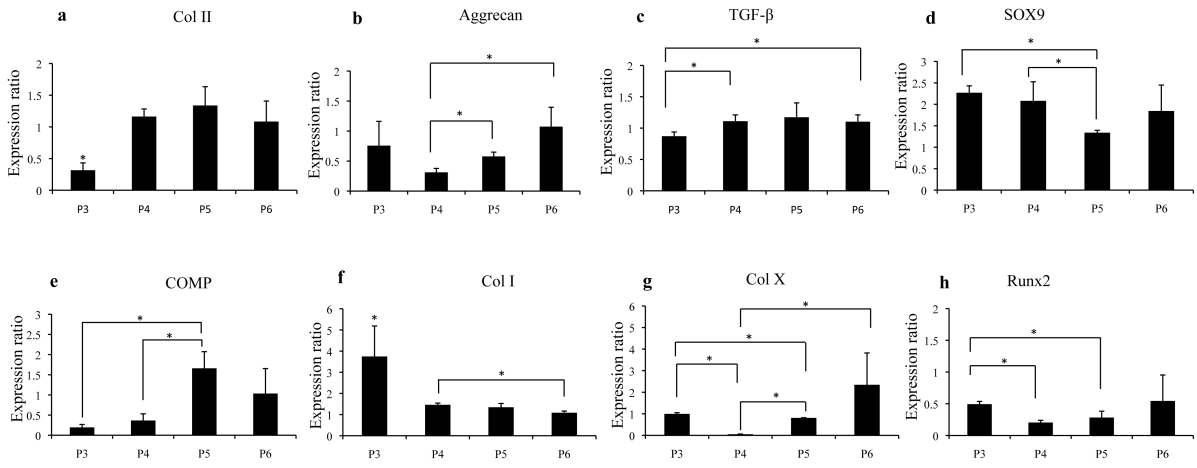

Figure 4 - Expression profile of chondrocyte-specific genes (a-e) and collagen type I (g) collagen type X (g) and Runx2 (h) observed during the expansion of hWJSCs. Level of mRNA transcripts fold induction of hWJSCs co-cultured with chondrocytes was normalized to values achieved form hWJSCs cultured alone during the expansion phase. Statistical analysis through the various time points ($p<0.05$) was performed.

3.4. Chondrogenic assessment of hWJSCs phenotype by micromass pellet assay

Histological analysis of micromass pellets of hWJSCs, previously expanded in co-culture and monoculture condition, was performed using Safranin O staining which marks cartilage extracellular cellular glycosaminoglycans (Figure 5). This analysis showed a non-homogeneous distribution of the cells within micromass pellets. The pellets displayed a positive staining for Safranin O at each cellular passage for both culture conditions. Nonetheless this positive staining, wharton's jelly stem cells cultured in absence of chondrocytes presented a fibroblast-like morphology than those expanded in co-culture.

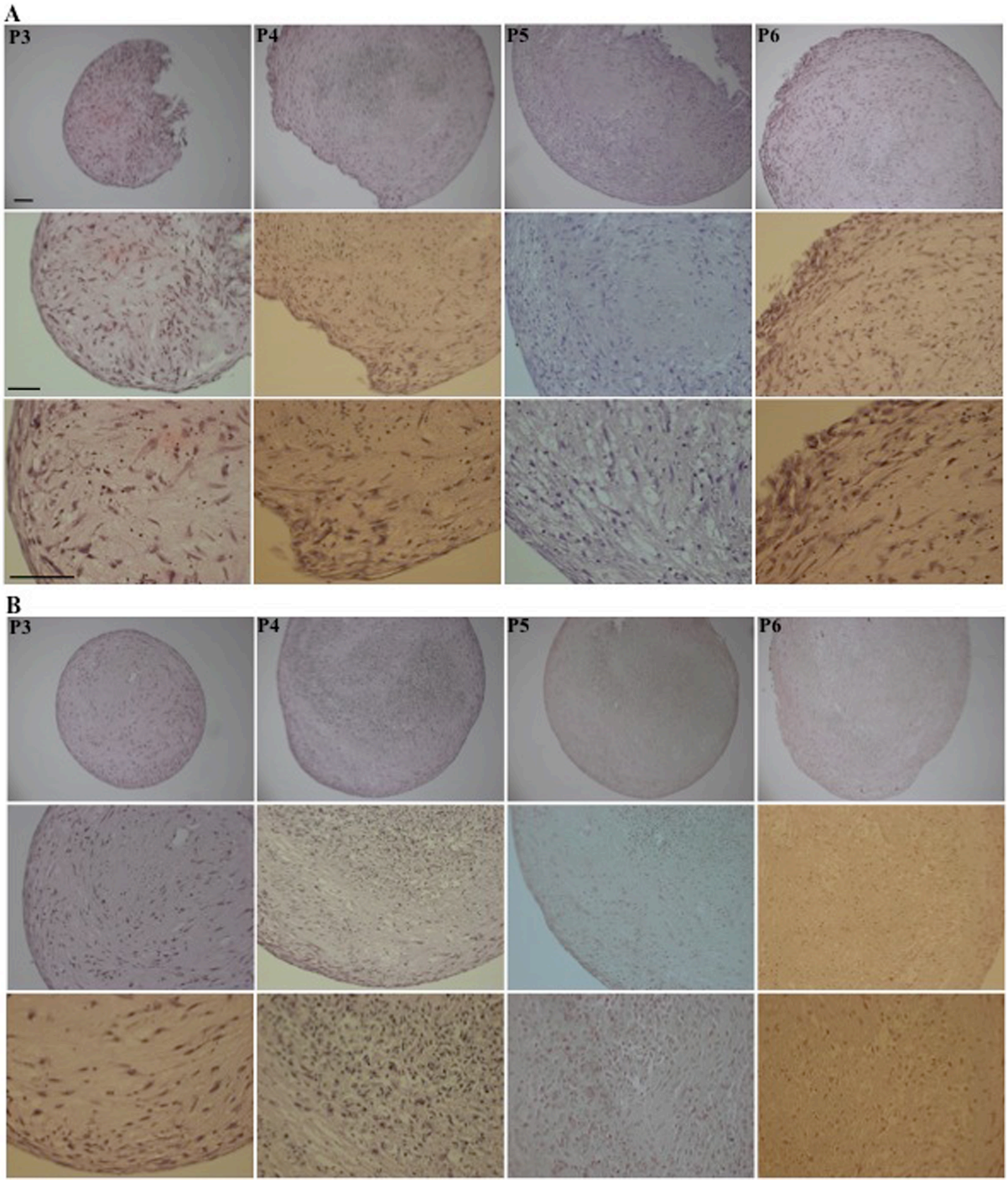

Figure 5 - Histological cross-sections of micromass culture of hWJSCs after being expanded in monoculture (A) or co-cultured with hACs (B) stained with Safranin O, which indicates the presence of sulfated glycosaminoglycans. P denotes passage number. Scale bar = 100 μm.

The results from immunohistochemistry localization of collagen type I and II can be appreciated in Figure 6. A more positive staining of type I collagen was detected in the micromass pellets made with cells expanded in

monoculture than those expanded in co-culture (Figure 6). A significant detection of collagen type II in pellets made with co-cultured hWJSCs was observed.

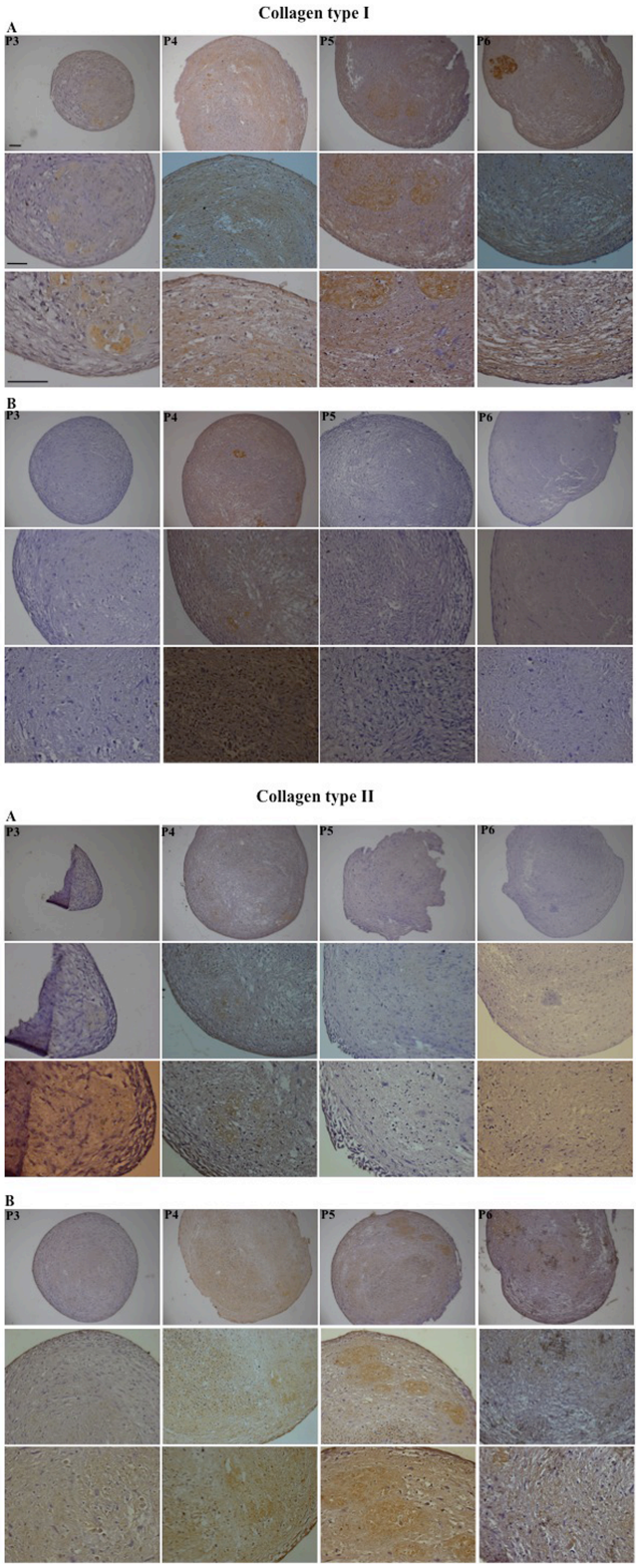

Figure 6. - Immunohistochemical detection of collagen type I and type II in hWJSCs cultured in micromass pellets after expansion in monoculture (A) and co-cultured with hACs (B). P denotes passage number. Scale bar = 100 μm.

4. DISCUSSION

One of the difficulties to engineer and repair cartilage is the lack of sufficient number of cells when using autologous chondrocytes. In the last years, this field of research has shown an increasing interest in using alternative cell sources and valid culture systems [6, 21].

Mesenchymal stem cells (MSCs), found in several tissues such as bone marrow [22] adipose tissue [23] and umbilical cord [24], have demonstrated a multilineage potential and have been considered as a potential alternative to autologous chondrocytes. Barry el al. [25] showed the chondrogenic differentiation ability of MSCs from bone marrow with the drawback risk of hypertrophy induction. Currently, adipose stem cells are an abundant source of adult stem cells but their chondrogenic potential is known to be inferior to those from bone marrow [26].

Like human umbilical cord stem cells [27-30], we suggest that hWJSCs could be a useful cellular source for cartilage regeneration therapies. This cell type combines most of the ESC and adult MSCs "stemness" markers for long expansion periods [10] showing the ability to differentiate along mesenchymal lineages [14, 31]. Harvested from umbilical cords, hWJSCs are easily accessible, presenting high levels of proliferation during *in vitro* expansion making possible to obtain a plentiful amount of cells in a short period of time [31, 32].

Cartilage tissue engineering relies not only on the selection of an adequate biomaterial scaffold but also on the existence of efficient approaches that control cell differentiation and neo-tissue assembly. In this perspective, chondrocytes have been employed in several studies using co-culture systems, with direct or non-direct cell contact, to achieve either chondrogenic or osteogenic differentiation of mesenchymal stem cells [8, 33, 34].

The use of human articular chondrocytes in a non-direct co-culture system with hWJSCs has not been reported in the literature so far. Furthermore, the increased awareness of the application potential of this cell type in cartilage tissue engineering has also motivated this study. As first result, co-culture of hWJSCs in alpha medium supplemented with 10% of fetal bovine serum for almost 36 days did not show statistically significant differences in proliferation ratio compared to those cultured in the same conditions but in absence of chondrocytes (Figure 3). This result differs from the data found in previous studies which have shown that soluble factors released

from chondrocytes enhanced the survival and proliferation of mesenchymal cells [8, 35, 36] but none of these studies have used hWJSCs. In the absence of data, we first report that cellular growth kinetics of hWJSCs in co-culture conditions is fairly analogous to those in monoculture. (Figure 3A). In both conditions, hWJSCs achieve approximately 20 duplications (corresponding to a 64-fold increase of the initial cell number) in about 36 days of culture. It is well known that chondrocytes expanded *ex-vivo* undergo phenotype changes with the different expression of several chondrogenic gene markers depending on the expansion time [37, 38]. For this reason, each time hWJSCs were counted and re-plated in the polystyrene culture plates, in average every 8 days, hACs were also detached by enzymatic digestion with trypsin. Fresh hACs at first passage number were re-plated in the insert wells. This was performed to avoid further expansion of human articular chondrocytes thus preventing their possible dedifferention [37]. As shown in Figure 2A, human articular chondrocytes seeded in transwell inserts are macroscopically positive for Alcian Blue, Toluidine Blue and Safranin O staining, demonstrating their ability to deposit cartilage extracellular matrix components. Optical microscopy images of hACs showed their typical polygonal shape demonstrating a possible phenotype maintenance of the cells (Figure 2B). Chondrogenic phenotype of human articular chondrocytes was evaluated by real time PCR and amplified DNA products were set on on 1% agarose gels (Figure 2C). It should be noticed the low level of expression of collagen type I indicating the lack of cellular high proliferation ratio, indicative of chondrogenic phenotype of the cells, known to present a restricted replication value [37]. When plated in monolayer (2D surfaces), chondrocytes hardly preserve the expression of collagen type II [37]. This behavior was also observed in our cells, confirmed by the faded line of the amplified DNA fragment. Nevertheless, we observed that hACs at passage one were able to express other typical cartilage marker genes such as aggrecan, COMP and TGF-beta1, as well as the cartilage master transcription factor SOX9, which is known to have an essential role in maintaining the chondrogenic phenotype in articular chondrocytes [37]. With this characterization, we assumed that the human articular chondrocytes plated in the multiwell inserts maintained their chondrogenic phenotype characteristics.

The identification of suitable markers for verifying the multipotencial of MSCs and indicating the differentiation status of these cells under specific conditions is of utmost importance. According to recent reports, the expression of CD73, CD90, and CD105 on the cell surface of bone marrow mesenchymal stem cells (BM-MSCs)

was down-regulated after differentiation [39, 40]. CD105, a 180 kDa integral membrane glycoprotein known as endoglin, is a receptor for TGF-beta1 and -beta3 and modulates TGF-beta signaling by interacting with related molecules, such as TGF-beta1, -beta3, BMP-2 and 7. It is speculated that these members of the TFG-beta superfamily are mediators of cell proliferation and differentiation and play regulatory roles in cartilage and bone formation. The disappearance of the CD105 antigen during osteogenesis suggests that this protein, like others in the TFG-beta superfamily, may be involved in the regulation of osteogenesis. Recent studies reported that members of the TGF-beta family control the differentiation of MSCs [41, 42]. However, whether CD105 plays a functional role during the process of stem-cell differentiation has not yet been clarified. Therefore, we evaluated the potential effect of chondrocyte soluble factors over the expression profile of MSCs markers (CD44, CD73 and CD105) in hWJSCs co-cultured with hACs for several passages (Figure 3C). No significant differences were observed at phenotypic level in co-culture conditions.

Real time polymerase chain reaction allowed the relative quantification of several genes expressed by hWJSCs, either in co-culture or monoculture conditions during expansion phase. Relative fold induction for each gene is displayed in Figure 4. Real time RT-PCR analyses illustrate the up-regulation of chondrogenic phenotype specific gene markers in hWJSCs co-cultured with chondrocytes.

Type II collagen expression fold shows an opposite behavior. The expression fold of this cartilage marker gene begins to be 0.3 at P3 presenting a considerable augment of expression fold in the following cell passage (1.2). This fold of expression remained high and constant until passage 6. A similar trend of relative fold induction was observed for aggrecan. There was a decay on the fold of aggrecan relative expression from the initial cell passage (P3) to the subsequent passage number (P4) but it then increased until P6. Cartilage master transcription factor SOX9 [21, 37, 43] (Figure 4) was up regulated in hWJSCs in co-culture conditions relatively to those cultured alone, presenting at P3 and P4 the highest values of relative fold induction, respectively 2.4 and 2.2. With further cell expansion we noticed a minor decrease of relative fold until passage 6. Relative fold induction values of TGF-beta1 gradually increased from passage 3 up to passage 5 (0.9 until 1.2) presenting a plateau behavior for the next passage (1.1 fold). Cartilage oligomeric matrix protein assumes a very important role in cartilage phenotype maintenance [44]. COMP relative expression shows a slow increase from passage 3 to 4 (0.2 up to 0.4 in this

order) with a subsequent significant enhance up 1.6 and 1.1 fold, respectively at passage 5 and 6. On the other hand, collagen type I expression presents its higher value of expression, 4 fold, at passage 3. With further cell expansion, collagen type I expression presents low values being possible to perceive a statistically significant continuous reduction tendency of expression until the end of the study. Collagen type X and Runx2 relative expression seems to proceed in a homologous way. hWJSCs relative expression fold of these two genes shows a significant decrease from passage 3 to 4 followed by a slight increase at P5. Cells culture at passage 6 present higher gene expression values, but not in a statistically significant way. The gene expression data suggest that human articular chondrocytes can be responsible for a possible chondrogenic commitment of hWJSCs when expanded in 2D monolayer surface. With these results, we first report that co-cultured hACs provide anabolic soluble factors to hWJSCs towards the chondrogenic phenotype without the need of adding exogenous growth factors. It was not our aim to determine which factors could lead to this commitment. Further research should focus on the identification of important factors that are responsible to drive stem cells into specific phenotypes. Using different cell types, Elisseeff [36] and Chun [45] suggested that TGF-beta and Cytokine-like 1 (Cytl1) could modulate a chondrogenic phenotype, respectively in human embryonic stem cells and mesenchymal stem cells [45]. Cytl1 protein oversecreted by chondrocytes showed the ability to promote chondrogenic differentiation of mesenchymal cells.

The use of chondrocytes collected and isolated from femoral condyles of patients undergoing partial knee arthroplasty is a limitation of the current study. It is remarkably difficult to use healthy human articular chondrocytes in cartilage research. Knee arthropasties, commonly performed worldwide, can provide, however, relatively healthy cartilage tissue, which after digestion/isolation allow obtaining a source of primary adult articular chondrocytes for cartilage research and therapy. Our data showed that chondrocytes obtained from biopsies from cartilage not totally healthy could promote the chondrogenic differentiation of hWJSCs by committing these cells into the chondrogenic phenotype during the 2D expansion.

With Safranin O staining (Figure 5) we were able to mark glycosaminoglycans, characteristic components of cartilage extracellular matrix or in cells exhibiting chondrogenic phenotype due to a successful chrondrogenic differentiation process. Figure 5A shows that expansion in monoculture conditions, hWJSCs display a fibroblast-

like morphology. This cell morphology can be also observed in the external region of hWJSCs micromass pellet expanded in co-culture (P4, P5 and P6). Still, in this culture condition, clusters of cells with round shape and more positive Safranin O staining can be observed in the pellet core.

Immunolocalization of collagens type I and II in the micromass pellets, maintained in culture for 3 weeks, was performed to assess the chondrogenic phenotype level accomplished by human wharton's jelly in both culture conditions during expansion phase.

The localization of collagen type I extracellular matrix protein can be observed in Figure 6. It is clear the presence of collagen type I in cells expanded in monoculture (Figure 6A). From the beginning (passage 3) we can observe more than one cellular region positively stained which demonstrate incomplete/reduced chondrogenic differentiation of the cells. After expansion in co-culture condition, micromass pellets of hWJSCs show a minimal detection of this protein.

Collagen type II is one of the main cartilage extracellular matrix proteins. Results of immunolocalization of this ECM protein in hWJSCs pellets can be observed in Figure 6. It is clear that in both cases hWJSCs are positive for collagen type II. Differences can, however, be observed when cells were expanded in different conditions. A closer look allows to observe several cell nodules with round shape and positive staining for this chondrogenic extracellular matrix protein in micromass pellets of wharton's jelly stem cells expanded in co-culture at all passages. These positive circular cellular zones are not visible in wharton's jelly stem cells cultured alone. Their positive staining for collagen type II is substantially low within the pellet. Collagen type I and II detection (Figure 6) corroborate the results of the safranin O staining, showing greater detection of cartilage ECM components in hWJSCs expanded in co-culture with chrondrocytes, comparable to those from hWJSCs cultured alone. The release of soluble factors by hACs during co-culture may modulate the deposition of ECM proteins during *in vitro* micromass pellet culture.

These results, in addition to the real time RT-PCR data showing positive modulation of collagens type I and II, aggrecan, Sox9, COMP, TGF-beta1 as well as the low values for collagen type X and Runx2, confirm the hypothesis that chondrocytes are able to secret soluble active growth factors with capacity to influence hWJSCs phenotype in co-culture. Uncertain about which are the metabolic soluble factors secreted by the chondrocytes

and their possible mechanisms of action on the chondrogenic commitment of hWJSCs, the results of this study are of utility in future investigations using co-culture strategies for influencing stem cell commitment and differentiation. However, additional *in vitro* studies using other sources of MSCs (e.g. from bone marrow or adipose tissue) are necessary to demonstrate the superiority of our co-culture system and *in vivo* experiments must be performed to validate the potential of the proposed strategy.

5. CONCLUSIONS

Our study can provide a proof-of-principle that *in vitro* expansion of hWJSCs in monolayer with a non direct co-culture system using articular human chondrocytes can be a valid model to commit a stem cell source for a possible strategy in cartilage tissue engineering using mesenchymal stem cells. During the expansion phase, hWJSCs co-cultured with human adult articular chondocytes not only maintained their proliferation ability but were also able to present an up-regulation expression of cartilage genes such as collagen type II, aggrecan, COMP, TGF-beta1 as well as of the master transcription factor SOX9. Histological and immunohistochemical analysis of micromass pellets cross-sections confirmed a more positive staining for glycosaminoglycans and collagen type II in those expanded in co-culture with chondrocytes. These results indicated that co-culture of hACs with hWJSCs enhanced the chondrogenic phenotype commitment of hWJSCs. Although promising results were obtained during *in vitro* expansion and using the micromass pellet model, corroboration should be done in *in vivo* environment to demonstrate that the proposed hypothesis is sufficient to induce a stable phenotype.

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CHAPTER IV

DUAL EFFECT OF PLATELET LYSATE ON HUMAN ARTICULAR CARTILAGE: A MAINTENANCE OF CHONDROGENIC POTENTIAL AND A TRANSIENT PRO-INFLAMMATORY ACTIVITY FOLLOWED BY AN INFLAMMATION RESOLUTION

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DUAL EFFECT OF PLATELET LYSATE ON HUMAN ARTICULAR CARTILAGE: A MAINTENANCE OF CHONDROGENIC POTENTIAL AND A TRANSIENT PRO-INFLAMMATORY ACTIVITY FOLLOWED BY AN INFLAMMATION RESOLUTION [*]

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Abstract

Platelet rich plasma (PRP), a cocktail of platelet growth factors and bio-active proteins, has been proposed as therapeutic agent to restore damaged articular cartilage. We report the biological effect of the platelet lysate (PL), a PRP derivative, on primary human articular chondrocytes cultured under both physiological and inflammatory conditions. When added to the culture medium, PL induced a strong mitogenic response in the chondrocytes. The *in vitro* expanded cell population maintained a chondrogenic re-differentiation potential as revealed by micromass culture *in vitro* and ectopic cartilage formation *in vivo*. Furthermore, in chondrocytes cultured in the presence of the pro-inflammatory cytokine IL-1α, the PL induced a drastic enhancement of the synthesis of the cytokines IL-6 and IL-8 and of NGAL, a lipocalin expressed during chondrocyte differentiation and inflammation. These events were mediated by the p38 MAP kinase and NF-κB pathways. We observed that inflammatory stimuli activated phospo-MAP kinase-activated protein kinase 2 (MAPKAPK-2), a direct target of p38. The pro-inflammatory effect of the PL was a transient phenomenon, after an initial up regulation we observed significant reduction of the NF-κB activity together with the repression of the inflammatory enzyme ciclooxygenase-2 (COX-2). Moreover, the medium of chondrocytes cultured in simultaneous presence of PL and IL-1α, showed a significant enhancement of the chemoattractant activity versus untreated chondrocytes. Our findings support the concept that the platelet products have a direct beneficial effect on articular chondrocytes and could drive in sequence a transient activation and the resolution of the inflammatory process, thus providing a rational for their use as therapeutic agents in cartilage inflammation and damage.

