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Original Article

Effect of mesenchymal stem cells combined with chondroitin sulfate in an *in vitro* model of osteoarthritis

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Abstract: Osteoarthritis (OA) is a degenerative joint disease affecting the whole joint structure. The specific molecules responsible for the inflammatory processes involved in the development of OA have been the focus of many studies. Adipose tissue-derived mesenchymal stem cells (ASCs) constitute a promising cell-based therapy which could be used as an alternative to or in combination with drug therapies. Chondroitin sulfate (CS) plays a protective role in the joint by decreasing concentrations of pro-inflammatory cytokines and therefore has an important part in moderating chondrocyte metabolism. The aim of this study is to use an *in vitro* model of OA to evaluate the combined effectiveness of CS and ASCs as a treatment. We give a detailed discussion of the roles of cytokines and other key molecules involved in OA. In addition, we report the effects of treating inflamed chondrocytes with ASCs and CS on the expression of specific cartilage genes. Findings show that both treatments reduced expression of all genes associated with the pro-inflammatory cytokines we analyzed. However, we saw no increase in the expression of the specific genes encoding for cartilage matrix proteins, such as collagen type II and aggrecan. This study shows the effectiveness of combining ASCs and CS in the treatment of OA.

Keywords: Mesenchymal stem cells, chondroitin sulfate, osteoarthritis

Introduction

In 2020, OA was on track to become the fourth leading cause of disability [1]. OA is a progressive disease which destroys joint cartilage and current thinking is that inflammation is a major factor in its development and progression, especially in the early stages [2]. Emerging experimental evidence points to a variety of pro-inflammatory cytokines, secreted by cells in response to inflammation, as critical mediators of the disturbed metabolism and enhanced catabolism in joint tissues affected by OA [3]. Cytokines mediate cartilage destruction through the upregulation of inflammatory or catabolic genes and the downregulation of anti-inflammatory or anabolic genes in articular chondrocytes [4]. In particular, IL-1 reduces the expression of type II collagen (Col2A1) [5] and increases the production of matrix metalloproteinases (MMPs) [6], prostaglandin E_2 (PGE_2), cytokines, chemokines, reactive oxygen species, and nitric oxide (NO) [7]. These substances enhance the catabolic activity of chondrocytes thereby destroying the cartilage matrix.

Chondroitin sulfate (CS) is a major component in the extracellular matrix of many connective tissues [8]. Commonly referred to as a "symptomatic slow-acting drug in OA" (SySADOA), CS is used extensively in the management of OA patients [9]. *In vitro*, CS has been shown to have anti-inflammatory and anti-catabolic effects on chondrocytes [10] and is considered to be a structure/disease modifying anti-osteoarthritis drug (S/DMOAD) [11].

Cellular therapies for treating the various stages of OA have been thoroughly researched for over two decades. Tissue engineering, using stem cells, emerged as an alternative method for treating OA within the last 10 years. In this way, the extensive research into mesenchymal stem or stromal cells (MSCs) has focused

mainly on their regenerative potential [12]. Stem cells are capable of secreting a wide range of trophic mediators which can exert paracrine effects on other cell types, and in this regard, adipose tissue-derived MSCs (ASCs), which can be collected easily using liposuction [13], provide an interesting alternative to bone marrow stem cells (BMCs).

Injected or infused MSCs display two main activities: immunomodulation and trophic mediation. The immunomodulatory action of these cells has been shown to be mediated by both the secretion of bioactive molecules and by cell-cell contact and suggested mechanisms involve: the suppression of T-cell proliferation in response to alloantigens or mitogens; inhibition of B-cell proliferation; dendritic cell maturation; and the generation of regulatory T-cells [14]. Amongst the bioactive molecules we find several which are involved in MSC immunomodulation [15]: nuclear factor kappa B (NF κB), transforming growth factor beta (TGF-β); indoleamine 2,3-dioxygenase (IDO); and interleukin 6 (IL-6), in addition to others involved in the renewal of the extracellular matrix, such as type II collagen [16].

The aim of this work is to study the effects of ASCs and CS on inflammatory mediators and certain proteolytic enzymes related to cartilage catabolism that are induced by tumor necrosis factor (TNF).

