Sanjurjo-Rodríguez, et al. J Regen Med 2014, 3:2
http://dx.doi.org/10.4172/2325-9620.1000116

Journal of Regenerative Medicine

Research Article A SCITECHNOL JOURNAL

Human Cartilage Tissue Engineering Using Type I Collagen/Heparan Sulfate Scaffolds

C. Sanjurjo-Rodríguez[1], A.H. Martínez-Sánchez[2], T. Hermida-Gómez[2], I. Fuentes-Boquete[1], S. Díaz Prado[1] and F.J. Blanco[3]

Abstract

Introduction: Cartilage engineering may provide a promising alternative solution to current methods of cartilage repair. The aim of this study was to evaluate the suitability of type I collagen (Col I) scaffolds with and without heparan sulfate (HS) to support attachment, proliferation and chondrogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs).

Materials and Methods: hBMSCs were cultured in Col I, Col I+1%HS, Col I+2%HS and Col I+3%HS scaffolds in both chondrogenic and non-differentiation media for 15 and 30 days. The resulting neo-tissues were analyzed using histochemistry, immunohistochemistry, electron microscopy (EM) and molecular biology. Collagen released into the media by the constructs was also measured by dye-binding assays.

Results: Our results showed successful growth and proliferation of hBMSCs on all scaffolds analyzed. Better results were obtained in chondrogenic cultures after 30 days, in which we observed oval/rounded cells along the scaffolds, and extracellular matrix (ECM) by EM; this ECM was strongly positive for proteoglycan (PG) safranin O staining and staining for type II (Col II). Greater total collagen release was found in supernatants of chondrogenic cultures than in controls. The best results for all analyses were found in scaffolds including HS.

Conclusions: We conclude that the Col I and HS scaffolds used in this study are suitable supports for hBMSCs to differentiate toward chondrocyte-like cells in chondrogenic medium. We observe that the addition of HS to Col I scaffolds improves the chondrogenic phenotype of the cells, with Col I+3%HS being the best scaffold.

Keywords

Cartilage tissue engineering; Scaffold; Mesenchymal Stem Cells (MSCs); Collagen

Abbreviations

3-D: Three-Dimensional; 20%DMEM: Dulbecco´s Modified Eagle´s Medium and 20% of foetal bovine serum; Agg: Aggrecan; ALP: Alkaline Phosphatase; APM1: Adipose most abundant gene transcript 1; cDNA: Complementary Deoxyribonucleic Acid; Col: Collagen; Col I: Type I collagen; Col II: Type II collagen; Col X: Type X collagen; DMEM: Dulbecco´s Modified Eagle´s Medium; DSC: Differential Scanning Calorimetry; ECM: Extracellular Matrix; EM: Electron Microscopy; FABP4: Fatty Acid Binding Protein 4; FBS: Foetal Bovine Serum; GAGs: Glycosaminoglycans; hBMSCs: Human Bone Marrow Mesenchymal Stem Cells; HE: Hematoxilin-Eosin; HS: Heparan Sulfate; LPL: Lipoprotein Lipase; MMP13: Matrix Metalloproteinase-13; MMT: Modified Masson´s Trichrome; MSCs: Mesenchymal Stem Cells; OA: Osteoarthritis; OP: Secreted phosphoprotein 1; PCNA: Proliferating Cell Nuclear Antigen; PG: Proteoglycan; PTHrP: Parathyroid hormone-related protein; qPCR: Real time quantitative polymerase chain reaction; REL: Gene Relative Expression Levels; RT-PCR: Reverse Transcription Polymerase Chain Reaction; S: Number of passage; SE: Standard Error; SEM: Scanning Electron Microscopy; SO: Safranin O; SOX9: [SRY (Sex Determining Region Y)-box9]; TBP: TATA Binding Protein; TEM: Transmission Electron Microscopy; TGFβ: Transforming Growth Factor β; VK: Von Kossa

Introduction

The self-regeneration capability of hyaline cartilage is very limited, due to its avascular nature and lack of innervation [1]. Other factors that contribute to the weak capability for self-repair of this cartilage are low metabolic activity, low cell number, and the inability of chondrocytes to migrate because of their extracellular matrix (ECM) [2,3].Therefore, most cartilage lesions do not heal spontaneously and may predispose the joint to subsequent development of secondary osteoarthritis (OA) [4].

OA is a degenerative joint disease characterized by deterioration of the integrity of the hyaline cartilage and subchondral bone[5], due to interactions among many factors. There are currently no satisfactory treatments for OA [6]. The available methods to treat OA target the elimination of the pain and inflammation produced during the pathologic process [7]. To date, none of the treatments have achieved hyaline cartilage regeneration, although a fibro cartilaginous tissue has been induced [3] that differs from native joint cartilage in structure and functionality. The course of the disease may finally make it necessary to replace the damaged zone with prosthesis [2]. To avoid joint replacement by surgery, in recent years cell therapy and tissue engineering have been suggested as alternative clinical approaches.

Tissue engineering is a multidisciplinary field integrating engineering and life science, with the goal of developing biological substitutes that restore, maintain or improve the function of damaged tissues having a limited capability for self-repair. To achieve tissue regeneration, three approaches have been studied individually or in combination: cell therapy, induction factors, and scaffolds or biomaterials [8].

***Corresponding author:** Silvia Díaz Prado, PhD Bs. Grupo de Terapia Celular y Medicina Regenerativa, Instituto de Investigación Biomédica de A Coruña (INIBIC), Complexo Hospitalario Universitario de A Coruña (CHUAC), SERGAS, Departamento de Medicina, Facultade de Ciencias da Saúde, Universidade da Coruña, Campus de A Coruña, 15071 A Coruña, Spain. Tel: 34-981 176399; Fax: 34-981 176398; E-mail: s.diaz1@udc.es

Francisco J Blanco, PhD MD. Grupo de Bioingeniería Tisular y Terapia Celular (GBTTC-CHUAC), Servicio de Reumatología, Instituto de Investigación Biomédica de A Coruña (INIBIC), Complexo Hospitalario Universitario de A Coruña (CHUAC), SERGAS, Departamento de Medicina. Universidade de Santiago de Compostela C/As Xubias S/N, 15006 A Coruña, Spain, Tel: 34-981 176399; Fax: 34-981 176398; E-mail: fblagar@sergas.es

Received: July 20, 2014 **Accepted:** October 10, 2014 **Published:** October 16, 2014

The use of chondrocytes in tissue engineering has disadvantages, including low availability, dedifferentiation when cultured *in vitro*, and a limited proliferative capability, making their use unsuitable for treating OA [9]. Because of their properties, mesenchymal stem cells (MSCs) have been suggested as a promising alternative to chondrocytes for the treatment of cartilage defects [10,11]. *In vitro* chondrogenesis of MSCs requires growth factors, and cell-cell and cell-matrix interactions [12]. Members of the transforming growth factor β (TGFβ) superfamily play a major role in cartilage development and repair, enhancing chondrocyte proliferation and increasing ECM synthesis. Further, TGFβ-1 and -3 promote chondrogenesis of MSCs [13].

Scaffolds are natural or synthetic temporary substrates which should provide a suitable three-dimensional (3D) structure to support cell viability and proliferation, allow cellular differentiation, and maintain a specific phenotype [8,14].

Collagen (Col) is a natural biodegradable material that can be reconstituted into fibrous structures simulating native ECM in tissues [15]. Even though we might expect type II Col (Col II), the main protein in the cartilage ECM, to be the most appropriate compound for scaffolds to be used in cartilage tissue engineering, Col I is immunologically less reactive than Col II. Col I have demonstrated competence for supporting cartilage regeneration [16] and allow the growth of chondrocytes with the correct hyaline phenotype [2]. However, to improve cell viability, Col biomaterials must be supplemented with other compounds [15]. On the other hand, heparan sulfate (HS) and proteoglycans (PGs) are known to be involved in chondrocyte differentiation by means of interactions with numerous chondroregulatory molecules [17].

In this study, we used Col I without HS and Col I scaffolds supplemented with HS to establish that they enable human bone marrow MSCs (hBMSCs) to differentiate towards chondrocytes and form chondrogenic constructs useful for cartilage tissue engineering. This study provides information about the potential efficacy of these scaffolds to allow cell transportation to the interior of damaged tissue to enhance regeneration.

Materials and Methods

Isolation and culture of hBMSCs

Bone marrow samples used to isolate hBMSCs were obtained from 12 patients (8 females and 4 males, mean age 76.58 ± 7.97) undergoing total hip replacement due to OA. Samples were provided by the Rheumatology Service at Complexo Hospitalario Universitario de A Coruña (CHUAC). The donors were not selected and the samples submitted were processed as they arrived at the laboratory. This study was approved by the Ethics Committee of Clinical Investigation of Galicia (Spain) and each donor in the study gave informed consent according to the guidelines of the local ethics committee.

hBMSCs were extracted by washing the bone marrow with Dulbecco´s Modified Eagle´s Medium (DMEM; Lonza, Barcelona, Spain) supplemented with 20% foetal bovine serum (FBS; LabClinics, Barcelona, Spain) (20%DMEM). Isolated cells were cultured in a 5% humidified CO_2 atmosphere at 37 °C. The resultant cell suspension was subjected to a pre-plating technique [18]. When the cells became 80% confluent at the fourth or fifth passage (S4-S5) a pool of hBMSCs from seven donors was used for both, phenotypic characterization by flow cytometry and multipotent differentiation, as previously described by Díaz-Prado et al. [19]. Characterization was made to confirm the identity of the cells before seeding on the scaffolds, following the International Society for Cellular Therapy standards [20]. hBMSCs from the other 5 donors were used as a pool to seed on the scaffolds.

