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Research Article

Chondroinductive Activity from Commercially Available Demineralized Bone Matrix Sponge on Mesenchymal Stem Cells

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Abstract

osteoarthritis (OA) evolution occurs due to the limited regenerative capacity of articular chondrocytes, which leads to the loss of function of the joint. For this reason our group working to develop new strategies to treat this disease. This study aimed to demonstrated the feasibility of using a human-based scaffold for the induction of mesenchymal stem cell to chondrocytes. The ATTC-PCS-500-12 (human mesenchymal stem cells, MSC) line was seeded in a human-scaffold commercially produced in a tissue bank in the presentation of a demineralized bone matrix sponge (DBMs), they were cultivated for 14 days under two in vitro conditions: i) culture medium with 10% of FBS, and ii) culture medium with 10% of FBS plus BMP-7 100 ng/ml. After the period of cell culture, an immunofluorescence assay was carried out for observing the expression of the characteristic proteins of the articular chondrocytes: SOX9, AGGRECAN and COL2A1. The results showed that the proteins expression was similar for the two in vitro conditions tested. In conclusion we established that the use of DBMs scaffold encourages the MSC differentiation to phenotype chondrocyte-like, without the need to add an extra growth factor to promote the differentiation of MSC to chondrocyte.

Keywords: Mesenchymal stem cell, Demineralized bone matrix, Chondro-inductive activity

Introduction

Osteoarthritis (OA) of the knee and other weight-bearing joints, is a pathology resultantly of mechanical of damage in articular cartilage superficies that by itself has a poor intrinsic capacity to repair and with the past of time progressing to the loss of joint mobility. The OA is and will continue to be the major health problem around the world, It is predicted that the population aged over 60 years will expand significantly by the year 2050 [1,2]. Unfortunately, the regenerative capacity of articular cartilage is limited, which reduces the treatment strategies to nonsteroidal anti-inflammatory drugs or surgery for those with severe symptoms [3]. Thus, most patients treated for early focal osteochondral lesions will ultimately require total joint replacement in the future as the disease advance [4].

The optimal osteochondral graft must address not only the damaged cartilage but also the underlying bone to allow an adequate subchondral repair and to support the overlying neo-cartilage. The tissue-engineered graft should produce tissue that is qualitatively similar to the native tissues at the site of the defect and incorporate quickly and fully into the host joint [5].

At the beginning of present century, was reported the use of scaffolds for growing chondrocytes, in most of them were not ideal for producing regeneration of cartilage and adequate mechanical integrity at the site of the joint defect [4,6,7]. Nowadays, the most recent research efforts have focused on the implementation of scaffolds that offer positive long-term results, like the use of: 3D bioprinting to fabricate cell-derived scaffolds [8]. decellularized cartilage matrix scaffolds with laser-machined micropores [9] and the use of thermosensitive hydrogels as scaffolds [10], These studies has been reported that the tissues obtained are inferior to native hyaline cartilage in terms of its mechanical properties due to the lack of structure and organization of articular cartilage. Therefore, it is proposed to continue investigating to find a definitive solution to these problems.

The use of BMP-7 has been reported as a growth factor for induction of three-dimensional in vitro chondrogenesis in human mesenchymal stem cells [11]. For this reason we used the BMP-7 as a positive control for the differentiation to MSC in our research and we proposed as aim of this work: if the demineralized bone matrix sponge (DBMs) possesses chondro-inductive activity in vitro, despite the osteo-inductive characteristics described for demineralized bone matrix (DBM) [12].

Study Design

Construct fabrication

The construct was fabricated using a MSC cell line isolated from human bone marrow, this cells line was purchased by our working group from the American Type Culture Collection (ATCC, PCS-500- 012,). The MSC were by seeding onto the DBMs scaffold (BioSponge, Biograft). The scaffold was cut with a Precision Tar-

