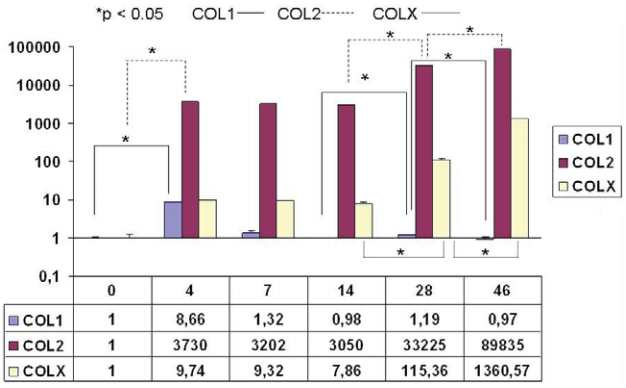
entiation of both of them confirmed by staining with Oil Red O and Alizarin Red respectively. We carried out RT-PCR and immunohistochemistry analysis to test expression of SOX9, type II collagen, type I collagen and C-20 aggrecan which are proteoglycan compounds of extra-cellular matrix and all of them were positive as soon as after 4 days in culture. Expressions of type X collagen and MMP-13 proteins also were measured raising their expression as well as increase the time in culture.



	0	4	7	14	28	46
COL1	1	8,66	1,32	0,98	1,19	0,97
COL2	1	3730	3202	3050	33225	89835
COLX	1	9,74	9,32	7,86	115,36	1360,57

Figure 1. COL1, COL2 and COLX gene expression by RT-PCR of spheroids produced at 4, 7, 14, 28 and 46 days using our chondrogenic medium.

Conclusions: Join together our results strongly suggest that the MSC pool from umbilical cord stromal tissue is an excellent source of cells susceptible to differentiate to chondrocyte-like cells which could be a new cellular therapy against cartilage degradation.

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PENTOSAN POLYSULFATE PROMOTES PROLIFERATION AND CHONDROGENIC DIFFERENTIATION OF ADULT HUMAN BONE MARROW DERIVED MULTIPOTENT MESENCHYMAL PRECURSOR STEM CELLS

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Purpose: To determine if the anti-osteoarthritis drug, pentosan polysulfate (PPS), promoted proliferation and chondrogenic differentiation of mesenchymal precursor stem cells (MPC) in vitro.
Methods: Human STRO-3+ MPC were maintained in monolayer or micromass cultures (mmc) for up to 10 days in the absence and presence of PPS at concentrations of 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, and 20 μg/ml. MPC proliferation and viability was assessed using the WST-1 mitochondral dehydrogenase cleavage assay and by the incorporation of 3H-Thymidine into macromolecular DNA in both culture systems. The capacity of PPS to protect MPC from IL4/INF-γ induced apoptosis was also evaluated using flow cytometry. Proteoglycan (PG) biosynthesis was monitored in mmc by the incorporation of 35SO4 into glycosaminoglycans (35S-GAGs) on days 4, 5 and 6 post culture initiation. The relative stimulatory effects of hyaluronan (HA) or dextran sulfate (DS) on PG synthesis was also examined and compared to PPS in day 5 mmc. The effects of PPS on the levels of bound and unbound HA in MPC in mmc was quantified by an ELISA and the deposition of type II collagen in the matrix assessed using a COL-II Mab and immuno-staining of the fixed cultures on day 10. The ability of PPS to modulate MPC osteogenic or adipogenic differentiation was investigated by co-culturing the cells in the respective inductive culture media for 28 days. Gene expression by MPC in micromass cultures was determined on days 7 and 10 by Real Time and RT-PCR.
Results: On applying the WST-1 assay to day 6 monolayer cultures it was found that PPS markedly stimulated MPC proliferation with significant effects being observed at concentrations of 1–10 μg/ml ($p<0.01$). However, DNA synthesis by MPC after 4 days in mmc, showed a maximal stimulation by PPS at 1–2.5 μg/ml ($p<0.005$). A 38% reduction in MPC apoptosis was obtained in the presence of 1–10 μg/ml PPS. In 4–5 day MPC mmc, 5 μg/ml PPS stimulated PG synthesis by 100% more than control ($p<0.005$) while HA and DS at 5 μg/ml and greater inhibited PG synthesis ($p<0.005$). In 10 day MPC mmc, PPS promoted maximal Type II collagen deposition in the matrix at 5 μg/ml while maximal deposition of HA, relative to controls, occurred with 20 μg/ml PPS ($p<0.005$). The results of the bioassays examining cartilage matrix production by MPC in the presence of PPS were supported by the Real Time and RT-PCR gene expression studies using primers for Aggrecan, COL-II, SOX-9 and other genes. Notably, while able to stimulate chondrogenic differentiation of MPC, PPS at concentrations in excess of 1.0 μg/ml inhibited osteogenic differentiation of MPC ($p<0.01$).
Conclusions: These results confirmed that PPS at low in-vitro concentrations promoted early proliferation and chondrogenesis of MPC while also blocking its potential to undergo osteogenic differentiation. On the basis of the published literature and our preliminary unpublished findings we propose that the promotion of MPC chondrogenesis by PPS may be mediated by the ability of this agent to enhance degradation of IGFBP-3 by ADAM-12s.

