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Research Article

Chondrogenic Differentiation of Human Wharton's Jelly and Bone Marrow-Derived Mesenchymal Stem Cells: Focus on the Role of an Acrylamide-Free Cross-Linked Hyaluronic Acid Hydrogel

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Abstract

Hyaluronic Acid (HA) is a natural biopolymer commonly used to reduce inflammatory process in degenerative joint disease such as Osteoarthritis (OA). In the last years, its possible use as biocompatible scaffold for cartilage tissue engineering was highlighted. In this field, to achieve promising results, recent studies have underlined the advantages of combining tissue engineering and cell therapy approaches. To date, the mainly cells employed for cartilage regeneration are Mesenchymal Stem Cells (MSCs) isolate from bone marrow. However, since their use has several limitations, there was a growing interesting in MSCs from perinatal tissue, such as umbilical cord, as possible source for cartilage regeneration. Therefore, the aim of this study was to investigate the role of an innovative acrylamide-free cross-linked HA Hydrogel formulation (HyLink®) on chondrogenic differentiation process employing both human umbilical cord Wharton's jelly- and Bone Marrow-derived MSC (hUC-WJ-MSC s and hBM-MSCs). Our data showed that the HyLink improved the chondrogenic differentiation in both mesenchymal cellular models as revealed by the increase of Glycosaminoglycans (GAGs) deposition and the mRNA levels of chondrogenic markers (SOX9, COL2a1 and ACAN). In addition, the results demonstrated that hUC-WJ-MSC have a greater cartilage differentiation potential compared to the MSCs isolated from bone marrow. Overall, our results demonstrate that HyLink improves chondrogenic differentiation process both in hBM-MSCs and hUC-WJ-MSC . Since the latest showed greater cartilage differentiation potential, we suggest that MSCs isolated from perinatal tissue might be a promising cell source for regenerative medicine in the orthopedic field.

Keywords: Cartilage; Hyaluronic acid; Mesenchymal stem cells; Regenerative medicine; Osteoarthritis

Introduction

Osteoarthritis (OA) is one of the most degenerative joint diseases characterized by a progressive articular cartilage destruction and loss [1]. At present there are no effective treatments for OA and the only available approaches have the scope to alleviate pain and improve daily functioning in OA patients [2]. These include physical therapy, weight loss, lifestyle changes, surgical options (such as arthroplasty) and intra-articular injections of anti-inflammatory compounds, such as corticosteroids and Hyaluronic Acid (HA) [3]. HA is an endogenous high-molecular-weight biopolymer produced by the type B synoviocytes and synovial fibroblasts. It represents one of the main components of synovial fluid and cartilage matrix where, thanks to its viscoelastic properties acts as joint lubricant and shock absorber to protect cartilage [4,5]. However, due to the inflammatory process, OA patients usually shown low levels of HA in the affected joint [6,7]. For this reason, several intra-articular HA formulations, derived from avian, bacterial and acrylamide-based ones, have been approved as viscosupplementation procedure to decrease inflammation, restore joint lubrication and exert chondroprotective effects [8,9]. Recently, acrylamide and its derivatives commonly used in the synthesis of different scaffolds, was shown to be a neurotoxicant, reproductive toxicant and carcinogenic [10]. For these reasons, in recent times, the chemical-pharmaceutical technologies have been focused on the development of innovative acrylamide-free products.