Keywords

Platelet-rich plasma (PRP), Platelet lysate (PL), Articular cartilage, Inflammation, Osteoarthritis (OA) and Regenerative Medicine

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1. INTRODUCTION

In the last decades the biology of articular cartilage has been the object of an increasing interest by tissue engineering scientists seeking a continuous improvement in the repair and regeneration of this tissue through the implant of cells with a chondrogenic potential. The low yield of healthy human articular chondrocytes (HAC) obtained from articular cartilage biopsies, together with the cell limited proliferation potential and the difficulty to maintain their cartilage commitment when cultured in monolayer, continues to demand further research to develop alternative and better culture methodologies (1, 2). According to standard protocols, the monolayer expansion of articular chondrocytes to be used in cell therapy is made in the presence of high concentrations of autologous human serum or fetal calf serum (FCS) (3). Recently, the disadvantages of these conventional culture methods have been overcome thanks to the development of media based on the addition of several growth factors (GFs), that can better sustain cell proliferation rate as well as assure maintenance of a chondrogenic re-differentiation potential by the expanded cells *in vitro* (1, 4, 5). Due to its high spectrum content of growth factors, Platelet-Rich Plasma (PRP), a natural concentrate of autologous blood platelet growth factors, has been proposed as a cell culture medium supplement (6). Obtained with a simple, low cost and minimally invasive method, PRP was introduced into clinical therapies in 1998 to augment bone grafts. Since then, PRP has been applied in distinct fields of medicine aiming to the improvement of tissue regeneration (7-9).

Given its excellent safety profile and ease of preparation, the use of PRP in orthopedic surgery will likely continue to grow. Despite the increasing number of clinical reports, the complex orchestra of signals activated in cells and in particular in human articular chondrocytes, as result of their exposure to the platelet released factors remains unclear (6, 8-14). Therefore, further laboratory research is needed to fully understand the mechanism of action of this cocktail of factors and to recognize the cascade of the events that support the efficacy of PRP treatment.

In a damaged tissue, the initial inflammatory response plays a key role triggering tissue repair and homeostasis, but can be detrimental in the long term, causing fibrosis. NF-kB, the main transcription factor controlling inflammation, induces the expression of many mediators of the immune and acute phase responses (15-18). One of the mediators is Cyclooxygenase-2 (COX-2), an enzyme that plays an essential role in the inflammatory

cascade leading to the acute phase response. COX-2 expression was recently described also in the differentiating growth plate and during chondrocyte differentiation and inflammation (12, 19-23). In previous publications on avian, murine and human cartilage, we described the expression of several proteins related to stress conditions in fully differentiated chondrocytes. In particular we reported that extracellular lipocalins (chicken-Ex-FABP, murine-Sip24, human-Ngal) were constitutively expressed in differentiated chondrocytes, and strongly overinduced by stimulation with inflammatory agents (23-26). Further, we recently described that the signaling pathway leading to the expression of Sip 24 lipocalin (Ngal homologue in human species) in murine cartilage, involves p38 activation/NFkB, and COX-2 expression (26). In current study we evaluated the effect of Platelet Lysate (PL), a PRP derivative, in the longterm expansion and maintenance of cartilage commitment of primary HAC *in vitro* and *in vivo.* We also investigated the activation of the p38MAPK and NF-κB pathways and their downstream products IL6, IL8, NGAL and MK2 direct target of p38, by PL in HAC cultured under both physiological and inflammatory conditions.

2. MATERIALS AND METHODS

2.1. Platelet lysate preparation

Platelet lysate was prepared according to Zaky and El Backly (27, 28) with minor modifications. In brief, platelet rich plasma (PRP) was obtained from healthy human blood donors from Blood Transfusion Center of the San Martino Hospital, Genoa-Italy, according to the protocol approved by the institutional ethics committee. After serial centrifugations, platelet pellet was resuspended in plasma at a concentration of about 1x 10^7platelets/ml. To obtain platelet membrane rupture, PRP preparations were immersed in liquid nitrogen for 1 minute before transferring at 37°C for 6 minutes. The cycle was repeated three times and platelet membranes removed by centrifugation. Supernatants (PL) were harvested, divided in aliquots and stored at –80°C until use.

2.2. Chondrocyte isolation and Culture

Bioptic material was collected from femoral condyles of patients undergoing partial knee arthroplasty (4 females and 4 males with a 65 year median age). All patients signed an informed consent approved by the Ethical Committee of San Antonio Hospital, Recco, Italy. Human articular chondrocyte (HAC) were isolated as already described by (2, 29). Single articular chondrocytes were released by repeated enzymatic digestions (1mg/ml hyaluronidase, 400U/ml collagenese I, 1,000 U/ml collagenase II, 0,25% tripsin in PBS). Isolated cells were pooled and cultured in adhesion in Coon's modified Ham's F12 containing 10% FCS supplemented with 2mM glutamine, 100U/ml penicillin, 100mg/ml streptomycin (all from Euroclone). After reaching semiconfluence the cells were trypsinized, seeded on culture dishes ($10x10^3$ cells/cm^2) and cultures continued either in Coon's modified Ham's F12 supplemented with 10%FCS or with 5% PL. During culture, cells were monitored using a bright field microscope (Axiovert 40 CFL, Germany) equipped with a digital camera (Canon Power Shot G8, Japan).

2.3. *Growth kinetics*

Number of cell doublings performed by HAC cultured in the presence of 10% FCS versus 5% PL was calculated considering the number of cells plated and recovered at each passage. Cells were detached from dishes by digestion with trypsin/EDTA (Invitrogen Life Technologies, Carlsbad, CA) before they reached confluence, counted and always re-plated at $2.5x10^4$ cells/cm^2 in a 60 mm x 15 mm cell culture dish. Six different primary cell cultures were tested.

2.4. Micromass pellet culture

The chondrogenic potential of HAC expanded in the presence of either FCS or PL was investigated at different culture passages by the micromass pellet culture. $2.5x10^5$ cells were pelleted in 15 ml conical tubes and cultured for 21 days in chondrogenic medium (10ng/ml hTGF-B1 (Prepotech,), 10^{-7} M dexamethasone (Sigma)

and 50 μg/ml ascorbic acid (Sigma) according to (30). Medium was changed every two days. Cartilage formation was evaluated by histology.

2.5. Ectopic cartilage formation in athymic mice

3D micromass pellets were maintained for 2-3 days *in vitro* before subcutaneous implantation in athymic mice (CD-1 nu/nu; Charles River Italia, Calco, Italy). Groups of 3 pellets for each culture condition were implanted in 6 different mice with comparable results. All animals were maintained in accordance with the standards of the Federation of European Laboratory Animal Science Associations (FELASA), as required by the Italian Ministry of Health. Animals were sacrificed, and implants were harvested after 4 weeks for the histological analysis of cartilage formation.

2.6. Histology and Immuno-histochemistry

Pellets and implants were fixed in 4% formaldehyde in PBS, dehydrated in ethanol, and paraffin-embedded. Cross-sections (5 μm) were cut, de-waxed and stained with toluidine blue for detection of sulfated glycosaminoglycan. For immuno-histochemical analysis, sections were de-waxed and treated with methanol: H_2O_2 (49:1) for 30 min, then treated with 1 mg/ml hyaluronidase in PBS pH 6.0, for 30 minutes at 37°C and washed with PBS. Slices were then incubated with goat serum for 1 hour to reduce non-specific binding. Type II collagen antibody diluted 1:250 (CIICI anti-COLLII, DSHB, University of Iowa) was added and incubated for 1 hour at room temperature. The procedure was performed using a Histomouse Kit (Zymed Laboratories, USA). The immunobinding was detected with biotinylated secondary antibody and streptavidin-peroxidase. The peroxidase activity was visualized by AEC chromogen substrate. Histology and immunohistochemistry slides were observed at different magnifications and images acquired with the Axiovert 200M microscope (Carl Zeiss, Germany).

2.7. Total RNA extraction and semi-quantitative reverse transcriptase – PCR

HAC were cultured in presence of 10% FCS or 5% PL, at different time (P0, P1 and P4), the total RNA was extracted from HAC using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. In brief, 0.5 ml TRIzol reagent were added to each cell dish. Semiquantitative RT-PCR was performed starting from 5 mg RNA that was reverse-transcribed into cDNA (Superscript, Invitrogen). The PCR was performed using a commercial amplification buffer (Platinum PCR Supermix, Invitrogen). Levels of transcripts for type I and type II collagens, the cartilage-specific transcription factor (SOX9) and the housekeeping enzyme-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were assessed. Primers for each gene were designed as reported in Table 1. Amplified products were resolved on 1% agarose gels and stained with ethidium bromide. Photographs were acquired under UV light. PCR analysis was performed with three different primary cell cultures.

Table 1 - Primers used for evaluating the gene expression of HAC by semi-quantitative Real Time-Polymerase Chain Reaction

Gene	Forward 5'	Reserve 5'	Annealing temperature (°C)	Segment (bp)	N° of Cycles
GAPDH	CCATCTTCCAGGAGCGAGAT	CTGCTTCACCACCTTCTTGAT	59	568	28
Collagen type I	TCTGCGACACAAGGAGTC	CGACCCACACTTCCATCACT	55	590	28
Collagen Type II	TCTGCAACATGGAGACTA	GAAGCAGACAGGCCCTATGT	58	517	32
SOX9	ATCTGAAGAAGGAGAGCGAG	TCAGAAGTCTCCAGAGCTTG	58	360	30

2.8. Western Blot analysis

Confluent HAC (at passage 1) were treated for 16 hours with medium supplemented with 5% PL, or 200U/ml IL-1α, or 5% PL + 200U/ml IL-1α or without any supplement. Media were then removed, cells extensively washed with PBS in order to remove residual PL before being transferred to serum-free medium (no FCS) for 24 hours. Conditioned serum-free culture media were collected and quantified by Bradford assay (31). After, 50 µg of proteins of each sample were loaded on 4-12% NuPAGE Bis-Tris gel (Invitrogen), electrophoresis and blot were performed according to a protocol previously optimized in our laboratory (19). Blot membranes were incubated

with specific primary antibodies against IL-6, IL-8, (1:200; Santa Cruz Biotechnology, CA, USA) and NGAL (1:1000; R&D Systems, Abingdon, UK) (n=6).

Western Blot analysis for IL-6, IL-8 and NGAL, secreted by cells cultured in the presence of the p38 inhibitor SB203580 was also performed (Calbiochem, Germany). To this purpose, cells were incubated in the presence of 5 μM SB203580 for 2 hours under the same conditions as described above. Images were scanned using the Epson perfection 1260 scanner and band densities were quantified using the image J software. Different pixel mean densities were analyzed. Means and standard deviations were obtained for statistical analysis (n=4).

To analyzed the phospho-(MK2), confluent HAC (at passage 1) were treated for only 30 minute, with medium supplemented with 5% PL, or 200U/ml IL-1α, or 5% PL + 200U/ml IL-1α or without any supplement, in absence and presence of the p38 inhibitor SB203580, as previously described. Cell layers were collected in RIPA buffer containing a protease and phosphatase inhibitor cocktail. The total protein of cellular lysate was quantified by the method of Bradford. Equal amounts of total protein (60 μg) were loaded on 4-12% NuPAGE Bis-Tris gel (Invitrogen) and electrophoresis was performed. Gels were blotted onto polyvinylidene fluoride membrane (GE Healthcare, Amersham Bioscience), immunoprobed with phosphoMK2 (Thr334) antibody (Cell Signalling) at a dilution of 1:100, and developed using ECL+(GE Healthcare, Buckinghsmshire, UK). Immunofluorescence was performed on HAC treated as decsribed above. Cells were fixed in 3% paraformaldehyde in PBS, pH 7,6 containing 2% sucrose for 5 min. After washing in PBS, cells were permeabilized with a solution (20mM Hepes, 300mM sucrose, 50mM NaCl, 3mM MgCl2 and 0,5% Triton X-100) for 5 minutes at 0°C. Non-specific binding was prevented by incubation with pure Goat serum for 30 minutes at 0°C. Slides were incubated with phosphoMK2 (Thr334) antibody at a dilution of 1:100 for 2 hours at 0°C. After rinsing in PBS, secondary fluorescence-labeled antibody Alexafluo-488 (Invitrogen) was added for 30 minutes Finally, samples were washed and counterstained with DAPI (Vectashield Mounting Medium- Vector) to visualize nuclei.

2.9. Quantitative RT-PCR

HAC at passage 1 were incubated with serum free media supplemented with 5% PL, or 200U/ml IL-1α, or 5% PL and 200U/ml IL-1α, or without any added supplement. After 16 hours incubation, RNA was extracted (as described previously) and cDNA was synthesized starting from 1 μg of total RNA. It was used an Oligo(dT)20, random hexamers mix and a Superscript III first-strand synthesis system supermix for RT-PCR (Invitrogen). cDNAs were diluted 5 to 20 times, then subjected to PCR analysis. Gene expression levels were quantified by real-time quantitative RT-PCR (qPCR), using Taqman Probe and the ABI Prism 7700 Sequence Detector (Applied Biosystems, Branchburg, New Jersey, USA), according to the manufacturer instruction. Primers and probes used for the reference gene (GAPDH) and the genes of interest are described in Table 2 (n=3).

Table 2 - Primers used for evaluating the gene expression of HAC by quantitative Real-Time PCR

Gene	Forward 5'	Reverse 5'	Probe
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC
IL-6	TGACGACCTAAGCTGCACTT	GGGCTGATTGGAAACCTTATTA	TCTTGCCATGCTAAA
IL-8	CGTGGCTCTCTTGGCAGC	TTAGCACTCCTTGGCAAAACTG	TTCCTGATTTCTGCAGCTCCTGTGAAGGT
NGAL	GGCCTCCCTGAAAACCACAT	CACCTGTGCACTCAGCCGT	CCAATCGACCAGTGTATC
COX2	AATTGCTGGCAGGGTTGCT	GGTCAATGGAAGCCTGTGATACT	TAGGAATGTTCCACCCGCA

2.10. NF-kB activity assay

To assess NF-kB activity (22), the binding of the NF-κB p65 subunit to the NF-κB binding consensus sequence 5'-GGGACTTTCC-3' in cell lysate was measured with Trans-Am NF-κB kit (Active Motif, CA). Cell extracts from HAC expanded in the presence of the different medium supplements, were prepared as recommended by the manufacturer. The Trans-Am kit employs 96-well plate coated with an oligonucleotide containing the NF-κB binding consensus sequence. The active form of the p65 subunit present in cell lysates was detected using Abs specific for an epitope accessible only in the activated subunit bound to its target DNA. Specificity was checked by measuring the ability of soluble wild type or mutated oligonucleotides to inhibit binding. Results were expressed as the absorbance values observed in the presence of the mutated oligonucleotide minus absorbance

values observed in the presence of the wild-type oligonucleotide. The NF-κB activity was measured in triplicate samples and assayed in triplicate.

2.11. Chemotatic assay

Boyden chamber chemotaxis assay of HUVEC (Human Umbilical Vein Endothelial Cells), Human BMSCs (Bone Marrow Stromal Cells), obtained from iliac crest marrow aspirates from healthy donors after informed consent (32), and HAC were made as previously described (33). HAC culture media were supplemented with 5% PL, or 200 U/ml IL-1α, or 5% PL + 200 U/ml IL-1α or without any supplement. After 16 hours, media were removed, and cells extensivily washed in PBS before transferring to serum-free culture medium. After 24 hours, 3 ml of conditioned culture media was collected and concentrated to 1 ml using Centricon Centrifugal Filter devices (Millipore Corporation, Bredford, MA, USA). The two compartments of the Boyden chamber were separated by 12 μm pore-size polycarbonate filters coated with 5 μg /50 μl filter of type IV collagen solution (in H_2O, 0.1% CH_3COOH). Filters were air dried and stored at 4°C until used. The different human cells were extensively washed with PBS and resuspended in Coon's modified Ham's-F-12 serum-free media to the final dilution of 1.2 x 10^6 cell/ml before seeding in the upper compartment of chemotaxis chamber. The concentrated conditioned media used as chemoattractant were placed in the lower chamber. After 6 hours of incubation at 37°C, the filters were recovered, fixed in ETOH and stained by toluidine blue after removal of the non-migrating cells on the upper surface. Chemotaxis was quantified counting five to ten random fields of migrated cells, for each filter, under a microscope at a 160x magnification, or by densitometric scanning of the stained filter. Each experimental point was run in triplicate. Mean and standard deviations were calculated from three different experiments.

2.12. Statistical analysis

All data are presented as mean and standard deviations. Numbers of cell doublings and Western Blot are expressed as a mean ± standard deviation with $n = 6$ for each culture condition. Results of semi-quantitative

PCR, Real Time-PCR and NF-κB and Western Blot for p38 analysis are expressed as mean ± standard deviation with $n = 3$ for each group. Single factor analysis of variance (ANOVA) was used to determine statistical significance within a data set. If ANOVA detected a significant difference within the data set, Tukey's honestly significantly different (HSD) multiple comparison tests was used to determine significant differences between groups and conditions. All tests were conducted with a confidence interval of 95% ($p < 0.05$).

3. RESULTS

3.1. Articular chondrocytes expanded *in vitro* in the presence of either FCS or PL display a different phenotype

Primary human chondrocytes were cultured in the presence of either FCS or PL for 45 days. Cells cultured in the presence of FCS showed a continuous, but minimal growth throughout the all time period (Fig. 1α). At the end of the culture, the total number of doublings performed by the chondrocytes was around 4. On the contrary, cells maintained in the presence of PL presented an initial growth burst (15 doublings during the first 20-25 days of culture), followed by a growth phase at a reduced, but still constant, rate. After 45 days, the number of doublings performed by the chondrocytes cultured in the presence of PL was higher than 20. Cell morphology changed in the two culture conditions were monitored (Fig. 1B). In the presence of FCS, at 15 days cells maintained their characteristic polygonal shape and filled the whole plastic surface, whereas at 45 days they were flattened and more dispersed on the surface of the plastic plate. In the presence of PL, after 15 days culture, the cells already presented a fibroblastic shape and rapidly covered the entire plastic surface. Cells maintained the elongated fibroblast morphology during culture.

We evaluated the expression of some specific genes by the cultured HAC during their 2D expansion in both conditions (Fig. 1C). The initial chondrocyte population (passage 0), was positive for the expression of chondrogenic markers, such as type II collagen and SOX 9, as well as for the expression of type I collagen. In chondrocytes cultured in the presence of FCS, the expression of these genes was maintained at the time of the first cell passage (P1) corresponding to 2 cell doublings. In the same culture, after an additional doubling (3 total

doublings, corresponding to P4), we observed a loss of the type II collagen expression whereas the expression of the SOX 9 and of the type I collagen was maintained. In chondrocytes cultured in the presence of PL, at the time of the first cell passage (P1), already corresponding to 10 cell doublings, we observed expression of type I collagen and SOX 9, but only trace amount of type II collagen. After 20 doublings (corresponding to P4), cells were still positive for type I collagen, with very low trace amount of SOX 9.

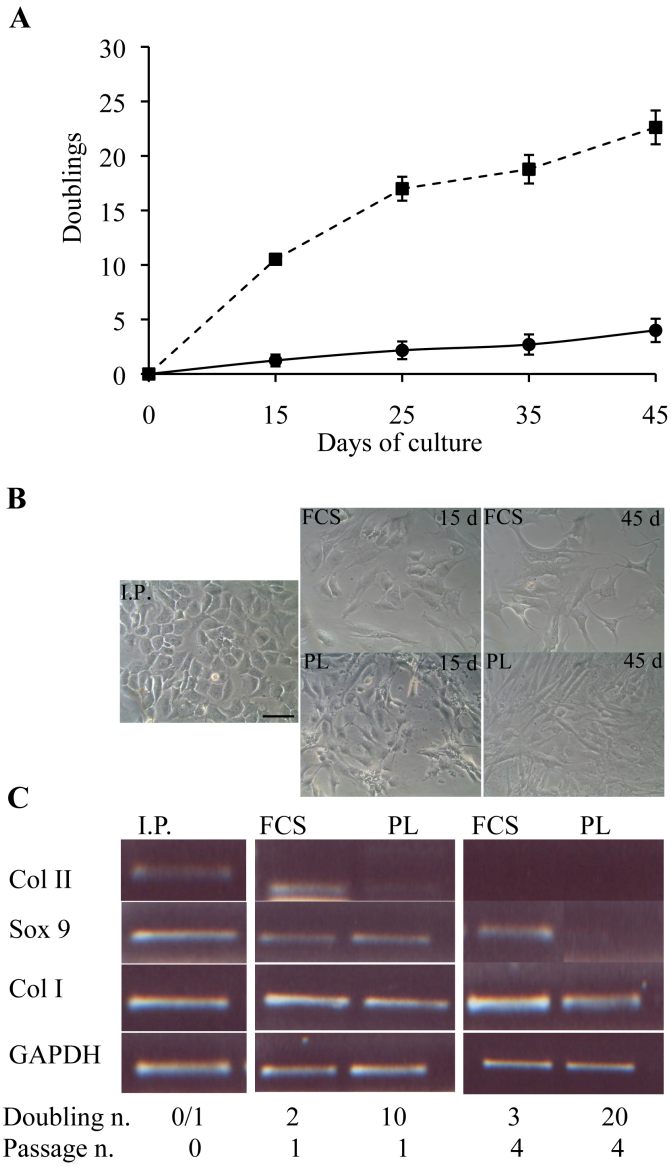

Figure 1 – Effects of PL on growth, morphology and gene expression of human articular chondrocytes. A) Growth kinetics plotted as number of cell duplications versus time of culture, Black line indicate chondrocytes expanded in presence of 10% FCS, Dashed line chondrocytes expanded in presence of 5% PL (n=6). B) Light microscopy images of chondrocyte morphology at different times of culture. I.P: chondrocytes isolated from the articular cartilage biopsy and plated (24 hours after initial plating). FCS; chondrocytes cultured in 10% FCS for 15 days (passage1) and 45 days (passage 4). PL: chondrocytes cultured in presence of 5% PL for 15 days (passage1) and 45 days (passage 4). C) Expression of different marker genes evaluated by RT-PCR analysis. Number of cell doublings and corresponding culture passages are indicated. Scale bar = 100 mm

3.2. Articular chondrocytes expanded *in vitro* in the presence of PL maintain their re-differentiation chondrogenic potential

To compare chondrogenic potential and cartilage matrix deposition capacity of the articular chondrocytes expanded *in vitro* in medium supplemented with either PL or FCS, we performed micromass pellet cultures with cells at passage 1 and 4 (Fig. 2). All cell populations formed the typical three-dimensional spherical aggregates. Nonetheless, aggregates formed by the cells cultured in presence of PL were smaller than those made by cells cultured in the presence of FCS (Fig. 2A). Micromass pellets formed by chondrocytes expanded in the presence of FCS presented some metachromatic toluidine blue staining after 2 and 4 cell doublings (1st and 4th passage), suggestive of the presence of cartilage proteoglycans. To note that, in the micromass pellet made by the chondrocytes that underwent 3 doublings (P4), the metachromatic toluidine blue positive staining was mostly located at the periphery of the pellet, whereas the inner part was mainly negative, probably due to a poor diffusion of medium nutrients through the cell mass. Type II collagen was detected by the specific antibodies in the micromass pellet made by the 2 doublings (P1) chondrocytes (Fig. 2A, left column), but not by the 3 doublings (P4) chondrocytes (Fig. 2A, third column). On the contrary, chondrocytes expanded in the presence of PL after 10 doublings (P1) maintained a chondrogenic potential and presenting the typical chondrocyte round shape and showing a positivity for metachromatic staining for cartilage matrix proteoglycans and for type II collagen (Fig. 2A, second column). Instead, in the micromass pellet formed by the 20 doubling (P4) chondrocytes cultured in the presence of PL, the relative ratio matrix/cells decreased, the metachromatic staining was almost negligible (Fig. 2A last column) and only trace amount of type II collagen were detected (Fig. 2A forth column).

When micromass pellets made by the 10 doubling chondrocytes (P1) in the presence of PL were subcutaneously implanted in immunodeficient mice, they formed a well differentiated cartilage tissue (Fig. 2B middle column). Explanted pellets exhibited an extracellular matrix with metachromatic stain and cartilaginous nodules with a strong and uniform positivity for collagen type II. The implanted micromass pellets made by the 20 doubling chondrocytes (P4) in presence of PL, showed a poor toluidine blue metachromatic staining and a very low level of uniformly distributed type II collagen (Fig. 2B, right column). Similarly implanted micromass pellets from

chondrocytes expanded with FCS (2 doublings) showed only a slight positivity for toluidine blue as well as only trace amount of type II collagen (Fig. 2B, left column). Pellets made with chondrocytes expanded for longer time in presence of FCS are not represented in the figure because the cell number required to perform the assay was not reached in these culture conditions.