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CHARACTERIZATION OF microRNA EXPRESSION PROFILES IN NORMAL AND OSTEOARTHRITIC HUMAN CHONDROCYTES

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Purpose: Osteoarthritis (OA) is a multifactorial disease characterized by destruction of the articular cartilage due to environmental, mechanical and genetic components. The genetics of OA is complex and is not completely understood. Recent work has demonstrated the importance of microRNAs (miRNAs) in cartilage function. MiRNAs are a class of small noncoding RNAs that regulate gene expression and are involved in different cellular process: apoptosis/proliferation, development, glucose and lipid metabolism.
The aim of this study was identify and characterize the expression profile of miRNAs in normal and OA chondrocytes and to determine their role in the chondrocyte biology.
Methods: Chondrocytes were obtained from 1 healthy donor and from 1 OA patient. After the first passage, the cells were moved to aggregate culture for a week. Evaluation of *in vitro* aggregate culture was carried out using histochemical (hematoxylin-eosin, Alcian Blue, Safranin O and Masson Tricromic) and immunohistochemical (Aggrecan, Collagen Type I and II) stainings. MicroRNAs were extracted with miRVana Isolation kit and analyzed at the CNIO Core Facilities (Madrid, Spain) using the Agilent Human miRNA Microarray. Raw microarray data were normalized and analyzed using Agilent FeatureExtraction (FE) Software and GeneSpring GX10. Of the 723 human miRNAs, a list of the differentially expressed miRNAs in normal and OA chondrocytes was bioinformatically analyzed (using *Sanger miRBase*, *microRNA.org*,

miRNAMap and *miRGen*) combining gene expression information with miRNA target prediction.
Results: We identified 7 up-regulated miRNA in both normal and OA chondrocytes (miR-663, miR-638, miR-125b, miR-23a, miR-23b, miR-103, miR-210) that through bioinformatic analysis could be related with the chondrocyte phenotype and with the hypoxia condition of the aggregate culture. In addition, 4 miRNA were up-regulated in normal chondrocytes (miR-18a, miR-801, miR-370, miR-224) and related with growth factors. Finally, 5 miRNA were up-regulated in OA chondrocytes (miR-656, miR-138, miR-369-3p, miR-143, miR-181d), related with inflammatory pathways and transcription factors implicated in the regulation of chondrogenesis.
Conclusions: The data reported can be relevant to better understand the molecular mechanism involved in the biology and in the pathophysiology of the chondrocytes. This preliminary study could help to improve the understanding of the pathogenesis of multifactorial diseases such as osteoarthritis and could have important diagnostic and therapeutic potential.
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MULTILINEAGE DIFFERENTIATION POTENTIAL OF HUMAN AMNIOTIC MEMBRANE-DERIVED STEM CELLS IS USEFUL FOR HUMAN ARTICULAR CARTILAGE REPAIR