In addition to the above, the cell therapy has been employed to improve pain and function in OA [11]. The first cell-based therapy approved for cartilage regeneration was based on the injection of autologous or fetal chondrocytes, the principal cell type found within cartilage [12-14]. However, given the limited availability of this cells source, Mesenchymal Stem Cells (MSCs) were recently proposed as alternative ideal option for cartilage regeneration [15]. Indeed, it is well established that MSCs from adult tissue are undifferentiated cells with a high proliferative capacity and the ability to differentiate into various cell lineages including adipocytes, osteoblasts and chondrocytes [16]. MSCs can also exerts anti-inflammatory and immunosuppressive effects that may be useful for OA treatment [17]. Among the various sources of MSCs, Bone Marrow MSC (BM-MSCs) are the most commonly employed [18-22], although it is well established that these cells are associated with several limitations such as the invasive and painful bone marrow harvesting procedure, the limited number of isolated cells, the differentiation potential influenced by the donor age and the high immunological reactivity once transplanted *in vivo* [23,24]. Therefore, besides these, perinatal stem cells from extra-embryonic tissue, such as placenta, amniotic fluid and umbilical cord, could represent a valid alternative source. Indeed, their peculiar features that make them promising candidates for cell therapy in regenerative medicine and tissue engineering [25]. In the field of tissue engineering, biocompatible and biodegradable scaffolds are frequently used with the aim to mimic the *in vivo* microenvironment and provide mechanical support to the cells [26]. As regard the chondrogenic tissue engineering, among the possible useful scaffolds, the HA based ones seem to be promising since it is a naturally occurring biomaterial with a low toxicity and able to supports MSCs migration [27].

Hence, considering the growing interest in the last years in MSCs of perinatal origin and that little is known about their potential regenerative in cartilage defects, the purpose of this study was to investigate the role of an innovative formulation of acrylamide-free cross-linked HA hydrogel during the chondrogenic differentiation of both human umbilical cord Wharton's Jelly- and Bone Marrow-derived MSCs (hUC-WJ-MSC and hBM-MSCs respectively).

Material and Methods

Cell cultures and Phenotype Characterization

This study was approved by the University Institutional Review Board and Ethical Committee (G. d'Annunzio University Chieti-Pescara) and adhered to the tenets of the Declaration of Helsinki. Furthermore, after a detailed explanation of the study, the written informed consent was obtained from each patient to collect the human samples necessary for the cell's isolation. hUC-WJ-MSC were isolated through spontaneous migration from Wharton's Jelly fragments obtained from healthy mothers-derived umbilical cords processed within 4 hours. Briefly, small pieces of Wharton's Jelly fragments (2-4 mm in length) were placed directly into a 25 cm^2 flask in Dulbecco's modified Eagle Medium low glucose (DMEM L-GLU, Gibco-Life Technologies, Waltham, MA, USA), 10% fetal bovine serum (FBS, Gibco-Life Technologies), 1% L-glutamine (L-GLU), and 1% of penicillin/streptomycin (P/S) (Sigma-Aldrich). hBM-MSCs were obtained from bone marrow harvested by patients undergoing orthopedic surgery procedure. Briefly, the cells were separated by Histopaque®-1077 (Sigma-Aldrich) from 20 ml of aspirates, resuspended and placed in a Petri dish with control medium (CTRL) composed of alpha minimum essential medium (α-MEM, Gibco-Life Technologies, Waltham, MA, USA), 10% FBS, 1% L-GLU, 1% P/Sand 3 ng/ml of β-FGF.

Following 10 days of culture (5% CO_2 and 37 °C) both cellular types were characterized by cytometric analysis for the expression of the typical mesenchymal stem markers. The analyses were performed using the FACSVerse (BD Biosciences, San Jose, CA, USA) and the FACSsuite software (BD Biosciences, San Jose, CA, USA). Briefly 10^6 cells per sample were incubated with the following conjugated primary antibody: FITC-conjugated anti-CD44, anti-CD45, anti-CD105, anti-CD166, and PE-conjugated anti-CD29were obtained from Ancell (Bayport, MN); anti-CD14-FITCwas obtained from MiltenyiBiotec (BergischGladbach,Germany); anti-HLA-DR-PE, anti-HLA-ABC-FITC,anti-CD90-FITC, anti-CD73-PE, anti-Sox-2-FITC, anti-SSEA-4-FITC, anti-OCT4-PE, anti-CD34-PerCP were obtained from Becton Dickinson (BD, San Jose, CA). Data were analyzed by the FlowJo software v8.8.6 (TreeStar, Ashland,

OR). The mean fluorescence intensity (MFI) ratio values were calculated (i.e, dividing the MFI of positive events by the MFI of negative events).