Materials and methods

Ethical disclosure

The authors state that the experimental procedures performed in this work were approved by the Medical Committee of the University Hospital of León. Written consent was obtained from all patients in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Materials

In this study, human cells were obtained from three patients with OA symptoms (n = 3 donors; a 74-year-old male and two females aged 67 and 55 years). Chondrocytes were obtained from femoral cartilage. ASCs were obtained from infrapatellar adipose tissue. As a control, we used healthy chondrocytes sourced from Innoprot® (Bizcaia, Spain).

Methods

Isolation and culture of ASCs and chondrocytes: ASCs were isolated from adipose tissue obtained from the infrapatellar deposit of fatty tissue at the knee joint, known as Hoffa's fat pad [17]. Cells were collected and plated in 25 cm^2 culture flasks (IWAKI®, Japan, Code: 3100-025).

Cartilage samples were isolated from femoral biopsies and incubated in 0.25% trypsin solution (Sigma Aldrich-Merck®, Germany, Code: 59427C) for 30 minutes at 37°C and 5% CO_2. After centrifugation, samples were incubated with 0.025% collagenase II (Sigma Aldrich-Merck®, Germany, Code: C6885) for 8 hours at 37°C. Cells were then resuspended in culture medium consisting of DMEM (Sigma Aldrich-Merck®, Germany, Code: D6429) supplemented with 10% fetal bovine serum (FBS) (Gibco Thermo Fisher Scientific®, USA, Code: 1267-6029) and 1% antibiotic-antimycotic solution (Gibco Thermo Fisher Scientific®, USA, Code: 15240112) at 37°C in 5% CO_2, 90% humidity. This culture medium was renewed every 2-3 days.

Co-cultures of ASCs and chondrocyte: ASCs and chondrocytes were co-cultured (ratio 1:1) in a 6-well plates. After 24 h when they had reached 80% confluence, the original medium was removed and replaced. We then added either TNF (Cusabio Technology®, USA, Code: CSB-AP002141HU) (25 ng/mL) or CS (Bioibérica®, Spain), or a combination of TNF and CS to the culture medium [10, 18] as shown in **Table 1**. The dose of CS used in these experiments was 200 ng/mL and it was chosen on the basis that it has given the best results in previous experiments conducted in our laboratory (not shown here) and it is also recommended by other authors [18]. Cells and media were collected to analyze the inflammation effects after 12 hours in culture.

ASCs characterization

Flow cytometry analyses: In order to confirm the identity of the ASCs, the expression of different surface markers: mouse anti-CD73, anti-CD90 and anti-CD105 (1:100) (Abcam®, UK, Codes: ab175396, ab181469, ab114-14) was determined. Cells were stained with streptavidin-Alexa 488 antibodies (1:100) (Invitrogen®, USA, Code: S11223). About 1×10^4

Table 1. Distribution of samples in 6-well plate

Well	Experiment 1	Experiment 2
1	ASCs	ASCs + CS
2	Chondrocytes	Chondrocytes + CS
3	Chondrocytes + ASCs	Chondrocytes + ASCs + CS
4	ASCs + TNF	ASCs + TNF + CS
5	Chondrocytes + TNF	Chondrocytes + TNF + CS
6	Chondrocytes + ASCs + TNF	Chondrocytes + ASCs + TNF + CS

When cell confluence was reached, 5 µL of TNF (25 ng/mL) was added and incubated for 12 hours (Experiment 1, wells 4-6). In experiment 2 when cell confluence was reached, TNF (25 ng/mL) and CS (200 ng/mL) were added to the medium.

events (minimum) were used for fluorescence capture.

Confocal characterization: Cells were sub-cultured on an 8-well Nunc Lab-Tek chamber slide system (Thermo Fisher Scientific®, USA, Code: 154534PK) (2×10^3 cells/well). Cells were fixed with 2% paraformaldehyde for 15 minutes prior to incubation with primary mouse anti-CD73, anti-CD90 and anti-CD105 antibodies (1:100) (Abcam®, UK, Codes: ab175-396, ab181469, ab11414) overnight at 4°C, after which they were treated with secondary biotinylated anti-mouse antibodies (1:100) (Abcam®, UK, Code: ab97044). Cells were then stained with streptavidin-Alexa 488 antibodies (1:100) (Invitrogen®, USA, Code: S11223). Finally, chamber slides were mounted using a Vectashield mounting medium (Vector Laboratories®, USA) containing DAPI. After staining, cells were imaged with a confocal microscope (Zeiss®, Germany).

