Anti-inflammatory and anti-catabolic effect of non-animal stabilized hyaluronic acid and mesenchymal stem cell-conditioned medium in an osteoarthritis coculture model

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Abstract. Previous clinical studies have reported the clinical effectiveness of non-animal stabilized hyaluronic acid (NASHA) and adipose-derived mesenchymal stromal/stem cells (MSC) in the treatment of knee osteoarthritis (OA). Unlike MSC secreted mediators, *in vitro* anti-inflammatory effects of NASHA have not been evaluated. We aimed to evaluate and compare the anti-inflammatory effect of NASHA and MSC conditioned medium (stem cell-conditioned medium; SC-CM), in an explant-based coculture model of OA. Cartilage and synovial membrane from seven patients undergoing total knee arthroplasty were used to create a coculture system. Recombinant IL-1β was added to the cocultures to induce inflammation. Four experimental groups were generated: i) Basal; ii) IL-1β; iii) NASHA (NASHA + IL-1β); and iv) SC-CM (SC-CM + IL-1β). Glycosaminoglycans (GAG) released in the culture medium and of nitric oxide (NO) production were quantified. Gene expression in cartilage and synovium of *IL-1β*, matrix metallopeptidase 13 (*MMP13*), ADAM metallopeptidase with thrombospondin type 1 motif 5 (*ADAMTS5*) and tissue inhibitor of metalloproteinases 1 (*TIMP1*) was measured by reverse transcription-quantitative PCR. Media GAG concentration was decreased in cocultures with NASHA and SC-CM (48 h, $P<0.05$; 72 h, $P<0.01$) compared with IL-1β. Production of NO was significantly lower only in SC-CM after 72 h ($P<0.01$). In cartilage, SC-CM inhibited the expression of *IL-1β*, *MMP13* and *ADAMTS5*, while NASHA had this effect only in *MMP13* and *ADAMTS5*. In synovium, SC-CM decreased the expression level of *MMP13* and *ADAMTS5*, while NASHA only decreased *ADAMTS5* expression. Both NASHA and SC-CM increased *TIMP1* expression in cartilage and synovium. Treatments with NASHA and SC-CM were shown to be a therapeutic option that may help counteract the catabolism produced by the inflammatory state in knee OA. The anti-inflammatory mediators produced by MSC promote a lower expression of inflammatory targets in our study model.

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Introduction

Osteoarthritis (OA) is a chronic and progressive disease that affects more than 20% of people over 45 years (1). The pathology of knee OA includes progressive articular cartilage loss, sclerotic changes in subchondral bone, and osteophyte formation. The cells in the OA joint change their phenotype as a result of aging and exposure to mechanical stress, oxidative stress, and inflammation (2,3). It is now well-recognized that there is a major inflammatory component to this disease and it is thought that inflammatory mediators may play an important role in joint degradation (4). Particularly, interleukin-1β (IL-1β) is known as a major inducer of articular cartilage extracellular matrix (ECM) degradation, promoting the production and activation of different factors that act as mediators and/or effectors of progressive cartilage loss (5).

To date, there is no treatment that cures OA. Thus, the development of therapeutic alternatives that can prevent or delay the destruction of cartilage and tissues involved in the knee joint or stimulate its adequate repair is required (6). The usefulness of treatments focused on the repair of focal lesions is very limited in OA, because more generalized cartilage defects and joint inflammation are present. Therefore, intra-articular treatment options seem more viable as therapeutic alternatives for the management of knee OA (7). Some of the advantages of using intra-articular injections are increased local bioavailability, reduced systemic exposure, fewer adverse events, and reduced cost (8).

The intra-articular injection of hyaluronic acid (HA) for knee OA has been widely studied. One of the most recent available products is non-animal stabilized HA (NASHA, Durolane HA®; Bioventus LLC). NASHA is a type of HA that is produced through bacterial synthesis of HA. It is subsequently purified and stabilized in a carefully controlled process involving molecular cross-linking (9). Available clinical evidence supports the use of NASHA as an effective and safe procedure for symptom relief in knee OA (10). However, the anti-inflammatory effect at the cellular and molecular level of this type of HA in knee OA has not been studied.

On the other hand, results from clinical studies suggest a clinical improvement for intra-articular injection of mesenchymal stromal/stem cells (MSC) in knee OA (11,12). Along with their differentiation potential into several lineages, including chondrogenic lineage, MSC influence their micro-environment by secreting trophic mediators (13-15). The paracrine capacities of MSC raise the attractive approach of possibly basing future therapies on their secreted factors rather than on the cells themselves.

In vitro stimulation with inflammatory factors provides an option to further use the MSC trophic effects since they need to be activated to exert such action (16-18). The effect of the mediators produced by stimulated MSC on inflammation and catabolic processes involved in OA have been tested before (16,17,19). The aim of this study was to evaluate the anti-inflammatory and anti-catabolic effect of the NASHA and compare it with the conditioned media from adipose-derived MSC in an *in vitro* coculture model of cartilage and synovium.

Materials and methods

Patients and samples. Cartilage and synovial tissue explants were harvested as surgical waste from patients with OA undergoing total knee arthroplasty (n=7) from April 2017 to December 2018. Usage of human tissues was approved by the Research Ethics Committee of the University Hospital and School of Medicine of Universidad Autonoma de Nuevo Leon (approval no. OR17-00002). All patients had advanced knee OA diagnosis (Kellgren-Lawrence grade IV), disabling knee pain and required total knee prosthesis. The cartilage and synovial explants for the coculture assays were obtained from seven male patients with a median of 71 years and an interquartile range of 60-79 years. Patients with joint infection were excluded and patients in whom the tissue sample was insufficient were eliminated. Informed consent was obtained from all patients. To obtain the explants, the joint tissue from the femoral condyles and synovial capsule was recovered, from which cuts of 6-8 mm in diameter were made by means of the Osteochondral Autograft Transfer System (OATS®, Arthrex Inc.). Efforts were made to obtain cartilage explants from samples without erosion or exposure of the subcondral bone which were identified in non-weight bearing zones and at the periphery of the femoral condyles. Most of synovial samples from which explants were obtained were characterized for presenting friability, redness, hyperemia and synovial hypertrophy. The obtained explants were washed three times with 1X sterile phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc.), once in 70% ethanol and once in sterile PBS 1X with gentamicin 50 μg/ml.