3-(4, 5-dimetHylthiazolyl-2-yl)-2,5-diphenyltetrazolium Bromide (MTT)

The effect of acrylamide-free cross-linked HA coating on hBM-MSCs and hUC-WJ-MSC metabolic activity was assessed by MTT assay (cat. M211281G, Sigma Aldrich) according to the manufacturer's instructions. Cells were seeded in a 96-well plate at the density of 3000 cells/well and were incubated with the CTRL and Chondrogenic Medium (CM) in presence or absence of HyLink coating (50µL/cm^2; HyLink®: Seikagaku Corporation Tokyo, Japan, through MDM S.p.A in Italy). The CM used for the differentiation process was composed of Dulbecco's modified Eagle Medium high glucose (DMEM H-GLU, Gibco-Life Technologies, Waltham, MA, USA), 10% FBS, 1% L-GLU, 1% P/S, 1% ITS-X (cat. 41400045, ThermoFisher Scientific, Waltham, MA, USA), sodium pyruvate 1% (cat. 11360070, ThermoFisher Scientific, Waltham, MA, USA), 0.05 mM of acid ascorbic 2-phosphate (cat. 66170-10-3, Sigma-Aldrich, Saint Louis, USA), dexamethasone 100 nM (cat. D4902-25MG, Sigma-Aldrich, Saint Louis, USA) and TGF-β1 10 ng/ml (cat. 100-21, Peprotech, Cranbury, NJ, USA).

After 7 days of culture, the spectrophotometric analysis was carried out at a wavelength of 540 nm using a microplate absorbance reader (SpectraMAX 190, Molecular Devices, San Jose, CA, USA)

HyLink coating and Chondrogenic induction

hUC-WJ-MSCs and hBM-MSCs were seeded (15000 cells/well in six well plates) and induced to chondrogenic differentiation with CM for 28 days (5% CO2 and 37° C) in the presence or absence of the acrylamide-free cross-linked HA coating (50µL/cm^2; HyLink®: Seikagaku Corporation Tokyo, Japan, through MDM S.p.A in Italy)

Alcian Blu Staining

Alcian Blu staining (cat. 04-163802A, Bio-Optica) was performed to evaluate the effect of HyLink coating on Glycosaminoglycans (GAGs) deposition during chondrogenic differentiation. Following manufacturer's instructions, the quantification analyses were performed at a wavelength of 620 nm using a microplate reader (SpectraMAX 190; Molecular Devices, San Jose, CA, USA).

RNA isolation and qRT-PCR

The effect of HyLink on chondrogenic differentiation was also evaluated through qRT-PCR in both hUC-WJ-MSC and hBM-MSCs. Total RNA was isolated following 28 days of chondrogenic induction by using Trizol reagent (ThermoFisher Scientific) and then was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The TaqMan Universal Master Mix II and TaqMan Gene Expression Assay (ThermoFisher Scientific) probes for human SOX9 (Hs00165814_m1), human COL2a1 (Hs00264051_m1), human ACAN (Hs00153936_m1), and GAPDH (Hs02786624_g1) were used. qRT-PCR experiments were performed using the QuantStudio 7 pro Real-Time PCR system (ThermoFisher Scientific). The relative gene expression was calculated using the comparative 2−DDCT method.

Statistical analysis

Data were expressed as mean ± error standard (SEM). Statistical analysis was performed using the Mann-Whitney test. The α value was set at 0.05. Analyses and graphs were performed using GraphPad Prism Software Analysis (version 9).

Results

Cells Phenotype Characterization

Adherent cell populations from hUC-WJ-MSC and hBM-MSCs were generated and characterized as reported in Figure 1. The cytometric analyses confirmed that both cellular types were positive for the expression of the mesenchymal markers (CD73, CD90, and CD105) and for the surface adhesion molecules (CD29, CD44, and CD166). On the contrary, hUC-WJ-MSC and hBM-MSCs did not express the hematopoietic molecular markers (CD14, CD34 and CD45) while they were highly positive for the intracellular stemness markers SSEA-4 and Sox-2 but expressed low -levels of OCT4. As expected, both cellular types were also positive for the MHC class I antigen (HLA-ABC) but did not express the MHC class II antigen (HLA-DR).