A pool of hBMSCs (7 donors) was differentiated toward chondrocyte, adipocyte and osteoblast lineages for 21 days using commercial media (hMSC commercial chondrogenic differentiation medium, Bullekit adipogenic differentiation medium and Bullekit osteogenic medium, Lonza). Differentiation to the three lineages was compared with a negative control of cells cultured in 20%DMEM. All differentiations were done in duplicate. Adipogenic, osteogenic and chondrogenic differentiation were evaluated by histochemistry, immunohistochemistry and molecular biology (Supplementary Figures 1 and 2).

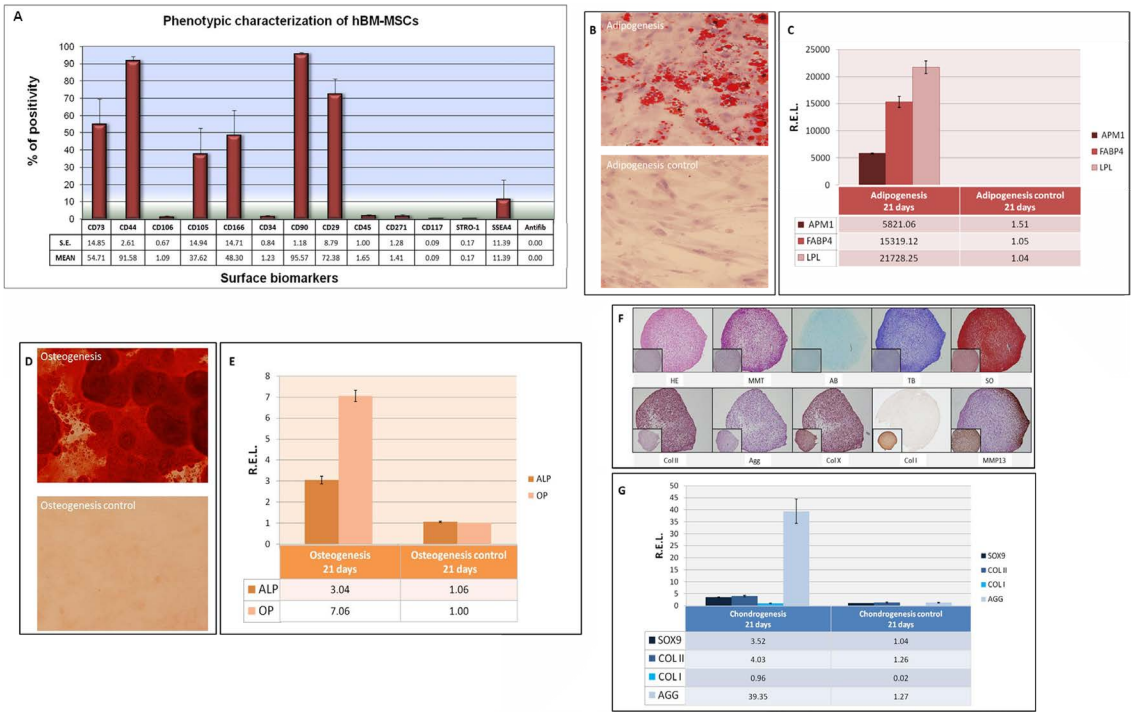
Characteristics of scaffolds

Four different scaffolds with a 1 cm^2 surface, sterilized with γ rays at 25 kGy, were employed. They were composed of Col I from horse tendon mixed with different concentrations of HS. Col I and HS were both prepared by Opocrin S.p.A., (Corlo di Formigine, Modena, Italy). HS concentrations were 0%, 1%, 2%, and 3% in the four scaffolds. Characterization of HS batches used showed 100% purity, a low molar ratio of sulfate ions to carboxylate ions (0.77), high molecular weight (25 kD) and very low anticoagulant activity (<1 APTTU/mg): this last parameter assures lack of hemorrhagic adverse effects. The porosity of the scaffolds increased from 148.3 ± 57.9 μm (in Col I without HS) to 406.9 ± 155.2 μm (in Col I +3%HS).

The behavior of the scaffolds as a function of temperature was evaluated by differential scanning calorimetry (DSC). As described by Mentink et al. [21], when Col in the hydrated state is heated, the crystalline triple helix of the Col is transformed into an amorphous random coil, resulting in shrinkage of the Col. As previously reported [22], *Tonset* (the intersection point between the baseline and the linear section of the ascending endothermic curve) can be considered as a representative parameter of the denaturation temperature of the polymer. Scaffolds using Col I displayed a denaturation temperature near 45.3 ± 0.3 °C. DSC analysis demonstrated that both the presence of HS and its concentration affected the thermal behavior of scaffolds. The Col denaturation temperature of Col I +3% HS increased over 50 °C, indicating higher thermal stability of this scaffold, while a percentage of HS lower than 2% did not notably affect the Col thermal transition [Characterization data supplied by OPOCRIN SpA and by Dr. Barbara Ruozi (TEFARTI group, Life Sciences Dept., University of Modena and Reggio Emilia, Italy)].

Cell culture on the scaffolds

A pool of hBMSCs (5 donors) was seeded on the scaffolds (2×10^5cells/cm^2) and cultured in normoxia conditions (5% humidified CO_2 atmosphere at 37 °C). Chondrogenic differentiation was induced by culturing the hBMSCs in the chondrogenic medium: hMSC Commercial chondrogenic differentiation medium (Lonza), with 10 ng/ml of TGFβ-3 (ProSpec-Tany Technogene Ltd, Rehovot, Israel) and 100 nM of parathyroid hormone-related protein (PTHrP) (provided by P. Esbrit from Fundación Jiménez Díaz). As a negative control for non-differentiation, hBMSCs were cultured in 20%DMEM with only PTHrP. In addition, each type of scaffold was cultured without cells in chondrogenic medium, to serve as negative controls for further Col assays and EM analyses. All scaffolds were seeded in triplicate for the different mediums and times of culture.



Supplementary Figure 1

NAME	FORWARD PRIMER	REVERSE PRIMER	G/C CONTENT		AMPLICON
			Forward primer	Reverse primer	
SOX9	5´gtacccgcacttgcacaac 3´	5´tcgctctcgttcagaagtctc 3´	58%	52%	72 bp
COL2A1	5´gtgtcagggccaggatgt 3´	5´tcccagtgtcacagacacagat 3´	61%	50%	116 bp
AGG	5´gcctacgaagcaggctatga-3´	5'-gcacgccataggtcctga-3'	55%	61%	136 bp
FABP4	5´ggatgataaactggtggtgga 3´	5´cacagaatgttgtagagttcaatgc 3´	48%	40%	125 bp
APM1	5´ggtgagaaaggagatccaggt 3´	5´tgctgagcggtatacataggc 3´	52%	52%	147 bp
LPL	5´agaacatcccattcactctgc 3´	5´ccatttgagcttcaacatgagt 3´	48%	41%	107 bp
ALP	5´gacggacccgtcactctc 3´	5´gtgcccgtggtcaattct 3´	67%	56%	109 bp
OP	5´cgcagacctgacatccagt 3´	5´ggctgtcccaatcagaagg 3´	58%	58%	136 bp
TBP	5´gcccatagtgatctttgcagt 3´	5´cgctggaactcgtctcacta 3´	48%	55%	142 bp

Supplementary Figure 2

Culture medium was changed three times a week, and supernatants were analyzed to measure Col released to the medium by the cells.

After 15 and 30 days of culture, each scaffold sample was subjected to three analyses to assess the chondrogenic differentiation: histological and immunohistochemical techniques, EM studies, molecular biology assays and measuring Col in the culture supernatants.

Histological analysis of constructs

For histochemical and immunohistochemical evaluation, scaffolds were fixed and embedded in paraffin after 15 and 30 days of culture. We separated the analyses of the intensity of staining due to the scaffold from that of the ECM and only took into consideration the positivity due to the ECM.

For cell morphological evaluation, hematoxylin-eosin (HE) and modified Masson's Trichrome (MMT) stains were used. To detect sulfated glycosaminoglycans (GAGs) in the ECM, scaffold sections were stained with safranin O (SO). Von Kossa staining (VK) was used to detect ECM calcification.

Immunohistochemical analyses were performed by incubating deparaffinized sections with primary antibodies to detect the presence of Col I (monoclonal clone), Col II (monoclonal clone), Col X (monoclonal clone), aggrecan (Agg) (monoclonal clone), matrix metalloproteinase-13 (MMP13) (monoclonal clone) (all from Thermo Fisher Scientific, Madrid, Spain) and proliferating cell nuclear antigen (PCNA) (monoclonal clone) (Calbiochem, Madrid, Spain). The peroxidase/DAB ChemMateTM DAKO EnVision™ kit (Dako, Barcelona, Spain) was used to determine antigen-antibody interaction.

Histological and immunohistochemical stainings were evaluated using analiSIS® software (version D; Olympus, Germany). Staining intensity and percentage of cells were measured in four different areas of each sample to obtain an average value.

Results were expressed semiquantitatively according to staining intensity: negative or absent (– or 0), weak (+ or 1), moderate (++ or 2) and strong (+++ or 3). In scoring staining intensity, 3 or +++ was assigned to those analyzed samples with metachromasia or higher stain intensity.