Cartilage-synovial tissue coculture. Cartilage and synovial tissue explants from the same donor were distributed in 12-transwell plates for coculture. In each well, an explant of cartilage and synovial tissue was placed; such explants were divided by a polystyrene membrane transwell insert with a pore diameter of 1.0 μm (Corning Incorporated). Cartilage explant was placed within the insert and the synovial tissue explant was placed at the bottom of the plate. Each coculture was maintained under the following conditions: 1) Basal: Complete DMEM/F12 (Gibco) [added with gentamicin 50 μg/ml and fungizone 0.25 μg/ml (Gibco), supplemented with fetal bovine serum (FBS) 10% (Gibco)], 2) Interleukin-1β (IL-1β): Complete DMEM/F12 with 10 ng/ml of human recombinant protein IL-1β (R&D Systems Inc.), 3) NASHA: Complete DMEM/F12 added with NASHA (hyaluronic acid, 1 mg/ml, Durolane HA) and 10 ng/ml of IL-1β; and 4) Stem cell-conditioned medium (SC-CM): Complete DMEM/F12 added with conditioned stem cell culture media (1 ml) and 10 ng/ml of IL-1β. All the cultures were incubated at 37˚C, with 5% CO_2 upon 0, 48 and 72 h for further RNA isolation and supernatants were storage to -80˚C until required for additional analysis.

MSC derived from adipose tissue. Adipose tissue samples were obtained from total or partial hip arthroplasty surgeries (n=5). The adipose tissue samples used to isolate MSC were derived from five male patients with a median of 70 years and an interquartile range of 67-77 years. MSC isolation was performed following a method previously described (20). Briefly, after the isolation, cells were plated in a 75 cm^2 culture flasks, changes of complete culture medium (DMEM/F12 added with gentamicin 50 μg/ml and fungizone 0.25 μg/ml, supplemented with FBS 10%) were made every 72 h until the cells reached 80-90% confluence and were ready to undergo a subculture. The cells from the different donors received a maximum of 2 subcultures, then they were pooled and maintained in new 75 cm^2 culture flasks until 80-90% confluency (approximately $2x10^6$ cells), and were stimulated with IL-1β (10 ng/ml) in 5 ml of complete DMEM/F12 medium for 24 h; the culture medium of the stimulated confluent cells (stem-cell conditioned medium, SC-CM) was stored at -80˚C and was used to supplement the cocultures of the cartilage and synovial tissue explants.

Immunophenotyping of the adipose-derived MSC by flow cytometry. The immunophenotype of the MSC in culture was evaluated within passages 2 and 3. Adherent cells cultured in 75 cm^2 bottles were detached with 0.75% trypsin/0.5 mM EDTA (Gibco). Cell pellet was obtained and resuspended in complete DMEM medium and incubated at 37˚C for 20 min. Subsequently, the cells were centrifuged and the cell button was diluted at approximately 200,000 cells per tube in 50 μl of staining buffer (SB, Becton Dickinson Pharmingen) or in a 0.5% bovine serum albumin (BSA, Sigma-Aldrich; Merck KGaA) solution in PBS. The antibody was added following the manufacture's recommendation and then the mix was incubated for 20 min in the dark. Then the samples were washed with 0.5 ml of 0.5% BSA in PBS and centrifuged at 1,800 x g (730 x g), for 1 min at 4˚C. The button of stained cells from each tube were resuspended in 250 μl of 0.5% BSA in PBS

and analyzed for three-color immunofluorescence by flow cytometry (FACSAria; BD Biosciences).

Differentiation potential of the adipose-derived MSC. To confirm the multilineage differentiation potential of the isolated MSC, we investigated the ability of those cells to differentiate into chondrogenic, adipogenic, and osteogenic lineages (21). Briefly, for adipocyte differentiation, the cells were induced with an adipogenic differentiation medium containing dexamethasone (1 mM), indomethacin (60 mM), 3-isobutyl-1-metyl-xanthine (500 mM; IBMX), and insulin (5 mg/ml) for 3 weeks. Differentiation into adipocytes was demonstrated by Oil-Red-O staining for the presence of lipid droplets. For chondrocyte differentiation, MSC were maintained as micromass cultures; the formed pellets were induced in the presence of a medium containing TGF-β1 (10 ng/ml), dexamethasone (0.1 μM), ascorbic acid (50 μg/ml), and insulin-transferrin-selenium solution (ITS, 10 μg/ml) for 3 weeks. The cells were differentiated toward chondrocytes, as shown by glycosaminoglycan (GAG) deposition and Alcian Blue staining. To evaluate the osteoblastic potential, cells were stained with Alizarin Red to detect mineralization after a cultivation period of 14 days with an osteogenic medium containing dexamethasone (0.1 μM), β-glycerophosphate (10 mM), and ascorbic acid (50 μg/ml).