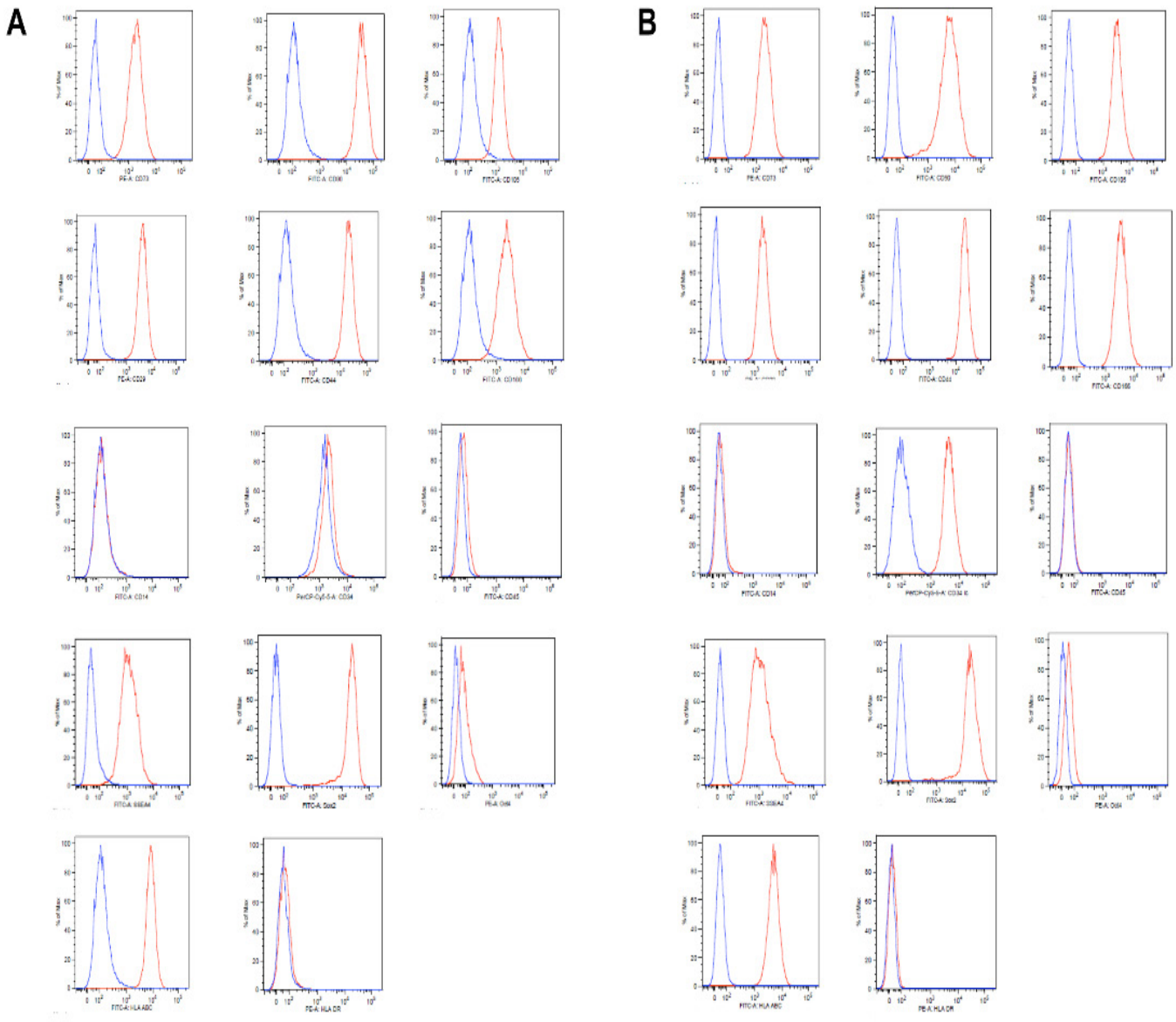

Figure 1: Cells phenotype characterization. Representative histograms of the CD73, CD90, CD105, CD29, CD44, CD166, CD14, CD34, CD45, SSEEA4, Sox2, OCT4, HLA ABC and HLA DR expression evaluated through cytometric analysis in (A) hUC-WJ-MSC and (B) hBM-MSCs.