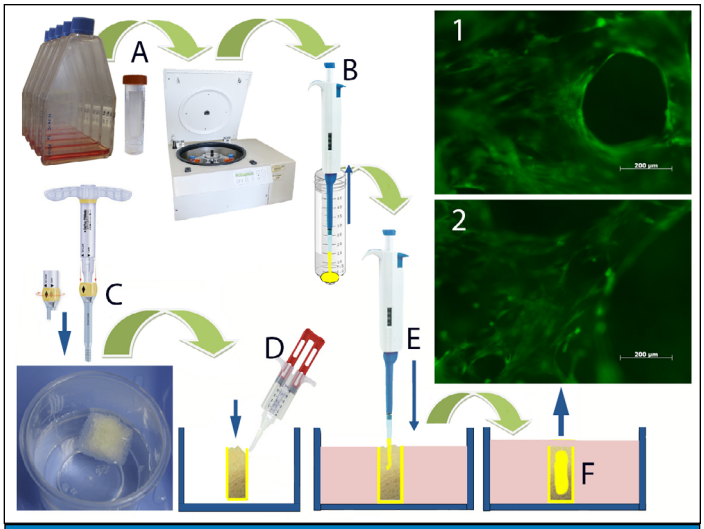

Figure 1: Diagram of the process of seeding the MSC cells in the demineralized bone matrix. A) Harvest of the MSCs, using culture flasks, by applying of trypsin and concentrating the MSCsutilizing 50 mL tubes and centrifugation; B) The cellular button obtained was mixed with the fibrin glue before being deposited in the demineralized bone matrix; C) Cut of demineralized bone matrix, (6mm diameter and 5mm height) by a Precision Targeting System (COR, DePuySynthes); D) Sealing the walls of the demineralized bone matrix with the matrix fibrin glue; E) Using a 200µl micropipette tip, were injected $2.5x10^5$ MSC inside to demineralized bone matrix; F) After spreading the MSC in the demineralized bone matrix, the construct was covered with culture medium according to the assigned experimental condition; and G Microfotography of calcein staining (green color), which indicates the viability of MSC in the demineralized bone matrix, 1) only media, 2) media plus BMP-7.

geting System (COR, DePuySynthes; 6mm to diameterand 5mm height) and placed in a 6-well cell culture plate. The MSC cells were seeded by slowly dispensing the cell solution ($2.5x10^5$ cells) with a fibrin glue (TISSEEL), the fragment of DBMs was sealed in the outer walls with human fibrin glue. Two versions of the construct were fabricated and cultivated for 14 days: 1) DBM with MSC, DMEM+10% FBS, and 2) DBMs plus the MSC and DMEM+10% of FBS plus BMP-7 (100ng/mL). The cultures were maintained at 37°C with 5% CO_2, and change of medium every other day (Figure 1).

Immunofluorescence assay

The constructs were fixed with 2% paraformaldehyde, followed by a solution of PBS/albumin 1%/triton 0.3% for 20 minutes to block unspecific binding sites. We used the following primary antibodies at a dilution of 1:500: anti-AGGRECAN, conjugated with FITC (Novusbio laboratories), anti-SOX9 (Santa Cruz), anti-COL2A1 (Santa Cruz), and were incubated overnight at 4°C. Later, both slides were washed two times with PBS + 0.1% triton and the secondary antibodies were added: Anti-IgG FITC (Santa Cruz) and Anti IgG1 FITC (Santa Cruz), diluted to 1:1000, following, the slides were incubated for 2h at 37°C and later, the slides were washed again with PBS + 0.1% triton to remove excess secondary antibody. For the isotype-controls we used anti-mouse IgG2a FITC (Santa Cruz), anti-mouse IgG1FITC (Santa Cruz), and ant-rabbit IgG1FITC (ABCAM). Finally, the slides were mounted with DAPI. The images were recorded using a pyramid microscope with UV lamp Carl Zeiss Axio system image Vission 4.8.2. All experiments were performed in triplicate for immunofluorescence and calcein staining. For obtain the percentage of fluorescence for each fluorochrome, 10 photos of each sample were taken and Image J program (NIH) was used to obtain the mean of the fluorescence percentage for each sample.

Statistical analysis

The data of this study were compilated in an Excel data base (Microsoft Office for PC), and processed with the STATISTICA ver. 10 software. The percentages of fluorescence obtained for the markers for articular cartilage: Sox9, AGGRECAN and COL2A, were analyzed. A Kolmogorov-Smirnov test was applied to establish if the samples presented a normal behavior and as a result of this, a T test for two independent samples was applied to establish if there were statistically significant differences between the experimental conditions.

Results

After 14 days of seeded the MSCs in the DBM, the cells were observed viable according to the calcein staining (Figure 1). As a result of the immunofluorescence, it was observed that for the two experimental conditions, positive cells were found for AGGRECAN, COL2A1 and SOX9 markers (Figure 2).

As a result of the quantification of the percentage of fluorescence for each of the markers analyzed by immunofluorescence, an average of 55.5 ± 7.70% was found, for the COL2A1 marker with the MSC seeded in the DBM, and when comparing this marker with the MSC seeded in DBM plus BMP-7 (45.8 ± 3.4%), no statistically significant differences were found between these two experimental conditions ($p = 0.361$). In the same way, when comparing the AGGRECAN marker, for MSC seeded in the DBM (47.9 ± 4.4%), against MSC seeded in DBM plus BMP-7 (45.1 ± 5.0%), no statistically significant differences were found ($p = 0.696$). The last comparison was made for the SOX9 marker, between the MSC seeded in the DBM (51.5 ± 4.2%), against MSC seeded in DBM plus BMP-7 (51.6 ± 3.7%), again, no differences were found. statistically significant ($p = 0.850$), Figure 3.