Total RNA extraction and gene expression analysis by reverse transcription-quantitative (RT-q)PCR. Total RNA was isolated from the cartilage and synovial explants samples at the indicated culture times using the commercial kit RNeasy Lipid Tissue Mini kit (Qiagen GmbH) following the manufacturer's recommendations. The quality of the samples obtained from RNA was evaluated by spectrophotometric quantification in the NanoDrop 2000 equipment (Thermo Fisher Scientific Inc.). From each total RNA sample obtained, complementary DNA synthesis (cDNA) was performed by means of a retrotranscription reaction with the GoTaq Probe 2-Step RT-qPCR System commercial kit (Promega Corporation). From the synthesized cDNA, the expression of different genes related to the inflammatory response and the ECM synthesis in cartilage and synovium was evaluated through the use of TaqMan probes (Applied Biosystems; Thermo Fisher Scientific, Inc.) specific for each gene in each coculture test and in each experimental group (4 groups) in triplicate. The expression assays were performed in the 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The list of genes evaluated is shown in Table I. Data analysis was carried out by means of the ΔΔCq method for relative expression, using β-2-microglobulin (*B2M*) as endogenous gene to normalization (22).

Release of GAG. The sulfated GAG (chondroitin sulfate, heparan sulfate, keratan sulfate and dermatan sulfate) were quantified by means of a spectrophotometric analysis using the metachromatic probe dimethylmethylene blue (DMB) using the Rheumara® commercial GAG detection kit (Astarte Biologics). The binding of DMB to sulfated GAG induces a change in the absorption spectrum, which is directly proportional to the amount of sulphated GAG. Measurements were performed in 96-well plates in the Cytation3 HT multimodal plate reader (BioTek Instruments Inc.) at a wavelength of 525 nm. The results obtained were normalized with respect to the wet weight of the cartilage explants. The presence of sulphated GAG from each coculture trial (7 trials) and from each experimental group (4 groups) was evaluated 5 times.

Nitric oxide production. Nitric oxide (NO) was determined in the culture supernatants by using the Griess reagent (Sigma-Aldrich), which converts available nitrate into nitrite through nitrite reductase. Absorbance at 540 nm was determined in 96-well plates in the Cytation3 HT multimodal plate reader (BioTek Instruments Inc.). Sodium nitrate was used to generate a standard curve with which the concentrations of the samples to be evaluated were determined. The production of NO from each coculture trial (7 trials) and from each experimental group (4 groups) was evaluated 5 times.

Statistical analysis. This study was an experimental, prospective, *in vitro* study. Sample size was calculated for the comparison of related means, based on an alpha value of 0.05 in two directions and a β value of 0.08; resulting in 7 trials per experimental group. The data are presented as the mean ± standard deviation (SD) of seven independent experiments performed in triplicate unless otherwise mentioned. The numerical variables were analyzed with the parametric ANOVA test with the Tukey post hoc test for multiple comparisons to observe the possible differences between the experimental groups. $P<0.05$ was considered to indicate a statistically significant difference. Data were processed with GraphPad Prism software version 5.00 for Windows (GraphPad Software, Inc.).

Results

Immunophenotyping of human adipose-derived MSC. The analysis by flow cytometry of the MSC revealed the surface markers that should be positive for this type of cells ($CD73^+/CD90^+/CD105^+$). In the histogram of the positive markers, only a well-defined peak is distinguished, which demonstrates the specificity of the test (Fig. 1A). The expression of the markers in the sample analyzed is also plotted; as expected, the expression of the CD105 was the one that varied more between individuals and passages. The markers CD73 and CD90 were the most stable. In Fig. 1A, a representative image of the analysis of the samples is shown, which is expressed as dot-plot and shows the purity of the cell population. Most of the cells are located in a very concentrated area and in the quadrant corresponding to those that are double positive.

Assessment of pluripotency in the human adipose-derived MSC. We assess the ability of isolated human adipose-derived MSC to differentiate into adipocytes, chondrocytes, and osteoblasts. The differentiated MSC to adipocytes underwent production of vacuoles containing lipid droplets detected with Oil-Red-O staining as red areas (Fig. 1B). The induced chondrogenic differentiation was detected by the presence of GAG within the extracellular matrix stained with Alcian Blue (Fig. 1B). Lastly, MSC developed a more round polygonal appearance and the mineralized foci were detected as red

Table I. Analyzed genes by reverse transcription-quantitative PCR. The corresponding code is presented for each of the TaqMan probes used.

Gene	Symbol	Code[a]
Interleukin-1 β	*IL-1β*	Hs01555410_m1
Matrix metallopeptidase 13	*MMP13*	Hs00233992_m1
ADAM metallopeptidase with thrombospondin type 1 motif 5	*ADAMTS5*	Hs00199841_m1
Tissue inhibitor of metalloproteinases 1	*TIMP1*	Hs00171558_m1
β-2-microglobulin	*B2M*	Hs99999907_m1

[a]Applied Biosystems; Thermo Fisher Scientific, Inc.

spotted areas stained with Alizarin Red after osteogenic differentiation (Fig. 1B). These data confirmed that the isolates of adipose-derived MSC used in this study are efficiently capable to initiate a multilineage differentiation process.

Release of GAG to the culture medium. The presence of sulfated GAG was detected in the culture media of all four experimental groups at 48 and 72 h (Fig. 2). A greater release of GAG was detected in the treatment group stimulated with IL-1β with respect to the basal, NASHA and SC-CM group at 48 h (P<0.05) and 72 h (P<0.01). It was observed that the level of GAG released to the culture medium was maintained at the level of the basal group (68.3±5.1 μg/g of cartilage) after supplementation with NASHA (76.9±1.5 μg/g of cartilage) or with SC-CM (70.6±2.9 μg/g of cartilage) at 72 h, with no significant changes between these two last groups.

Quantification of NO. The concentrations of nitrite, as an index of NO production, increased markedly in the groups in which inflammation with IL-1β was induced (IL-1β, NASHA and SC-CM) compared with the basal group at 48 and 72 h (P<0.001; Fig. 3). A decrease in the production of NO was observed in the groups treated with NASHA and MSC with respect to the IL-1β group at 72 h; nevertheless, only in the SC-CM group a significant decrease in the NO levels was detected (IL-1β, 33.1±9.8 μM vs. SC-CM, 23.9±5.4 μM; P<0.05).