Effect of HyLink coating on hUC-WJ-MSC and hBM-MSCs viability

Once confirmed the mesenchymal stem cells phenotype of both hUC-WJ-MSC and hBM-MSCs, the effect of HyLink coating (50 µl/cm^2) on cell viability was evaluated using the MTT assay. As clearly shown in Figure 2, the coating did not exert a cytotoxic effect as it did not induce a significant decrease on cells metabolic activity of both hUC-WJ-MSC and hBM-MSCs.

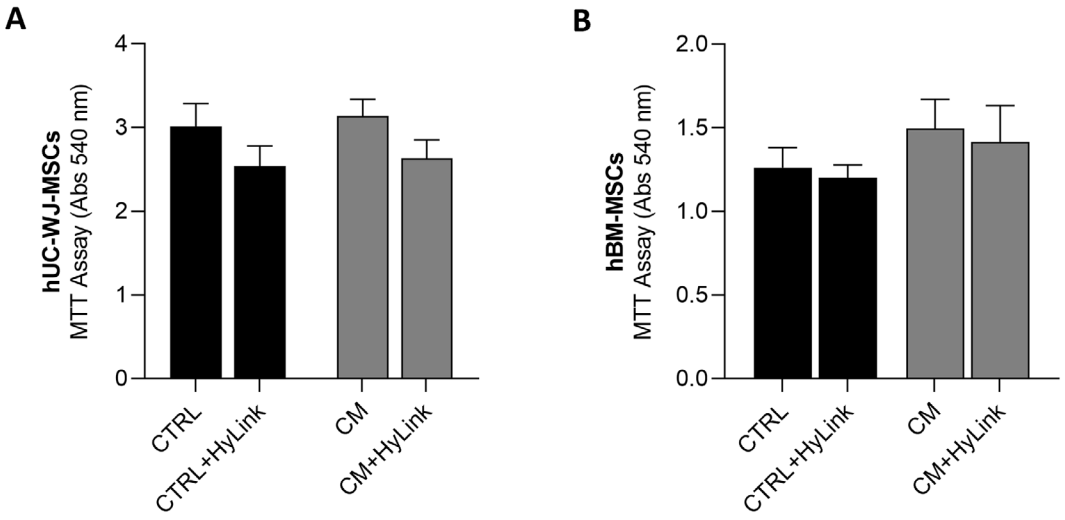

Figure 2: Cells metabolic activity. Effects of HyLink coating on **(A)** hUC-WJ-MSC and **(B)** hBM-MSCs following 7 days of culture. Results are shown as mean ± error standard (SEM) (n≥3).

Effect of HyLink coating on GAGs deposition

In order to investigate the effect of HyLink on hUC-WJ-MSC and hBM-MSCs chondrogenic differentiation, GAGs deposition was assessed after 28 days of cultured cells with CM in presence or absence of coating. Interestingly, compared to cells treated with the CM alone, the HyLink coating induced a significant increase of Alcian Blue staining both in hUC-WJ-MSC and hBM-MSCs (Figure 3). Of note, it is important to point out that in the absence of the coating we found a significant basal higher chondrogenic differentiation ability in hUC-WJ-MSC compared to hBM-MSCs (p<0,005).