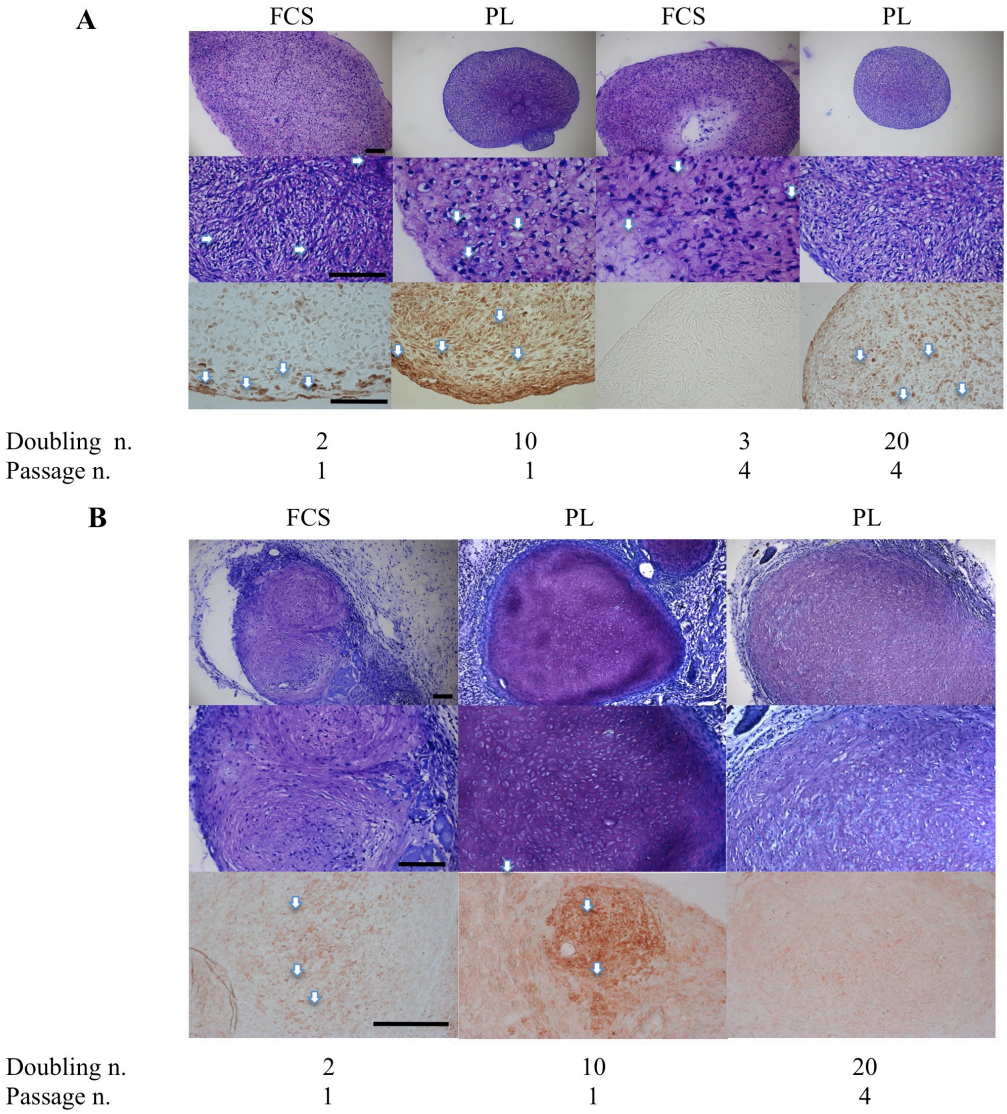

Figure 2 – Histological staining of 3D micromass pellet cross-sections. A: upper and middle row panels) Different magnifications of toluidine blue staining of micromass pellets formed *in vitro* by human articular chondrocytes that underwent a different number of cell duplications in different culture conditions. Columns 1 and 3, chondrocytes cultured in presence of 10% FCS. Columns 2 and 4 chondrocytes cultured in presence of 5% PL; A: lower row panels) Immunohistochemical analysis of micromass pellets for type II collagen expression; B: upper and middle row panels) Different magnification of toluidine blue staining of tissues formed *in vivo* following subcutaneous implantation of micromass pellets in nude mice. Implanted micromass pellets were formed by human articular chondrocytes in culture in presence of 10% FCS after 2 duplications (first column) and in presence of 5%PL after 10 and 20 duplications (central and right column respectively); B: lower row panels show the immunolocalization of collagen type II in the same samples. Scale bars = 100 µm.

3.3. PL promotes pro-inflammatory cytokine expression and secretion in cultured articular chondrocytes

We evaluated the effect of PL on mRNA expression and protein secretion of the pro-inflammatory cytokines interleukin 6 (IL-6) and interleukin 8 (IL-8) as well as of the Neutrophil-gelatinase associated lipocalin (NGAL), in HAC cultured under normal and inflammatory conditions (Fig. 3). Confluent chondrocytes (at P1) were treated for 16 hours with serum-free medium supplemented with 5% PL or 200 U/ml IL-1α or 5% PL + 200U/ml IL-1α or without any supplement. At the end of the incubation time, culture media were removed and replaced with serum free media (no supplements). After additional 24 hours of incubation, media were collected and secreted proteins were detected by western blot and quantified. In a parallel experiment, gene expression levels were measured and quantified, in lysates of the cells after 16 hours of the different treatments (Fig. 3). The secretion of both IL-6 and IL-8 increased when chondrocytes culture medium was supplemented with IL-1α, as compared to controls (p<0.05) (Fig. 3A). The addition of PL alone to the medium did not show a relevant effect on the secretion of these cytokines. Interestingly, when the medium was supplemented with PL plus IL-1α, the IL-6 and IL-8 levels were significantly higher then with IL-1α alone. Unlike IL-6 and IL-8, the NGAL secretion was significantly (p<0.05) induced by both PL and IL-1α (Fig. 3A lower row). When the medium was supplemented with both PL and IL-1α, an additive effect on NGAL secretion was observed. (Fig. 3A last row).

The analysis of mRNA expression levels confirmed the results (Fig. 3B). After IL-1α and IL-1α+PL treatment, IL-6, IL-8 mRNA levels showed a significant increase (p<0.05) as compared to the untreated control. The analysis of the NGAL mRNA transcript after the 16 hours of treatment revealed an apparent discrepancy between the mRNA transcript levels and the amount of protein secreted. Nonetheless when we analyzed the NGAL mRNA transcript levels after 40 hours from the beginning of the treatment, (the same time we collected the secreted protein), we observed a reduction of the transcripts in the IL-1α treated cultures and an increased transcript level in the only PL treated cultures (data not shown). This two-phase kinetic would suggest the involvement of a secondary PL induced mediator specifically causing NGAL gene expression, neo-synthesis and secretion.

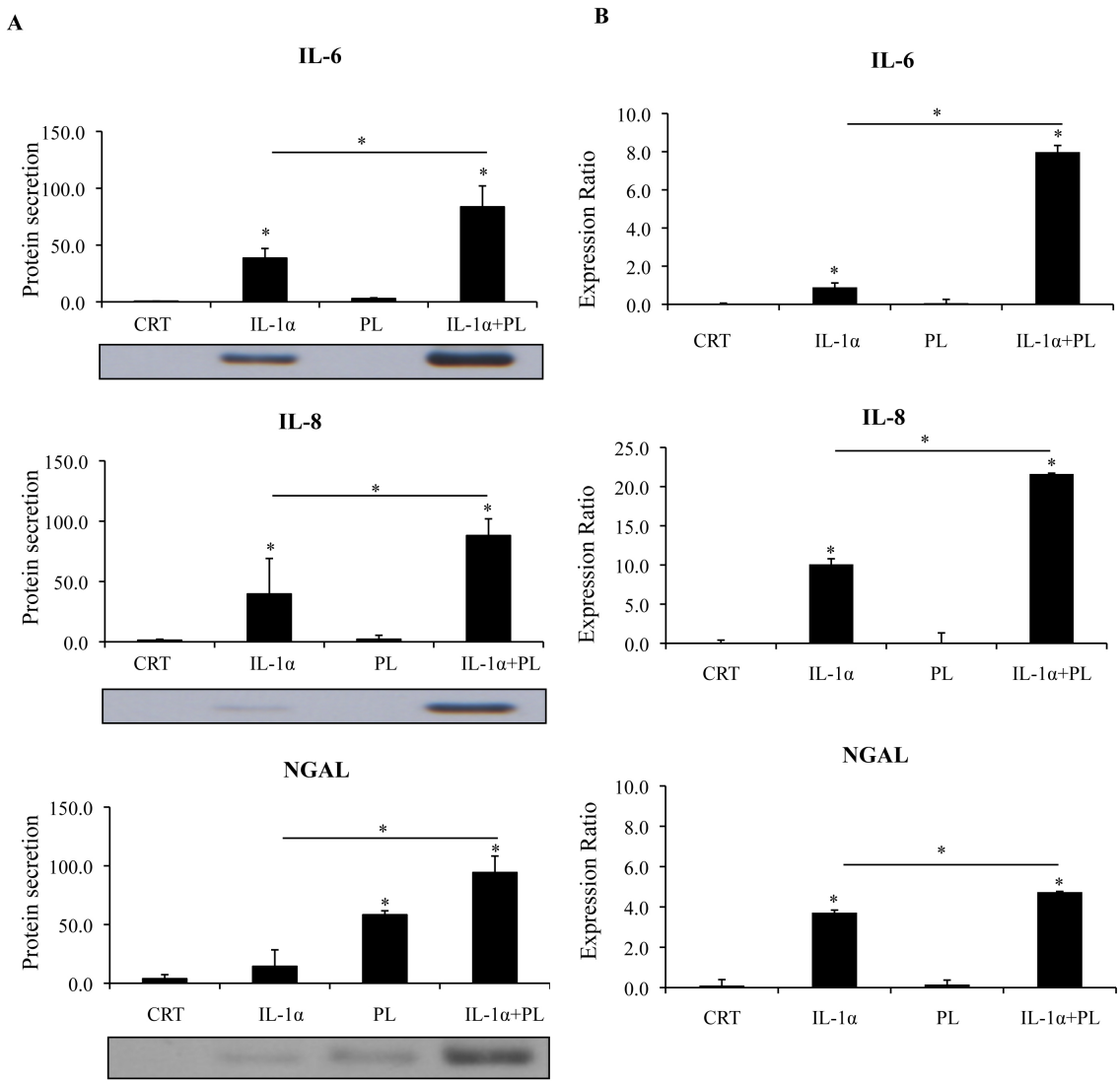

Figure 3 - Response of human articular chondrocytes to the PL treatment in a normal or an inflammatory microenvironment. A) Identification of protein in the chondrocyte conditioned culture media, was performed after the cell different treatments. Western Blot analysis of IL-6, IL-8 and NGAL secretion was performed and the corresponding band intensity was determined by densitometric analysis (n=6). B) Level of IL-6, IL-8 and NGAL mRNAs in the treated chondrocytes quantified by Real Time RT-PCR, Results are expressed as means standard deviations with n=3 for each bar. * Represents significant difference (p<0.05) comparing each condition with the control.

3.4. The pro-inflammatory effect of PL is associated with the activation of p38

Recently, we described that a common pathway was activated in cartilage differentiation and in cartilage inflammation. In particular we reported that the p38/NF-κB inflammatory pathways were activated in both circumstances leading to the expression of cyclooxygenase-2 (COX-2), an inflammation-related enzyme (22, 26,

34). Knowing that the p38 pathway was activated in the inflammatory condition, we treated HAC, cultured in the presence of PL either alone or in association with IL-1α, with the p38 specific inhibitor SB203580 (SB) (Fig. 4). This approach caused a reduced production of IL-6 and IL-8 by IL-1α and of IL-1α+PL treatment. NGAL secretion was also partially reduced.

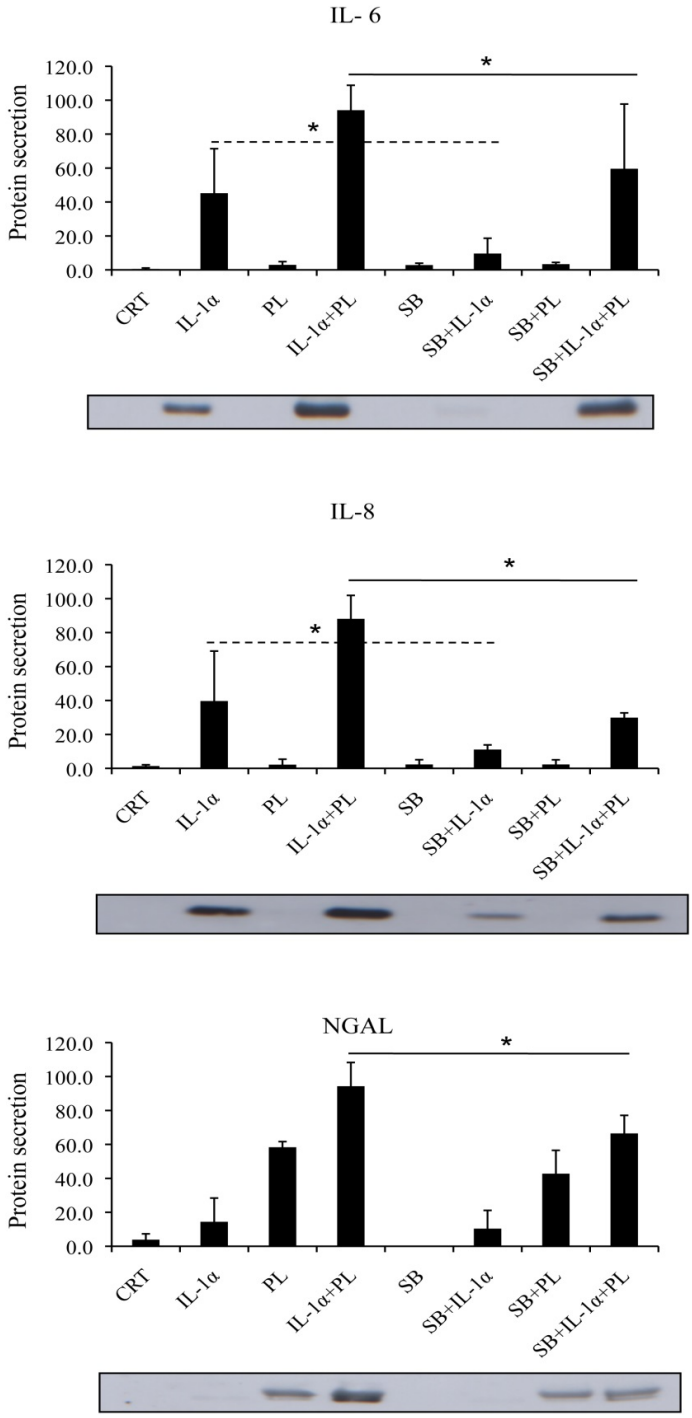

Figure 4 - Western Blot analysis of IL-6, IL-8 and NGAL secretion in the absence or in the presence of the p38 inhibitor SB203580. Quantification was made using band intensity measurements for all the conditions. Results are expressed as mean standard deviations with n=3 for each bar.

To confirm the p38 pathway activation, we detected the phosphorylated MK2 expression in our cellular system. MK2 kinase activity increase following phosphorilation of residues Thr222, Ser272, Thr334 by p38 (35). We assayed the Thr334 phosphorylation status of MK2 in treated HAC by western blot and by immunofluorescence. We observed that MK2 Thr334 phosphorilation was maximal following 30 minutes of IL-1α and IL-1α+PL stimulation, and was drastically reduced in presence of SB203580 inhibitor (Fig. 5A). Immunofluorescence analysis was performed in a parallel cell culture experiments. Representative images confirmed the presence of active MK2 only under inflammatory stimuli (Fig. 5B)

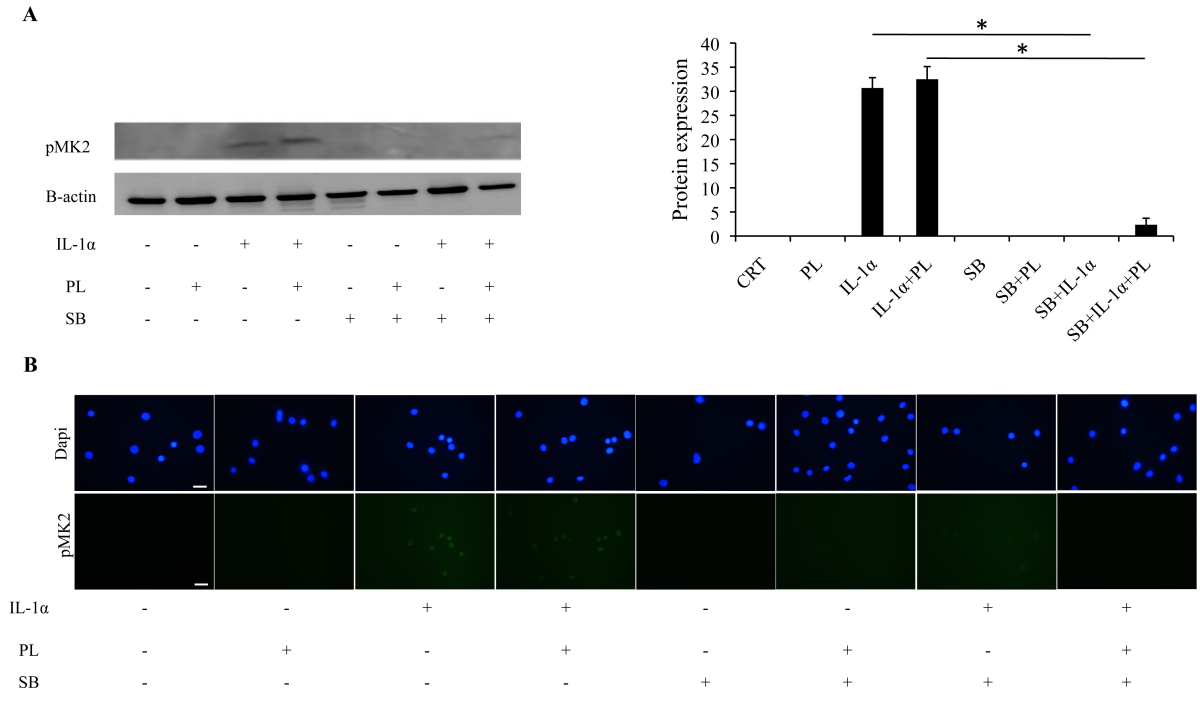

Figure 5 - Analysis of phosphorylated MK2 expression in human articular chondrocytes. A) Western Blot analysis of pMK2 after the addition of PL in normal and inflammatory conditions in absence and presence of p38 inhibitor SB 203580 (n=2). B) Representative images of pMK2 immunofluorescence detection under the above mentioned analyzed conditions. Scale bar = 25 μm. * Represents significant difference ($p<0.05$).

3.5. PL modulates the activation of NF-κB and COX-2 gene expression thus promoting the resolution of the inflammatory response

We examined the activation of NF-κB in HAC treated with PL under physiologic and inflammatory conditions. After 4 hours stimulation with IL-1α and IL-1α+PL we detected the up-regulation of NF-κB as compared to medium supplemented with PL alone (p<0.05) or to untreated control (p<0.05) (Fig. 6A). PL plus IL-1α caused a slight increased of on NF-κB activation as compared to HAC treated with IL-1α alone. After 40 hours the activity of NF-κB in the presence of PL+ IL-1α was subverted with a significant reduction as compared to IL-1α alone (p<0.05) (Fig. 6B).

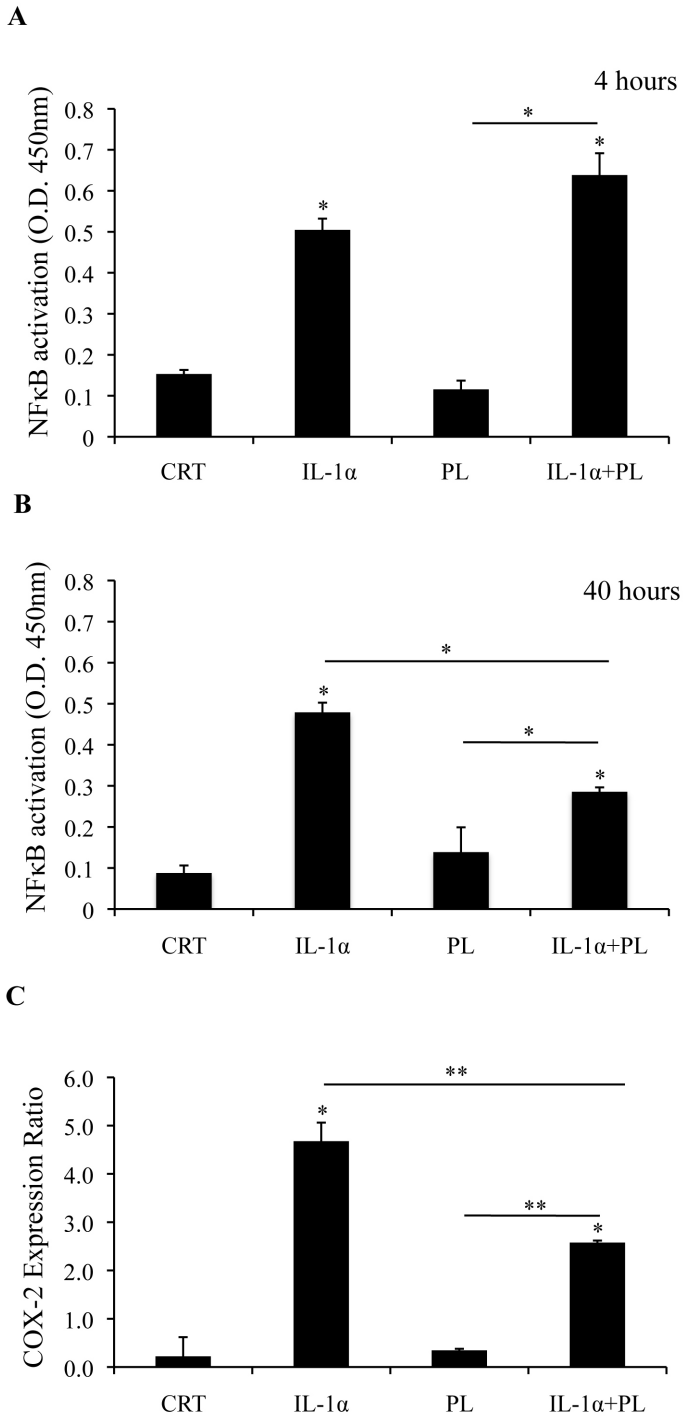

Figure 6 - NF-κB pathway activity in human articular chondrocytes under normal and inflammatory conditions after 4 (A) and 40 (B) hours treatment. Cells lysates were tested for binding of the activated p65 NF-kB subunit to an NF-kB consensus sequence using the Trans-Am NF-kB ELISA kit. Results were expressed as specific binding, meaning, observed absorbance values in the presence of the mutated oligonucleotide minus those observed in the presence of the wild-type oligonucleotide. Results are expressed as mean standard deviations with n=3 for each bar. *Represents significant difference (p<0.05) comparing each condition with the control; C) Expression of cyclooxygenase-2 (COX-2) by real-time (RT-PCR), in chondrocytes maintained under normal and inflammatory conditions. *Represents significant difference (p<0.05) comparing each condition with the control (n=3).

3.6. Medium conditioned by PL treated chondrocytes has a strong chemotactic activity versus chondrogenic lineage cells

We evaluated a possible chemotactic activity of the medium conditioned by the PL treated chondrocytes both in the presence and in the absence of an inflammatory stimulus (Fig. 7). The chemotactic effect of the conditioned media was tested versus human endothelial cells (HUVEC), primary articular chondrocytes (HAC), and bone marrow stromal cells (BMSC). We first compared the medium conditioned by untreated chondrocytes (control) and the medium conditioned by chondrocytes treated with PL (Fig. 7A). The chemotactic effect of chondrocytes conditioned medium on HUVEC was not influenced by PL treatment, whereas migration was doubled in HAC ($p<0.05$). On the contrary, the same medium showed an inhibitory effect on the migration of the BMSC. This panel of different activities suggest a selective recruitment of cartilage cells.

We decided to selectively test the chemotactic activity of HAC conditioned medium (24h treatment) on untreated HAC in the presence/absence of IL-1α (Fig. 7B). Compared to media from untreated or from IL-1α treated chondrocytes, the treatment with PL, in both culture conditions, showed an enhanced chemotactic potential on HAC ($p<0.05$). The higher activity was observed in the medium conditioned by the PL+ IL-1α despite the inactivity of IL-1α alone.