Expression of genes related to inflammation and matrix turnover in cartilage and synovial membrane explants

IL-1β. The recombinant IL-1β markedly increased the expression of *IL-1β* in the IL-1β, NASHA, and SC-CM groups, both in cartilage (P<0.001) and in the synovium (P<0.05) showing a clearly inflammatory effect (Fig. 4A, B). Treatments with NASHA and SC-CM decreased the expression of *IL-1β* at 72 h in both cartilage and synovial membrane. In cartilage, only the treatment with the conditioned culture medium of MSC significantly decreased the expression of *IL-1β* (P<0.05) at 72 h. In synovial membrane, the treatment with SC-CM also decreased the expression level of *IL-1β* compared to the treatment with NASHA. Despite this, the difference shown was not statistically significant (NASHA, 5.2±5.7 vs. SC-CM, 3.1±3.1; P>0.05).

MMP13. The expression of *MMP13* was significantly increased in the IL-1β group compared with its basal expression levels after exposure to recombinant IL-1β in cartilage and synovial explants (P<0.05; Fig. 4C, D). However, the expression of *MMP13* significantly decreased in both cartilage and synovium (P<0.05) after 72 h of treatment with SC-CM. Treatment with NASHA exhibited a significantly downregulation of *MMP13* only in cartilage at 72 h (P<0.05).

ADAMTS5. In the cartilage explants, both treatments significantly decreased the expression of *ADAMTS5* at 48 and 72 h (P<0.05 and P<0.01, respectively) despite the inflammatory influence of recombinant IL-1β (Fig. 4E). In explants of synovial tissue, the same behavior was detected only at 72 h (P<0.05; Fig. 4F).

TIMP1. Conversely to the effect of IL-1β in the aforementioned genes in cartilage explants, the expression of *TIMP1* was decreased as a consequence of the inflammatory stimulation compared with basal level (Fig. 4G). It can be distinguished how in both cartilage and synovial membrane explants, treatments with NASHA and SC-CM recovered the expression level of *TIMP1* (Fig. 4G, H), and the difference was significantly higher than in the IL-1β group at 48 and 72 h (P<0.01).

Discussion

Exposure of synovial and cartilage explants in coculture to NASHA and SC-CM, resulted in positive changes in gene expression profiles and release of agents consistent with an anti-inflammatory and anti-catabolic activity in articular tissues. However, only the mediators produced by the MSC previously stimulated with IL-1β were able to significantly decrease a greater number of inflammation and matrix degradation targets studied in the *in vitro* model used.

It is known that the tissues involved in the OA joint produce a wide variety of catabolic and proinflammatory mediators that lead to the destruction of the extracellular matrix of these structures (23); therefore, is important to evaluate these processes in tissues other than articular cartilage. While *in vitro* inflammation models exist for some inflammatory diseases, to date, there is no simply and readily available OA model. As earlier mentioned, we used an *ex vivo* coculture system of cartilage and synovial membrane explants using inserts that allowed the interaction of the two types of tissue through different molecule production to better represent the joint environment, unlike what happens in an isolated culture

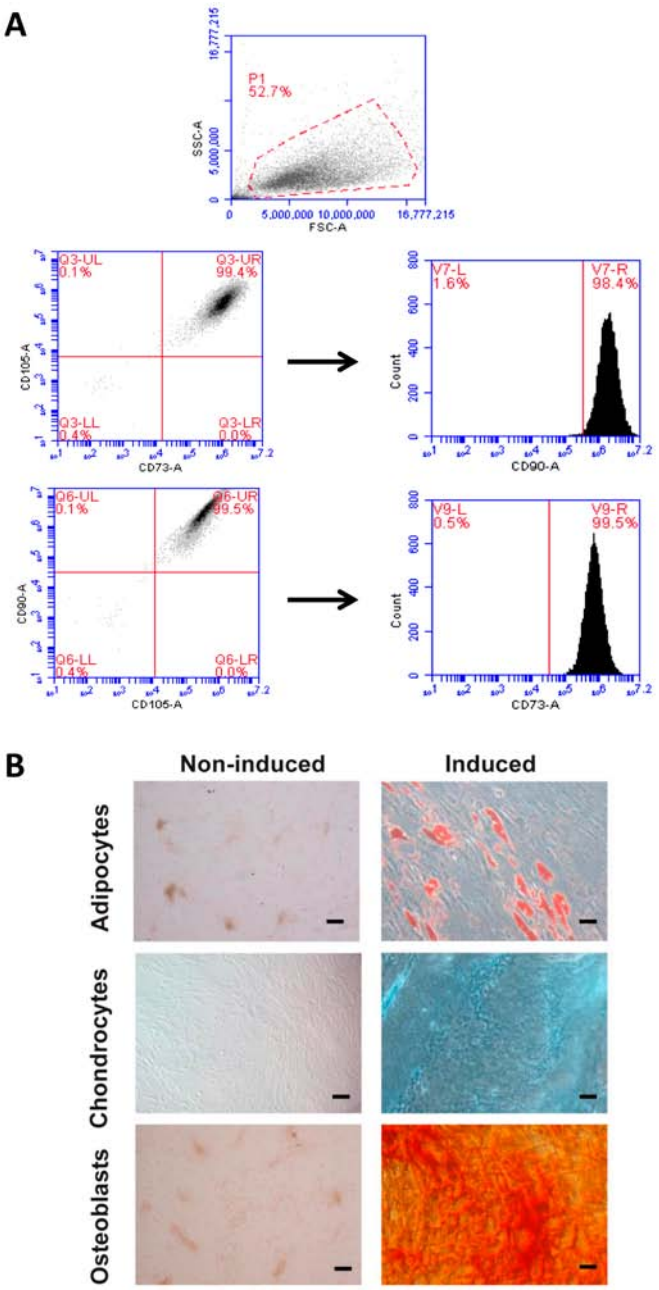

Figure 1. Characterization of adipose-derived MSC. (A) Flow cytometry analysis of mesenchymal positive stem cell markers (CD90, CD73 and CD105). Representative dot-plots of one cell isolate are shown. (B) Assessment of multilineage differentiation potential in isolated MSC. Cells treated with adipogenic medium show red-stained fat droplets. Micromass cultures were analyzed for proteoglycan content by Alcian Blue staining. Differentiation to osteoblasts was evidenced by Alizarin Red staining showing mineral deposition of calcium. Scale bar, 50 μm. MSC, mesenchymal stromal/stem cells.