Discussion

The DBM is an osteoconductive and osteo-inductive commercial biomaterial with medical approval used in bone defects with a long track record of clinical use in diverse forms [1]. True to its name and as an acid-extracted organic matrix from human bone sources, DBM retains much of the proteinaceous native to bone, with small amounts of calcium-based solids, inorganic phosphates and some trace cell debris [13]. Many of DBM`s proteinaceous components, e.g., growth factors, are known to be potent osteogenic agents. DBM was the original source for discovery of several key growth factors that were subsequently cloned and recombinantly expressed to become FDA-approved clinical therapeutic proteins: BMP-2 and BMP-7 in bone repair [13].

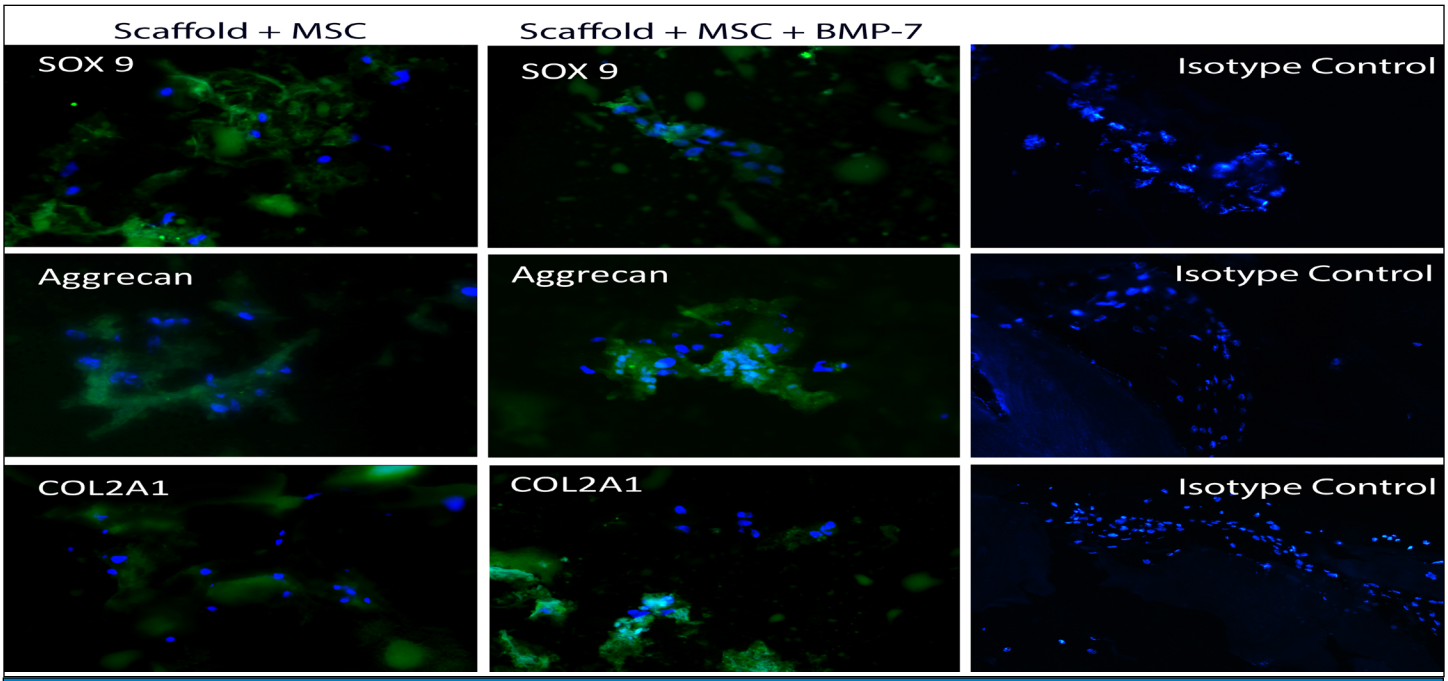

Figure 2: Inmunofluorescence of MSC seeded in the bone matrix fragment, in two different experimental conditions after 14 days in cellular culture. The nuclei of each cell appear in blue (stained with 4`6-diamidino-2-phenylidole (DAPI) and in green marking indicates positivity for some one of different cartilage markers that we used (COL2A1, AGGRECAN and SOX9). The positive markers are in green labeling with fluorescein isothiocynate fluorochorme (FITC).