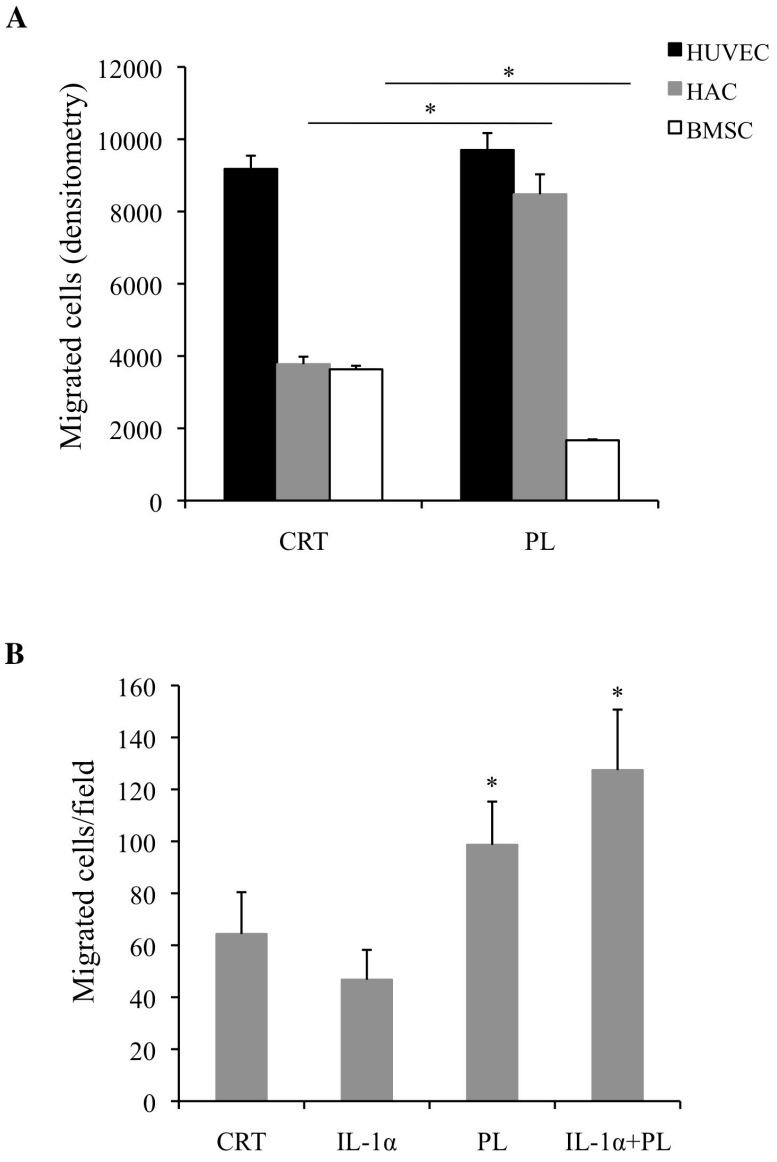

Figure 7 - Chemotactic activity of human chondrocyte conditioned media. A) Response of HUVEC (black bar), HAC (gray bar) and BMSC (white bar) cells to conditioned media from human articular chondrocytes cultured with 10 % FCS (crt) or 5% PL; B) Media from human articular chondrocytes treated for 24 hours with PL under physiological and inflammatory conditions presenting a chemotactic effect on HAC. Results are expressed as mean standard deviations with n=3 for each bar. Both panels * show significant difference (p<0.05) comparing each condition with the control.

4. Discussion

Platelet Rich Plasma (PRP) and Platelet Lysate (PL) are natural growth factor reservoirs responsible for providing signals that activate a cascade of event during the processes leading to the repair of damaged tissues. The use of platelet derived products, such as PRP, in medicine has significantly increased in the last years. Following its

initial use in dentistry and tendon disorders (36, 37), nowadays PRP is being adopted also for the treatment of articular cartilage lesions and the associated inflammatory status (11, 38, 39). The goal of our study was to investigate how platelet derived products could have a beneficial effect on cartilage regeneration by studying its effect on primary human articular chondrocytes (HAC) cultured in both physiological and inflammatory environments.

Here we report that HAC expanded, in the presence of FCS lost the polygonal morphology and showed a reduced duplication rate. The initially expression of type II collagen was halted during the first four culture passages whereas the expression of the cartilage transcription factor SOX9 was maintained (40). Nevertheless the capacity of the cells to form cartilage in the micromass pellet culture *in vitro* and in the ectopic implants *in vivo* was observed only in first passage cells (2 doublings). On the contrary, in the presence of PL, articular chondrocytes showed an elongated fibroblastic shape and maintained a high proliferation rate throughout the all culture. This was accompanied by a rapid loss of type II collagen expression, whereas SOX9 expression was still present in the cells even after 10 doublings. The extended expression of the SOX-9 suggested that, regardless of the high proliferation capacity, not usual in somatic cells, chondrocytes could have kept their natural somatic memory (1, 40). In fact, micromass pellets formed *in vitro* by the cells after 10 doublings exibited a cartilage phenotype, and after 20 duplications, SOX9 expression as chondrogenic "memory" marker was essentially lost. These findings were confirmed by the histological analysis of micromass cell pellets implanted *in vivo*. The implanted pellets obtained from 10 doublings HAC in the presence of PL, showed cartilage nodules positive for type II collagen. Micromass pellets formed by cells after 20 doublings maintained the expression of type I collagen and presented only trace amounts of metachromatic toluidine blue staining and type II collagen expression.

It should be noticed the stunning increase of the number of cell doublings at the same passage of culture, triggered by PL. Furthermore, chondrocytes expanded in the presence of FCS lost the ability to re-differente earlyer then chondrocytes expanded in PL. Adult HAC have a finite capacity to form stable cartilage *in vivo* and this capacity was progressively lost during *in vitro* expansion (40-42). Our data suggested that PL supports chondrocytes expansion and maintain cartilage phenotype.

Several studies have underlined the importance of specific growth factor (EGF, PDGF, FGF-2, TGFB1) during the expansion and redifferentiation of human condrocytes (5, 43, 44). In the present study we analyzed the effect of crude PL as a source of multiple growth factors able to influence cell proliferation and cell redifferentiation capacity. Our *in vitro* analysis wanted to mimic the clinical use of PRP in cartilage lesion, where fresh PRP, containing several growth factors, improved cartilage repair (38, 45).

In joint, under physiological conditions, chondrocytes, the extracellular matrix and cell secretoma (i.e. the whole joint micro-environment) are responsible for the maintenance of tissue homeostasis. However, when lesions occur, profound changes perturb the microenvironment. The pro-inflammatory cytokine interleukin-1α produced in the inflamed joints by activated synovial cells and infiltrating macrophages, is one of the most potent catabolic factors in knee diseases (46, 47). This molecule is responsible for the induction of several mediators of cartilage degeneration such as IL-6 and IL-8. In this condition the cells near to the site of the injury are not able to organize an acceptable regenerative response and, as a consequence, the cartilage normal structure and composition are not restored. We compared the effects of PL on HAC maintained under physiological and inflammatory conditions (i.e. in the presence of IL-1α). The addition of PL itself did not modify the secretion profile of the pro-inflammatory cytokines IL-6 and IL-8, but caused an increased secretion of these factors enforcing IL-1α activity.

Neutrophil-gelatinase associated lipocalin (NGAL) is an acute phase protein with an antimicrobial activity, expressed at low levels in several human tissues. The synthesis of NGAL was shown to be enhanced in several types of cells as a consequence of inflammation and malignancy. In cartilage, NGAL expression is activated in osteoarthritis and during neoplastic transformation of chondrogenic lineage cell, in agreement with the finding that IL-1 induced NGAL mRNA transcription via a NF-κB dependent pathways (48, 49). As expected, the exposure of HAC to the inflammatory cytokine IL-1α induced NGAL expression. Moreover, the addition of PL alone or in combination with the inflammatory stimulus to HAC incremented NGAL levels. The different regulation of the this protein compared to the expression of the pro-inflammatory cytokines IL-6 and IL-8, confirm, once more, that PL is important for the maintenance of the chondrogenic potential. In fact, as we previously described

NRL and Sip24, the NGAL homologues in rat and mouse respectively, are associated not only with an inflammation status but also with a physiological proliferation and differentiation of chondrocytes. (25).

Since the inflammation phase response and the associated release of pro-inflammatory cytokines is controlled by different pathways, we examined the p38 MAP kinase and nuclear NF-κB pathways in HAC treated with PL under physiologic and inflammatory conditions.

Our previous observations made in mouse chondrocytes (19, 22), revealed that a common pathway present during differentiation and inflammation drives p38 activation in chondrocytes. The involvement of p38 in the induction of IL-6, IL-8 and NGAL was here demonstrated also in cultured human chondrocytes by repeating the different cellular treatments in presence of the p38 specific inhibitor SB203580. This drug drastically reduced the synthesis of IL-6, IL-8 and NGAL protein and gene expression.

The mechanism by which these downstream effects are mediated is signal dependent and could involve the transduction of signals via MAPK-activated protein kinases (MAPKAPKs) (50). MAPKAPK2 (MK2), is one of several kinases directly activated by p38 MAPK. Recently active MK2 was described in OA cartilage tissue and in OA chondrocytes (35). In our cell system in presence of IL-1α and IL-1α+PL induced a drastic increase of phospho-MK2. Total reduction of MK2 was observed when HAC were cultured in presence of p38 inhibitor. These data confirm that the inflammatory response in HAC was mediated by the p38 MAP kinase pathway.

The inhibition of NF-κB by the PRP has been recently reported in a human immortalized chondrocyte cell line (51). Moreover, in primary human osteoarthritic chondrocytes it was observed that PRP inhibited the translocation of NF-κB to the nucleus (52). Here, we extend these observations and report that, in primary HAC, PL, in combination with IL-1α, drives the initial transient activation of NF-κB (4 hours treatment) with consequent up-regulation of IL-6 and IL-8, whereas at longer time of treatment (40 hours) causes a reduction of the NF-κB activity. Based on these data, we suggest that both the activation of p38, and the activation and the subsequent inhibition, of NF-κB, are involved in the inflammation response and resolution controlled by PL.

In line with the observed reduction of NF-κB activity after an extended PL+IL-1α treatment, we found that also cyclooxygenase-2, an enzyme activated during the acute phase response, was significantly reduced. Several

studies demonstrated that in the skeletal systems, COX-2 is expressed during fracture callus formation, being its function essential for bone healing (53). In cartilage the activation of COX-2 was described not only in differentiated growth plate but also during inflammation (54-56). Ulivi et al. showed that in a chondrocyte cell line, COX-2 was expressed via p38/NF-κB activation and nuclear translocation during both differentiation and inflammatory response (22). In this context, our observation that PL in the presence of IL-1α was able to reduce not only NF-κB but also COX-2 expression indicates to an early attempt to obtain the resolution of the inflammation process. We therefore propose that in cartilage the platelet products (PRP and PL) initially enhance the inflammatory response, thus promoting a transient inflammation of the human articular chondrocytes, but trigger a cascade of events leading to inflammation resolution.

In clinic, PRP is used for cartilage repair by one step approach to recruit progenitors cells to the cartilage defect and guide them towards forming of hyaline cartilage taking advantage of microfracture or matrix-induced chondrogenesis procedure. For this reason, we evaluated the effect of PL on the migration of chondrocytes and progenitors cells (BMSC). We demonstrated that chondrocyte-conditioned media, had a moderate chemotactic effect on human BMSCs, whereas the supernatants of PL pre-stimulated chondrocytes caused a selective and strong chemotactic activity versus human chondrocytes, suggesting that PL can promote a direct selective migration of chondrocyte in cartilage lesion. This fact could, in presence of suitable environment, lead to cartilage repair process. This chemotactic activity is strongly enhanced by the concomitant presence of the inflammatory stimulus.

5. Conclusions

The use of PRP has been proposed for the treatment of articular cartilage lesions and the associated inflammatory status. Nonetheless, how the platelet-derived products could exert a beneficial effect in the cartilage repair process remains unclear. Our study showed that: *i*) PL, a PRP derivative, can significantly stimulate and sustain the proliferation of primary human articular chondrocytes maintaining their chondrogenic re-differentiation potential for at least 10 doublings; *ii)* in a mimicked inflamed environment, PL is first playing a role

as a pro-inflammatory agent, acting synergistically with the well known pro-inflammatory cytokine IL-1α thus enhancing the initial inflammatory response. Subsequently, PL contributes to the down modulation of the NF-κB signal pathway and the COX-2 expression, thus triggering the resolution of the inflammation; *iii*) Finally, PL is a source of growth factors able to induce a selective chondrocytes recruitment.

These findings could explain the beneficial effects of the PRP treatment in cartilage repair and in the OA treatment.

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CHAPTER V

HYALURONIC ACID MICROPARTICLES AS INTRAARTICULAR INJECTABLE DRUG CARRIER FOR CARTILAGE REPAIR: EFFECT ON HUMAN ARTICULAR CHONDROCYTE BEHAVIOR

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HYALURONIC ACID MICROPARTICLES AS INTRAARTICULAR INJECTABLE DRUG CARRIER FOR CARTILAGE REPAIR: EFFECT ON HUMAN ARTICULAR CHONDROCYTE BEHAVIOR*

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Abstract

Human articular cartilage has no or very low ability of self-repair and untreated cartilage lesions may lead to the development of osteoarthritis (OA). Hyaluronic acid (HA), one of the major components of the extracellular matrix (ECM) of this connective tissue, has been used in cartilage regenerative field in different preparations. In the present paper we describe the production and characterization of HA microparticles prepared by water-in-oil emulsion. The developed microparticles showed a regular and circular shape. To evaluate their potential medical applications in knee joints we studied their effect on the cell viability (MTT assay), proliferation (Dna content) and phenotype by means of Flow Cytometry and Real Time PCR analysis of cartilage resident cells, (chondrocytes). The presence of the microparticles in direct and non-direct contact with primary human articular chondrocytes (hAC) did not altered significatly their viability and proliferation kinetics up to 28 days of *in vitro* culture. During this time, hAC cultured in direct contact with HA microparticles showed a significant increase in the expression values of cartilage-related genes such as aggrecan, SOX9 and COMP. No significant changes in the expression of hyaluronic acid receptor - CD44 - were observed. Together, our study seems to indicate that HA microparticles could have good potential as intraarticular injectable drug-carrier for cartilage tissue engineering and regenerative medicine applications.

Keywords HA microparticles; Human articular chondrocytes; cell phenotype

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R. C. Pereira, R.L. Reis and H.S. Azevedo, "*Hyaluronic acid microparticles as intraarticular injectable drug carrier for cartilage repair: effect on human articular chondrocyte behavior*", submitted

1. INTRODUCTION

Articular cartilage is a connective tissue with origin in mesenchymal cells that exists in the entire articular surface of bones (1). Chondrocytes are the unique cell type that composes adult articular cartilage, and this fact, confers apparent tissue simplicity. Adult articular chondrocytes are the only responsible for secretion and deposition, around themselves, of a hydrated and organized pericellular and extracellular matrix (ECM) composed of hyaluronic acid (HA), proteoglycans and collagen I, II and VI responsible for the mechanical properties of this connective tissue (2-7). Chondrocytes in adult articular cartilage do not divide but assume an important role in the maintenance of tissue integrity through the balance of anabolic and catabolic activities. Due to limited capacity for self-repair, regeneration after injury is nonexistent. Disability caused by joint pain is one of the major serious problems affecting people worldwide. Pain generally results from degeneration of the cartilage from the joint due to osteoarthritis (OA) (1, 8-10). This is initially characterized by the degradation and loss of cartilage tissue, hypertrophic bone changes, subchondral bone remodeling, and, at the final clinical stage of the disease, chronic inflammation (11, 12). Discovered over 75 years ago, HA is a water-soluble polyanion with a linear structure composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. It plays a complex biological role due to its interactions with several HA-binding proteins, molecules of cartilage ECM, as well as, with cells regulating pathways of development via modulation of cellular activities. The special physiochemical and biological properties of HA and its non-immunogenic nature have made this biopolymer a useful substance in biomaterials research (13-16). To date, several authors have suggested with success several biomaterials shapes composed only by this biopolymer or blended with other polymers aiming cartilage repair/regeneration (17-20). Other study has shown that different molecular weight of HA modulate the behavior of articular chondrocytes (21). Due to its intrinsic protective physiochemical functions, HA has also been proposed as intraarticular supplementation in OA treatment (22-25). The signaling pathways associated with known biological effects of HA on articular chondrocytes changes have not been clearly defined. Yet, it has been shown that HA oligosaccharides are incorporated in articular chondrocytes through interactions with HA main receptor, the CD 44 that involves anabolic and catabolic pathways (26, 27).

Other studies using several processing techniques of polymers have shown the possibility to synthesize HA microparticles for cartilage drug delivery applications and repair strategies (28-30). In this context, our work have the main aims: i) to develop and characterize HA microparticles by water-in-oil emulsion for possible injectable carriers in delivery of therapeutic or signaling molecules (e.g. soluble growth factors) in cartilage repair strategies; ii) to analyze the effect of HA microparticles in direct and non-direct contact on hAC viability and proliferation over 28 days of culture, iii) to study the influence of the microparticles on hAC phenotype by the gene expression analysis of cartilaginous gene markers (e.g. collagen type I and II, aggrecan, SOX9, and COMP) and by the quantification of CD44 citofluorimetric expression.

2. MATERIALS AND METHODS

2.1. Microparticles production

HA microparticles were produced using a water-in-oil-emulsion technique. Briefly, an emulsion composed of 95 ml of mineral oil and 5 ml of Span 85 (both from Sigma-Aldrich, St. Louis, MO) was prepared in a thermal bath at 35 °C adapted from Yan *et al.* (30). Then, 1% (w/v) of HA (235 KDa, LifeCore, USA) was dissolved at 4 °C overnight under smooth rotation. The homogeneous polymer solution was then dropped with a syringe with a 23 Gauge needle into the water-in-oil emulsion. The obtained HA microparticles were kept under mechanical agitation at 600 rpm for 10 hours. After that, the microparticles were centrifuged at 1800 rpm for 10 minutes. Mineral phase was removed and the microparticles deposited in the bottom of the tubes were washed with n-hexane and centrifuged at 1500 rpm for 8 minutes. This procedure was repeated tree times to assure total absence of the mineral phase. After the final wash, HA microparticles were resuspended in a solution of 50 mg of adipic dihydrazide acid (ADH, Sigma-Aldrich, St. Louis, MO) and 25 mg of ethyl-3-[3-dimethylamino] propyl carbodiimide (EDCI-HCl) in 90% (v/v) of isopropanol (Sigma-Aldrich, St. Louis. MO). This solution was kept under slow magnetic agitation for 4 hours. After this time, HA microparticles were centrifuged and washed with isopropanol 90% (v/v). At the end, HA microparticles were sterilized by ethanol 70%, and stored under sterile conditions for further analysis and cellular culture.

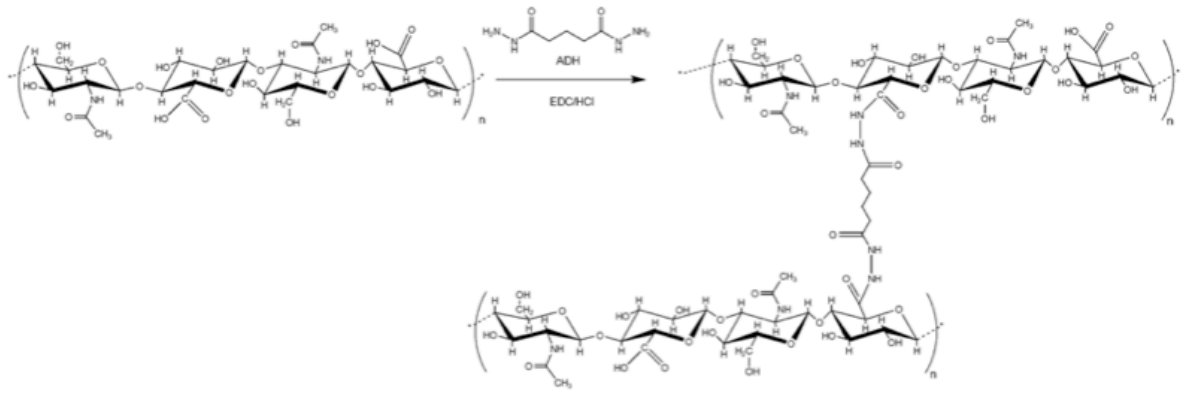

Figure 1 - Schematic illustration of hyaluronic acid chemical composition and chemical crosslink reaction with adipic dihydrazide acid/ ethyl-3-[3-dimethylamino] propyl carbodiimide. (adapted from (30)).

2.2. Microparticles morphological and size analysis

The microparticles morphology was evaluated by scanning electron microscopy (SEM). Briefly, dried samples were mounted on aluminum stubs and sputter coated with Pt/Pd target (80:20) generating a thin film with 6 nm of thickness (208 HR High Resolution Sputter Coater, Cressington). The HA microparticles were imaged on an ultra-high resolution field-emission gun scanning electron microscope (FEGSEM, FEI Nova 200 NanoSEM).

2.3. Human articular chondrocyte (hAC) isolation and culture

Bioptic material to perform articular chondrocyte isolation was collected from femoral condyles and tibiae plates of patients undergoing partial knee arthroplasty. All patients had signed an informed consent approved by the Ethical Committee of São Marcos Hospital, Braga, Portugal. Briefly, articular cartilage was cleaned of connective tissue as well as of subchondral bone and minced in small fragments and rinsed in fresh phosphate buffered saline solution (PBS, pH 7.4) according to previously published procedures (31). Chondrocytes were individually released by consecutive enzymatic digestions as follows: 400 U/mL collagenase I, 1000 U/mL collagenase II (Worthing Biochemical, Lakewood, NJ), 0.25% trypsin (Invitrogen, UK), 1 mg/mL hyaluronidase (Sigma, USA) in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) at 37 °C.

In this work three different conditions were studied: direct contact; non-direct contact and control (absence of HA microparticles). It was studied the effect of the HA microparticles on the behavior of hAC as a function of time

(direct contact conditions). For culturing hAC in non-direct contact with HA microparticles, it was used a transwell system (Corning incorporated, Transwell®) where cells, were plated in the bottom of the well with the transwell chambers with HA micropartilces above. Cells shared the same medium but no direct cell-material contact due to the physical separation of the cells by a polycarbonate membrane with pore size of 0.4 μm. Primary human articular chondrocytes were plated at density of 8000 cells/cm^2 and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) with phenol red at 37 °C supplemented with 10% fetal bovine serum (Biochrom AG, Germany), 5 mM L-Glutamine (Sigma, St. Louis, MO) and 1% of antibiotic-antimicotic mixture (Invitrogen, St. Louis, MO).

2.4. Human articular chondrocytes viability (MTT assay)

The viability of hAC in direct and non-direct contact with HA microparticles, as well as those in absence of microparticles (control), was evaluated at different time intervals using thiazolyl blue staining (MTT, Sigma-Aldrich, St. Louis, MO). Briefly, at each time point the medium was removed in triplicate and replaced with 0.5 ml fresh serum-free medium supplemented with 25 μl MTT stock solution (5 mg/ml). In the case of hAC cultured in direct contact with HA micropartilces, to avoid any kind of artifact, particles were removed from the medium before the MTT test. After 3 hours of incubation, the medium was collected and the converted dye was solubilized with 1 ml of absolute ethanol. Dye absorbance was measured at 570 nm with background subtraction at 670 nm (32).

2.5. Human articular chondrocytes proliferation (DNA quantification)

Human articular chondrocytes proliferation in non-direct and direct contact with HA microparticles was determined using a fluorimetric double-strand DNA (dsDNA) quantification kit (Quant-iT PicoGreen dsDNA reagent, Molecular Probes, Invitrogen). For this, all samples were scraped in Dnase ultrapure water, collected at 1, 3, 7, 14, 21 and 28 days of culturing, transferred into 1.5 mL microtubes and stored in a −80 °C. Prior to dsDNA quantification, cells were sonicated for 15 minutes. Samples and standards (ranging from 0 to 2 μg/mL) were prepared and mixed with a PicoGreen solution in a 200:1 ratio and were placed on opaque 96-well plate.

DNA quantification of each sample/standard was made in triplicate. The plate was incubated for 10 minutes in the dark, and fluorescence was measured on a microplate reader (Synergy HT, BioTek Instruments) using an excitation wavelength of 485/20 nm and an emission of 528/20 nm. Standard curve was created, and DNA values were obtained.

2.6. RNA extraction and quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Human articular chondrocytes cultured in direct contact and in absence of HA microparticles (control) were collected at each time point, washed in PBS, immersed in Trizol® reagent (Invitrogen, St. Louis, MO) and kept at -80 °C for subsequent RNA extraction. Afterwards, total RNA was extracted as previously described (31). Briefly, samples were incubated at 4 °C for 10 minutes with chloroform (Sigma-Aldrich, St. Louis, MO) and after that, centrifuged at 13000 rpm for 15 minutes. 700 µL of the supernatant were collected and an equivalent volume of isopropanol (Sigma-Aldrich, St. Louis, MO) was added. After RNA precipitation for 1 hour, samples were centrifuged at 13000 rpm at 4 °C for 15 minutes. Supernatant was removed and 700 µl of 70% ethanol was added. Eppendorfs tubes were again centrifuged at 13000 rpm and at 4 °C for 5 minutes, and the supernatant was removed. Pellets were dried at room temperature and, after that, were ressuspended in 40 µl of DNase/RNase-free distilled water (Gibco/10977-015). RNA content and integrity was assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA). Isolated RNA was transcribed into cDNA using the iScript cDNA synthesis kit. Relative gene expression quantification was performed by real-time quantitative RT-PCR using a BioRad CFX96 real-time PCR detection system (BioRad Laboratories, USA) and SYBR Green IQ Supermix (Bio-Rad Laboratories, CA, USA). Primer Express software was used to generate forward and reverse oligonucleotides listed in Table 1. cDNA (2 µl for total volume of 25 ml per reaction) was analyzed for the gene of interest and for the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression level of each target genes (e.g collagen type I, collagen type II, aggrecan, Sex determining region Y-box9 (SOX9) and cartilage oligomeric matrix protein (COMP)) was calculated using the -2^{DDCt} method as described Livak *et al.* (33). Each sample was repeated three times for the gene of interest.

Table 1 – Primers used for evaluating the gene expression of human articular chondrocytes by quantitative real-time polymerase chain reaction.