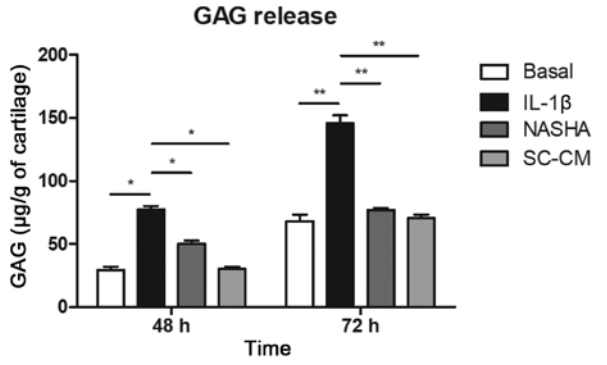

Figure 2. Effect of NASHA and SC-CM on GAG released into the culture media. Data were normalized using the wet weight of cartilage based on seven different experiments performed in quintuplicate. Data are expressed as the mean ± SD. *P<0.05 and **P<0.01. NASHA, non-animal stabilized hyaluronic acid; GAG, glycosaminoglycan; SC-CM, stem cell-conditioned medium..

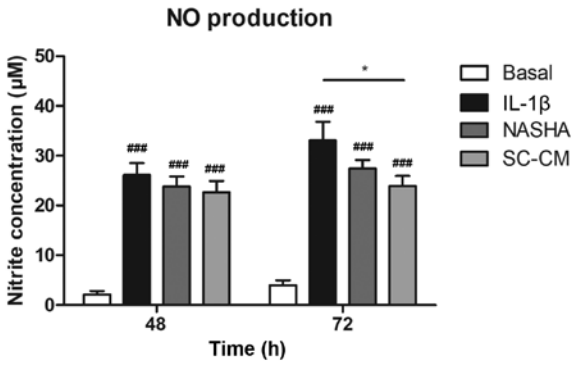

Figure 3. Effect of NASHA and SC-CM on NO production. Data represent the analysis of seven different experiments performed in quintuplicate. Data are expressed as the mean ± SD. *P<0.05; $^{###}$P<0.001 vs. basal. NASHA, non-animal stabilized hyaluronic acid; NO, nitric oxide; SC-CM, stem cell-conditioned medium.

of cartilage or synovium (16,24). Additionally, models based on explant culture have the main advantage that they can be used to examine the response of cells in their natural extracellular matrix, without affecting the cell phenotype (25).

The release of GAG has been reported to be useful as a measure of ECM degradation in cultures of cartilage explants after an inflammatory stimulus (e.g., IL-1β) (26-28). In our model, we detected an increase of GAG release induced by IL-1β (10 ng/ml) and both NASHA and SC-CM were able to protect articular tissues against ECM degradation. This effect might be directly related with the reduction of gene expression of ECM degrading enzymes such as *MMP13* and *ADAMTS5*.

The inflammatory effects of IL-1β include triggering a signaling pathway that stimulates the release of NO. As consequence, cartilage degradation is produced due to an increased synthesis of proteolytic enzymes such as MMP13 and ADAMTS5 (29). In our study, both NASHA and SC-CM reduced the IL-1β-induced production of the inflammatory mediator NO but only the SC-CM showed a meaningful significantly difference. This outcome could be a consequence of the decreased gene expression of *MMP13* and *ADAMTS5* in our cartilage and synovial explant coculture model. It has been reported that human SC-CM might produce this protective action as a consequence of *iNOS* downregulation (17), since NO inhibits ECM synthesis (30).

In OA, anabolic and catabolic processes are significantly affected as a result of a homeostasis imbalance. Important markers of catabolism in OA are MMP13 and ADAMTS5, which are commonly elevated. We found an increased IL-1β-induced expression of *MMP13* in cartilage and synovial explants. This effect has been previously reported over *MMP13* and other MMP in similar OA explant-based culture models (16,31). On the other hand, *ADAMTS5* was not