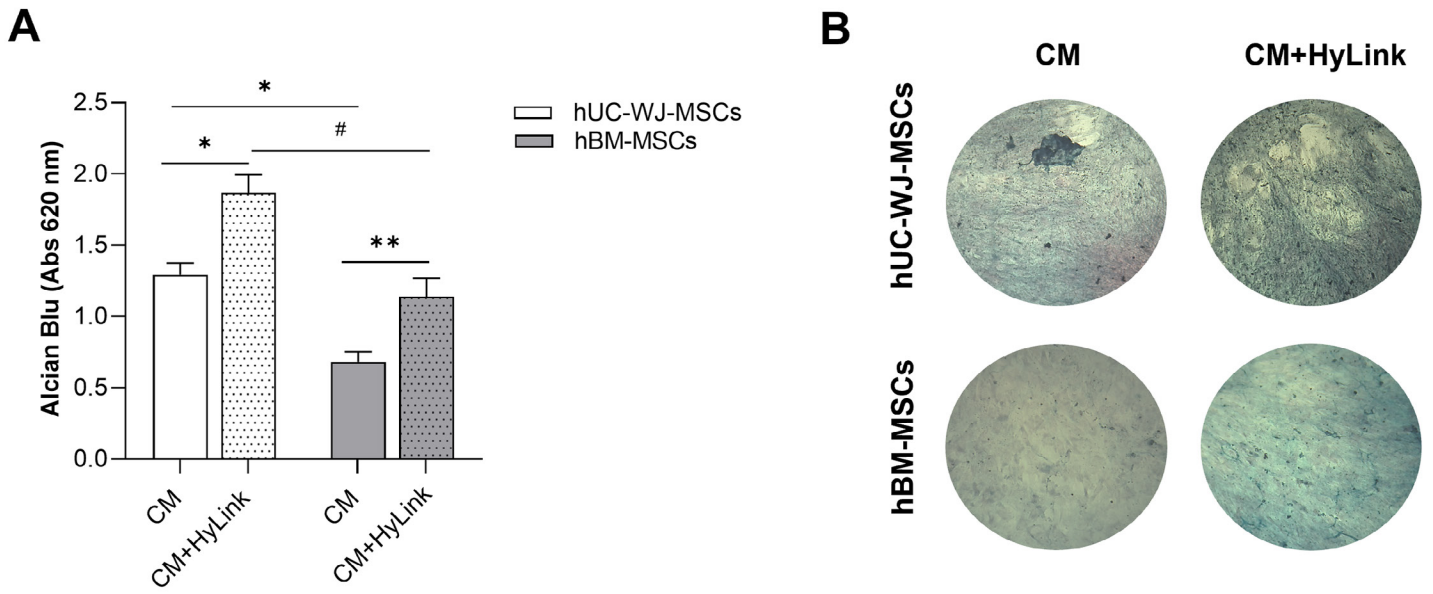

Figure 3: Chondrogenic differentiation: Alcian Blue staining. Effect of HyLink on GAGs deposition: **(A)** Relative quantification and **(B)** representative images of Alcian Blue staining in hUC-WJ-MSC (grey bars) and hBM-MSCs (black bars) differentiated for 28 days in CM. Results are mean ± error standard (SEM) (n≥3). (*p<0.05 vs CM hUC-WJ-MSC : **p<0.05 vs CM hBM-MSCs; #p<0.05 vs CM+HyLink hUC-WJ-MSCs).

hUC-WJ-MSC and hBM-MSCs responsiveness to HyLink coating

The mRNA expression of specific chondrogenic markers was then evaluated in hUC-WJ-MSC and hBM-MSCs treated with CM in presence or absence of HyLink coating. As revealed by the graphs in Figure 4A, the SOX9, ACAN and COL2a1 mRNA expression was significantly improved in hUC-WJ-MSC cultured on coating. These results were also confirmed in hBM-MSCs (Figure 4B) which did not show mRNA expression of the typical chondrogenic marker COL2a1, indirectly confirming the potential higher capability of the perinatal hUC-WJ-MSC to chondrogenic differentiation (Figure 4B).