The percentage of cells in the scaffold area studied was grouped as 1-24% (scored as 1), 25-49% (scored as 2), 50-74% (scored as 3) and 75-100% (scored as 4). PCNA was also expressed as a percentage of cells immunostained: 1-24% (scored as 1), 25-49% (scored as 2), 50-74% (scored as 3) and 75-100% (scored as 4).

Electron microscopy

All EM studies were performed by the *Servizo de Apoio á Investigación* (*SAI*), at the Universidade da Coruña (UDC).

Transmission electron microscopy: Scaffolds were analyzed by transmission EM (TEM) to study the ultra structure of the cells. As the negative control, we used scaffolds without cells cultured in chondrogenic medium. The scaffolds were fixed with glutaraldehyde cacodylate buffer and post-fixed with OsO_4. The samples were dehydrated with a gradual acetone sequence and then embedded in Spurr (Electron Microscopy Sciences, Hatfield, USA). The ultra structure was studied using a transmission microscope model JEOL JEM 1010 (Jeol, Tokyo, Japan).

Scanning electron microscopy: To evaluate the morphometry of cells cultured in chondrogenic medium, scaffolds with and without cells were analyzed by scanning EM (SEM). Both scaffolds were fixed with glutaraldehyde cacodylate buffer and post-fixed with OsO_4. The samples were then dehydrated with ethanol and critical point drying in CO_2 (Balzers, Liechtenstein, Germany). Finally, the samples were metalized with gold and visualized using a scanning microscope model JEOL JSM 6400 (Jeol).

Molecular studies of cell differentiation

Total RNA isolation: Constructs of each kind of scaffold were sliced and the slices were introduced into tubes with zirconia glass beads and frozen in liquid nitrogen. Tubes were then placed on a Mixer Mill MM200 (Retsch, Haan, Germany) to disintegrate the constructs. Isolation of total RNA from the homogenate was accomplished using Trizol (Invitrogen™, Barcelona, Spain) reagent, following the manufacturer's protocol.

cDNA synthesis: DNase I was used for DNase treatment (Fermentas, City, Spain). The RT-PCR reaction was performed from the total RNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™) following the manufacturer's instructions.

Quantitative Real Time PCR analysis

Using the primers shown in Supplementary Figure 2, qPCR analyses were performed on a LightCycler® 480 Instrument (Roche, Mannheim, Germany) and the LightCycler 480 SYBR Green I Master (Roche), following the manufacturer's instructions. The initial enzyme activation at 95 °C for 10 min was followed by 60 cycles of target amplification consisting of three sequential steps: 95 C for 10 s, 61°C for 5 s, and 72C for 7 s. After amplification, a melting curve analysis was performed, following three subsequent steps: 95 °C for 5 s, 65 °C for 60 s and 97 °C for 1 s. Finally, a cooling step was done at 40 °C for 20 s.

The TATA binding protein (*TBP*) was used as the housekeeping gene to normalize the amount of target cDNA. Primers for SOX9 [SRY (sex determining region Y)-box 9] (*SOX9*), Agg (*AGG*) and alpha 1 Col II (*COL II*) were used to evaluate chondrogenesis; fatty acid binding protein 4 (*FABP4*), adipose most abundant gene transcript 1 (*APM1*), and lipoprotein lipase (*LPL*) to evaluate adipogenesis; and, alkaline phosphatase (*ALP*) and secreted phosphoprotein 1 (*OP*) to evaluate osteogenesis. For data analyses, the LightCycler® 480 Relative Quantification software (Roche) was used. Gene relative expression levels (R.E.L.) were calculated by the $2^{-\Delta\Delta Ct}$ method [23].

Measurement of collagen released

Total Col released by cells cultured on the scaffolds was measured every 3-4 days using the Sircol™ Soluble Collagen Assay (Biocolor, Carrickfergus, UK), following the manufacturer´s protocol. Absorbance was measured on a spectrophotometer (Infinite® 200 PRO NanoQuant, Tecan, Männedorf, Switzerland). For each sample, concentrations of soluble Col were measured in the total volume of supernatant of the culture media. To avoid including Col from the degradation of scaffolds, the absorbance of control supernatants from scaffolds cultured without cells was measured and subtracted from the data for supernatants from scaffolds with cells. Subsequently, the data were compared to the control groups cultured in 20%DMEM.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 for

Windows. p values<0.05 are considered statistically significant. Results are expressed as the mean ± standard error (S.E.).

Results

Cellular evaluation

Localization, morphology, viability and proliferation of cells in constructs: To study the percentage of cells relative to the area of scaffold analyzed, the localization of cells, and cell morphology, we interpreted the results obtained from HE and MMT staining. Cells were located on the surface and inside the scaffolds in chondrogenic cultures, but primarily on the surface of their control counterparts (Figure 1, Table 1).

In our evaluation of cells in the constructs we determined that scaffolds with cells cultured in chondrogenic medium showed high cell numbers and ECM volume in all cases. At 15 days, the highest amount of ECM and number of cells in chondrogenic medium was detected throughout the scaffolds composed of Col I +1%HS (more than 80%) and Col I +3%HS (more than 75%) (Figure 1, Table 1). At 30 days, all the chondrogenic-stimulated supports showed homogenous distribution of ECM as well as cells (more than 80% of cells) throughout the entire scaffold, indicating that cells were able to grow on the surface and inside the scaffolds. The Col I+3%HS scaffold showed the highest percentage of cells: >90% (Figure 1, Table 1). In the controls with non-differentiating medium (20%DMEM) the percentage of cells in culture was very low: <1% in Col I +1%HS, >10% in Col I +2% and >5% in Col I +3%HS (Figure 1, Table 1). After 15 and 30 days in 20%DMEM culture, the Col I without HS scaffold was highly degraded; we detected less than 1% of cells and no ECM. In the rest of the control scaffolds, only CoI I+1%HS showed an increase in the percentage of cells (Figure 1, Table 1).

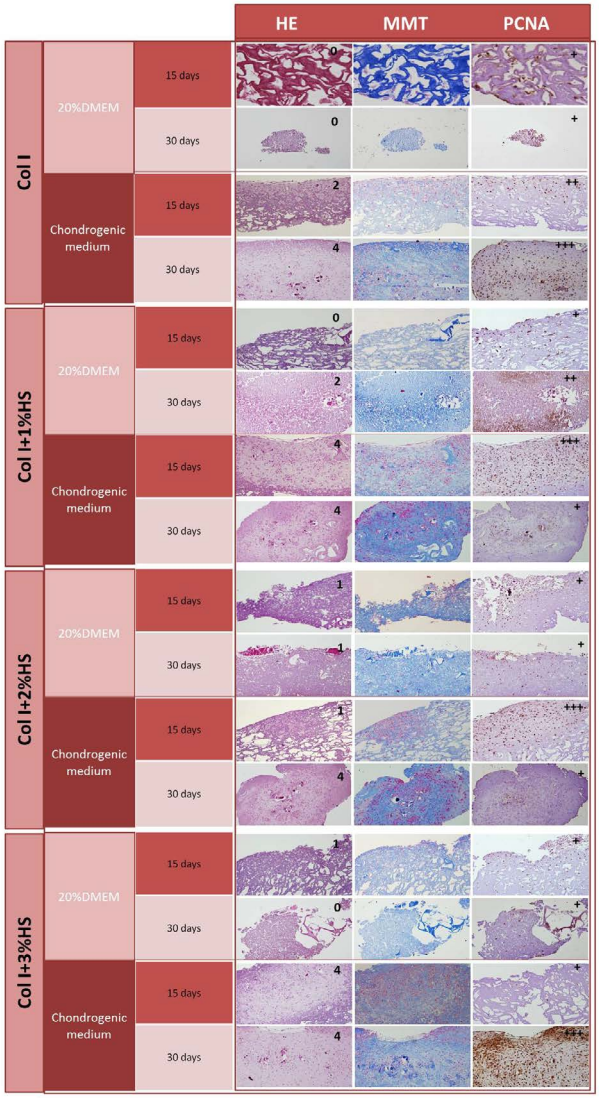


Figure 1: Chart showing results of HE (Hematoxylin-Eosin) and MMT (Modified Masson's Thrichrome) staining, and proliferation cell nuclear antigen (PCNA) immunostaining of differentiated (Chondrogenic medium) and non-differentiated (20%DMEM) human bone marrow mesenchymal stem cells at 15 and 30 days of culture. Scores of % cells and % cells immunostained with PCNA in the scaffold area studied are grouped as: 1-24% (1), 25-49% (2), 50-74% (3) and 75-100% (4). Magnification 100x.

Cell morphology was also observed using HE staining. At both 15 and 30 days of culture, scaffolds containing hBMSCs cultured in chondrogenic medium showed uninucleated and non-vacuolated cells and the presence of oval/rounded cell aggregates, except for Col I+2%HS (Figure 1, Table 1). Scaffolds containing hBMSCs cultured in 20%DMEM alone showed cytoplasmic eosinophilia and nuclear pyknosis after 15 days of culture; cells with fibroblastic morphology became necrotic after 30 days, except for those in Col I+1%HS scaffolds (Figure 1, Table 1).