Gene of interest	Forward	Reserve
GAPDH	5'-ACAGTCAGCCGCATCTTCTT-3'	5'-ACGACCCAAATCCGTTGACTC-3'
Collagen type I	5'-CATCTCCCCTTCGTTTTTGA-3	5'-CCAAATCCGATGTTTCTGCT-3'
Collagen Type II	5'-GACAATCTGGCTCCCAAC-3'	5'-ACAGTCTTGCCCCACTTAC-3'
SOX9	5'-TACGACTACACCGACCACCA-3'	5'-TTAGGATCATCTCGGCCATC-3'
COMP	5'-CCCACAGACCCTTCCAAGTA-3'	5'-GGGGACAACTGGAGTGAAAA-3'
Aggrecan	5'-TGAGTCCtTCAAGCCTCCTGT-3'	5'-TGGTCTGCAGCAGTTGATTC-3'

2.7. Flow cytometry analysis (FACs)

The phenotype of hAC cultured in direct contact of HA microparticles and in absence of them (control) was assessed by flow cytometry. Briefly, harvested cells were collected, washed with PBS and incubated with fluorescent monoclonal antibodies against CD44, (BD Biosciences Pharmingen, USA) for 30 minutes at 4 °C. Unlabeled controls were included in all experiment to evaluate the unspecific binding. Samples were analyzed using a FACScalibur (Becton-Dickinson, USA) with CellQuest analysis software (BectonDickinson, USA).

2.8. Statistical analysis

Results of Real Time analysis are expressed as mean ± standard deviation with $n = 3$ for each group. Single factor analysis of variance (ANOVA) was used to determine statistical significance within a data set. If ANOVA detected a significant difference within the data set, Tukey's honestly significantly different (HSD) multiple comparison tests was used to determine significant differences between groups and conditions. All tests were conducted with a confidence interval of 95% ($p < 0.05$).

3. RESULTS

3.1. Characterization of HA microparticles

HA microparticles produced by a water-in-oil-emulsion and crosslinked with ADH and EDCI-HCl presented a spherical and smooth conformation with some heterogeneous size distribution (Figure 2).

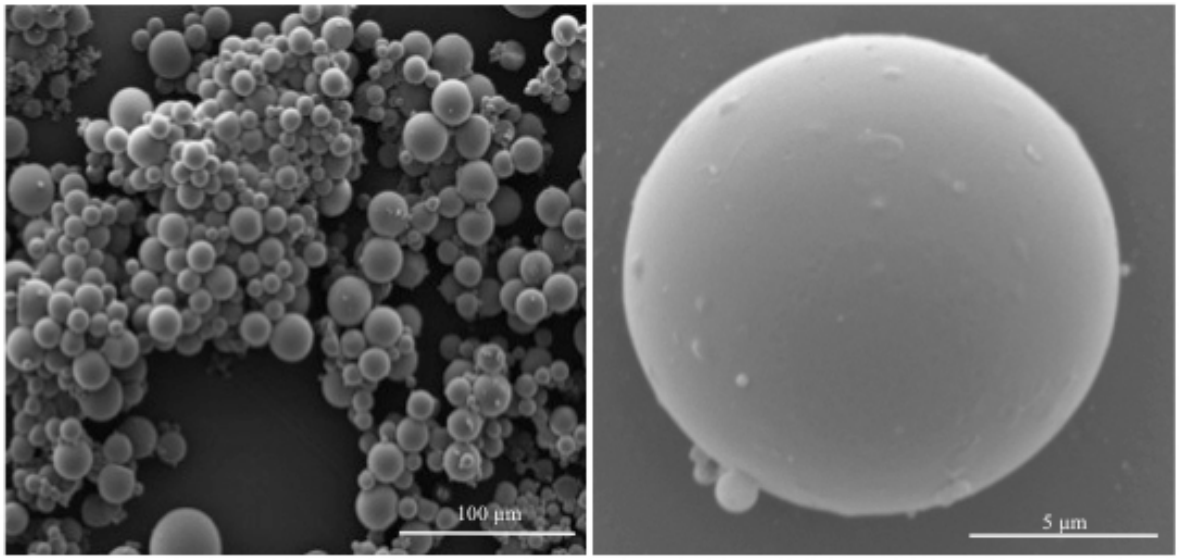

Figure 2 – Scanning electron microscopy (SEM) images of HA microparticles.

3.2. hAC Viability (MTT assay) and Proliferation (DNA content)

In the present study, the influence of direct and non-direct contact of HA microparticles on the viability and proliferation profiles of primary hAC was analyzed. The results of MTS assay and DNA content indicate that hAC in the different culture conditions (direct and non-direct contact, and control) remained viable and were able to proliferate up to 28 days *in vitro*. The cellular viability in direct, non-direct and absence of HA microparticles (control) up to 28 days was confirmed using MTT analysis. MTT assay results demonstrated that in all culture conditions HA microparticles were clearly non-cytotoxic (Figure 3). These results, Figure 3 A a) shows that HA microparticles in non-direct contact present higher cell viability values as compared to those obtained in direct contact (Figure 3A b).

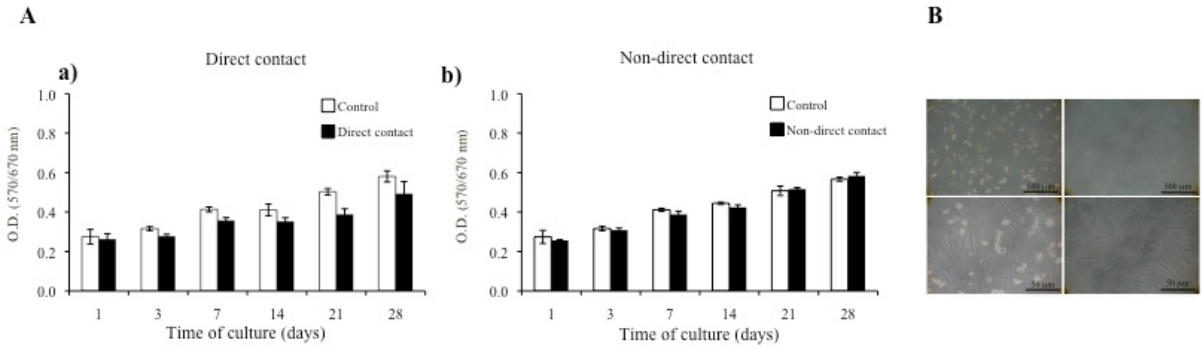

Figure 3 – Viability of hAC over time for the studied culture conditions revealed by MTT staining A); a) Viability absorbance values for human articular chondrocytes in direct culture with HA microparticles and b) Viability absorbance values for human articular chondrocytes in non-direct culture with HA microparticles. (B) Light microscopy images showing chondrocyte morphology in 2D culture.

The results of DNA quantification (Figure 4) indicate that the cell culture method (direct and non-direct contact) did not have a significant effect on cell proliferation. The number of cells, for both cases, increased as a function of time (Figure 4). DNA results of hAC in non-direct contact with HA microparticles over time (Figure 4 a) show a slight but not significant higher values in their proliferation ratio compared to those obtained in direct contact microparticles (Figure 4 b).

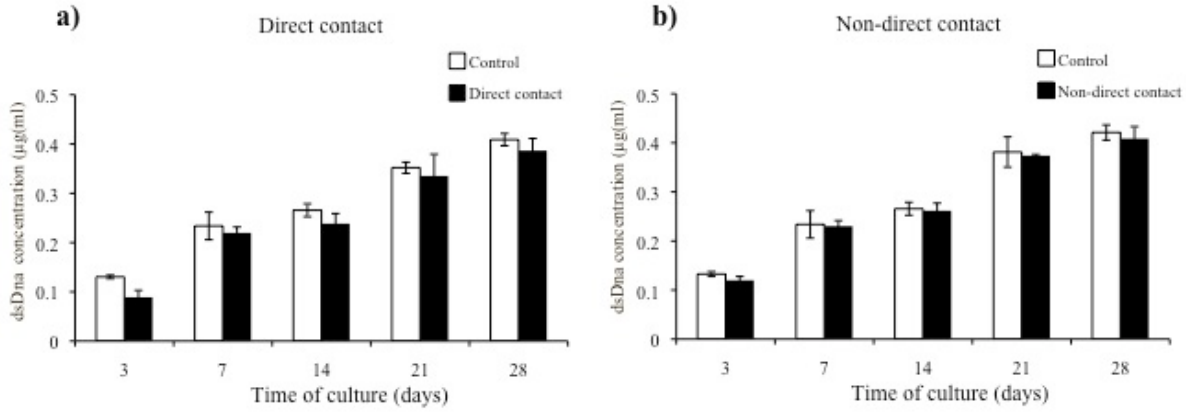

Figure 4 - Amount of dsDNA that correlates with the cell number quantified over time of culture for the analyzed conditions. A) Human articular chondrocytes cultured in direct contact with HA microparticles and B) Human articular chondrocytes cultured in non-direct contact with HA microparticles.

3.3. hAC phenotype analysis

Real-time polymerase chain reaction was used to simultaneously amplify and quantify specific sequences of DNA fragments. In order to quantify variations in gene expression by hAC, in direct contact with HA microparticles and

without (control), quantitative PCR was performed. The expression profile of several genes by hAC was normalized by GAPDH that was chosen as reference housekeeping gene (Figure 5). The threshold cycle (Ct) value for each sample was determined in the exponential phase of amplification. Generally, it was observed an up-regulation of gene expression for all analyzed genes when hAC were cultured in direct contact with HA microparticles compared to hAC culture alone (without microparticles). It was observed in the absence of HA particles, from day 7 up to 28 days of culture, a significant decrease ($p< 0.05$) of collagen type II transcripts in hAC. Expression of collagen type II (Figure 5 a) by hAC cultured in direct contact with HA microparticles presented a slight decrease for the same period of time but it was not significant. The same behavior was observed for the expression of aggrecan in hAC cultured without HA microparticles. This gene expression results presented a significant decrease ($p< 0.01$) over time (Figure 5 b). Moreover, it is also observed a significant increase of this gene expression after 28 days of culture in direct contact with HA microparticles ($p< 0.01$). The same trend occurred for SOX9 expression (Figure 5 c). With time, cells cultured in absence of HA microparticles presented a significant decrease of fold induction ($p< 0.05$). At 28 days, hAC cultured in direct presence of HA miroparticles, showed significant augment ($p< 0.05$) of transcripts then those cultured in absence of it. COMP fold of expression did not present significant differences over time between culture conditions (Figure 5 d). However, either at 7 and 28 days, a significant increase ($p< 0.01$) of fold induction was observed in hAC cultured in direct contact conditions compared to those cultured without HA microparticles Collagen type I (Figure 5 e) presents constant values of expression without significant changes in both culture conditions over time.

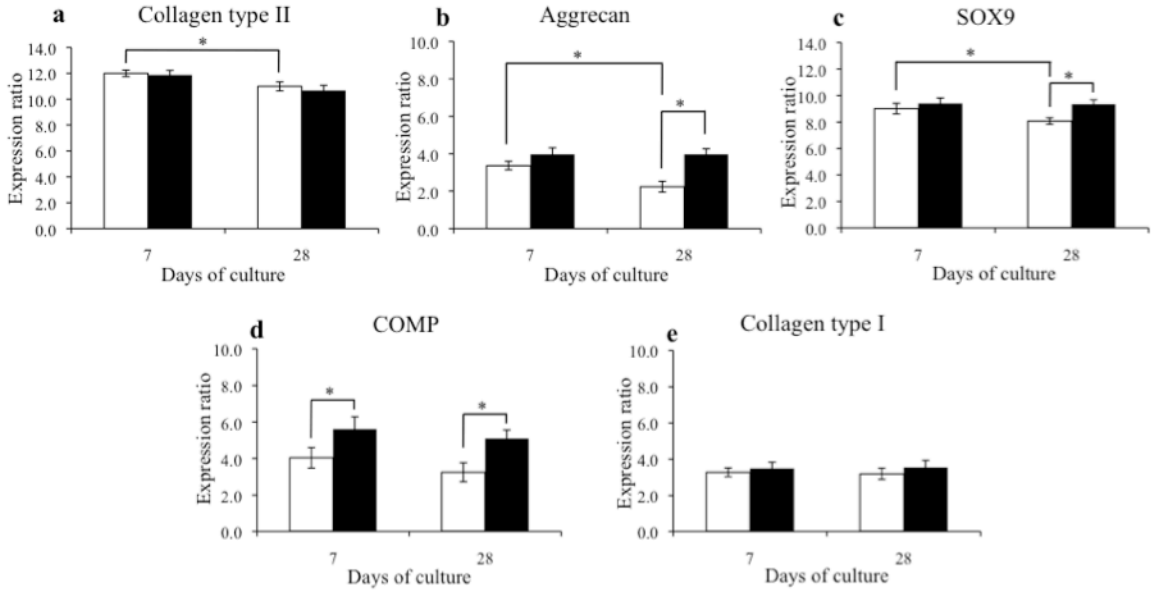

Figure 5 - Expression profile of chondrocyte-specific genes (a-d) and collagen type I (e) observed in hAC over time of culture; white column – cells cultured in absence of HA microparticles (control condition); black column – cells cultured in direct contact with HA microparticles. Level of mRNA transcripts fold induction of hAC was normalized for GAPDH expression. Statistical analysis through the various time points ($p<0.05$) was performed.

3.4. Flow cytometry analysis of hAC

Cluster of differentiation (CD) antigen expression of hAC during the *in vitro* culture in absence and presence of HA microparticles in direct contact condition was assessed by flow cytometry (Figure 6). All conditions were tested for the expression of CD44 over time. No significant differences on the expression of CD44 were observed for hAC cultured in presence and absence of HA microparticles.

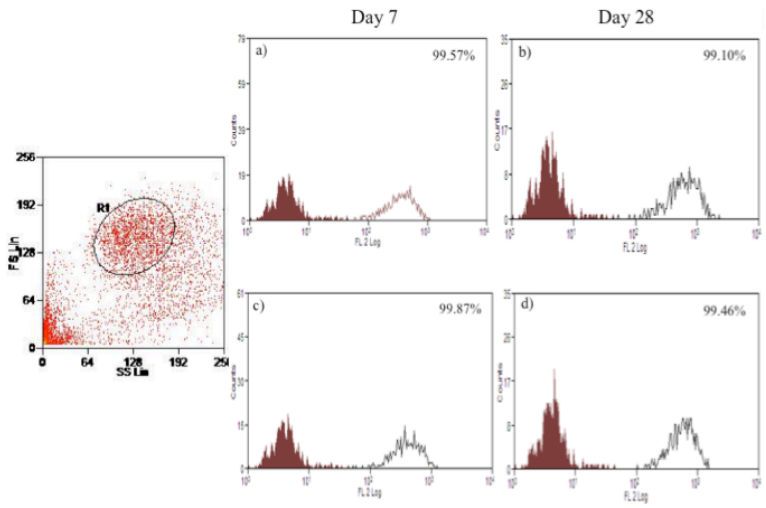

Figure 6 - Flow cytometry analysis of the expression of CD44 surface marker on hAC over time: a and b, CD44 expression on hAC in absence of HA microparticles at 7 and 28 days respectively; c and d, hAC CD44 expression in direct contact with HA microparticles at 7 and 28 days respectively.

4. DISCUSSION

Adult chondrocytes have, as primary role, the maintenance of the normal function of articular cartilage with the production and sustain of the complex pericellular and ECM. In this tissue, with limited self-renewing properties, cell-cell and cell-matrix interactions are essential for the tissue homeostasis. Hyaluronic acid (HA), a ubiquitous constituent of cartilage extracellular matrix has functional influence on cell behavior. This biopolymer bounds to cell through the membrane receptor interacting with cell-cell ligants receptors, and cell pericellular/ECM interactions. It has been demonstrated that HA as a fundamental role on maintenance of ECM structure as well as in cellular functions such as proliferation, adhesion, migration, and differentiation. The special physicochemical and biological properties of HA and its non-immunogenic nature have made this biopolymer a particular useful tool for engineering bioactive matrices in cartilage regenerative field.

In this context, the purpose of this study was to produce and characterize HA microparticles and to evaluate their effect on primary hAC viability, proliferation, chondrogenic phenotype plasticity, and CD 44 expression.

In this work it was used the water-in-oil emulsion to produce HA microparticles, adapted from (30) (Figure 1). Alterations to the aforementioned protocol did not induced significant differences on HA microparticles size and

surface texture. The developed microparticles showed a regular and smooth surface as demonstrated by SEM images (Figure 2).

To access possible deleterious effects of the developed microparticles on the target cells, hAC viability and proliferation assays were performed.

Viability results for both studied conditions (Figure 3A a and b) indicate that HA microparticles did not show any kind of cytotoxicity on hAC behavior. All conditions demonstrated to be clearly non-cytotoxic. MTT results for all culture conditions showed a slightly increase trend over time of culture, as consequence of normal cell increasing viability with growth. In particular, chondrocytes cultured in direct contact with HA microparticles (Figure 3 A, b) presented lower values of viability than those in absence of the biomaterial (Figure 3 A, a). This fact can be related to less physic space to cell grow and proliferate due to the presence of the micropaticles on the plastic surface or to an effective lower proliferation of the cells caused by HA cellular interaction. In fact, we strongly believe that the direct contact of HA microparticles could induce a decrease of cellular proliferation. These findings were corroborated by proliferation results obtained by measuring the DNA content of the expanded cells for the same studied conditions (Figure 4 a and b). Cells cultured in direct contact of HA microparticles did not show any morphological changes in their shape as confirmed by optical microscope images (Figure 3 B).

It is well established that during *in vitro* monolayer expansion of chondrocytes, independently of their origin (e.g. nasal, auricular or articular), cells tendency loss their phenotype and gradually start a process of dedifferentiation (34, 35). Dedifferentiation and chondrogenic potential of heterotopic chondrocytes is, in fact, one of the drawbacks of monolayer passages necessary to achieve a sufficient number of chondrocytes. Several authors correlated this degree of dedifferentiation to the number of cell doublings/passages (36, 37).

In the process of dedifferentiation, primarily articular chondrocytes dedifferentiate to fibroblast like cells, gaining their proliferation rate but showing a simultaneously significant time-dependent decrease in the expression and synthesis of cartilage specific markers such as aggrecan, Sox9, Collagen type II and COMP (36, 38). This phenomenon is independent of primary cell sources (38-41) and culture conditions (38, 42, 43). A commonly accepted explanation to this fact can be attributed to the loss of cell – pericelluar and ECM interactions. Transfer

chondrocytes from their natural three-dimensional (3D) embedded matrix to a two dimensional (2D) plastic layer results on increase of cell proliferation as well as diminished chondrogenic phenotype. The proliferation of hAC, in direct and non-direct contact with HA microparticles, was accessed by DNA quantification (Figure 4 a and b). The obtained results indicate no significant differences on hAC proliferation for both conditions with maintenance of the cellular growth tendency over the 28 days of *in vitro* culture. Nonetheless, it is worth noting that hAC proliferation values in direct contact with HA microparticles, tend to be lower than the remaining culture conditions (non-direct contact and in absence of HA microparticles - control condition), Figure 4 a and b white columns) indicating a slight proliferation reduction. We hypothesize that this small difference on DNA amount in the presence of HA microparticles could induce some phenotype changes on hAC. During the expansion of hAC, the degree of dedifferentiation has been correlated to the number of cell division or passages. Chondrocyte proliferation and simultaneous maintenance of the intrinsic chondrogenic differentiation potential are regarded as the two primary goals to be achieved in order to accomplish the requests of the clinical applications and concomitant tissue repair. It has been reported that dedifferentiated chondrocytes lose their specific synthetic profile, and instead of cartilage-specific gene markers expression (e.g. type II collagen, SOX9, aggrecan, COMP among others) it is observed an increasing expression of nonspecific articular chondrogenic genes (e.g. type I collagen) (44). Chondrocyte dedifferentiation during cellular monolayer expansion is observed in all heterotopic (articular, auricular, nasoseptal, and costal) chondrocyte cultures (34, 42, 45, 46). Chondrocyte redifferentiation and/or maintenance of intrinsic somatic phenotype are possible in multiple 3D culture systems with the use of different biomaterials formulations and polymer blends. This phenomenon can be explained by the fact that dedifferentiation frequently is involved on cell proliferation capacity (35, 38, 41, 46).

Our Real Time results (Figure 5) indicate that over time, the responsible genes for chondrogenic phenotype modulation and sustain (e.g. collagen type II, aggracan, Sox9 and COMP), are up regulated when hAC were cultured in the direct presence of HA microparticles. This fact is related with concomitant decrease on cell proliferation, denoting that HA microparticles cell interactions are responsible for the high maintenance of the somatic cellular phenotype. Indeed, results show that HA microparticles in direct contact with hAC, led to a significant increase ($p<0.01$ and $p<0.05$) of aggrecan, SOX9 and COMP gene expression folds compared to

values obtained for the same in *vitro* culture time in the absence of microparticles (Figure 5 b and c). It is well known and documented that the transcription factor SOX9 is the major responsible factor for chondrogenic phenotype sustain (47-49). In fact, Malpeli *et al.* defend that the maintenance of SOX9 expression on *ex vivo* expanded chrondroytes is the main responsible for their phenotype modulation commitment in a future cellular application (38). Furthermore, it has been described that aggrecan and COMP genes play a key role in the cellular ability to deposit ECM proteins responsible for cell – cell and cell – pericellular interactions and consequently tissue homeostasis (42, 50-55). The significant increase of cartilage specific genes (Figure 5), that will encode for ECM proteins instead of dedifferentiation signs present in hAC cultured in absence of HA microparticles, leaded us to raise the hypothesis that the developed biomaterial might change also the expression of the cellular main receptor of HA, CD44. The CD44 is a member or non-integrin family of cell surface transmembrane proteins that has a large variety depending on the cell types (56).

In cartilage, the retention of ECM properties during chondrogenesis is mediated via CD44, where HA binds to cell receptors (57, 58). In this connective tissue, cell-matrix interactions are fundamental for tissue differentiation and maintenance of the intrinsic properties and homeostasis. Knudson *et al.* (59) reported that the capacity of chondrocytes to retain endogenous pericellular matrix properties is related with the level of cell surface CD44 expression. In our work, we have observed that the expression of the HA receptor CD44 did not present significant changes in the presence of HA microparticles (Figure 6). At day 7, 99.57% of hAC cultured in absence of HA microparticles (control), expressed CD44. The same behavior was observed for cells cultured in direct contact conditions, presenting 99.87% of positivity for this glycoprotein. As a function of time, the expression of CD44 was not altered. In absence of HA microparticles, control condition, hAC maintained their positivity, 99.10% as well as those cultured in direct contact conditions, obtaining 99.46% of expression. The presence of HA microparticles in direct contact condition, did not altered the expression of HA cell receptor over time. We do think that inexistence of significant effect on the expression of CD44 might be related to the internal cellular signals times. The significant differences observed at mRNA transcriptional level in aggrecan, Sox9 and COMP expression could be time dependent to encode for protein production as well as the CD44 transmembrane expression augment/reduction. Ishida *et al.* (26) reported that CD44 plays an important role in normal and

abnormal functions of cartilage through the adhesion to HA. This fact is responsible for a diverse of stimulatory signals on chondrocyte proliferation and matrix synthesis.

In our study we have demonstrated that: i) HA microparticles produced by a water-in-oil emulsion do not negatively affect primary hAC viability; ii) HA microparticles are responsible for a slight decrease of hAC proliferation ration compared to those cultured in their absence; iii) The presence of HA microparticles have a positive effect on hAC being responsible for the up-regulation of aggrecan, SOX9 and COMP expression, proving the increase/maintenance of chondrogenic phenotype over time. However, our developed biomaterial didn't show any effect on CD44 expression.

5. CONCLUSIONS

The developed HA microparticles by water-in-oil emulsion presented regular and smooth shape with robust behavior in aqueous environment. The direct and non-direct contact conditions of HA particles with primary hAC were clearly non-cytotoxic as confirmed by of the increase cellular viability as a function of time up to 28 days. Cellular viability results were in agreement with hAC proliferation results showing an increasing trend over time for the both culture conditions. In more detail, hAC cultured in direct contact with HA microparticles showed a slight reduction on their proliferation ratio compared to the control. HA particles in direct contact with hAC have a clear positive effect their chondrogenic phenotype, as revealed by a significant increase on the expression of some chondrogenic gene markers such as collagen type II, aggrecan, SOX9 and COMP. Transcript levels of these genes were in the overall higher than those observed in the absence of HA particles, suggesting a possible chondrogenic "intrinsic protective" potential of HA microparticles in hAC. However, the positive outcome in the chondrogenic phenotype was not associated with CD44 expression. No significant changes in CD44 expression were observed in direct contact with HA microparticles over time compared to those in absence of microparticles. While further studies are needed, these findings indicate that the developed HA microparticles have a strong potential as a candidate for intraarticular injectable vehicles in knee cartilage regenerative therapies.

Acknowledgements

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CHAPTER VI

HYALURONIC ACID/POLY-LYSINE NANO COMPLEXES FOR CARTILAGE REPAIR STRATEGIES

CHAPTER VI

HYALURONIC ACID/POLY-LYSINE NANO COMPLEXES FOR CARTILAGE REPAIR STRATEGIES *

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Abstract

The development of new delivery systems for in situ administration of therapeutics agents in cartilage tissue engineering is an area with increasing interest. Hyaluronic acid (HA) is a naturally occurring polysaccharide that has benefit from huge successes in cartilage biomedical field as matrices and hydrogels. Poly-L-lysine (PLL) is biocompatible and has important cell adhesion properties that allied to its polycation charge consent the formation of polyelectrolyte complexes with the polyanion polymers. By this, we aimed to developed nano polyelectrolyte complexes as fruit of the combination of different ratios of opposite charged biodegradable and biocompatible polymers. We study the effect of different polymer concentrations on nano complexes size and structural stability. Polyelectrolyte complexes of HA and PLL could be use as potential carriers for small pharmacological bioactive agents to be deliver locally in inflammatory articular cartilage site.