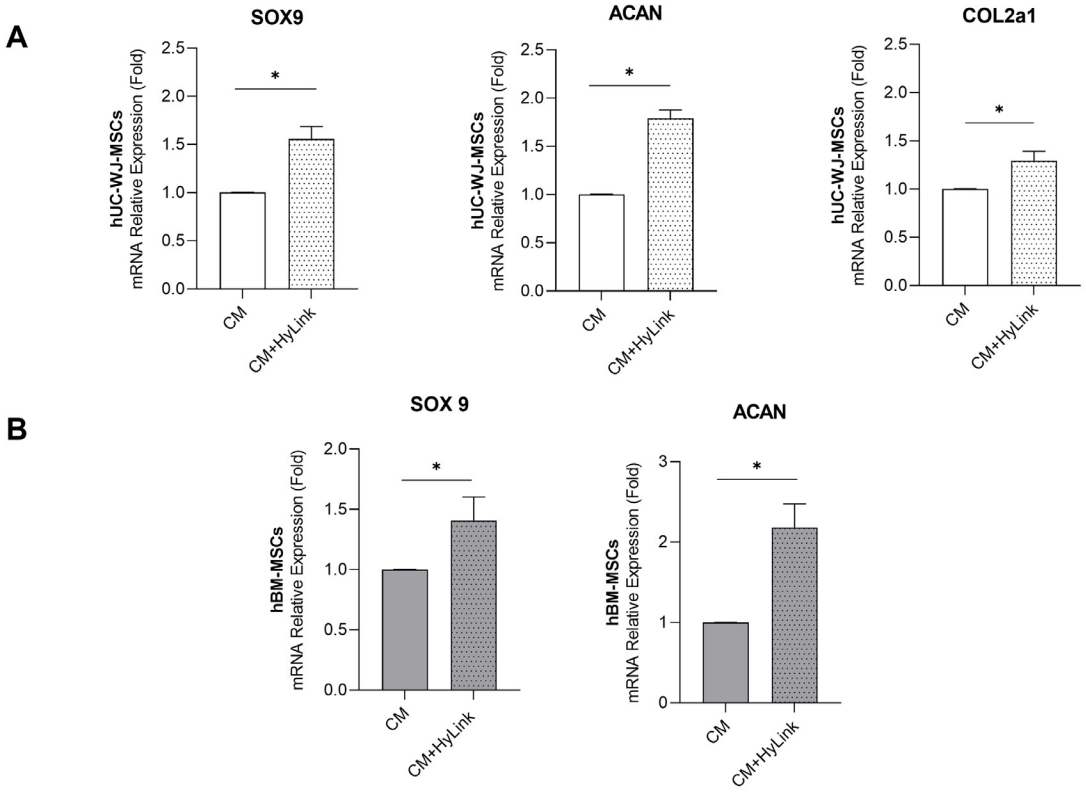

Figure 4: Chondrogenic differentiation: qRT-PCR. Effects of HyLink coating on the mRNA expression of the specific chondrogenic markers in **(A)** hUC-WJ-MSC (white bars) and **(B)** hBM-MSCs (grey bras) differentiated for 28 days in CM. Results are mean ± error standard (SEM) (n≥3). (*p<0.05 vs CM).

Discussion

OA is the most widespread joint disease worldwide, affecting an estimated 10% of men and 18% of women over 60 years of age [28]. Therefore, over the years both pharmacological and surgical therapeutic approaches have been proposed. Among the pharmacological treatment it is important to mention the injections of exogenous compounds such as corticosteroids, HA and growth factors. However, the typical dense consistency of the cartilage tissue due to the components of the matrix, together with the absence of vascularization, limits the transport and bioavailability of such injected compounds [29]. What concern the current surgical techniques [30, 31], such as allo-, xeno- and self-transplantation, these approaches are invasive and not always a complete structural integration of the transplanted tissue was achieved. For these reasons, great interest has recently been given to the development of alternative approaches for the treatment of traumatic and degenerative cartilage defects such as OA. As regard of this, different studies highlighted the possible application of cell-based therapy by using MSCs. These are undifferentiated, multipotent cells able to differentiate *in vitro* into osteoblasts, adipocytes and chondrocytes [11]. Although bone marrow is the most commonly source of MSCs used for the cartilage regeneration [32,33], they have numerous limitations. These include the invasive, difficult and painful harvesting practice, a limited number of isolated

cells (0.001% of the total cell population) and the differentiation potential influenced by the donor age. Furthermore, the use of allogenic BM-MSCs is unsuitable due to the high immunological reactivity in allogeneic transplants [23,24].