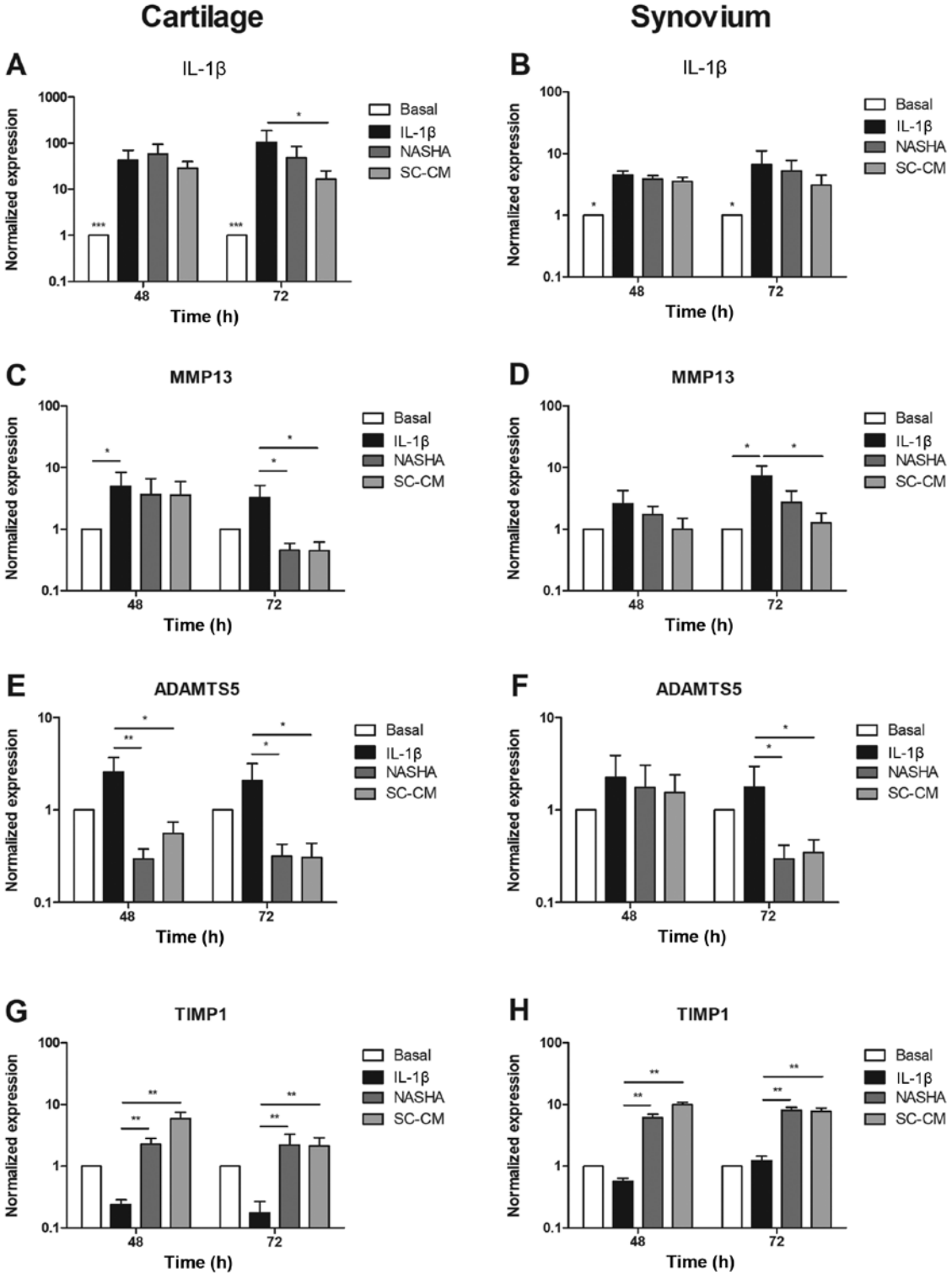

Figure 4.Effect of NASHA and SC-CM on the expression of genes related to inflammation and matrix degradation in cartilage and synovial membrane explants. (A) Relative expression of IL-1β in cartilage. (B) Relative expression of IL-1β in synovium. (C) Relative expression of MMP13 in cartilage. (D) Relative expression of MMP13 in synovium. (E) Relative expression of ADAMTS5 in cartilage. (F) Relative expression of ADAMTS5 in synovium. (G) Relative expression of TIMP1 in cartilage. (H) Relative expression of TIMP1 in synovium. All bars represent Log10 of change in gene expression with respect to their correspondent baseline expression. Data represent the analysis of seven different experiments performed in triplicate. Data are expressed as the mean ± SD. *P<0.05 and **P<0.01. NASHA, non-animal stabilized hyaluronic acid; SC-CM, stem cell-conditioned medium; MMP13, matrix metallopeptidase 13; ADAMTS5, ADAM metallopeptidase with thrombospondin type 1 motif 5; TIMP1, tissue inhibitor of metalloproteinases 1; IL-1β, interleukin-1β.

upregulated by IL-1β in explant tissues. These is in agreement with previous investigations who have found that *ADAMTS5* is not affected by inflammatory cytokines (i.e., IL-1β, TNF-α) especially in human chondrocytes and cartilage (32-34). Our results showed that SC-CM inhibited the expression of *IL-1β*, *MMP13* and *ADAMTS5* in cartilage, whereas NASHA produced a similar effect only in *MMP13* and *ADAMTS5*. Moreover, synovial tissues had also different decreased

expression levels of catabolic markers after SC-CM (*MMP13* and *ADAMTS5*) and NASHA (*ADAMTS5*) treatments. We also found a significant overexpression of *TIMP1* in IL-1β-treated cartilage and synovial explants after exposure to NASHA and SC-CM. TIMP1 is a natural inhibitor of different MMP, including MMP13 (35), and is thought to play a role in regulating ECM turnover by modulating the degradative capacity of these MMP. A higher *TIPM1* gene expression might indicate a protective effect against ECM degradation in articular tissues. Together, these results suggest that both NASHA and SC-CM exerts an anti-inflammatory and anti-catabolic effect by partially counteract the catabolic effect of proinflammatory cytokines through different pathways.

Several clinical studies have shown that the use of NASHA is well tolerated in patients in whom it has been administered intra-articularly, and that its clinical effectiveness remains for several weeks in the treatment of knee OA (36-38). However, to our knowledge, the ability of NASHA to reduce the inflammatory process associated with the development and progression of OA has not been evaluated. On the other hand, it has been shown that much of the chondrogenic potential of MSC is due to the secretion of soluble factors that act in a paracrine fashion and that play an important role in immunomodulation and tissue regeneration (17,39). Considering this characteristic, we could compare the anti-inflammatory effect of these two types of therapies in the same culture system.

A systematic review about the anti-inflammatory effect of intra-articular HA reported that high molecular weight HA can promote an anti-inflammatory response within the cell through binding sites of CD44, TLR-2 and TLR-4 receptors (40). The binding of the high molecular weight HA to CD44 receptor downregulates the expression of IL-8, IL-33, MMP, proteoglycans, and PGE_2 and suppresses NF-κB activation (40). It would be important to determine if NASHA exerts its anti-inflammatory effect over some of the targets reported in this study through the aforementioned mechanism.