To establish that cells were capable of proliferation after 15 and 30 days of culture, we looked for the presence of the PCNA proliferation marker in scaffolds containing hBMSCs using immunohistochemical staining. After 15 days, percentage of cells stained was <25% in the non-differentiation controls. In chondrogenic-stimulated scaffolds, the percentage was <50% of the cells stained for PCNA in Col I, <25% in Col I +3%HS and <75% in the remaining biomaterials (Col I +1%HS and Col I +2%HS) (Figure 1, Table 1), indicating a high level of cell proliferation. After 30 days, this marker was barely detectable in control scaffolds (Figure 1, Table 1). In 30-day-chondrogenic-stimulated scaffolds composed of Col I without HS the percentage of cells stained with PCNA was <75% and, in Col I +3%HS was >75%, both were higher than those at 15 days. While scaffolds composed of Col I +1%HS and Col I +2%HS was <25% after 30 days and lower than their respective intensities at 15 days (Figure 1, Table 1).

Molecular profile of the differentiated cells in the constructs

R.E.L. data from qPCR analyses showed that cells in most of the constructs expressed *SOX 9, AGG and COL II* at 30 days of culture (Figure 2). In all the constructs, the R.E.L. of *SOX9* was higher when cells were cultured in chondrogenic medium than when cells were not stimulated (p-values<0.05).

We did not find differences in the R.E.L. of *AGG*, with the exception of Col I and Col I+3%HS scaffolds. In Col I constructs, *AGG* expression was higher in control cells (p-value=0.036), while stimulated cells did not showed *AGG* expression. In Col I+3%HS, the R.E.L. of *AGG* was higher in stimulated constructs than in their counterpart controls (p-value=0.049) (Figure 2). No differences between any groups were detected in the R.E.L. of *COLII* (p-values>0.05) (Figure 2). Those results suggest that hBMSCs in chondrogenic-stimulated scaffolds were able to differentiate toward chondrocyte-like cells.

Table 1: Summary of Histological and Immunohistochemical Results.

Medium	Days	Scaffold	Stainings								
			Score of % Cells	Score of % PCNA	SO	Col II	Agg	VK	Col I	Col X	MMP 13
20%DMEM	15	Col I	0	1	0	1	0	0	1	3	1
		Col I + 1% HS	0	1	0	1	1	0	2	2	1
		Col I + 2% HS	1	1	1	3	3	1	0	3	1
		Col I + 3% HS	1	1	1	1	1	1	0	3	1
	30	Col I	0	1	1	3	2	1	1	0	0
		Col I + 1% HS	2	2	1	3	2	3	1	3	0
		Col I + 2% HS	1	1	1	3	0	1	1	0	0
		Col I + 3% HS	0	1	1	3	0	1	0	1	0
CHONDRO	15	Col I	2	2	2	2	1	0	1	2	0
		Col I + 1% HS	4	3	2	2	2	1	2	2	0
		Col I + 2% HS	1	3	1	2	2	1	1	3	0
		Col I + 3% HS	4	1	3	2	1	0	1	1	1
	30	Col I	4	3	3	3	2	1	1	3	0
		Col I + 1% HS	4	1	3	2	0	1	1	0	0
		Col I + 2% HS	4	1	3	1	0	1	1	1	0
		Col I + 3% HS	4	3	3	3	1	1	1	3	0

Summary table of histological and immunohistochemical results of culture of human bone marrow mesenchymal stem cells on various scaffolds, at 15 and 30 days in control medium (20%DMEM) and in chondrogenic medium (Chondro).
PCNA: Proliferating cell nuclear antigen; SO: Safranin O; Col: Collagen; Agg: Aggrecan; VK: Von Kossa; MMP13: Matrix metalloproteinase-13; HS: Heparan sulfate
Semiquantitative histological and immunohistochemical staining intensity results: negative or absent (– or 0), weak (+ or 1), moderate (++ or 2) and strong (+++ or 3). % cells in the scaffold area studied grouped as: 1-24% (scored as 1), 25-49% (scored as 2), 50-74% (scored as 3) and 75-100% (scored as 4). % cells inmunostained with PCNA in the scaffold area studied grouped as: 1-24% (scored as 1), 25-49% (scored as 2), 50-74% (scored as 3) and 75-100% (scored as 4). Hematoxylin-eosin (HE) and modified Masson's Trichrome (MMT) stains were used. To detect sulfated glycosaminoglycans (GAGs) in the ECM, scaffold sections were stained with Safranin O (SO).

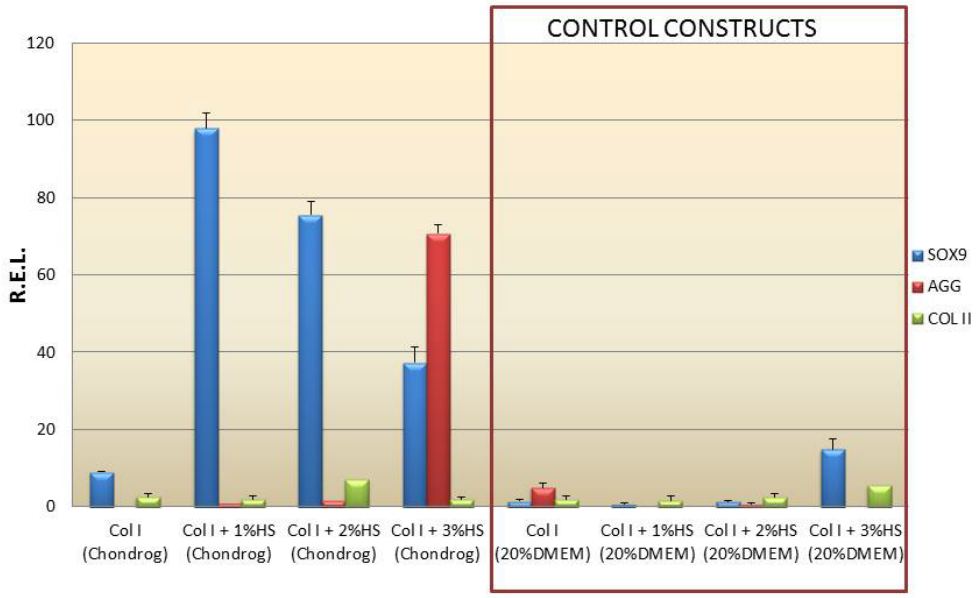


Figure 2: Relative expression levels (R.E.L.) of the characteristic chondrocytic genes, SOX9, AGG, and COL II, compared between constructs with human bone marrow mesenchymal stem cells differentiated in chondrogenic medium (Chondrog) and control constructs cultured in 20%DMEM at 30 days of culture.

Extracellular matrix evaluation

Assessment of characteristic cartilage extracellular matrix components: In control cultures at 15 days, SO staining showed a low amount of sulfated GAGs in Col I+3%HS and was negative in Col I and Col I +1%HS scaffolds (Figure 3, Table 1). At 30 days, all the non-differentiated scaffolds exhibited weak positive SO staining, indicating a slight increase. At 15 days in culture of biomaterials with chondrogenic-stimulated cells, weak positive SO staining was detected in Col I+2%HS, moderate in Col I and Col I+1%HS, and strong in Col I+3%HS scaffolds. At 30 days, SO staining was strong in all the scaffolds containing cells cultured in chondrogenic medium (Figure 3, Table 1).

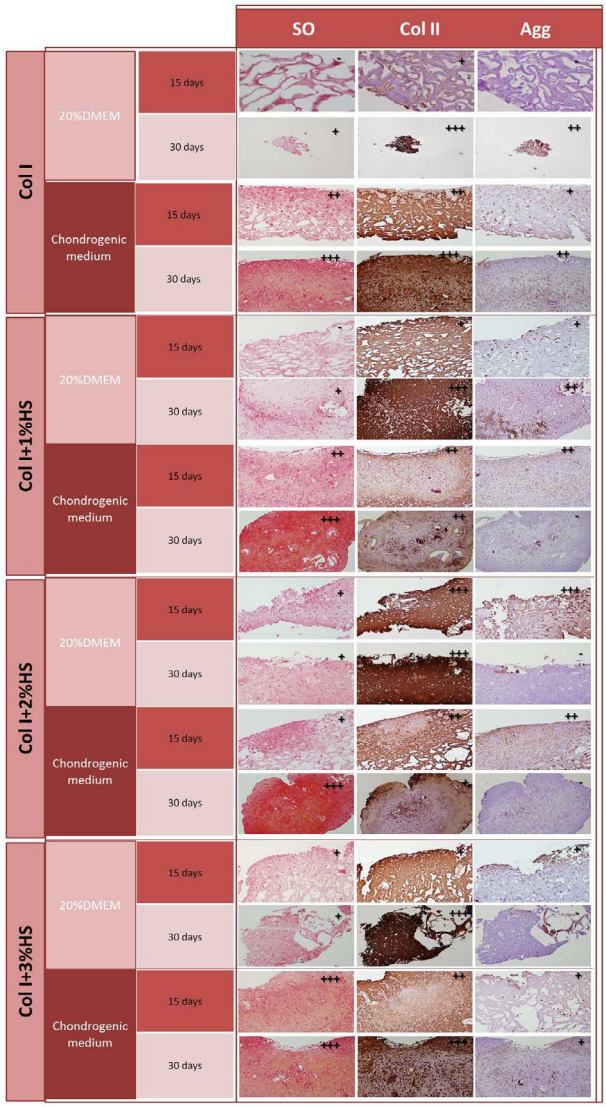


Figure 3: Chart showing results of Safranin O (SO) staining and immunostaining for Col II (type II collagen) and Agg (aggrecan) of differentiated (chondrogenic medium) and non-differentiated (20%DMEM) human bone marrow mesenchymal stem cells at 15 and 30 days of culture. Staining positivity scores range from absent (-) to strongly stained (+++). Magnification 100x.