Keywords

biomaterials; polyelectrolyte interactions; nano complexes

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R. C. Pereira[1,2], R.M.P. da Silva [1,2], H.S. Azevedo[1,2] and R.L. Reis[1,2] "Hyaluronic acid/poly-lysine nano complexes for cartilage repair strategies", *submitted*

1. INTRODUCTION

In recent years, nanocomplexes have been generated a considerable interest as vehicles for delivery of pharmacological drugs and genes in different research fields [1, 2]. In this context, electrolyte complexes have been receiving great attention for tissue engineering. The known electrostatic attraction between opposite charges molecules is being over time an excellent driving force for nano-complexes fabrication based on alternated adsorption of cationic and anionic polymers [2]. The possibility to control the number of interactions between cationic and anionic charges turns this technique applicable to fabricate ultra-thin complexes with thickness, composition, properties and charge required. Layer-by-layer assembled complexes have demonstrated to interact with several bioactive agents. Ladam et. al [3] showed that proteins interacted strongly with polyelectrolyte layers in a mechanism dependent of the multilayer charges and the type of protein. Also, antibodies were reported to retain their reactivity with respect to their antigens after incorporation in constructed layer-by-layer polyelectrolyte complexes [4]. This fact shows that constructed nano-multilayers delivered in the desirable environment have the select ability, depending on their charge, to interact with the presenting proteins. With these properties, research can be oriented to achieve definite multi complexes incorporating specific ligands that keep their biological activity and promote the selective adhesion to particular cell types. In particular, in the field of cartilage tissue engineering for articular knee joints repair strategies, the use of natural biomaterials to construct multi-layer nano-complexes is an appealing concept.

Since hyaluronic acid (HA) is a natural linear polysaccharide presented as major component in articular cartilage extracellular matrix, the use of this polymer as part of a polyelectrolyte complex could be very useful for the development of a nano-complexes to be locally delivery in the knee joint. This polysaccharide demonstrates exceptional rheological, hygroscopic and viscoelastic properties. HA structure consists in alternated disaccharide units of D-glucuronic acid and N-cetyl-D-glucosamine with beta (1 to 4) inter-glycosidic linkage [5-7]. The molecular weight changes from $1x10^5$ to $5x10^6$ Daltons (Da). HA, a negatively charged poly-anion, presents unique and excellent physicochemical properties, such as biodegradability, biocompatibility and viscoelastcity [7].

Due to intrinsic properties, HA has been widely used in articular cartilage environment in strategies that required the modification of the polymer mainly through carboxyl and hydroxyl groups in processes that include

esterification [8, 9] and chemical modification by crosslinking with carbodiimide [10]. However, in this case, chemical modifications of HA generally must be carried out in extreme alkaline or acidic solutions as well as at elevate temperatures which can create an obstacle to possible bioactive small molecules incorporation.

To achieve the formation of nanocomplexes by electrostatic interactions with hyaluronic acid we have used Poly-L-lysine (PLL). This natural polymer, with cationic charge, has been reported as non-toxic and biodegradable in humans. Over the years, PLL has been used as polycation component in polyelectrolyte complexation systems between anionic/cationic polymers, i.e. for cells encapsulation and *in vivo in situ* delivery [11]. It has been reported that the combination of PLL with other cationic polymers allowed the formation of permeable capsules/membranes that can be used for various applications [12-14]. Due to the highly positive charge, through charge/charge interaction, PLL is also used as stable carrier of DNA molecules [15, 16].

In order to design surfaces and freestanding structures using layer-by-layer techniques, comprising the alternate deposition of polyelectrolytes with opposite charges, at least one positively charged polymer should be considered. In this sense, we have chosen to study the electrostatic interactions between poly(L-lysine) (PLL) (polycation) and hyaluronic acid (HA) (polyanion) as a starting point.

The objectives of this work were to study the iontropic complexes formation properties of hyaluronic acid and poly-lysine molecules in presence of sodium carbonate and to characterize the impact of the different concentration ratios of both polymers. We aimed also to determine the effect of salt concentration and the proportion between components on the size distribution of the resultant nanocomplexes.

2. MATERIALS AND METHODS

2.1. Materials

Poly(L-lysine) hydrobromide was purchased from Sigma (USA) with two different molecular weights, that were labeled according to the molecular weight as PLL4.2kDa (Viscometry: M_v = 4.2 kDa) and PLL26kDa (MALLS:

M_w = 25.6 kDa). Hyalluronic acid Sodium salt (Lifecore, USA) of two different molecular weights was used, herein abbreviated as HA37kDa (GPC: $M_w = 3.7 \times 10^4$ Da, $M_w/M_n = 1.2$) and HA230kDA (GPC: $M_w = 2.3 \times 10^5$ Da, Mw/Mn = 1.7). Utrapure water (Milli-Q) was used throughout the experiments (c < 0.05 mS).

Sodium Chloride (NaCl) solutions with final concentration of 1M were performed using ultrapure water above mentioned.

2.2. Polyelectrolyte complexes mixture

To perform HA solutions, polymer was left under slow constant agitation at 4°C in NaCl solution for 24 hours to allow polymer total dissolution. After, solutions were filtered under in clean environment using a 0.22 μm filter. Same procedure was made to dissolve PLL. At the end, solutions were mixtured at different ratios to perform the following analysis.

2.3. Electrostatic Interactions between poly(L-lysine) and hyaluronic acid

For both dynamic light scattering (DLS) and electrophoretic mobility measurements, the original solutions were prepared in ultrapure water and filtered using a 0.20 μm disposable PES membrane filter (TPP, Trasadingen, Switzerland). Solution mixtures and dilutions were performed in a laminar flow chamber at room temperature, in order to avoid dust contaminants.

2.4. Dynamic light scattering (DLS)

The hydrodynamic diameter was determined by Dynamic Light Scattering (DLS) using a Zetasizer NanoZS Instrument (ZEN3600, Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He-Ne laser (λ_0 = 633 nm) and with non-invasive backscattering (NIBS) detection at a scattering angle of 173°. Both

measuring position and attenuator were adjusted automatically before each measurement. Measurements were performed at 25 ºC after an equilibration time of at least 2 min.

2.5. Electrophoretic mobility

The electrophoretic mobility was obtained by Laser Doppler Velocimetry (LDV) using a Zetasizer NanoZS Instrument (ZEN3600, Malvern Instruments, Worcestershire, UK) at a scattering angle of 17° and capillary folded cells (DTS1060, Malvern, Worcestershire, UK). The measurements were performed with automatic voltage selection at 25 ºC after an equilibration time of 2 min.

Cloud point measurements were done mixing polyelectrolytes with opposite charges at high ionic strength (NaCl, 1 M) to screen electrostatic interactions, and diluting with distilled water in order to progressively decrease salt concentrations. The solutions turbidity was monitored by UV-vis spectrophotometry in a multiwell microplate reader at room temperature. The absorbance was corrected to account polymer dilution and represented as a function of the salt concentration.

3. RESULTS AND DISCUSSION

The use of polyelectrolyte complexes is being proposed in different modes in tissue engineering [17-19]. The fact that polyelectrolyte complexes can be formed without the use of aggressive polymers cross-linkers, and the nontoxic nature of the dissociation products, allow these polymer complexes to be use e.g. as a single delivery system [18] or as scaffold coating with the aim of superior cellular behaviour [20-22]. In attempts to repair cartilaginous tissue, HA can play a positive effect on joint resident cells [23, 24]. The use of PLL described by different authors in analogous systems could enhance polyelectrolyte selective adhesive properties in cellular environments [25-27]. Polyelectolyte complexation with both polymers could be an alternative method to create structures with potential appliance on cartilage tissue engineering.

In the present study we aim to understand the electrostatic interactions between PLL and HA in solution. The objective was to study the effect of ionic strength (sodium chloride concentration), temperature, polyelectrolyte chain size, conformation and mixture conditions on the aggregation behaviour of polyelectrolytes complexes.

The possibility to analyse the complexes in solution allow a faster screening of the physical properties of the polyelectrolyte system that can be very useful for the design and construction of the layer-by-layer surfaces and free-standing semi-solid structures.

The first studies were performed using HA37kDa and PLL4.2kD, with molecular weight of the HA around ten-fold higher than the PLL molecular weight. Mixtures of solutions of HA and PLL remained clear at a NaCl concentration of 0.154 M, regardless of polymer concentration and polyelectrolyte relative amount. This fact seems to indicate that the electrostatic attractive forces are adequately screened at this salt concentration, and no macromolecular aggregation was observed. On the other hand, turbidity develops immediately when mixing solutions of HA and PLL previously dissolved in distilled water.

In Figure 1 it is possible to observe that a rapid mixture between HA and PLL solutions leads to colloidal particles at the submicron scale, with relatively narrow monomodal size distributions. Interestingly, the average hydrodynamic diameter (D_h) - calculated from the size distribution by volume - decreases when one of the polymeric mixture components dominates over the other. We thought that the surface would be preferentially composed by the component in excess, forming an electrostatic barrier, which could stabilize the colloidal particle, providing the absence of free molecules of the limiting polyelectrolyte component existed in solution.

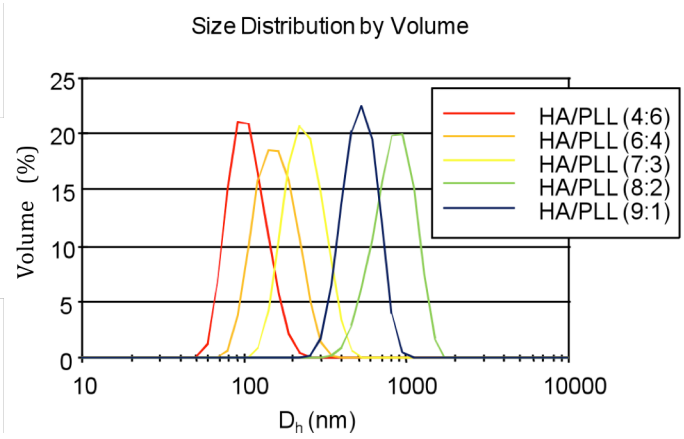

Figure 1 - Volume-Size distribution of aggregates obtained by one-shot addition of HA37kDa and PLL4.2kDa aqueous solutions at different polymer weight ratios.

In fact, a strong correlation is observed (Figure 2) between the electrophoretic mobility (μ) (which is related to the surface electrokinetic potential) and size (D_h); the maximum D_h is observed at the μ zero-crossing point.

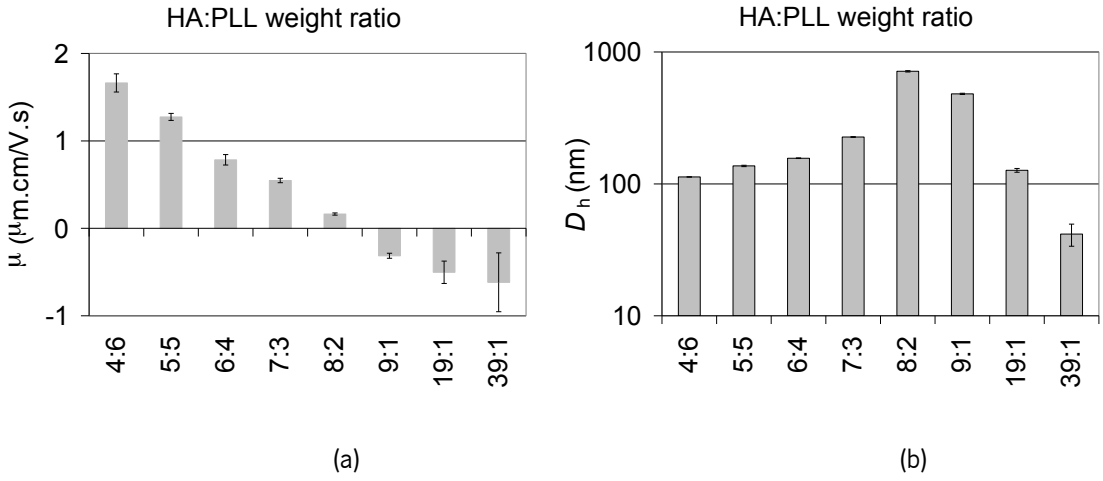

Figure 2 - (a) Electrophoretic mobility and (b) apparent hydrodynamic diameter (D_h) of polyelectrolyte aggregates as a function of HA37kDa and PLL4.2kDa weight ratios.

The colloidal size stability was evaluated following the D_h evolution for 10 days. It could be observed that dispersions were not at equilibrium and that the polyelectrolyte agglomeration rate was much lower for the mixtures in which a greater excess of PLL was used as it is demonstrated in Figure 3.

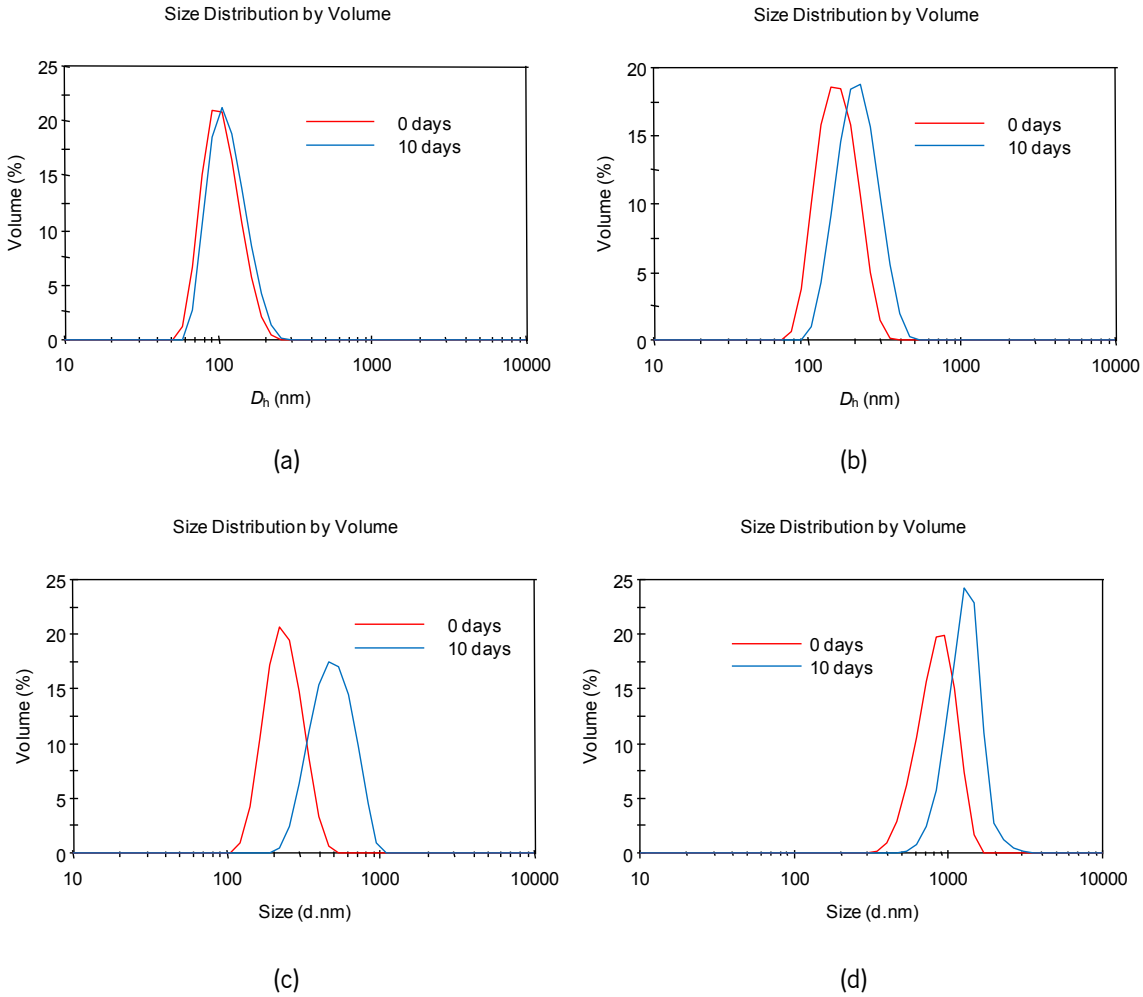

Figure 3 - Time evolution of volume-size distributions for aggregates produced by one-shot addition of HA37kDa and PLL4.2kDa aqueous solutions at HA/PLL weight ratios of (4:6)(a), (6:4) (b), (7:3) (c) and (8:2) (d).

We have also studied the influence of the polymer molecular size on the salt screening effect. The described system possesses a strong limitation to be used in physiological environments. The physiological ionic strength is around 0.154 M, and no interaction was observed for HA37kDa and PLL4.2kDa at this salt concentration. Since, the electrostatic forces act cooperatively to the aggregates cohesion, we hypothesized that, increasing the polymers molecular weight could increase the salt concentration required to cancel the aggregation between the oppositely charged polyelectrolytes. Being so, we have studied the effect of salt concentration on the turbidity of HA and PLL mixtures at several NaCl concentrations. Higher molecular weight polymers were used. However, the size ratio was roughly the same, being HA chains around ten-fold larger that the PLL chains.

Solutions of both polymers were prepared at high salt concentration (NaCl, 1 M) to cancel electrostatic attraction, thus precluding aggregation. The several studied mixtures were diluted with distilled water to reduce salt activity. Also, absorbance was recorded to detect solutions turbidity as shown in Figure 4.

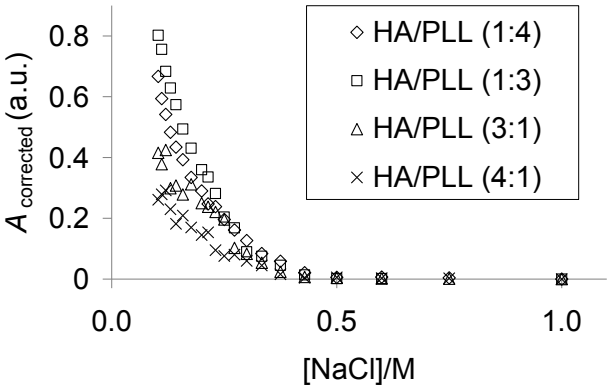

Figure 4 - Turbidimetric curves obtained by dilution of an initial HA230kDa/PLL26kDa solution at high ionic strength with distilled water. Absorbance (*A*) was corrected to account with the polymer dilution.

We have found that mixtures of higher molecular weight polyelectrolytes became turbid at higher salt concentration than those of lower molecular weight. In opposition to the previous systems, this higher molecular weight system can be used to design layer-by-layer surface coatings and free-standing structures that withstand the physiological ionic strength. It is also interesting to notice that the salt concentration at the turbidity onset is independent on the polyelectrolyte-mixing ratio.

4. Conclusions

The electrostatic interactions between PLL and HA were studied in solution. We have found that the aggregation behaviour depends on the ionic strength (sodium chloride concentration), polyelectrolyte chain size and mixture conditions. The interactions between HA and PLL will be thoroughly screened by using different molecular weight and polymer size ratios, other salts, pH, temperature and mixing conditions. The physical properties of the

polyelectrolyte system will be used to design and construct more sophisticated layer-by-layer surfaces and freestanding semi-solid structures. Due to the surface charge of the polyelectrolyte complexes, the inherent properties of the polysaccharides used, and the harmless disassociation products of the polyelectrolytes, we suggest that the development of these HA/PLL complexes can offer a possible cartilage repair system platform. Furthermore, these developed polyelectrolyte complexes systems can be used for peptide or protein drug delivery.

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CHAPTER VII

NOVEL INJECTABLE GEL (SYSTEM) AS A VEHICLE FOR HUMAN ARTICULAR CHONDROCYTES IN CARTILAGE TISSUE REGENERATION

CHAPTER VII

NOVEL INJECTABLE GEL (SYSTEM) AS A VEHICLE FOR HUMAN ARTICULAR CHONDROCYTES IN CARTILAGE TISSUE REGENERATION*

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Abstract

We developed a novel injectable carragenan/fibrin/hyaluronic acid-based hydrogel with *in situ* gelling properties to be seeded with chondrogenic cells and used for cartilage tissue engineering applications. We first analyzed the distribution within the hydrogel construct and the phenotype of human articular chondrocytes (HAC) cultured for 3 weeks *in vitro*. We observed a statistically significant increase in the cell number during the first two weeks and maintenance of cell viability throughout the cell culture together with the deposition/formation of a cartilage specific extra cellular matrix (ECM). Taking advantage of a new *in vivo* model that allow to investigate the integration between the newly formed cartilage and a preexisting cartilage in immunodeficient mice, we showed that the injectable hydrogel seeded with human articular chondrocytes was able to regenerate and repair an experimentally made lesion in bovine articular cartilage thus demonstrating the potential of this novel cell delivery system for cartilage tissue engineering.

Keywords

Cartilage tissue engineering, Cell culture, Biomaterial, Animal Model, ECM (extracellular matrix)

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1. INTRODUCTION

Tissue loss, as a result of trauma, congenital disorders and joint diseases, usually involving structural damage of articular cartilage surface, is a relevant clinical problem [1, 2]. In fact these pathologies result in severe pain and disability for millions of people worldwide and represent a major challenge for orthopedic surgeons [3-6]. Tissue engineering (TE) can be defined as the art of reconstructing tissues, [1] both structurally and functionally, with the knowledge that reconstruction can be performed either entirely or partially *in vitro* and then completed *in vivo* [3, 7-11].

During the past decade exciting new tissue engineering strategies have emerged that show the potential to regenerate damaged cartilage [3, 6, 12]. In particular, autologous chondrocyte transplantation (ACT), a standard clinical procedure to treat human articular cartilage defects [13, 14], has been successfully used on thousands of patients. However, ACT, in addition to some intrinsic limitations such as a poor *in vitro* expansion of chondrocytes and some complexity in the graft fixation, requires an invasive surgical technique. To overcome some of these problems, new materials and new models have been studied and proposed as cell delivery systems [15-17].

A very new and exciting approach to cell delivery for tissue engineering is using hydrogels that have the ability to be injected into the body as a solution and to form a gel immediately after injection, thus enabling the clinician to transplant the cell-support system in a minimally invasive manner. The use of materials that present an injectable formulation satisfies also the need of a perfect filling of the lesion, as they can be easily applied also in the presence of an irregular defect [16, 18, 19].

Biodegradable and biocompatible hydrogels with components and structure similar to the extracellular matrices (ECM) present in different districts of the body have found numerous applications in the fields of TE and in drug delivery [8, 9, 15, 18, 20, 21]. In particular, several scaffolds/hydrogels have been proposed in association with different cell types for articular cartilage regeneration [1, 15, 19, 22]. Although the use of synthetic hydrogels appears very promising, there are some important aspects that must be investigated before they can be widely used in the clinical practice [1, 17]. More specifically, the interactions of the cells with the hydrogels can interfere with cell adhesion, as well as with cell proliferation and migration, being the adhesion cell-type specific and

dependent on the interactions of specific cell receptors with components adsorbed or encapsulated into the materials (e.g. ECM macromolecules and growth factors).

Aim of this study was to investigate some of these aspects in the case of a new biodegradable carrageenan-based injectable gel. Recent papers described a new organ culture model where human articular chondrocytes were cultured within an experimentally made osteochondral defect in an articular cartilage biopsy subcutaneously implanted in an immunodeficient mouse [23, 24]. We adopted a similar approach, to show that carrageenan-based hydrogel, seeded with human articular chondrocytes (HAC), has the potential to regenerate and repair an articular defect.

2. MATERIALS AND METHODS

2.1. Hydrogel preparation

K and i -carrageenans (Sigma-Aldrich, Germany) were dissolved in bi-distillate water (0.8 and 1.2 % w/v) and the solution was heated at 65°C under constant stirring to obtain a final homogeneous preparation. Carrageenans are high molecular weight sulfated polygalactans derived from several species of red seaweeds (Rhodophyceae). The most common forms of carrageenan are lambda (l), kappa (k) and iota (i). Carrageenan has alternating disaccharide units composed of D-galactose-2-sulfate and D-galactose- 2,6-disulfate, being the galactose residues joined by -1,3 and -1,4 linkages. These polysaccharides form gels upon cooling in presence of specific ions due to the development of three-dimensional networks as the result of the formation of helical chains complexes. In the presence of K^+ ions, k-carrageenan forms strong crisp gels, whereas i–carrageenan in the presence of Ca^{2+} ions forms elastic gels [25, 26].

Fibrinogen (final concentration 3,000 UIK/ml-Baxter AG-Austria) and thrombin (final concentration 500U/ml-Baxter-AG-Austria) were dissolved in an aprotinin solution (10,000 KIE/ml-Baxter AG-Austria) and in 40 mM $CaCl_2$ respectively.

Human chondrocytes, harvested from a human knee biopsy (Fig. 1 A-B) and cultured in serum free medium (see following paragraph) were resuspended at a concentration of $2x10^6$ cells/ml in culture medium (Biochrom A.G. Berlin, Germany) containing 0.265 g/L $CaCl_2$ and 0.4 g/L KCl in order to trigger gel formation when mixing with the carrageenan polymeric solution. Cell suspension was supplemented with the thrombin solution (5,9% v/v) and with 0,75% (v/v) of hyaluronic acid before loading in syringe A of the injection system (Fig. 1 C). Syringe B was loaded with the carrageenan solution supplemented with the fibrinogen solution at 35% (v/v). The carrageenan/fibrinogen solution was mixed with the cells/thrombin/hyaluronic acid solution by activating the two syringes at the same time. The gel formed almost instantaneously.