ASCs differentiation: Isolated ASCs were cultured separately in either a) adipogenic medium, b) osteogenic medium and c) chondrogenic medium. Those cultured in adipogenic or osteogenic medium were both examined after 15 days. The production of lipids was confirmed in the former by staining with oil red O and in the latter, osteogenic differentiation confirmed by alizarin red staining. Confirmation of chondrogenesis was achieved after three weeks by alcian blue staining and confocal microscopy using anti-Col2a1 (Abcam®, UK, Code: 185430) (1:100). Immuno-histochemistry Col2a1 staining was performed as indicated in the previous paragraph.

Fluorescence-based proliferation assay: Chondrocytes and ASCs (cell density 1×10^6 cells/mL) were labeled with 5 µM of, respectively, CellTrace® green CFSE dye and violet proliferation tracking dye (Invitrogen®, USA, Codes: C34554, C34557). Cell proliferation was analyzed after 24 and 36 hours in culture with and without TNF (25 ng/mL) and CS (200 ng/mL) added to the culture media. A Nikon Eclipse TE2000-U inverted microscope (Nikon®, Japan) was used to capture two-dimensional, digital images of samples.

Flow cytometry proliferation assay: 1×10^5 cells (ASCs and chondrocytes) were seeded in 24-well plates and were analyzed at 12, 24 and 36 hours after induction of inflammation with TNF (25 ng/mL) and with addition of CS (200 ng/mL). The fluorescence of cells stained with Cell Trace CFSE dye (green) and violet cell proliferation Kit (Invitrogen®, USA, Codes: C34554, C34557) was determined using a Beckman Coulter CyAn® ADP flow cytometer (Dako-Agilent®, USA) counting at least 1×10^4 events per sample. The excitation wavelengths used were 488 and 405 nm, and the emission wavelengths were 630 and 450 nm, for green and violet stained cells respectively.

NF-κB activity assay: Chondrocytes and ASCs were seeded at 3×10^5 cells/well in 2 Nunc Lab-Tek chamber slide systems (Thermo Fisher Scientific®, USA, Code: 154461) and stimulated for 12 hours with TNF (25 ng/mL) either with or without CS (200 ng/mL) as indicated in **Table 1**. Cells were fixed with 2% formaldehyde in PBS for 15 minutes at room temperature and incubated overnight, at 4°C, with human antip65-NFκB pS529-FITC antibody obtained from Miltenyi Biotech® (USA, Code: 130-107-781). Finally, chamber slides were mounted using Vectashield mounting medium containing DAPI and examined under a confocal microscope (Zeiss®, Germany).

Quantitative real-time PCR: Total RNA was extracted using the GeneMatrix universal RNA purification kit (EurX®, Poland, Code: E3598-01). Reverse transcription was accomplished on 1 µg of total RNA using MultiScribe® RT (Applied Biosystems®, USA, Code: 4311235) following the manufacturer's instructions for the high capacity cDNA reverse transcription

Table 2. Gene primer sequences and conditions used for qRT-PCR

Gene	NCBI RefSeq	Primer sequence (5'-3') (Forward/Reverse)	Melting temperature (°C)	Optimal concentration
ACT-β	KR710455	CCCTCCATCGTCCACCGCAAATGCT	79.1	50 nM
		CTGCTGTCACCTTCACCGTTCCAGT	73.2	50 nM
IL-6	HUMIFNB2A	ATAACCACCCCTGACCCAA	55.9	50 nM
		CCATGCTACATTTGCCGAA	53.4	50 nM
TGF-β	NM_000660	CTCCCGCAAAGACTTTTCCCCAGACCT	64.0	300 nM
		CCACGGAAATAACCTAGATGGGCGCGAT	63.7	300 nM
IDO	M58159	CATCCTGATTCCTGCAAGCC	56.3	50 nM
		TCTGCTATGATAAAATGTGCTCT	52.0	50 nM
TNF	AB202113	CCTGAAAACAACCCTCAGACGCCACA	63.0	300 nM
		TCCTCGGCCAGCTCCACGTCCC	67.8	300 nM
MMP13	NM_002427	CCAGAACTTCCCAACCGTATTGATGC	72.3	50 nM
		TGCCTGTATCCTCAAAGTGAACAGC	69.1	50 nM
COL2a1	X16711	CCCATCTGCCCAACTGACC	58.5	50 nM
		CACCTTTGTCACCACGATCCC	58.2	50 nM
iNOS	AF045478	AACGTTGCTCCCCATCAAGCCCTT	63.3	50 nM
		AGCAGCAAGTTCCATCTTTCACCCACT	62.3	50 nM