The immunohistochemical analysis of the ECM for Col II, a typical marker for hyaline cartilage, was positive in every scaffold. At 15 days, positivity for Col II staining was moderate in the stimulated scaffolds, increasing at 30 days to strongly positive in Col I and Col I +3%HS scaffolds (Figure 3, Table 1). At 15 days in non-differentiated constructs, staining for Col II was weakly positive in Col I without HS, Col I+1%HS and in Col I+3%HS, but strongly positive in the other scaffolds. At 30 days, positivity of Col II staining was strong in all the control scaffolds, actually stronger than that of some of the stimulated constructs (Figure 3, Table 1).

At 15 days, the immunohistochemical analysis of the ECM for Agg, another important marker for hyaline cartilage, found this molecule present in all study groups in both 20%DMEM controls and chondrogenic-stimulated scaffolds, except for the control Col I without HS scaffold (Figure 3, Table 1). Col I+2%HS scaffolds in control cultures showed the overall highest positive staining at 15 days. At 30 days, positive staining for Agg was moderate in stimulated Col I and non-differentiated Col I and Col I+1%HS scaffolds, while stimulated Col I+1%HS and Col I+2%HS, and control Col I + 2%HS and Col I+3%HS scaffolds were negative for Agg immunostaining. Weakly positive staining for Agg was detected in stimulated Col I+3%HS scaffolds at 30 days (Figure 3, Table 1).

Assessment of other extracellular matrix compounds: At 15 days, VK positivity was weak in non-differentiated Col I+2%HS and Col I+3%HS, and in stimulated Col I+1%HS and Col I+2%HS scaffolds. We did not detect VK staining in non-differentiated Col I and Col I+1%HS and stimulated Col I and Col I+3%HS scaffolds (Figure 4, Table 1). At 30 days, all stimulated scaffolds were weakly positive for VK staining, indicating a slight staining increase in Col I and Col I+3%HS constructs. In the control scaffolds, positivity remained weak in all but Col I+1%HS, which varied from absent to strong staining (Figure 4, Table 1).

Immunodetection for Col I, a marker of fibroblastic differentiation

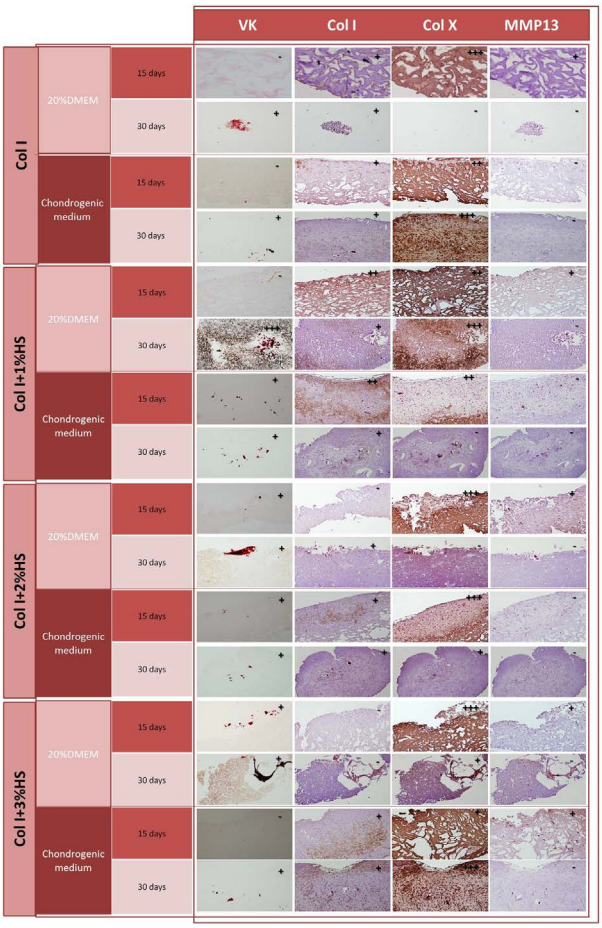


Figure 4: Chart showing results of Von Kossa (VK) staining and immunostaining for type I collagen (Col I), type X collagen (Col X) and metalloproteinase 13 (MMP13) of differentiated (chondrogenic medium) and non-differentiated (20%DMEM) human bone marrow mesenchymal stem cells at 15 and 30 days of culture. Staining positivity scores range from absent (-) to strongly stained (+++). Magnification 100x.

and undifferentiated MSCs, was also assessed. Non-differentiation controls were negative for Col I staining in 15-day-Col I +2%HS and Col I +3%HS scaffolds and weak in the remaining scaffolds, except for the 15-day-20%DMEM Col I+1%HS scaffold, which was moderate (Figure 4, Table 1). Stimulated scaffolds expressed weakly positive values at both 15 and 30 days, except for 15-day-stimulated Col I+1%HS scaffold (Figure 4, Table 1).

When immunohistochemically assessing Col X, a marker for hypertrophic chondrocytes, at 15 days, positivity for this marker was strong in stimulated Col I+2%HS, control Col I, Col I+2%HS and Col I+3%HS scaffolds (Figure 4, Table 1). In stimulated Col I and Col I+1%HS, and in 20%DMEM Col I+1%HS scaffolds, staining for Col X was moderately positive. Stimulated Col I+3%HS scaffolds stained weakly for Col X (Figure 4, Table 1). At 30 days in 20%DMEM, staining intensity for Col X increased from moderate to strong in Col I +1%HS, and decreased in Col I+2%HS and Col+3%HS scaffolds (Figure 4, Table 1). The stimulated scaffolds composed of Col I and Col I+3%HS showed increased staining for Col X, while the remaining scaffolds cultured in chondrogenic medium exhibited a decrease (Figure 4, Table 1).

Finally, the immunohistochemical analysis to detect MMP13 showed decreasing positivity in non-differentiated controls after 15 days, being absent at the end of culture at 30 days. Staining for MMP13 was absent in all the stimulated scaffolds, except for Col I+3%HS at 15 days, which had disappeared at 30 days (Figure 4, Table 1).

Cell and extracellular matrix morphometric and structural evaluation

Morphometric analysis: Those scaffolds with cells cultured in chondrogenic medium and their respective controls without cells were assessed by SEM. Samples were analyzed after 15 and 30 days of culture.

At 15 days, supports that were initially seeded with hBMSCs, showed a high number of cells over the entire studied area. Cell morphology was typically spherical, characteristic of chondrocytes when embedded in native hyaline cartilage. Chondrocyte-like cells in great number attached to the surface of both Col I and blended scaffolds (Col I and HS) (Figure 5). This result indicates that all the scaffolds possess the same properties of cell adhesion. At 30 days, cells had continued to proliferate because cell numbers had increased; cells had spread on the walls of the scaffolds and infiltrated inside the pores (Figure 5). Cells had the native (spherical) morphology of chondrocytes in cartilage, indicating that they were able to maintain the differentiated phenotype.The extracellular environment included large amount of fibrils and vesicles at 15 days in culture (Figure 5), surrounding and even covering the cells; these fibrils and vesicles were probably synthesized and released by the cells. ECM covered wide regions of the scaffolds, suggesting that differentiated cells recognized the surface of these biomaterials as native. At 30 days, the ECM covered nearly the entire surface of the scaffold, making it difficult to highlight the fibrils from the original biomaterial (Figure 5). We were also able to detect large quantities of cellular debris distributed over all the studied areas. Degradation of the biomaterials was gradual, without loss of integrity, suggesting these scaffolds may allow suitable hyaline cartilage formation.

Ultrastructural analysis: TEM analysis was performed only on biomaterials seeded with hBMSCs and cultured in chondrogenic medium for 15 and 30 days. At both time points we found cells on every scaffold, varying in size from 10 to 20 μm and showing rounded or ovoid morphology. Nuclei were prominent and well developed, with a diverse morphology from mostly spherical to very heterogeneous. Inside some of the nuclei, we could differentiate the nucleolus as a dense area usually located in the central region of the nucleus. We could also differentiate heterochromatin spread throughout the nucleus, and in some cases, associated with the nuclear membrane (Figure 5).

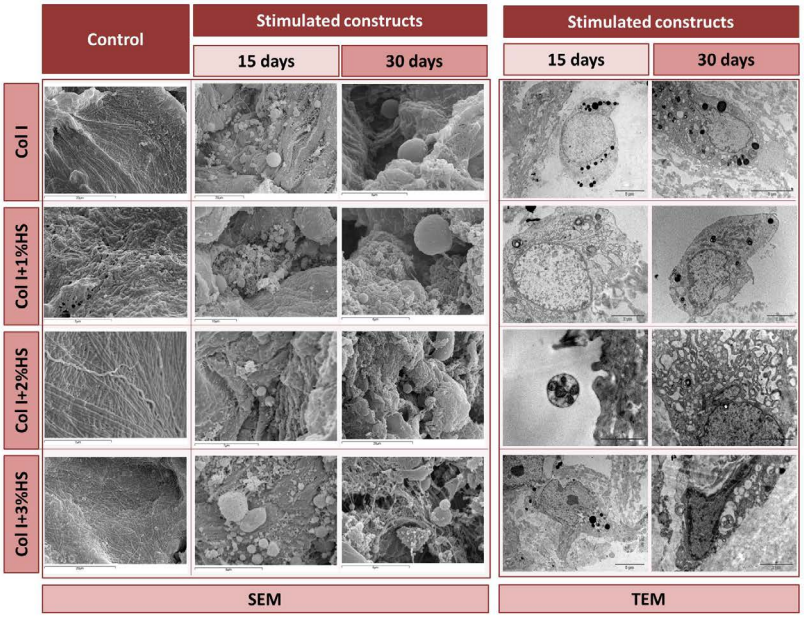


Figure 5: Scanning (SEM) and transmission (TEM) electron microscopy. Images show human bone marrow mesenchymal stem cells cultured for 15 and 30 days in chondrogenic medium (stimulated constructs). In SEM, images of controls without cells (control) are shown.