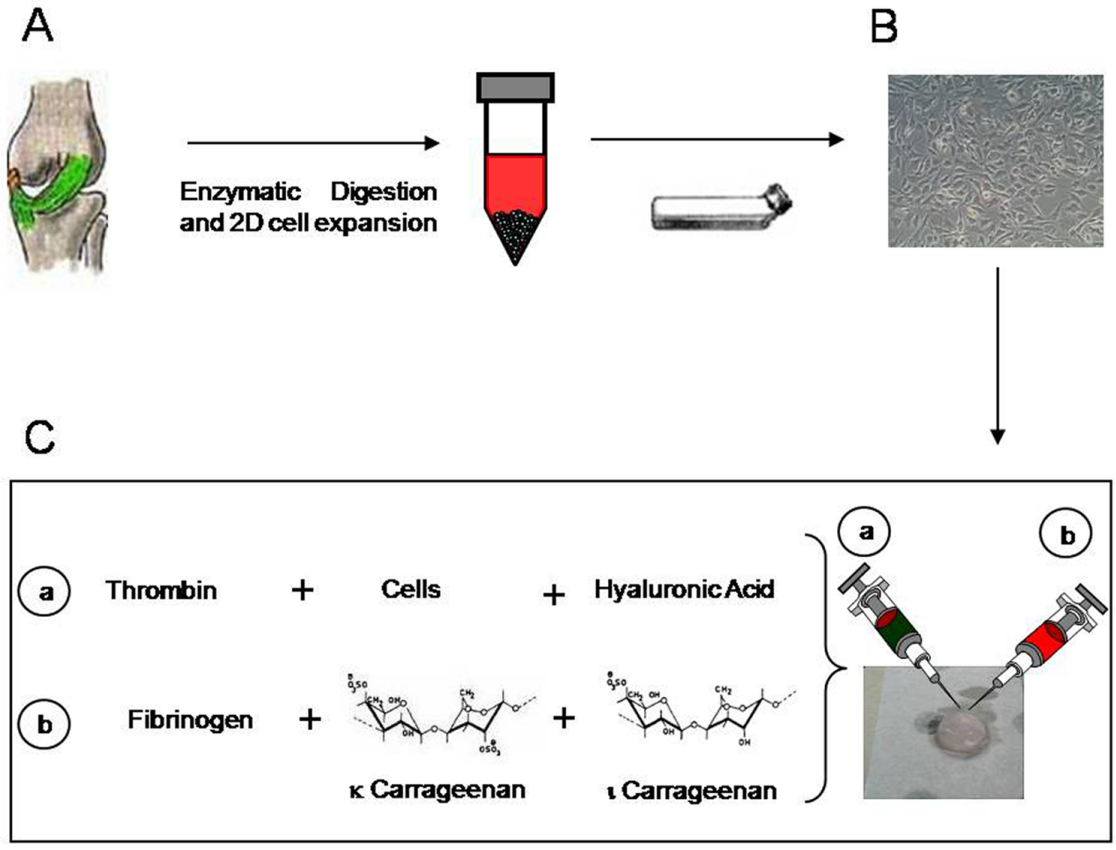

Figure 1 - Scheme of the procedure followed to form the hydrogel. Panel A) Human articular chondrocytes biopsy and digestions; Panel B) Light microscopy of a primary monolayer culture in serum free (SF) medium before the recovery of the *in vitro* expanded cells. Panel C) Biodegradable hydrogel system: content composition in "a" and "b" syringes and gross morphology of the formed gel.

2.2. *In vitro* cell culture

Articular cartilage biopsies were collected from male or female patients (age comprised between 61 and 75 years) undergoing total knee arthroplasty. Patients had signed an informed consensus approved by the Ethical Committee of the San Antonio Hospital, Recco, Italy.

Briefly, cartilage was cleaned of connective tissue and/or subchondral bone and cut in small fragments, according to published procedures [27]. Individual HAC were released by repeated enzymatic digestions with collagenase I (400 U/mL) collagenase II (1,000 U/mL) (Worthing Biochemical, Lakewood, NJ) hyaluronidase (1 mg/ml) (Sigma, St. Louis, MO) and 0.25% trypsin (Invitrogen Life Technologies, Carlsbad, CA) at 37°C. Cells were pooled, counted and seeded in culture dishes pre-coated for 24 hours with 10% fetal calf serum (FCS) in culture medium (Biochrom A.G. Berlin, Germany) to allow attachment of the serum fibronectin to the plastic dish surface. After 48 hours from seeding, the chondrocytes were enzymatically detached, collected, washed in PBS, counted and re-plated in dishes pre-rinsed several times with PBS to remove any residual trace of serum. The culture medium was then replaced with the serum-free (SF) medium described by Malpeli *et al.* [27] and the cells expanded *in vitro* in this culture condition. Medium was changed 3 times a week. After 4-5 cell doublings HAC were collected and either encapsulated within the tissue engineering construct and the culture continued in chondrogenic medium [28] for 3 weeks or pelleted by centrifugation (2.5 x 10^5 cells) and cultured in chondrogenic medium for 3 weeks.

2.3. Cell imaging

Over the course of the culture, the engineered constructs (n=3) were monitored using a bright field light microscope (Axiovert 10, Zeiss, Germany) equipped with a digital camera (Olympus DP 10, Olympus Optical Co. LTD, Japan). Images were acquired at 5 x and 10 x magnification.

To assess live cells distribution, the constructs were incubated with the nuclear staining Hoescht 33342 (Sigma-Aldrich, Germany) at the final concentration of 5 mg/ml in culture medium for 30 min.

Optical sections along the Z axis were then acquired by structured epifluorescent illumination using an Axiovert 200M microscope equipped with the Apotome module the filter set 49 and the AxiocamHR camera (Carl Zeiss, Jena, Germany). Image acquisition stacking of the optical sections and 3D reconstruction of the samples were performed by the Axiovision Software (Carl Zeiss) [29, 30].

2.4. MTT analysis

The viability of encapsulated HAC within the hydrogel was evaluated at different time intervals using the Thiazolyl Blue staining (MTT; Sigma-Aldrich) which absorbance data correlate to cell number. Briefly, in triplicate sample culture, at each time point the medium was removed and replaced with 0.5 ml of fresh serum-free medium supplemented with 25 µl of MTT stock solution (5mg/ml). After 3 hours of incubation, the medium was colleted and the converted dye was solubilized with 1 ml absolute ethanol. Dye absorbance was measured at 570 nm with background subtraction at 670 nm [31].

2.5. Semiquantitative reverse transcriptase – polymerase chain reaction

RNA was extracted by TRIzol® Reagent (Invitrogen, Carlsbad CA, USA) procedure [32]. Conditions for semiquantitative reverse transcription – polymerase chain reaction (RT-PCR) are described in Banfi et al. [33]. The mRNAs analyzed were aggrecan, α1 (I) collagen (Collagen Type I), α 1 (II) collagen (Collagen Type II) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences were as follows in Table 1.

Table 1 - Primers list for semi quantitative PCR.

Aggrecan	Forward 5' – ATGCCCAAGACTACCAGTGG – 3'
	Reverse 5' – GTGAGCTCCGCTTCTGTAGT – 3'
Collagen Type I	Forward 5' – TCTGCGACACAAGGAGTCTG – 3'
	Reserve 5' – CGACCCACACTTCCATCACT – 3'
Collagen Type II	Forward 5' – TCTGCAACATGGAGACTAGC – 3'
	Reverse 5' – GAAGCAGACAGGCCCTATGT -3'
GAPDH	Forward 5' – CCATCTTCCAGGAGCGAGAT – 3'
	Reverse 5' – CTGCTTCACCACCTTCTTGAT – 3'

Following a 4 minute denaturation step at 95°C, the adopted reaction profiles were: aggrecan, collagen type I and GAPDH: 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 25 cycles; Collagen type II: 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 30 cycles. All PCRs ended with 8 minutes of incubation at 72°C. The values of the cycles used were in the linear range of amplification as determined previously in preliminary conduct experiments. PCR products were run on 1% agarose gels and visualized by ethidium bromide. Reaction product sizes were: aggrecan, 448 bp; collagen type I, 599 bp; collagen type II, 517 bp and GAPDH, 450 bp.

2.6. Bovine organ culture defect model

Bovine articular cartilage was harvested from knees of 18-month-old cows, washed twice with PBS, and cut in regular cube of about 1.5 x 1.5 x 0.5 cm. Cartilage defects were created on the cartilage surface by a drilling device (0.4 cm) under sterile condition. The defects were then filled with hydrogel seeded or not seeded with $2x10^6$/ml of cells. Six engineered cartilage pieces were subsequently implanted subcutaneously in immunodeficient mice (nu/nu) and the animals were sacrificed after 4, 6 and 8 weeks to recover the cartilage pieces. All animals were treated and handled according to institutional guidelines.

2.7. Histology and Immunohistochemistry

Histology samples were rinsed three times in PBS and then fixed in 4% formaldehyde in PBS for 6 hours. After fixation, the samples were dehydrated by serial immersion in ethanol solutions (70%, 90%, 95%, and 100%) and xylene. Specimens were then embedded in paraffin and 5 mm sections were cut using a Microtome (Leica RM 2165). Sections were then dewaxed and stained with Toluidine Blue (E. Merck, Darmstadt, Germany), or Alcian Blue (Sigma-Aldrich, Chemical, St. Louis, USA) to reveal cartilage matrix components.

For immunohistochemistry analysis, serial sections of paraffin embedded samples were dewaxed and treated with methanol/H_2O_2 (49/1) for 30 minutes to inhibit endogenous peroxidases. Sections were then treated with 1

mg/ml hyaluronidase in PBS pH 6.0 for 20 minutes at 37°C and washed with PBS. After incubation with goat serum for 1 h to reduce non-specific binding, the specific antibody was added and incubated for 1 hour at room temperature. Sections were washed several times in PBS and challenged with biotinylated anti mouse IgG (Jackson Laboratory Inc., West Grove, PA, USA) and peroxidase-conjugated egg-white avidin (Jackson Laboratory Inc. West Grove, PA, USA). After additional washing of the sections with PBS and 50 mM Na Acetate pH 5.0, the peroxidase activity was visualized by enzymatic modification of the 3-amino-9-ethylcarbazole substratum (3-amino-9-ethylcarbazole 0.4% in dimethylformamide: 50 mM Na Acetate, pH 5: 30% H_2O_2; 100:900:1) performed in the dark at room temperature for 15 min. Sections were counterstained with Harri`s hematoxylin and mounted with Gel mount from Biomeda Corp. (Foster City, CA, USA). Slides were observed and images acquired with the Axiovert 200M microscope (Carl Zeiss).

The specific antibodies were an anti type II collagen monoclonal antibody (CIICI anti-COLLII, DSHB University of Iowa) and anti-human Tenascin monoclonal antibody (generous gift of Dr. E. Balza, Istituto Nazionale per la Ricerca sul Cancro, Genova).

2.8. Statistical analysis

All data were analyzed by One – way ANOVA followed by a post-hoc comparison with Turkey's HDS. Differences were accepted to be statistically significant at $p<0.05$. All errors are given as standard deviations.

3. RESULTS

3.1. *In vitro* culture of articular chondrocytes seeded into the injectable hydrogel

Human articular chondrocytes were expanded up to a maximum of 5 doublings in serum free medium. This culture condition allows the proliferation of chondrocytes also derived from the elderly donors maintaining at the same time the cell chondrogenic potential needed for the therapeutic application [34].

Cells grown in monolayer were detached and resuspended at a concentration of about 4.10^6/ml in a solution containing hyaluronic acid and trombin and loaded in the syringe A, of a DUPLOJECT syringe support (included in the Tissucol Kit-Baxter). The syringe B, was loaded with the solution containing fibrinogen and k and i-carrageenan. Upon mixing the two solutions formed almost instantaneously a gel containing the cells; for each sample, the total volume of the gel was about 250 ml. Chondrocytes embedded in this gel were cultured in 1.5 ml of chondrogenic medium [28] for 3 weeks.

Cell morphology and distribution within the gel were examined by microscopy at several time points during the *in vitro* culture. Immediately after the hydrogel formation, the cells appeared round and homogeneously distributed, but during the first week of culture, they showed a tendency to form clusters (Fig. 2 a-b). After 3 weeks we observed a transition of the encapsulated chondrocytes from an aggregate status to a more homogeneous and uniform distribution (Fig. 2 d-e).

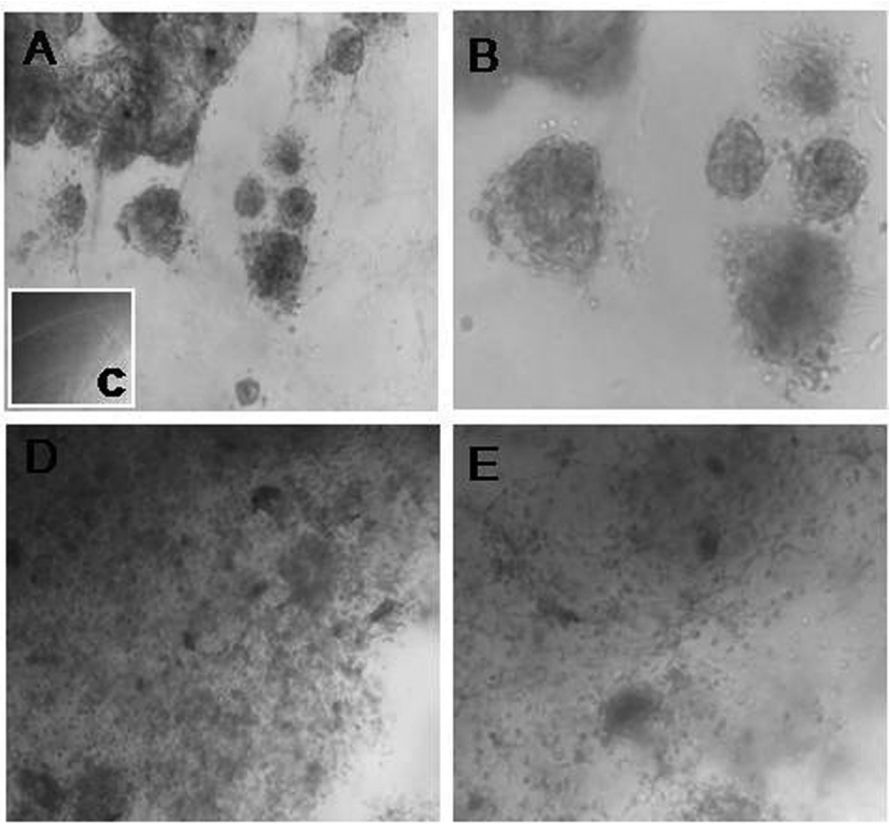

Figure 2 - Light microscopy images of the hydrogel containing the human chondrocytes after 1 week (A, B) and 3 weeks (D, E) of culture. A control hydrogel not seeded with cells after 1week of culture is shown in (C). Bar = 50 mm.

Optical sections of the specimens incubated with the Hoechst dye and subsequent 3D reconstruction allowed the identification and the visualization of the cells yielding an accurate three-dimensional distribution of these within the scaffolds (Fig. 3). A distribution over the total depth of the gel of human chondrocytes was observed both after 1 and 3 weeks of culture (Fig. 3).

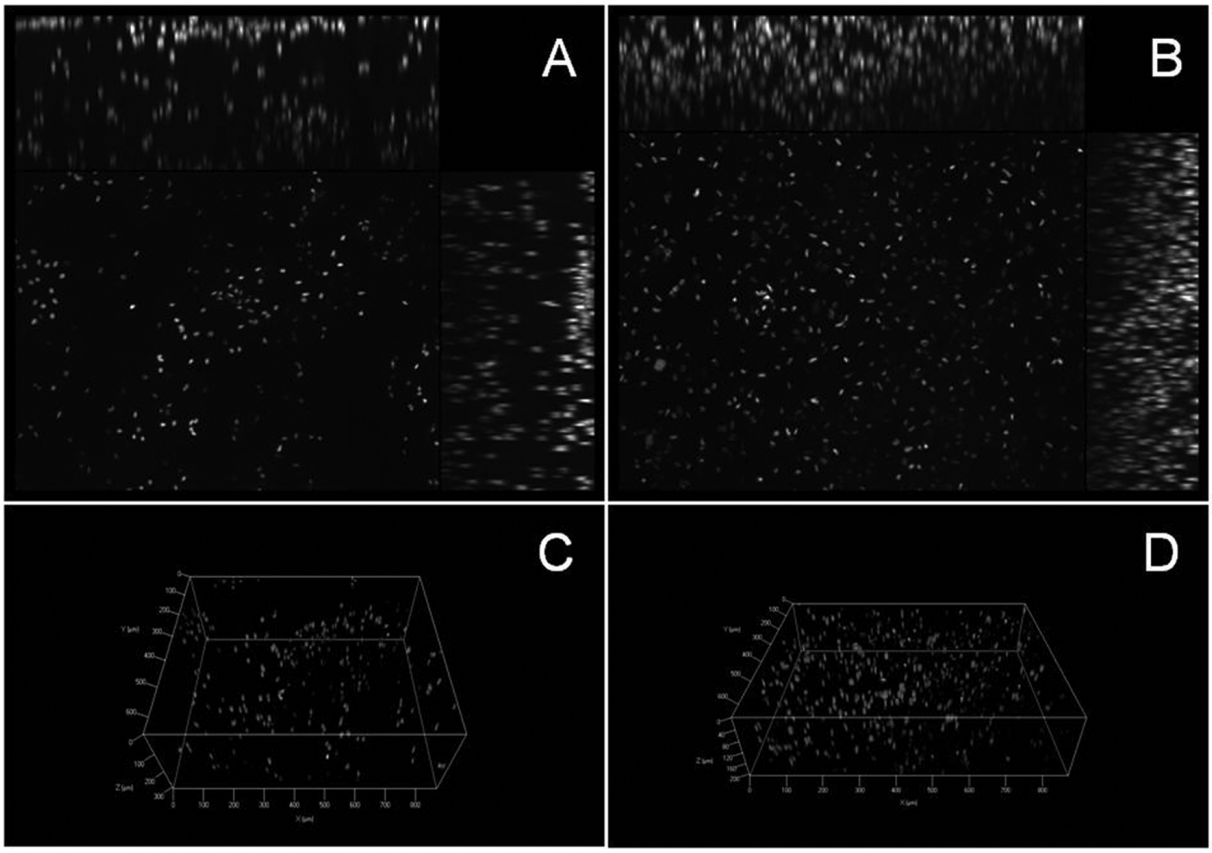

Figure 3 - Representative images of live human articular chondrocytes within the tissue engineered constructs at 1 (A, C) and 3 weeks (B, D) of culture after labeling of the cell nuclei with Hoechst and optical sectioning by structured epifluorescent illumination. Image projections along the X, Y and Z axes (A, B) and their respective 3D reconstructed stacks (C, D) are shown.

Viability of encapsulated cells within the polysaccharide biodegradable hydrogel during the 3 weeks culture was confirmed by the MTT analysis. The number of cells increased during the initial two weeks culture and remained viable over the remaining culture period (Fig. 4). These data demonstrated that human articular chondrocytes, after their expansion as a monolayer culture in serum free medium, once seeded within the biodegradable hydrogel, maintain a proliferation potential and remain viable.

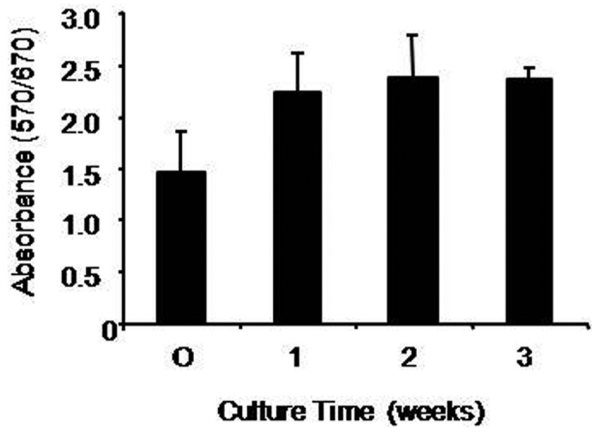

Figure 4 - Viability of human chondrocytes during the culture within the tissue engineered construct as revealed by MTT staining. Error bars represent means ± standard deviation. Statisticall analysis by ANOVA with significant differences accepted to be with $p<0.05$. T0 vs T3w ($p<0.05$); HSD (0,05) = 0,9; HSD (0,01) = 1,23; MS error = 0,1182; df error = 8 and P = 0,034.

3.2. *In vitro* cartilage formation

To test the capability of the hydrogel embedded cells to form cartilage, human articular chondrocytes expanded in monolayer, were seeded into the hydrogel and maintained for 3 weeks in chondrogenic medium. At the end of monolayer culture and before encapsulation into the hydrogel (T0), human articular chondrocytes expanded in the presence of serum free medium expressed the chondrocyte specific aggrecan and type II collagen mRNA and, as expected, also type I collagen mRNA (fig. 5). After 3 weeks of *in vitro* culture within the gel (TF), the encapsulated human articular chondrocytes maintained the expression of aggrecan and type II collagen, but stopped the expression of type I collagen.

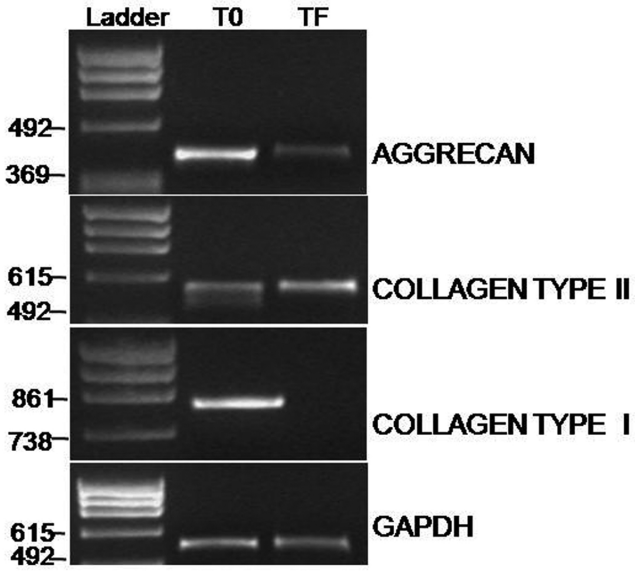

Figure 5 - Expression of chondrogenic marker genes by 3 dimensional cultured articular chondrocytes. Reverse-transcripted cDNA was amplified by PCR with specific primers for the housekeeping gene GAPDH (5698 bp); collagen type I (750 bp); collagen type II (517 bp) and aggrecan (448 bp). Ladder: DNA molecular weight markers (in base pairs) is shown on the left side; T0: expanded chondrocytes before encapsulation; TF: encapsulated chondrocytes after 3 weeks of *in vitro* culture.

As a further control, at the time of the establishment of the culture within the hydrogel an aliquot of the cells from the monolayer culture was used to establish a parallel *in vitro* micromass pellet culture [27] that was maintained in the same chondrogenic medium of the hydrogel culture. Histological analyses were performed for the hydrogel and the micromass *in vitro* culture after 3 weeks respectively.

Mucopolysaccharides and glycosaminoglycans, characteristic of the cartilage extra-cellular matrix revealed by toluidine (Fig. 6 b-d) and alcian blue (Fig. 6 a-c) were detected in micromass pellet, as described by Johnstone [28], and in hydrogel culture after 3 weeks. Noteworthy, the distribution of the cartilage matrix in the hydrogels was more homogeneous than in the micromass pellets where it was preferentially located at the peripheral regions.