kit (Applied Biosystems®, USA, 4368814). Gene expression of IL-6, TFG-β, IDO, MMP-13, COL2A1, iNOS and TNF was determined using qRT-PCR. Assays were performed using StepOnePlus RT-PCR (Applied Biosystems®, USA) in a total volume of 25 µL containing 0.7 µL DNA template, 1X SYBR® Green (EURx®, Poland, Code: E0401-02), 400 nM ROX and 0.30 U uracil-*N*-glycosylase (UNG) master mix (EURx®, Poland, Code: N8080096), and 300 nM of each primer.

Relative quantification was carried out by normalizing to the housekeeping gene, ACT-β. Primers were designed using an OLIGO7® primer design tool (**Table 2**) which was provided by Integrated DNA Technologies (Coralville®, USA).

ELISA: Chondrocytes and ASCs were stimulated with TNF (25 ng/mL) for 12 hours after which time the concentration of prostaglandin E_2 (PGE_2) was measured using a specific ELISA with a goat anti-Mouse IgG microtiter plate, following the manufacturer's instructions (Enzo Life Sciences Inc.®, USA). Measurements were conducted using a Multiskan® GO microplate spectrophotometer (Thermo Fisher Scientific®, USA) at 450 nm. Concentrations of PGE_2 were calculated by comparing them to established standards.

Statistical analysis

Each experiment was repeated three times and the final result expressed as a mean ± SD of the three values obtained. Statistical analysis was performed using IBM® SPSS® statistics (USA). To determine whether there were significant differences between cell samples subjected to the various experimental conditions we used ANOVA followed by post-hoc analysis for multiple group comparisons or Student's t-test for two group comparisons. Results with $P< 0.05$ were considered statistically significant.

Results

ASC characterization

ASCs derived from infrapatellar fat were characterized at passage 2 and 3. ASCs are adherent cells with fibroblastic morphology. After harvesting, cells reached 80% confluence in a T75 culture flask within 7-9 days.

Fluorescence microscopy confirmed that cells were positive for the surface markers CD105 (endoglin), CD73 (ecto-5'-nucleotidase) and CD90 (Thy1). In addition, flow cytometry revealed high levels of expression for these makers (over 97% in all cases) as can be seen in **Figure 1A-C**.

Confirmation of the tri-lineage differentiation potential of ASCs

The differentiation of cultured ASCs into osteocytes, chondrocytes or adipocytes was confirmed after 15 and 21 days. ASCs, which underwent adipogenic differentiation, were char-