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Purpose: Amniotic membranes have been used extensively as biologic dressings in ophthalmic, abdominal, and plastic surgery. Actually, there is growing evidence that the human amnion contains various types of stem cell. Human amniotic membrane (HAM) could be an alternative source to bone marrow. As amniotic membrane is highly abundant and readily available, it has the potential to be an important source of mesenchymal stem cells (MSCs) with multilineage differentiation potential for human articular cartilage repair. Given the minimal ethical and legal issues associated with its usage warrants further investigation into their functional potential in vivo. The aim of thisstudy was investigate the potential of mesenchymal cells derived from HAM to differentiate into chondrocytes, adipocytes and osteoblasts useful for regenerative medicine and cell therapy.
Methods: Human amniotic membranes were obtained from caesarean-sectioned mothers. Two different protocols of HAM isolation were performed. After monolayer expansion of the adherent cells, isolated from both protocols, we characterized them by flow citometry for MSC markers. Multipotentiality was studied by means of the differentiation towards adipocytes, osteoblasts and chondrocytes-like cells. Adipogenesis and osteogenesis were induced using commercial medium (Lonza Group Ltd, Switzerland). Chondrogenesis was performed using cells in aggregate culture and in a chondrogenic medium for 3 weeks. Histochemical (hematoxylin-eosin, Masson's thrichome, toluidine blue, safranin O, Oil-Red-O, Alizarin Red), immunohistochemical (type II and I collagens, aggrecan) and qPCR studies were performed in order to evaluate the multilineage potential. For qPCR analysis the following primers were used: Sox9, Agg and Col II (for chondrogenesis), LPL, FABP4 and AMP1 (for adipogenesis), ALP and OC (for osteogenesis).
Results: From each of the protocols we isolated two different populations of mesenchymal cells derived from HAM. Both populations were characterized by the presence of the same cell surface markers with the exception of CD44, CD73, CD105, CD166 and CD117 markers. However, only one of the two populations had a strong capacity to differentiate towards chondrocytes, adipocytes and osteoblast. In this population, after 21 days of culture, the differentiations showed to be consistent and were confirmed by qPCR analysis.
Conclusions: We isolated a high pluripotency mesenchymal stem cell population from HAM, suggesting that they may be very useful to repair articular cartilage injuries and to improve the understanding of the molecular events implicated in chondrogenesis. Further studies should be carried out to determine whether such *in vitro*-differentiated cells can function *in vivo*.
Project funded by Servizo Galego de Saúde (SERGAS), Xunta de Galicia (PS07/84) and Instituto de Salud Carlos III CIBER BBN CB06-01-0040. Claudia Cicione was supported by a Fellowship from the University of Rome "Sapienza", Italy, now she is contract by Fundación Complejo Hospitalario Universitario A Coruña, Spain. Emma Muiños Lopez is beneficiary of a Fellowship from Rheumatology Spanish Foundation, Spain. Silvia Diaz Prado is beneficiary of an Isidro Parga Pondal contract from Xunta de Galicia, Spain.

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THE EFFECT OF LOW-INTENSITY PULSED ULTRASOUND FOR SACFFOLD-FREE CHONDROCYTE PLATE IN VITRO AND IN VIVO

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Purpose: The aim of this study is to evaluate the effect of low-intensity pulsed ultrasound (LIPUS) for scaffold-free chondrocyte plate in vitro and in vivo.
Methods: Chondrocytes were collected from articular cartilage of Japanese white rabbits. For acquirement the number of cells, the collected primary chondrocytes (passage 0, P0) were cultured up to subconfluent in 500cm^2 square dishes. The cells were then condensed to the density at 10^7cells/cm^2 (passage 1, P1) on synthetic membranes with 0.2μm pore. The LIPUS application group was stimulated for 20 min/day. The mode of the applied ultrasound is a 200μs burst sine wave of 1.5 MHz repeating at 1kHz with an intensity of 30mW/cm^2. To investigate effect LIPUS stimulation on the matrix-synthesis of the constructs, mRNA expression of type II collagen (col2), aggrecan and typeIcollagen (col1) was studied using real-time polymerase chain reaction. Synthesis of type II collagen and proteoglycan was also assessed histochemically. We made full-thickness cartilage defect model in rabbit and tried to repair full thickness cartilage defect with allograft of the chondrocyte plate, and assessed histochemically.
Results: In our previous study, we presented that high-density culture with P1 chondrocytes more than 10^7cells/cm^2 could form a cell mass as scaffold-free cartilage under existing cell-cell interactions in rat model.
In this way, the chondrocytes (P1) prepared at 10^7cells/cm^2 detached from the membranes to form a plate of chondrocytes around the 7th day (day 7) of starting P1 culture. After forming the plate, the constructs were detached then by surgical pincers and moved to simple 6 well dishes for oxygen and nutrition diffusion. The expression of col2 and aggrecan mRNA was significantly higher in the group by stimulation of LIPUS (LIPUS group) than the group by no stimulation (sham group).