Therefore, the researchers in recent years have focused on alternative and more easily obtainable sources of stem cells such as the perinatal tissues which include placenta, amniotic fluid and umbilical cord [34]. Indeed, these cells are isolated from waste tissues with no ethical problems, present a low immunological reactivity and shown an intermediate differentiation potential between multipotent and pluripotent mesenchymal stem cells [35]. Even though several studies have been conducted in order to evaluate their regenerative features, little is known about their chondrogenic potential in regenerative medicine in the orthopedic field [36]. For these reasons, in the present study particular attention was given to the biological properties of MSCs isolated from Wharton's Jelly and to their chondrogenic differentiation ability. In fact, it was well established that these cells show a good proliferative capacity and a lower immunological reactivity compared to hBM-MSCs, characteristics that make them promising candidates for cell therapy in the field of regenerative medicine and tissue engineering [34]. In this context, the current literature has also highlighted the need to develop innovative system useful to promote the growth and the differentiation process of MSCs. These systems, defined scaffolds, allow to mimic *in vitro* the extracellular matrix characteristics and can be synthesized starting from natural or synthetic compounds such as collagen, gelatin and HA [37].

HA is a polysaccharide composed of repeated disaccharide units of N-acetyl-d-glucosamine and d-glucuronic acid, present in connective tissues, including cartilage and synovial fluid [38]. Its physiological functions are to preserve tissue hydration, lubricate and cushion shocks in the joints [39]. In the context of tissue engineering, the products derived from HA have shown a high biocompatibility, a reduced immunogenicity and a high biodegradability. HA-based scaffolds can be also synthesized in different forms including hydrogels, nets, fibers and porous sponges [40,41]. Interestingly, in order to limit the use of acrylamide, innovative chemical-pharmaceutical technologies have made it possible to synthesize derivatives in which the cross-linking HA is determined by residues of the safer cinnamic acid (cross-linked HA). This innovative formulation presents several advantages such as a controlled degradation, a greater viscoelasticity, better mechanical properties and good biological responses (M.D.M Spa, Italy). However, few studies have been reported how the use of the cross-linked HA could improve *in vitro* the chondrogenic differentiation of MSCs [42].

Therefore, the present work aimed to evaluate the effect of an innovative formulation of acrylamide-free cross-linked HA Hydrogel (HyLink) on the chondrogenic differentiation process of both immunophenotyped hUC-WJ-MSC and hBM-MSCs (Figure 1). It should be noted that, although this innovative formulation is characterized by the absence of acrylamide, its viscous properties have made it difficult to optimize the *in vitro* experimental protocols. Therefore, once standardized the experimental coating procedure, the potential cytotoxic effect of the HyLink coating was evaluated by MTT assay (Figure 2). Given that we did not find significant effects in term of cellular metabolic activity, the role of HyLink in improving hUC-WJ-MSC and hBM-MSCs chondrogenic differentiation was evaluated through Alcian Blue staining (Figure 3) and gene expression analysis of SOX9, COL2a1 and ACAN (Figure 4).

Interestingly, the obtained results showed that the HyLink coating was able to increase the GAGs deposition and the mRNA expression of the aforementioned chondrogenic markers, thus confirming the positive role of HyLink during the chondrogenic commitment. These results are in agreement with a previous study, conduct by Bauer and colleagues [42], in which they found an increased in the expression of cartilage-specific genes and the production of sulfated glycosaminoglycans in the osteoarthritic chondrocytes seeded over the HA scaffold. In addition, it is important to remark that in our study the hUC-WJ-MSC , also in the absence of the HyLink coating, have shown a greater cartilage differentiation potential compared to the MSCs isolated from bone marrow. Therefore, these data support the evidence that perinatal MSCs such as hUC-WJ-MSC could be a suitable source of mesenchymal cells potentially useful in regenerative medicine and tissue engineering in orthopedic field. Furthermore, despite this study was conducted by using a conventional bidimensional culture system, our data suggest that this innovate formulation of hyaluronic acid can be useful not only for the treatment of OA pain, but also as a scaffold able to promote chondrogenic differentiation in MSC, isolated both from bone marrow and Wharton's Jelly. Of note, these results paving the way for future studies focused on the development of Three-Dimensional (3D) culture, also employing specific bioreactors, in order to reproduce *in vitro* the cartilage tissue which may be particularly advantageous to better understand the molecular mechanisms underlying MSCs chondrogenic differentiation.

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