With the appropriate matrix, it may be possible to construct a single unit that has the capacity to repair both the chondral and subchondral defects [14]. This could improve the initial attachment and integration of the implanted construct into the joint, this due to the intrinsic characteristics described for the DBM such as: natural porous structure of the DBM scaffold, that provides a surface area and internal space for extracellular matrix secretion, as well, the good biocompatibility and biodegradability, low antigenicity, and no cytotoxicity provided by the DBM [15].

In this study, we have fabricated and characterized a novel osteochondral construct containing fragment of DBMs plus MSC. Two types of the construct were fabricated, with and without BMP-7, after 14 days of culture the expression of COL2A1, SOX9 and aggrecan, was similar between the two culture conditions, (Figure 2). In a similar study using adult cells, the chondrocyte phenotype was maintained for 6-week culture in a DBM-gelatin matrix [6], in another investigation, Wayne, et al. [7] reported that use the 3D scaffolds (PLA-alginate), stimulate the cartilage-like matrix quality, cell distribution, and proteoglycan staining. Biomechanically, experimental and control specimens exhibited similar behavior; however, both tissues were still immature compared with normal cartilage [7].

The DBMs construct produced in this study have a number of potential advantages. First it contains intrinsic growth factors that have a proven stimulatory effect on chondrogenesis and osteogenesis [16,17] For this Reason, the addition of extrinsic growth factors does not appear to be required. Second, demineralized bone matrix has an excellent biocompatibility without the toxic properties of others scaffolding materials. This inert material is not likely to create a host immune or inflammatory response due to the absence of cellular antigens [6]. Third, demineralized bone matrix retains both its native morphology and mechanical properties, making it structurally capable of "press fit" fixation into an appropriately prepared defect. Finally, it would help develop the zonal organization of articular cartilage, which is crucial for its normal function [18,19].

The main limitation of the present work focuses on the need to deepen the molecular analysis of BDM and the interactions with MSCs, for this reason, in this present short communication, we

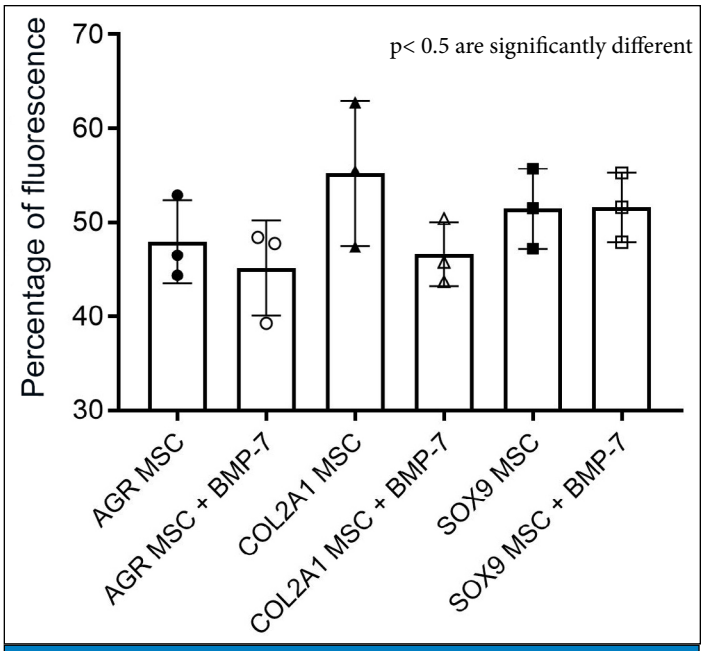

Figure 3: Comparison of the fluorescence averages of the markers: AGGRECAN (AGR), COL2A1 and SOX9, for articular cartilage evaluated for the two experimental conditions: i) culture medium with 10% of FBS and culture medium (MSC), and ii) culture medium with 10% of FBS plus BMP-7 100 ng/ml (MSC + BM-7).

present the first preliminary results with the intention to disseminate to the scientific community and will require further investigation before it can be widely applied to repair the osteochondral defects *in vivo*. Thus, further work on the culture environment and time will be needed to improve the consistency of the chondral product. Finally, *in vivo* studies will be required to define the ability of this tissue-engineering construct to unite with the surrounding joint surface.

Conclusion

Our results indicate that is feasible to use a human-based scaffold 3D (DBMs), for inducing MSC differentiation into chondral linage and promotes the expression of hyaline cartilage proteins: Sox9, AGR and COL2A.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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