We observed highly electron-dense bodies (black vesicles) containing glycogen scattered in the cytoplasm. Less electron-dense vesicles, which varied in intensity, could be packets of secretory material and lysosomes. Their number and size increased from 15 to 30 days of culture. Furthermore, lipid droplets occupied a great proportion of the cytoplasm, seen as large bright vesicles (Figure 5).

In some cases, the rough endoplasmic reticulum was heavily populated with ribosomes with swollen lumens, containing a large amount of secretory material, indicating a high level of biosynthesis (Figure 5). We found a large number of mitochondria at both 15 and 30 days in culture. The size and morphology of mitochondria were variable (Figure 5).

Extracellular areas also showed a great confluence of electron-dense vesicles in proximity to cell surfaces. It was sometimes possible to differentiate vesicles that were being released to the ECM. Both facts indicate activity between the cells and their ECM.

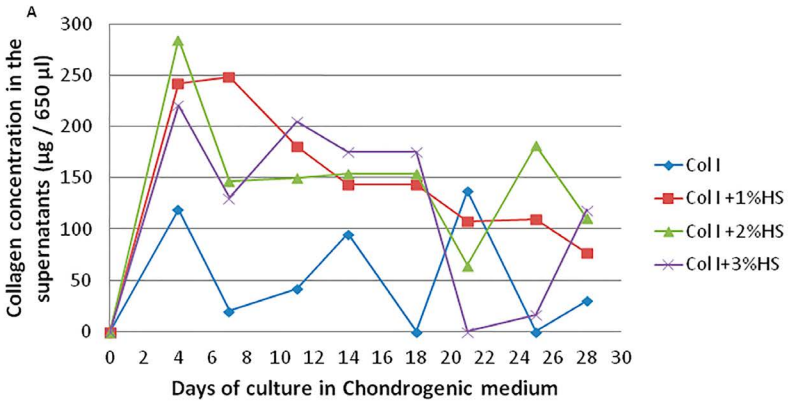
At 15 and 30 days in culture, we were able to clearly differentiate Col II fibrils in close proximity to the cell surfaces surrounding them (Figure 5). These fibrils differed from those constituting the scaffolds (Col I). These collagenous fibrils were irregularly arranged (normal for the ECM of cartilage), and defined a territorial zone of cartilaginous matrix.

Evaluation of total collagen released

As shown in Figure 6, cells cultured in chondrogenic medium released Col into the supernatant at almost all time points analyzed, the exception being from the 14th to 18th days of culture in the Col I scaffold, and from the 18th to 21st days in the Col I +3%HS scaffold, during which times we were unable to detect Col release. A tendency for a slight decrease in the Col concentration of supernatants from scaffolds composed of Col I with HS was noted over the culture period. This tendency to decrease was more gradual and constant in Col I +1%HS scaffolds. Col I +2%HS scaffolds presented the highest initial concentration of Col, but from the 18th to the 21st day of culture it showed a decrease, followed by a final increase at the 25th-28th day interval (Figure 6). Finally, supernatants from the Col I +3%HS scaffolds had an initial Col concentration, which stayed nearly constant until the 18th-21st days of culture, when no Col was detectable in the supernatant; in the final period Col did increase. Col concentration in the supernatants of the Col I scaffold varied considerably and was generally lower than in the other scaffolds.

On the other hand, analyses performed on culture supernatants of cells on scaffolds cultured in 20%DMEM showed no Col release. In a few exceptional cases, we detected a release of Col, which was not significant (in the 7th-11th day interval for Col I and in the 0-4th day interval, as well as in the 21st-25th day interval for Col I +3%HS) (Figure 6). Concentrations of Col in the supernatants were

A



B

DAYS (intervals)	0-4	4-7	7-11	11-14	14-18	18-21	21-25	25-28
Col I	0	0	1.01	0	0	0	0	0
Col I + 1%HS	0	0	0	0	0	0	0	0
Col I + 2%HS	0	0	0	0	0	0	0	0
Col I + 3%HS	5.73	0	0	0	0	0	0.09	0

C

DAYS (intervals)	0-4	4-7	7-11	11-14	14-18	18-21	21-25	25-28
Col I	119.33	20.12	41.45	95.21	0	137.34	0	30.05
Col I + 1%HS	241.90	248.40	180.53	143.59	143.49	107.85	109.95	76.67
Col I + 2%HS	284.93	146.51	150.12	154.35	154.35	64.66	181.62	110.61
Col I + 3%HS	220.91	130.40	204.65	175.47	175.47	0	16.03	118.71

Figure 6: (A) Graphic representation of total collagen released into the culture supernatant by chondrogenic-stimulated human bone marrow mesenchymal stem cells over the time of culture. The x-axis represents days of cell culture on the scaffolds in chondrogenic medium; the y-axis represents total collagen concentration measured in culture supernatants. B-C) Table showing µg of total collagen in the volume of culture supernatants (650 µl) from cells cultured in 20%DMEM (B) and in chondrogenic medium (C).

significantly higher ($p<0.05$) in scaffolds cultured in chondrogenic medium than in scaffolds cultured in 20%DMEM.

Discussion

Several therapies for repair of joint lesions have been developed. They are focused on the formation of a neo-tissue with biochemical, structural, morphological and functional properties similar to those of endogenous hyaline cartilage [24].

Tissue engineering provides a promising remedy for the treatment of tissue defects, using biodegradable scaffolds, cells and cell factors to construct 3D engineered tissues for *in vivo* implantation [25]. It is currently accepted that 3D *in vitro* culture systems could better mimic the *in vivo* situation than 2D cultures [26]. Biocompatible scaffolds that provide a suitable 3D structure are able to support cell viability and proliferation, maintain the cell-specific phenotype and allow cellular differentiation. The appropriate choice of both cells and biomaterials is the most important aspect of cell-based cartilage engineering [14]. To be selected for tissue engineering [8], the biomaterial supports should have biochemical and biophysical characteristics, such as non-cytotoxicity, porosity, ability to integrate with the surrounding cartilage, and biodegradability [4]. The synthesis of the neo-tissue should take place gradually, as the scaffold degrades, thereby maintaining the structural integrity of the newly formed cartilage [2]. The ideal scaffold should degrade at a rate that optimizes cellular growth and neo-tissue development. It should also have the potential to anchor to the injury site and the porosity to allow nutrient and differentiation factors to flow in and for waste exchange [26]. Although all the biomaterials we employed exhibited slow degradation, it seemed slower in the biomaterial composed only of Col I, while the Col I +3% HS scaffold showed the most rapid degradation.

A number of scaffolds have been fabricated for use in cartilage tissue engineering, such as fibrous structures, porous sponges, woven or non-woven meshes and hydrogels [27,28]. Natural biomaterials employed include Col [29,30], agarose [31,32], fibrin [33,34] and alginate [35,36], among others. Examples of synthetic scaffolds are poly-lactic glycolic acid (PLGA) [37,38] and a polymeric nanofiber [39,40]. These scaffolds are used alone or in combination to develop cell supports [24]. Scaffolds should not induce adverse reactions in the host [41].

Col is a protein found in a high concentration in a variety of tissues [42] and has several properties useful for tissue engineering. One such property is that Col can be degraded by in situ collagenases [43], its degradation could result in functional and structural restoration of the damaged tissue. Most current studies are focused on optimization of scaffolds using Col, sometimes in combination with other molecules, such as GAGs, to improve tissue function [42]. The attachment of GAGs to biomaterials may offer an appropriate environment for cellular signaling. GAG matrices modulate neo-synthesized tissue rejection by the host. The potential of these supports arises from several properties that make them compatible with the areas of implantation [44]. In this study, we used Col I biomaterials and compared them with Col I combined with HS. HS chains bind to a variety of ECM and basement membrane components and also function as co-receptors for many growth factors. These biocharacteristics indicate a major role for GAGs in basic biological phenomena like adhesion, migration, proliferation and differentiation of cells [44].

Cells have the capability to grow *in vitro* on a biomaterial with characteristics similar to those of tissues; this helps us to understand the behavior of cells in complex environments [42]. Although Col II is the major structural element of the cartilage ECM, Col I is immunologically less reactive when implanted in a host [45]. The use of mature cell types, such as chondrocytes, for cell therapy, provides a moderate quality repairing tissue. Moreover, autologous chondrocyte implantation is currently limited to healing focal damage [10]. In many cases, scaffolds are implanted with autologous chondrocytes, resulting in formation of a tissue that differs histologically from the original cartilage [45]. Furthermore, the use of autologous chondrocytes requires an invasive technique due to the need to extract healthy cartilage [24]. These issues have led to the necessity for improving our knowledge of alternative cells, such as MSCs.