Our findings are in line with those of previous research, which found that conditioned media from MSC stimulated with pro-inflammatory mediators are able to inhibit inflammatory processes by targeting catabolic and inflammatory factors (16,17). One of these reports suggest that the inhibitory effects of SC-CM could be partially supported by the fact that mediators produced by MSC stimulated with proinflamatory agents induce a reduction of NF-κB activation in OA chondrocytes (17).

Some of the limitations of this study include that we only show changes in gene expression of markers related to inflammation and matrix turnover. It would be important to confirm these changes at the protein level and with other specific markers of main ECM components such as collagen type II and aggrecan at both gene and protein level. Moreover, a further characterization of specific markers related to degradation and inflammation of cartilage and synovium would yield more information about these processes since only the GAG and NO were quantified.

This study evaluated the anti-inflammatory and anti-catabolic properties of NASHA compared to those of SC-CM. Both treatments showed similar favorable effects; however, SC-CM was able to counteract inflammatory effects over a higher number of the evaluated targets. The wide number of bioactive molecules probably works in favor of the MSC to achieve the positive effects observed. Further studies are needed to elucidate the exact mechanism by which each therapy exerts its positive effects.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MSM, SALS, FVC, VMPM and LFM conceived the original idea and designed the experiments. MSM, SALS and VPS performed the experiments. HGMR, FVC and CAAO analyzed the data. VPS, FVC and VMPM interpreted the data. MSM and VMPM wrote the manuscript. FVC and LFM edited the original article. FVC, LFM and VMPM critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. OR17-00002) by The Ethical Committee and Research Review Board (Research Ethics Committee) of the University Hospital and School of Medicine of Universidad Autonoma de Nuevo Leon. Informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Lawrence RC, Felson DT, Helmick CG, Arnold LM, Choi H, Deyo RA, Gabriel S, Hirsch R, Hochberg MC, Hunder GG, *et al*: Estimates of the prevalence of arthritis and other rheumatic conditions in the United States: Part II. Arthritis Rheum 58: 26-35, 2008.
2. Shen J, Abu-Amer Y, O'Keefe RJ and McAlinden A: Inflammation and epigenetic regulation in osteoarthritis. Connect Tissue Res 58: 49-63, 2017.
3. Jørgensen AEM, Kjær M and Heinemeier KM: The effect of aging and mechanical loading on the metabolism of articular cartilage. J Rheumatol 44: 410-417, 2017.
4. Tetlow LC, Adlam DJ and Woolley DE: Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: Associations with degenerative changes. Arthritis Rheum 44: 585-594, 2001.