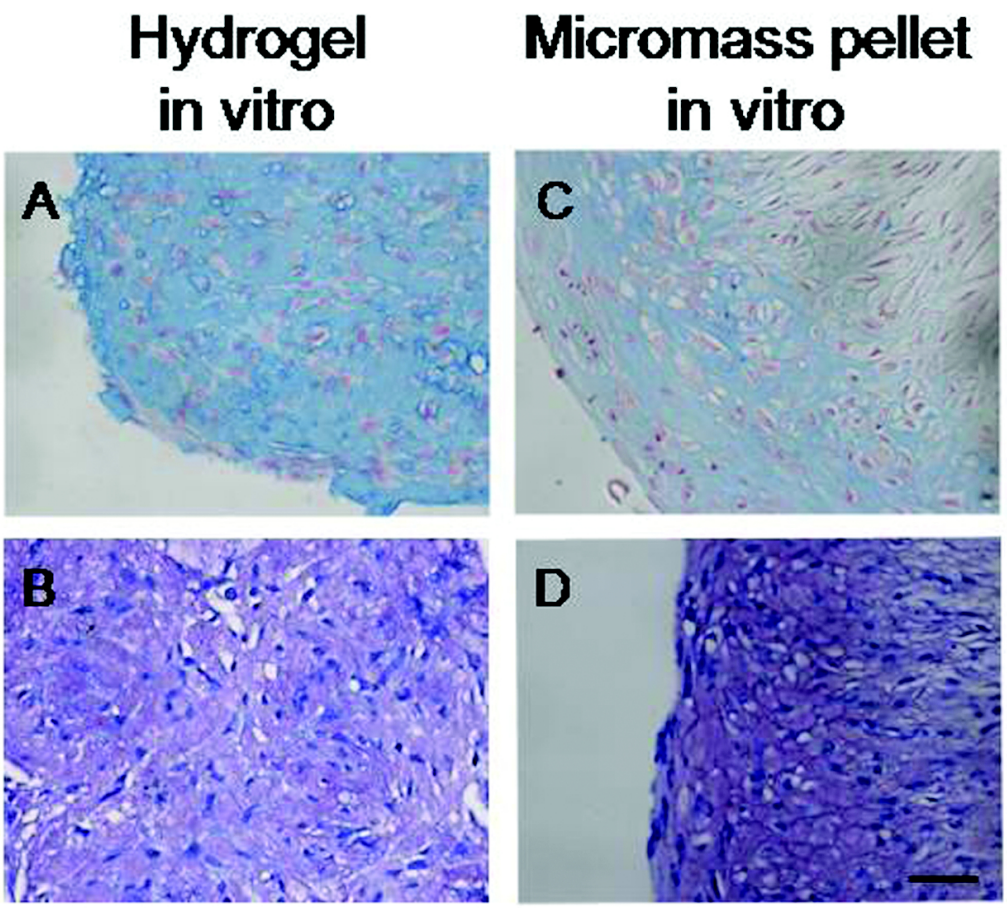

Figure 6 - Histological cross sections of the hydrogel (A, B) and micromass pellet (C, D) after 3 weeks of culture respectively. Sections were stained with alcian blue (A, C) and toluidine blue (B-D). Bar = 50 µm.

3.3. *In vivo* implantation of human articular chondrocytes-hydrogel constructs

The immunodeficient mouse model is a well established method to investigate *in vivo* engineering of cartilage and bone [5, 23, 35]. Nevertheless, in the case of the cartilage engineering, one of the major limit of this method is the lack of an appropriate articular cartilage surrounding the newly formed tissue and therefore the impossibility to investigate the integration of the newly formed cartilage with the pre-existing one. We used a organ culture model recently described [23, 24] in which the hydrogel including encapsulated HAC was directly injected into the defect created on the surface of the bovine articular cartilage layer (Fig. 7).

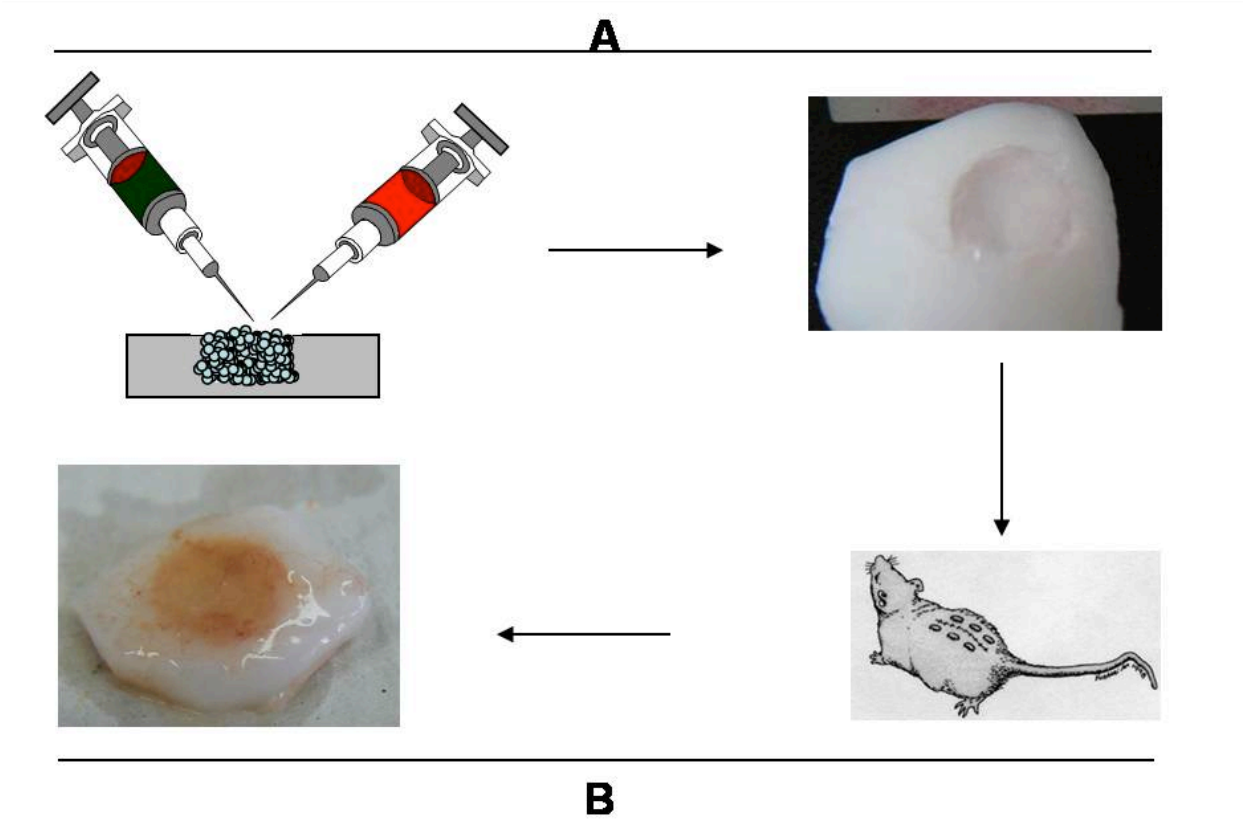

Figure 7 - Contained defect organ culture model: Bovine cartilage defects filled with hydrogel containing human articular chondrocytes (A); Organ construct model implanted for 6 weeks in an immunodeficient mouse (B).

The cartilage biopsy with the hydrogel was maintained in culture in chondrogenic medium for 1 week then implanted subcutaneously in the immunodeficient mouse. Histological analysis was performed on cross sections of the recovered samples after 4, 6 and 8 weeks of implantation (Fig. 8).

Macroscopic inspection of the retrieved implanted construct showed a smooth surface and a complete integration of the HAC-hydrogel with the surrounding cartilage (Fig. 8).

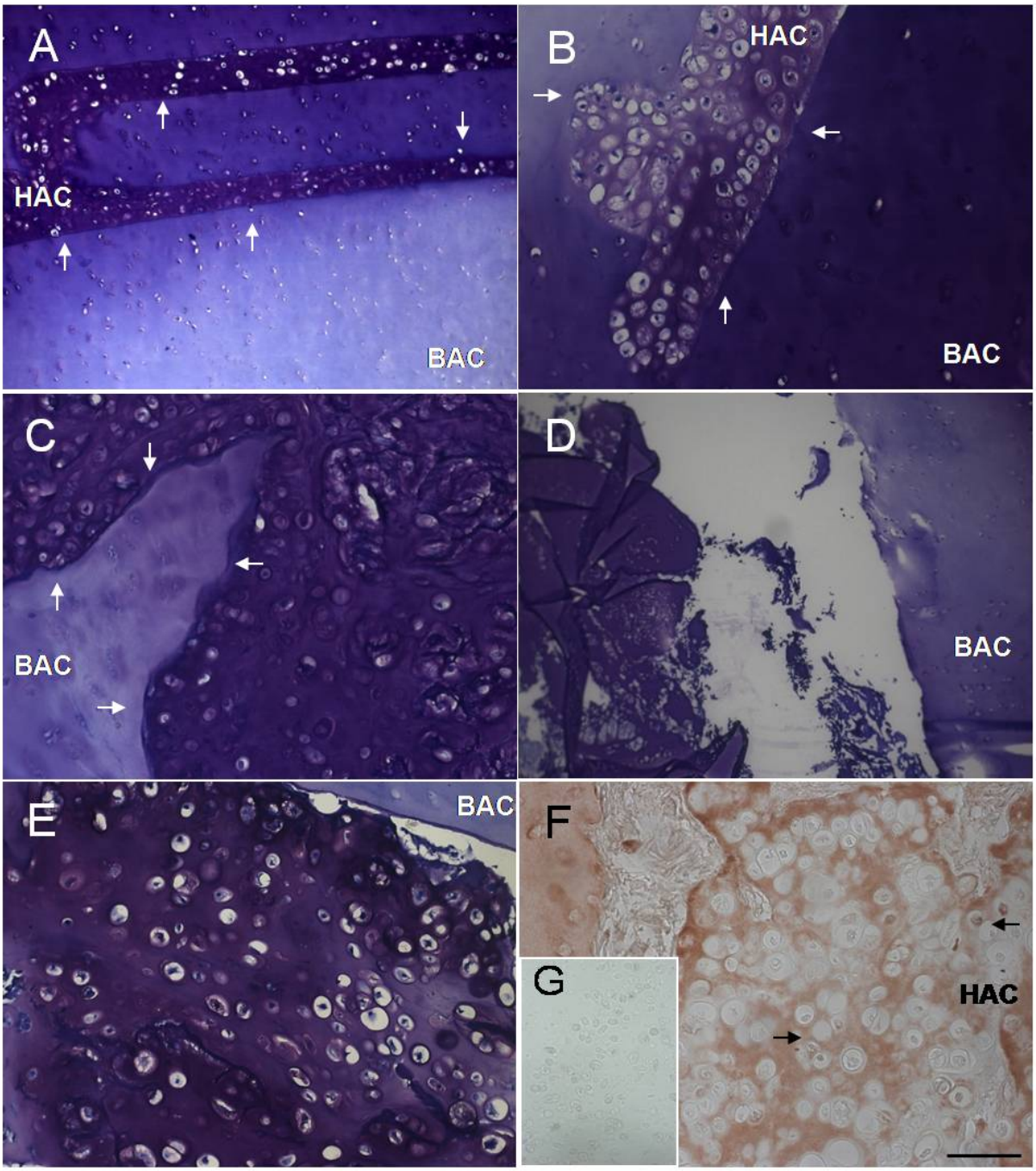

Figure 8 - Histological cross sections of the hydrogel within the bovine cartilage gap after 6 weeks from the implantation in immunodeficient mouse. Toluidine blue staining (A-E); Immunohistochemical staining showing localization of type II collagen (F), negative control, without primary antibody (G); control sample not seeded with cells (D). Bovine Articular Cartilage (BAC); Human Articular Cartilage (HAC). Bar = 50μm. Arrows indicated the newly human cartilage deposition and a good integration of the tissue.

The histological examination of the sample revealed the repair of the bovine cartilage defect by a cartilage tissue. The newly formed cartilage showed matrix deposition and uniform cellularity, it was clearly stained by toluidine blue (Fig. 8 a-c, e), and was positive for the presence of type II collagen (Fig. 8 f). Moreover, at the tissue

interface a good integration and grip between the newly formed human cartilage and the surrounding bovine cartilage was observed. In a control biopsy treated with the hydrogel without cells, we failed to detect a newly formed organized cartilage, and an integration of the tissue filling the gap with the bovine cartilage (Fig. 8 d). The origin of the neoformed cartilage was confirmed by immunolocalization of the human tenascin taking advantage of a specific monoclonal antibody which stained the human chondrocytes in the neoformed tissue and not those in the surrounding bovine cartilage (Fig. 9 a, b).

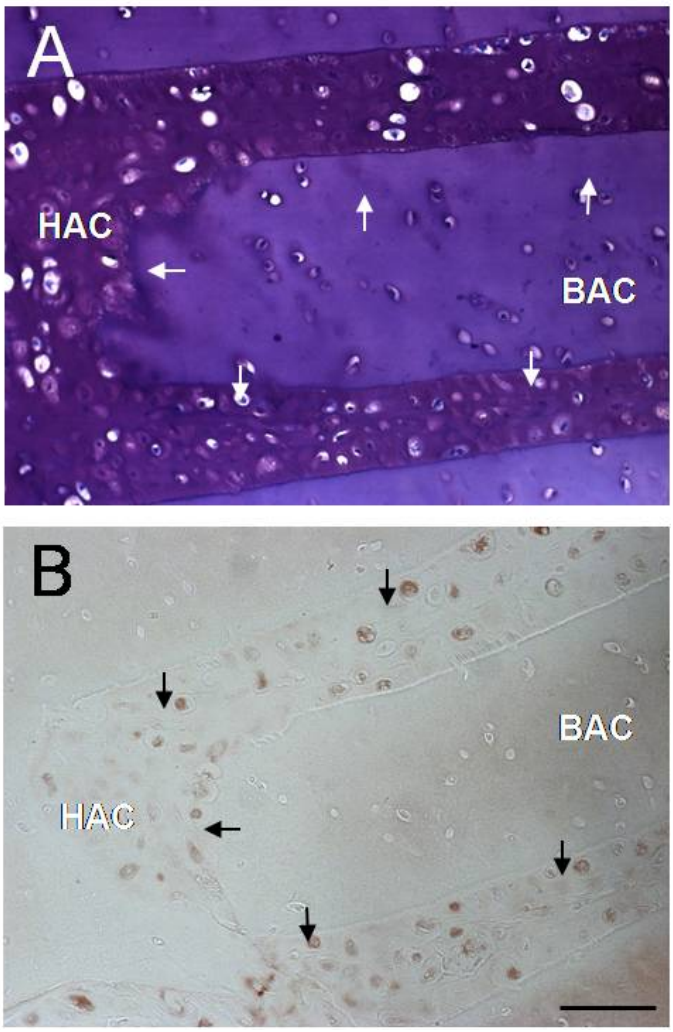

Figure 9 - Histological cross sections of the hydrogel within the bovine cartilage gap after 6 weeks from the implantation in immunodeficient mouse. The neo-cartilage formed shows a strong staining with toluidine blue (A). The specific anti human tenascin antibody recognizes only chondrocytes in the newly cartilage formed in the gap defect (B). Bovine Articular Cartilage (BAC); Human Articular Cartilage (HAC). Bar = 50μm. White arrows shown the area of integration of human articular cartilage in the bovine tissue. Black arrows show the human chondrocyte cells.

4. DISCUSSION

Cartilage tissue engineering is an emerging approach for the regeneration of cartilage tissue damaged due to disease or trauma. Since cartilage lacks spontaneous regenerative capabilities, it is essential to develop methodologies for the delivery of appropriate cells, biomaterials and factors to the defect site. The Articular Chondrocyte Transplantation technique [13, 14] is a commonly used method for cartilage repair, requiring a first arthroscopic intervention to harvest the healthy cartilage sample from which chondrocytes are isolated and expanded *in vitro*. With a second arthrotomy intervention, these cells are then delivered to the cartilage defect site under a periosteum or a synthetic membrane flap.

Goal of this study was to determine the possibility of delivering the human articular chondrocytes by encapsulating them in a novel easy to handle, biodegradable hydrogel, with *in situ* gelation properties and not affecting the cell chondrogenic differentiation. We developed a suitable injectable biomaterial obtained by the mixture of hyaluronic acid, fibrinogen, thrombin and carrageenan.

Carrageenans are heteropolysaccharides structurally related to agar [26]. They are extracted from red algae and come in three types k, i and l that can form upon cooling a variety of gel. The rationale of using a carrageenan hydrogel over gels made of synthetic polymers or other polysaccharides, was mainly-based on the ability of these polysaccharides to form a gel at conditions that allow the direct encapsulation of mammalian cells. These polysaccharides are water soluble when heated and gel upon cooling the solution in presence of electrolytes (Ca^{2+} and K^{+}) [9]. In the conditions described in our work we were able to mix the cell suspension with the polysaccharide solution and then form a gel at temperature ranging between 36 and 40° C with retention of cell viability. K and i carrageenan are commonly used to produce pellets, tablets or beads for drug delivery [25, 26, 36].

Hyaluronic acid (HA) is a major constituent of the cartilage extracellular matrix. Its biological properties and its rapid degradation, makes HA an ideal candidate for the development of new biomaterials [37, 38]. Fibrinogen/fibrin was selected because of its adhesive properties. Moreover, various studies reported the use of fibrin gel as injectable scaffold for generating new cartilage matrix [9, 11].

We performed preliminary studies with gels composed of only HA and fibrin. When the articular chondrocytes or other types of cells were encapsulated in this gel, within few days, we observed a drastic shrinkage of the scaffold. Indeed, these unpublished observations are in agreement with recently published data [11]. Therefore, to improve the quality of the hydrogel, we introduced carrageenan in the HA-fibrin gel. With this new gel composition, we observed no shrinking effects, on the contrary the gel presented a good elasticity as well as a better rigidity.

Chondrocytes suspended in a three-dimensional matrix, similar to their natural environment, retain their native phenotype and organize a cartilage extracellular matrix. Hydrogel scaffolds appear to satisfy this requirement. We encapsulated human articular chondrocytes into this novel polysaccharide/fibrin hydrogel and cultured them *in vitro* for 3 weeks. Our data indicate that this injectable scaffold sustains an initial cell growth without compromising the cell chondrogenic potential. The growth and the distribution of the cells within the hydrogel were qualitatively confirmed via images obtained by histology, bright field and epifluorescent light microscopy. In particular, structured epifluorescent illumination and optical sectioning allowed the acquisition of images through most of the hydrogel thickness without the need of any manipulation such as the dehydration required for histology that may introduce artifacts. This analysis revealed a uniform distribution of viable human articular chondrocytes throughout the hydrogel up to 3 weeks of culture.

After an encapsulation period of 3 weeks into the tissue engineering construct, cells not only maintained the expression of proteins characteristic of the chondrogenic lineage, but also down regulated the expression of type I collagen characteristically expressed by dedifferentiated articular chondrocytes and by articular chondrocytes expanded in monolayer culture in serum free medium.

We are aware that chondrocytes harvested from osteoarthritic patients are not the best source of cells for cartilage regeneration. Chondrocytes harvested from healthy tissue or other cell populations such as mesenchymal stem cells may have led to even better results.

To provide a definitive assessment concerning the suitability of a new biomaterial for cartilage tissue engineering, *in vivo* models are required. The “conventional” immunodeficient mouse model can support cartilage formation, but fails in investigating integration of the newly formed cartilage within a pre-existing cartilage. Therefore, in our

study we used a contained defect organ culture model, recently described by Mueller-Rath et al. [23] and Sculler et al. [24]. This experimental model mimics a real clinical intra-articular situation where the repair tissue is having direct contact with the surrounding cartilage. Seen the difficult to harvest human articular cartilage, we have been forced to used two different species to test the capability of human articular chondrocytes expanded *in vitro* to regenerate tissue in a bovine cartilage lesion.

Using this approach, we observed a good integration between the human cartilaginous tissue formed in the articular cartilage defect and the surrounding bovine cartilage after 6 weeks from the implantation.

5. CONCLUSIONS

In this study, we demonstrated the suitability for cartilage engineering of a newly developed injectable gel. Within this novel hydrogel the encapsulated human articular chondrocytes remained viable, proliferated, maintained the expression of the typical chondrogenic marker genes and deposited a cartilage extra cellular matrix. Taking advantage of a new *in vivo* model, we showed the integration of the newly formed cartilage with the surrounding pre-existing cartilage. We propose that the novel hydrogel we developed may be used with success to deliver by arthroscopy injection the *ex vivo* expanded articular chondrocytes to the site of the articular cartilage lesion and therefore to repair the articular cartilage damage with a minimally invasive technique.

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SECTION 4

CONCLUSIONS AND OUTLOOK

CHAPTER VIII

GENERAL CONCUSIONS AND FINAL REMARKS

Cartilage Tissue Engineering and Cell Therapies offer, nowadays, new means of treatment for quite a few cartilaginous pathological conditions. Still, the limitations of the current strategies of cartilage tissue engineering demonstrate the need of more exhaustive understanding and characterization of cell-cell and cell-extracelluar matrix mechanisms of interaction. In truth, this fact makes the research challenge continuously stimulating and appealing.

In Chapter III of this thesis it is provided the proof-of principle that hWJSCs in 2D monolayer with a non-direct co-culture system with articular human chondrocytes can be a valid model to commit a stem cell source for a possible strategy in cartilage tissue engineering using human pluripotent stem cells.

During the expansion phase, hWJSCs co-cultured with human adult articular chondocytes maintained their proliferation ability with an up-regulation expression of cartilage specific genes.

Histological and immunohistochemical analysis of micromass pellets confirmed a remarkable positive staining for glycosaminoglycans and collagen type II in those expanded with chondrocytes. Results indicated that co-culture of hACs with hWJSCs enhanced the chondrogenic phenotype commitment of hWJSCs. These results corroborate the validity of the study hypothesis. Although promising results were obtained during *in vitro* expansion using the micromass pellet model, studies *in vivo* should confirm these findings and demonstrate that the proposed hypothesis is sufficient to induce a stable phenotype. Human wharton's jelly cells should also be seeded onto 3D structures used in clinical practice and further implanted. The *in vivo* evaluation of cartilage tissue formation using 3D cell/constructs subcutaneously implanted in the back of nude mice should give clear evidences of the positive effects of our co-culture system approach.

The international scientific community has an increasing interest of platelet – rich plasma derivates effect in cartilage tissue. The chapter IV aimed to understand the real outcome of platelet plasma in primary adult articular chondrocytes. This chapter provides evidences that human articular chondrocytes culture with platelet lysate as media supplementation present higher values of growth rate over time compared to those in "normal standard" culture conditions. Even with a fast cellular growth kinetics cellular phenotype memoir as it was

demonstrated by *in vitro* and *in vivo* chondrogenic differentiation assays. Under simulated inflammatory environment, platelet lysate initially lead to an increased pro inflammatory cytokines secretion by human articular chondrocytes that gave raise to an over activation of NF-kB pathway. This pro-inflammatory effect vanished overtime as demonstrated by COX2 decrease of expression with simultaneous reduction of NF-kB activation values. We also observed a strong chemotactic effect of platelet lysate on articular chondrocytes, which could lead to possible selective host cell recruitment in the lesion site. These data clearly demonstrated that platelet lysate triggers chondrocyte 2D resolution of inflammation with enhancement of cell mobility. Future studies should be performed using human articular chondrocytes to better understand and to recognize which specific growth factors contained in the platelet-rich plasma derivate are responsible for the aforementioned data. These findings could also explain the beneficial effects of the platelet lysate in the current treatment of cartilage lesions.

In chapter V we aimed to produce hyaluronic acid microparticles and to study their effect on human articular chondrocytes. The developed HA microparticles presented regular and smooth shape with robust behavior in aqueous environment. The direct and non-direct contact conditions of HA particles with primary hAC were clearly non-cytotoxic as confirmed by the increase cellular viability as a function of time. Cellular viability results were in agreement with hAC proliferation data showing an increasing trend over time for both culture conditions. HA particles in direct contact with hAC have a clear positive effect on their chondrogenic phenotype, as revealed by a significant increase on the expression of some chondrogenic gene markers. Transcript levels of these genes were significantly higher than those observed in the absence of HA particles, suggesting a possible chondrogenic "intrinsic protective" potential of HA microparticles in hAC. While further studies are needed, these findings indicate that the developed HA microparticles have a strong potential as a candidate for intra-articular injectable vehicles in knee cartilage regenerative therapies.

In the chapter VI, nano complexes were formed by polyelectrolyte interactions between the polyanion polysaccharide hyaluronic acid and polycation poly-L-lysine. We have found that the aggregation behaviour depends on the solution ionic strength (sodium chloride concentration), polyelectrolyte chain size and mixture conditions. The interactions between HA and PLL were thoroughly screened by using different molecular weight and polymer size ratios, other salts, pH, temperature and mixing conditions. Future studies would be useful to

study the effect of different polymer concentration on nano complexes size, stability and final charge with the main aim of establishing the best proportions to produce nano carriers. The nano polyelectrolyte complexes of HA and PLL with specific size and charge could be used as potential carriers for pharmacological bioactive agents to be delivered locally in inflammatory articular cartilage site.

The last chapter of this thesis describes the development of a novel injectable hydrogel. This hydrogel can be prepared and mixed with cells/drugs using a very simple and fast technique with few reagents. *In vitro* experiments with human articular chondrocytes showed that encapsulated cells within the hydrogel maintained their proliferative capacity and mitochondrial activity as well as the maintenance of their typical "chondrocyte like" round shape revealed by clusters formation. More over, *in vitro* results demonstrated that cells within the hydrogel kept their somatic phenotype as demonstrated by expression of cartilage genes and transcription factors. To prove the efficacy of your system, an organ culture model was performed with bovine articular cartilage where a defect was created. Hydrogels combined with cells were injected within the induced lesion site and the entire model was implanted subcutaneously in nude mice being the results very promising regarding the formation of a functional *de nov*o cartilage tissue. It was proved by immunodetection that the neo-cartilage formed tissue had human origin which reinforced the functionally of our hydrogel. To mimic the clinical scenario, future studies with New Zealand rabbits could be performed.

In summary, the results of this thesis show the accomplishment of new evidences in the role of biochemical factors and extracellular matrix environment during chondrocyte culture that can augment knowledge in articular cartilage repair strategies.

In conclusion, it is therefore beyond doubt that the essential assignment of researchers in this field is to make more efforts to bring together: *i)* experimental results of cell optimized culture conditions, *ii)* improvements of using novel deliverable strategies (biomaterials and bioactive agents); and *iii)* availability of surgical techniques and analysis resources.

These events will place the research community close to the summit. This will overcome the current difficulties and will allow the enhancement of the short and long-term outcomes in cartilage tissue engineering.