In this study, we have seen that hBMSCs not only survive on Col I and HS scaffolds, but also proliferate and differentiate. Cells cultured with chondrogenic-stimulation medium were spread homogenously throughout the scaffold and a remarkable increase of the ECM occurred. We detected a high level of cell proliferation, the highest being in Col I +3% HS scaffolds. Data obtained from the PCNA assay also suggested high proliferation. Regarding cell differentiation, we successfully cultured hBMSCs on scaffolds composed of Col I and HS in chondrogenic medium, and after 30 days we observed oval/rounded cells and ECM. When no stimulus was present in the culture, the number of cells on the scaffolds decreased from 15 to 30 days, in spite of evidence of some proliferation. These cells, however, had a fibroblastic morphology, indicating that no differentiation took place. Also, in these non-stimulated cultures, cells were located only on the surface of the biomaterials and, after 30 days, little ECM was present.

The use of TGFβ in the culture of MSCs favors chondrogenic differentiation as well as ECM formation [46,47]. However, MSCs under chondrogenic induction by TGFs have shown a hypertophic phenotype [48].

In our study, the molecular characterization of the differentiated hBMSCs cultured in chondrogenic medium with TGFβ-3, expressed characteristic genes of hyaline cartilage, such as *SOX9, COL II* and *AGG*. Although there was no difference in gene expression of *COL II* between controls and stimulated cells, we found higher expression of *SOX9* and *AGG* in most of the differentiated constructs, suggesting that neo-formation of such tissue was really taking place in our biomaterials. These results were supported by immunohistochemical detection of Col II in the ECM, with Col II staining intensely in all the study groups at 30 days of culture. PGs were also strongly stained by SO in chondrogenic constructs and weakly in the non-differentiated ones. Agg, representing most of the PG in cartilage, was detected in all the constructs at 15 days, but in only a few stimulated constructs after 30 days. These results in contrast with the beginning of AGG expression at 30 days could indicate that hBMSCs were in an early state of differentiation.

Col X was present in all stimulated and non-stimulated groups we studied. Mwale et al. [49] found that type X Col was detectable in undifferentiated MSCs and concluded that Col X is not a good marker for chondrocyte hypertrophy during early MSC differentiation. Because MMP13 was absent in all the groups of our study, we could confirm that there was no hypertrophy.

After 30 days, we found weakly positive mineralization in both stimulated and control constructs on histochemical analysis with VK, except in Col I+1%HS control scaffolds. Different data were obtained by inducing chondrogenic differentiation of MSCs in scaffolds

composed of polycapralactone (PCL), where mineralization of the new ECM was detected after 45 days in culture [50]. Hellingman et al. [51] established that a higher level of mineralization *in vitro* does not imply mineralization *in vivo*. Another study showed that calcification can be due to some variations in the surface tension of oxygen or to the pH of the media [50]. In our study, it is important to note that the degree of calcification in the stimulated cells was lower than that of controls.

Col release to the culture medium by cells took place throughout the 30 days of culture on stimulated scaffolds, indicating a high production of Col due to the synthesis of new cartilage-like tissue. We detected no Col in the supernatant of scaffolds cultured in 20%DMEM, suggesting that chondrogenic differentiation of stimulated hBMSCs was successful. Col release was less in the scaffolds composed of Col I than in the other scaffolds with HS, indicating the Col I with HS scaffolds are more favorable for hBMSCs differentiation.

Finally, we confirmed chondrogenic differentiation in the scaffolds by TEM and SEM, detecting high amounts of electron-dense vesicles, the presence of which is characteristic of cells that are synthesizing ECM in neo-cartilage [52]. Many of the chondrocyte-like cells possessed a highly developed pericellular capsule with a band of densely bundled Col fibers, consistent with TEM of articular cartilage matrices *in vivo* [53]. The SEM analysis showed a gradual degradation of the biomaterials.

The results obtained in this study suggest that Col I +3% HS is the most suitable candidate for use as a scaffold for cartilage repair This can be due to three reasons: 1) the higher porous size probably provides a better environment for proliferation, 2) there is a positive balance between formation of neo-tissue and the degradation of scaffold and, 3) the higher HS concentration could be the responsible of the better chondrogenic phenotype.

We conclude that the Col I and HS scaffolds used in this study are suitable supports for hBMSC to differentiate toward chondrocyte-like cells at both 15 and 30 days in chondrogenic medium; these scaffolds enhance the maintenance of the typical chondrocyte phenotype. Furthermore, cells on these scaffolds were able to synthesize a cartilage-like ECM, giving rise to a cartilaginous-like tissue *in vitro*. Further studies are needed to highlight the usefulness of Col I and HS scaffolds for regenerating cartilage damage and their capacity for integration with surrounding host tissue.

Acknowledgements

We want to thank Bruna Parma from Opocrin, S.P.A. for supplying all the biomaterials; Dr Barbara Ruozi (TEFARTI group, Life Sciences Dept., University of Modena and Reggio Emilia, Italy) for supplying scaffold characterization data; P. Esbrit (Fundación Jiménez Díaz) for supplying the PTHrP; Julia Buján and Natalio García from the Department of Medical Specialties; the Department of Medical Specialties, Universidad Alcalá de Henares; Ada Castro-Couceiro, Ph.D. and Catalina Sueiro-López, Ph.D. from research support services of the Universidade da Coruña (SAI-UDC); and laboratory technicians: María José Sánchez-Dopico, Purificación Filgueira-Fernández and Noa Goyanes-Rey from INIBIC-CHUAC for their support and assistance.

This study was supported by grants: Servizo Galego de Saúde; Cátedra Bioiberica from Universidade da Coruña and Instituto de Salud Carlos III CIBER BBN; CAM (S2009/MAT-1472); REDICENT (Rede de Investigación en Células Nai e Terapia Celular); Universidade da Coruña (UDC); Clara Sanjurjo-Rodríguez is a beneficiary of a fellowship from Diputación de A Coruña, Spain.

References

1. Remya NS, Nair PD (2013) Engineering cartilage tissue interfaces using a natural glycosaminoglycan hydrogel matrix An in *vitro study*. Mat Sci Eng C-Mat Bio Appl 33: 575-582.
2. Negri S, Fila C, Farinato S, Bellomi A, Pagliaro PP (2007) Tissue engineering: chondrocyte culture on type 1 collagen support. Cytohistological and immunohistochemical study. J Tissue Eng Regen Med 1: 57-63.
3. Meretoja VV, Dahlin RL, Wright S, Kasper FK, Mikos AG (2013) The effect of hypoxia on the chondrogenic differentiation of co-cultured articular chondrocytes and mesenchymal stem cells in scaffolds. Biomaterials 34: 4266-4273.
4. Dhollander AA, Guevara Sánchez VR, Almqvist KF, Verdonk R, Verbruggen G, et al. (2012) The use of scaffolds in the treatment of osteochondral lesions in the knee: current concepts and future trends. J Knee Surg 25: 179-186.
5. Ishiguro N, Kojima T, Poole AR (2002) Mechanism of cartilage destruction in osteoarthritis. Nagoya J Med Sci 65: 73-84.
6. Khoshgoftar M, van Donkelaar CC, Ito K (2011) Mechanical stimulation to stimulate formation of a physiological collagen architecture in tissue-engineered cartilage: a numerical study. Comput Methods Biomech Biomed Engin 14: 135-144.
7. Steinert AF, Ghivizzani SC, Rethwilm A, Tuan RS, Evans CH, et al. (2007) Major biological obstacles for persistent cell-based regeneration of articular cartilage. Arthritis Res Ther 9: 213.
8. Gloria A, De Santis R, Ambrosio L (2010) Polymer-based composite scaffolds for tissue engineering. J Appl Biomater Biomech 8: 57-67.
9. Tritz-Schiavi J, Charif N, Henrionnet C, de Isla N, Bensoussan D, et al. (2010) Original approach for cartilage tissue engineering with mesenchymal stem cells. Biomed Mater Eng 20: 167-174.
10. Facchini A, Lisignoli G, Cristino S, Roseti L, De Franceschi L, et al. (2006) Human chondrocytes and mesenchymal stem cells grown onto engineered scaffold. Biorheology 43: 471-480.
11. López-Ruiz E1, Perán M, Cobo-Molinos J, Jiménez G, Picón M, et al. (2013) Chondrocytes extract from patients with osteoarthritis induces chondrogenesis in infrapatellar fat pad-derived stem cells. Osteoarthritis Cartilage 21: 246-258.
12. Varghese S, Hwang NS, Canver AC, Theprungsirikul P, Lin DW, et al. (2008) Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells. Matrix Biol 27: 12-21.
13. Kock L, van Donkelaar CC, Ito K (2012) Tissue engineering of functional articular cartilage: the current status. Cell Tissue Res 347: 613-627.
14. Cavallo C, Desando G, Columbaro M, Ferrari A, Zini N, et al. (2012) Chondrogenic differentiation of bone marrow concentrate grown onto a hylauronan scaffold: Rationale for its use in the treatment of cartilage lesions. J Biomed Mater Res A 101: 1559-1570.
15. Chan BP, Leong KW (2008) Scaffolding in tissue engineering: general approaches and tissue-specific considerations. Eur Spine J 17 Suppl 4: 467-479.
16. Zhang L, Yuan T, Guo L, Zhang X (2012) An in vitro study of collagen hydrogel to induce the chondrogenic differentiation of mesenchymal stem cells. J Biomed Mater Res A 100: 2717-2725.
17. Kirn-Safran CB, Gomes RR, Brown AJ, Carson DD (2004) Heparan sulfate proteoglycans: coordinators of multiple signaling pathways during chondrogenesis. Birth Defects Res C Embryo Today 72: 69-88.
18. Richler C, Yaffe D (1970) The in vitro cultivation and differentiation capacities of myogenic cell lines. Dev Biol 23: 1-22.
19. Diaz-Prado S, Muinos-Lopez E, Hermida-Gomez T, Esther Rendal-Vazquez M, Fuentes-Boquete I, et al. (2010) Multilineage Differentiation Potential of Cells Isolated From the Human Amniotic Membrane. J Cell Biochem 111: 846-857.
20. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315-317.
21. Mentink CJ, Hendriks M, Levels AA, Wolffenbuttel BH (2002) Glucose-mediated cross-linking of collagen in rat tendon and skin. Clin Chim Acta 321: 69-76.
22. Ruozi B, Parma B, Croce MA, Tosi G, Bondioli L, et al. (2009) Collagen-based modified membranes for tissue engineering: Influence of type and molecular weight of GAGs on cell proliferation. Int J Pharm 378: 108-115.

23. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

24. Fuentes-Boquete IM, Arufe Gonda MC, Díaz Prado SM, Hermida Gómez T, de Toro Santos FJ, et al. (2008) Cell and Tissue Transplant Strategies for Joint Lesions. Open Transplant J 2: 21-28.

25. Xu C, Lu W, Bian S, Liang J, Fan Y, et al. (2012) Porous collagen scaffold reinforced with surfaced activated PLLA nanoparticles. Scientific World Journal 2012: 695137.

26. Seo S, Na K (2011) Mesenchymal stem cell-based tissue engineering for chondrogenesis. J Biomed Biotechnol 2011: 806891.

27. Tritz J, Rahouadj R, de Isla N, Charif N, Pinzano A, et al. (2010) Designing a three-dimensional alginate hydrogel by spraying method for cartilage tissue engineering. Soft Matter 6: 5165-5174.

28. Yuan T, Zhang L, Feng L, Fan H, Zhang X (2010) Chondrogenic differentiation and immunological properties of mesenchymal stem cells in collagen type I hydrogel. Biotechnol Prog 26: 1749-1758.

29. Zheng L, Fan HS, Sun J, Chen XN, Wang G, et al. (2010) Chondrogenic differentiation of mesenchymal stem cells induced by collagen-based hydrogel: an in vivo study. J Biomed Mater Res A 93: 783-792.

30. Cao H, Xu SY (2008) EDC/NHS-crosslinked type II collagen-chondroitin sulfate scaffold: characterization and in vitro evaluation. J Mater Sci Mater Med 19: 567-575.

31. Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F (2004) Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials 25: 3211-3222.

32. Mauck RL, Soltz MA, Wang CCB, Wong DD, Chao PHG, et al. (2000) Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng 122: 252-260.

33. Scotti C, Mangiavini L, Boschetti F, Vitari F, Domeneghini C, et al. (2010) Effect of in vitro culture on a chondrocyte-fibrin glue hydrogel for cartilage repair. Knee Surg Sports Traumatol Arthrosc 18: 1400-1406.

34. Sage A, Chang AA, Schumacher BL, Sah RL, Watson D (2009) Cartilage outgrowth in fibrin scaffolds. Am J Rhinol Allergy 23: 486-491.

35. Herlofsen SR, Kuechler AM, Melvik JE, Brinchmann JE (2011) Chondrogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells in Self-Gelling Alginate Discs Reveals Novel Chondrogenic Signature Gene Clusters. Tissue Eng Part A 17: 1003-1013.

36. Endres M, Wenda N, Woehlecke H, Neumann K, Ringe J, et al. (2010) Microencapsulation and chondrogenic differentiation of human mesenchymal progenitor cells from subchondral bone marrow in Ca-alginate for cell injection. Acta Biomater 6: 436-444.

37. Kang SW, Lee SJ, Kim JS, Choi EH, Cha BH, et al. (2011) Effect of a scaffold fabricated thermally from acetylated PLGA on the formation of engineered cartilage. Macromol Biosci 11: 267-274.

38. Han SH, Kim YH, Park MS, Kim IA, Shin JW, et al. (2008) Histological and biomechanical properties of regenerated articular cartilage using chondrogenic bone marrow stromal cells with a PLGA scaffold in vivo. J Biomed Mater Res A 87A: 850-861.

39. Alves da Silva ML, Martins A, Costa-Pinto AR, Costa P, Faria S, et al. (2010) Cartilage tissue engineering using electrospun PCL nanofiber meshes and MSCs. Biomacromolecules 11: 3228-3236.

40. Casper ME, Fitzsimmons JS, Stone JJ, Meza AO, Huang Y, et al. (2010) Tissue engineering of cartilage using poly-epsilon-caprolactone nanofiber scaffolds seeded in vivo with periosteal cells. Osteoarthritis Cartilage 18: 981-991.

41. Niknejad H, Peirovi H, Jorjani M, Ahmadiani A, Ghanavi J, et al. (2008) Properties of the amniotic membrane for potential use in tissue engineering. Eur Cell Mater 15: 88-99.

42. Parenteau-Bareil R, Gauvin R, Berthod F (2010) Collagen-Based Biomaterials for Tissue Engineering Applications. Materials 3: 1863-1887.

43. Tedder ME, Liao J, Weed B, Stabler C, Zhang H, et al. (2009) Stabilized collagen scaffolds for heart valve tissue engineering. Tissue Eng Part A 15: 1257-1268.

44. Pieper JS, van Wachem PB, van Luyn MJA, Brouwer LA, Hafmans T, et al. (2000) Attachment of glycosaminoglycans to collagenous matrices modulates the tissue response in rats. Biomaterials 21: 1689-1699.

45. De Franceschi L, Grigolo B, Roseti L, Facchini A, Fini M, et al. (2005) Transplantation of chondrocytes seeded on collagen-based scaffold in cartilage defects in rabbits. J Biomed Mater Res A 75: 612-622.

46. Janjanin S, Li WJ, Morgan MT, Shanti RA, Tuan RS (2008) Mold-shaped, nanofiber scaffold-based cartilage engineering using human mesenchymal stem cells and bioreactor. J Surg Res 149: 47-56.

47. Lisignoli G, Cristino S, Piacentini A, Toneguzzi S, Grassi F, et al. (2005) Cellular and molecular events during chondrogenesis of human mesenchymal stromal cells grown in a three-dimensional hyaluronan based scaffold. Biomaterials 26: 5679-5686.

48. Bian L, Zhai DY, Mauck RL, Burdick JA (2011) Coculture of Human Mesenchymal Stem Cells and Articular Chondrocytes Reduces Hypertrophy and Enhances Functional Properties of Engineered Cartilage. Tissue Eng Part A 17: 1137-1145.

49. Mwale F, Stachura D, Roughley P, Antoniou J (2006) Limitations of using aggrecan and type X collagen as markers of chondrogenesis in mesenchymal stem cell differentiation. J Orthop Res 24: 1791-1798.

50. Abrahamsson CK, Yang F, Park H, Brunger JM, Valonen PK, et al. (2010) Chondrogenesis and mineralization during in vitro culture of human mesenchymal stem cells on three-dimensional woven scaffolds. Tissue Eng Part A 16: 3709-3718.

51. Hellingman CA, Koevoet W, van Osch GJ (2012) Can one generate stable hyaline cartilage from adult mesenchymal stem cells? A developmental approach. J Tissue Eng Regen Med 6: e1-1e11.

52. Danišovic L, Lesný P, Havlas V, Teyssler P, Syrová Z, et al. (2007) Chondrogenic differentiation of human bone marrow and adipose tissue-derived mesenchymal stem cells. J Appl Biomed 5: 139-150.

53. Wilson R, Diseberg AF, Gordon L, Zivkovic S, Tatarczuch L, et al. (2010) Comprehensive Profiling of Cartilage Extracellular Matrix Formation and Maturation Using Sequential Extraction and Label-free Quantitative Proteomics. Mol Cell Proteomics 9: 1296-1313.

Author Affiliations

Top

[1]Grupo de Terapia Celular y Medicina Regenerativa, Instituto de Investigación Biomédica de A Coruña (INIBIC), Complexo Hospitalario Universitario de A Coruña (CHUAC), SERGAS, Departamento de Medicina, Facultade de Ciencias da Saúde, Universidade da Coruña, Campus de A Coruña, 15071 A Coruña, Spain.

[2]Grupo de Bioingeniería Tisular y Terapia Celular (GBTTC-CHUAC), Servicio de Reumatología, Instituto de Investigación Biomédica de A Coruña (INIBIC), Complexo Hospitalario Universitario de A Coruña (CHUAC), SERGAS, C/As Xubias S/N, 15006 A Coruña, Spain.

[3]Grupo de Bioingeniería Tisular y Terapia Celular (GBTTC-CHUAC), Servicio de Reumatología, Instituto de Investigación Biomédica de A Coruña (INIBIC), Complexo Hospitalario Universitario de A Coruña (CHUAC), SERGAS, Departamento de Medicina. Universidade de Santiago de Compostela, C/As Xubias S/N, 15006 A Coruña, Spain.