5. Goldring MB, Otero M, Tsuchimochi K, Ijiri K and Li Y: Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. Ann Rheum Dis 67 (Suppl 3): iii75-iii82, 2008.
6. Li MH, Xiao R, Li JB and Zhu Q: Regenerative approaches for cartilage repair in the treatment of osteoarthritis. Osteoarthritis Cartilage 25: 1577-1587, 2017.
7. Jones IA, Togashi R, Wilson ML, Heckmann N and Vangsness CT Jr: Intra-articular treatment options for knee osteoarthritis. Nat Rev Rheumatol 15: 77-90, 2019.
8. Evans CH, Kraus VB and Setton LA: Progress in intra-articular therapy. Nat Rev Rheumatol 10: 11-22, 2014.
9. Agerup B, Berg P and Akermark C: Non-animal stabilized hyaluronic acid: A new formulation for the treatment of osteoarthritis. BioDrugs 19: 23-30, 2005.
10. Leighton R, Fitzpatrick J, Smith H, Crandall D, Flannery CR and Conrozier T: Systematic clinical evidence review of NASHA (Durolane hyaluronic acid) for the treatment of knee osteoarthritis. Open access Rheumatol 10: 43-54, 2018.
11. Gupta PK, Chullikana A, Rengasamy M, Shetty N, Pandey V, Agarwal V, Wagh SY, Vellotare PK, Damodaran D, Viswanathan P, *et al*: Efficacy and safety of adult human bone marrow-derived, cultured, pooled, allogeneic mesenchymal stromal cells (Stempeucel®): Preclinical and clinical trial in osteoarthritis of the knee joint. Arthritis Res Ther 18: 301, 2016.
12. Pers YM, Rackwitz L, Ferreira R, Pullig O, Delfour C, Barry F, Sensebe L, Casteilla L, Fleury S, Bourin P, *et al*: Adipose mesenchymal stromal Cell-based therapy for severe osteoarthritis of the knee: A phase I dose-escalation trial. Stem Cells Transl Med 5: 847-856, 2016.
13. Timmers L, Lim SK, Hoefer IE, Arslan F, Lai RC, van Oorschot AA, Goumans MJ, Strijder C, Sze SK, Choo A, *et al*: Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. Stem Cell Res 6: 206-214, 2011.
14. Le Blanc K and Mougiakakos D: Multipotent mesenchymal stromal cells and the innate immune system. Nat Rev Immunol 12: 383-396, 2012.
15. Caplan AI: Mesenchymal stem cells: Time to change the Name! Stem Cells Transl Med 6: 1445-1451, 2017.
16. van Buul GM, Villafuertes E, Bos PK, Waarsing JH, Kops N, Narcisi R, Weinans H, Verhaar JA, Bernsen MR and van Osch GJ: Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture. Osteoarthritis Cartilage 20: 1186-1196, 2012.
17. Platas J, Guillén MI, del Caz MD, Gomar F, Mirabet V and Alcaraz MJ: Conditioned media from adipose-tissue-derived mesenchymal stem cells downregulate degradative mediators induced by interleukin-1β in osteoarthritic chondrocytes. Mediators Inflamm 2013: 357014, 2013.
18. Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Pescatori M, Stubbs AP, van Ijcken WF, Dahlke MH, Eggenhofer E, Weimar W and Hoogduijn MJ: Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clin Exp Immunol 162: 474-486, 2010.
19. Khatab S, van Osch GJ, Kops N, Bastiaansen-Jenniskens YM, Bos PK, Verhaar JA, Bernsen MR and van Buul GM: Mesenchymal stem cell secretome reduces pain and prevents cartilage damage in a murine osteoarthritis model. Eur Cell Mater 36: 218-230, 2018.
20. Hernandez-Hurtado AA, Borrego-Soto G, Marino-Martinez IA, Lara-Arias J, Romero-Diaz VJ, Abrego-Guerra A, Vilchez-Cavazos JF, Elizondo-Riojas G, Martinez-Rodriguez HG, Espinoza-Juarez MA, *et al*: Implant composed of demineralized bone and mesenchymal stem cells genetically modified with AdBMP2/AdBMP7 for the regeneration of bone fractures in ovis aries. Stem Cells Int 2016: 7403890, 2016.
21. Ciuffreda MC, Malpasso G, Musarò P, Turco V and Gnecchi M: Protocols for in vitro differentiation of human mesenchymal stem cells into osteogenic, chondrogenic and adipogenic lineages. Methods Mol Biol 1416: 149-158, 2016.
22. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45, 2001.
23. Sellam J and Berenbaum F: The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nat Rev Rheumatol 6: 625-635, 2010.
24. Osterman C, McCarthy MB, Cote MP, Beitzel K, Bradley J, Polkowski G and Mazzocca AD: Platelet-Rich plasma increases Anti-inflammatory markers in a human coculture model for osteoarthritis. Am J Sports Med 43: 1474-1484, 2015.
25. Johnson CI, Argyle DJ and Clements DN: In vitro models for the study of osteoarthritis. Vet J 209: 40-49, 2016.
26. Choi CH, Kim TH, Sung YK, Choi CB, Na YI, Yoo H and Jun JB: SKI306X inhibition of glycosaminoglycan degradation in human cartilage involves down-regulation of cytokine-induced catabolic genes. Korean J Intern Med 29: 647-655, 2014.
27. Behrendt P, Preusse-Prange A, Klüter T, Haake M, Rolauffs B, Grodzinsky AJ, Lippross S and Kurz B: IL-10 reduces apoptosis and extracellular matrix degradation after injurious compression of mature articular cartilage. Osteoarthritis Cartilage 24: 1981-1988, 2016.
28. Gierman LM, van El B, van der Ham F, Koudijs A, Stoop R, Verheijen JH, Kloppenburg M, van Osch GJ, Stojanovic-Susulic V, Huizinga TW and Zuurmond AM: Profiling the secretion of soluble mediators by end stage osteoarthritis synovial tissue explants reveals a reduced responsiveness to an inflammatory trigger. PLoS One 8: e62634, 2013.
29. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP and Fahmi H: Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 7: 33-42, 2011.
30. Abramson SB: Osteoarthritis and nitric oxide. Osteoarthritis Cartilage 16 (Suppl 2): S15-S20, 2008.
31. Haltmayer E, Ribitsch I, Gabner S, Rosser J, Gueltekin S, Peham J, Giese U, Dolezal M, Egerbacher M and Jenner F: Co-culture of osteochondral explants and synovial membrane as in vitro model for osteoarthritis. PLoS One 14: e0214709, 2019.
32. Koshy PJ, Lundy CJ, Rowan AD, Porter S, Edwards DR, Hogan A, Clark IM and Cawston TE: The modulation of matrix metalloproteinase and ADAM gene expression in human chondrocytes by interleukin-1 and oncostatin M: A time-course study using real-time quantitative reverse transcription-polymerase chain reaction. Arthritis Rheum 46: 961-967, 2002.
33. Pratta MA, Yao W, Decicco C, Tortorella MD, Liu RQ, Copeland RA, Magolda R, Newton RC, Trzaskos JM and Arner EC: Aggrecan protects cartilage collagen from proteolytic cleavage. J Biol Chem 278: 45539-45545, 2003.
34. Simental-Mendía M, Vilchez-Cavazos F, García-Garza R, Lara-Arias J, Montes-de-Oca-Luna R, Said-Fernández S and Martínez-Rodríguez HG: The matrix synthesis and anti-inflammatory effect of autologous leukocyte-poor platelet rich plasma in human cartilage explants. Histol Histopathol 33: 609-618, 2018.
35. Brew K and Nagase H: The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. Biochim Biophys Acta 1803: 55-71, 2010.
36. Leighton R, Akermark C, Therrien R, Richardson JB, Andersson M, Todman MG and Arden NK; DUROLANE Study Group: NASHA hyaluronic acid vs methylprednisolone for knee osteoarthritis: A prospective, multi-centre, randomized, non-inferiority trial. Osteoarthritis Cartilage 22: 17-25, 2014.
37. Altman RD, Akermark C, Beaulieu AD and Schnitzer T; Durolane International Study Group: Efficacy and safety of a single intra-articular injection of non-animal stabilized hyaluronic acid (NASHA) in patients with osteoarthritis of the knee. Osteoarthritis Cartilage 12: 642-649, 2004.
38. Estades-Rubio FJ, Reyes-Martín A, Morales-Marcos V, García-Piriz M, García-Vera JJ, Perán M, Marchal JA and Montañez-Heredia E: Knee viscosupplementation: Cost-effectiveness analysis between stabilized hyaluronic acid in a single injection versus five injections of standard hyaluronic acid. Int J Mol Sci 18: pii: E658, 2017.
39. Singer NG and Caplan AI: Mesenchymal stem cells: Mechanisms of inflammation. Annu Rev Pathol 6: 457-478, 2011.
40. Altman R, Bedi A, Manjoo A, Niazi F, Shaw P and Mease P: Anti-inflammatory effects of Intra-articular hyaluronic acid: A systematic review. Cartilage 10: 43-52, 2019.