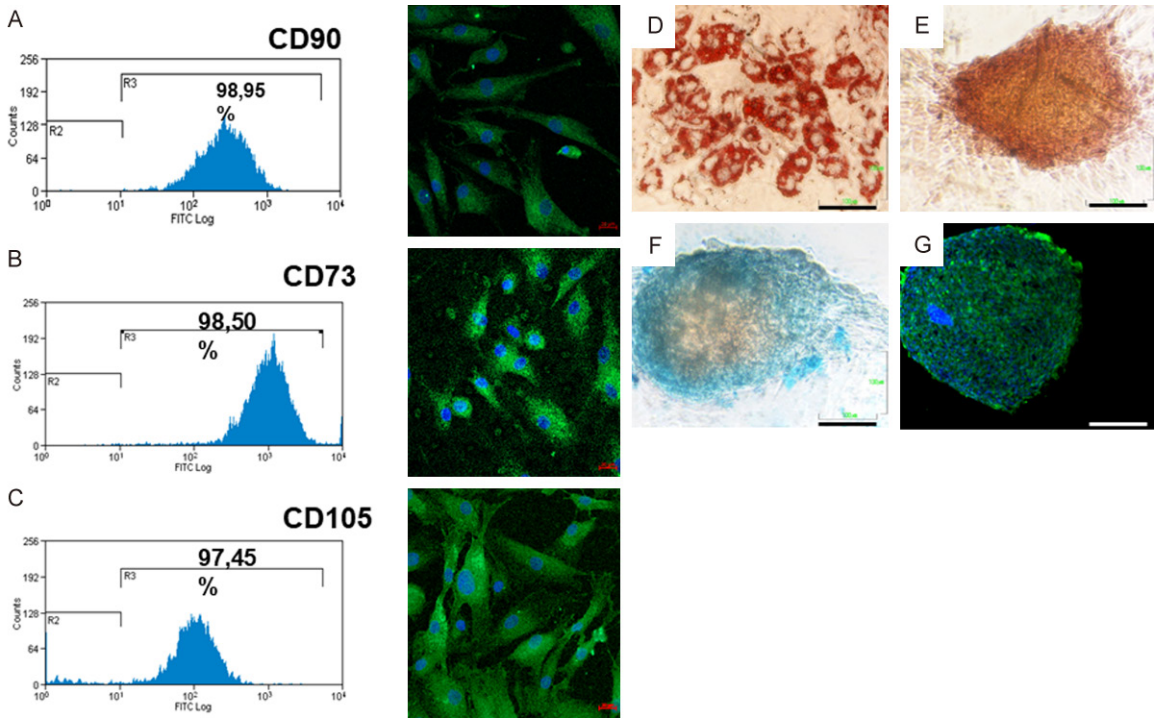

Figure 1. Immunophenotyping analysis of ASCs by flow cytometry and immune-fluorescence. (A) ASCs were positive to CD90 (98.95% expression), (B) to CD73 (98.50% expression) and (C) to CD105 marker (97.45% expression). In the fluorescence analysis (magnification 40×), all surface proteins are present in cells with the highest fluorescence intensity in the case of CD105. Nuclei were stained with DAPI. Tri-lineage differentiation potential of ASCs after 2 weeks of culture in differentiation medium (magnification 20×). (D) Adipocytes stained with oil red O. Presence of intracytoplasmic lipid-rich droplets. (E) Osteocytes visualized with alizarin red S. Matrix mineralization is clearly visible in induced cultures. (F) Extracellular matrix of chondrocytes stained with alcian blue. (G) Positive cells stained with anti-Col2a1.

acterized by an accumulation of cytoplasmic triglycerides. The presence of these lipid droplets was verified by oil red O staining (**Figure 1D**). Similarly, under osteogenic conditions, dark ECM deposits were detected after the culture period. These deposits stained orange-red with alizarin red S proving them to be mineralized calcium deposits (**Figure 1E**). Spherical nodules were observed in chondrogenic cultures and deposits of acid mucopolysaccharides were confirmed with alcian blue staining (**Figure 1F**). In **Figure 1G** green positive stain for Col2a1 was evident.

Cell proliferation in co-cultures of ASCs and chondrocytes

In order to study the behavior of ASC-chondrocyte co-cultures, a proliferation analysis was carried out using fluorescence microscopy and flow cytometry. Cell proliferation was analyzed at 12, 24 and 36 hours with no stimulation (control), stimulate with TNF or stimulated with TNF + CS. As described previously, to enable visualization by various methods, ASCs and chondrocytes were stained violet and green, respectively. The results obtained using confocal microscopy and flow cytometry are shown in **Figure 2**. Chondrocyte proliferation rates were higher than those seen for ASCs under all experimental conditions. The proliferation rates for each cell line were different due to the larger size of ASCs compared to chondrocytes. In fact, chondrocyte proliferation rates were higher than those seen for ASCs under all conditions.

The addition of 25 ng/mL of inflammatory agent (TNF) slightly reduced the viability of chondrocytes (Experiment 1, wells 4-6), but this stabilized where TNF was added in the presence of CS (Experiment 2, wells 4-6). The addition of CS alone (Experiment 2, wells 1-3) had no measurable effect on the viability of either cell line (**Figure 2C**).

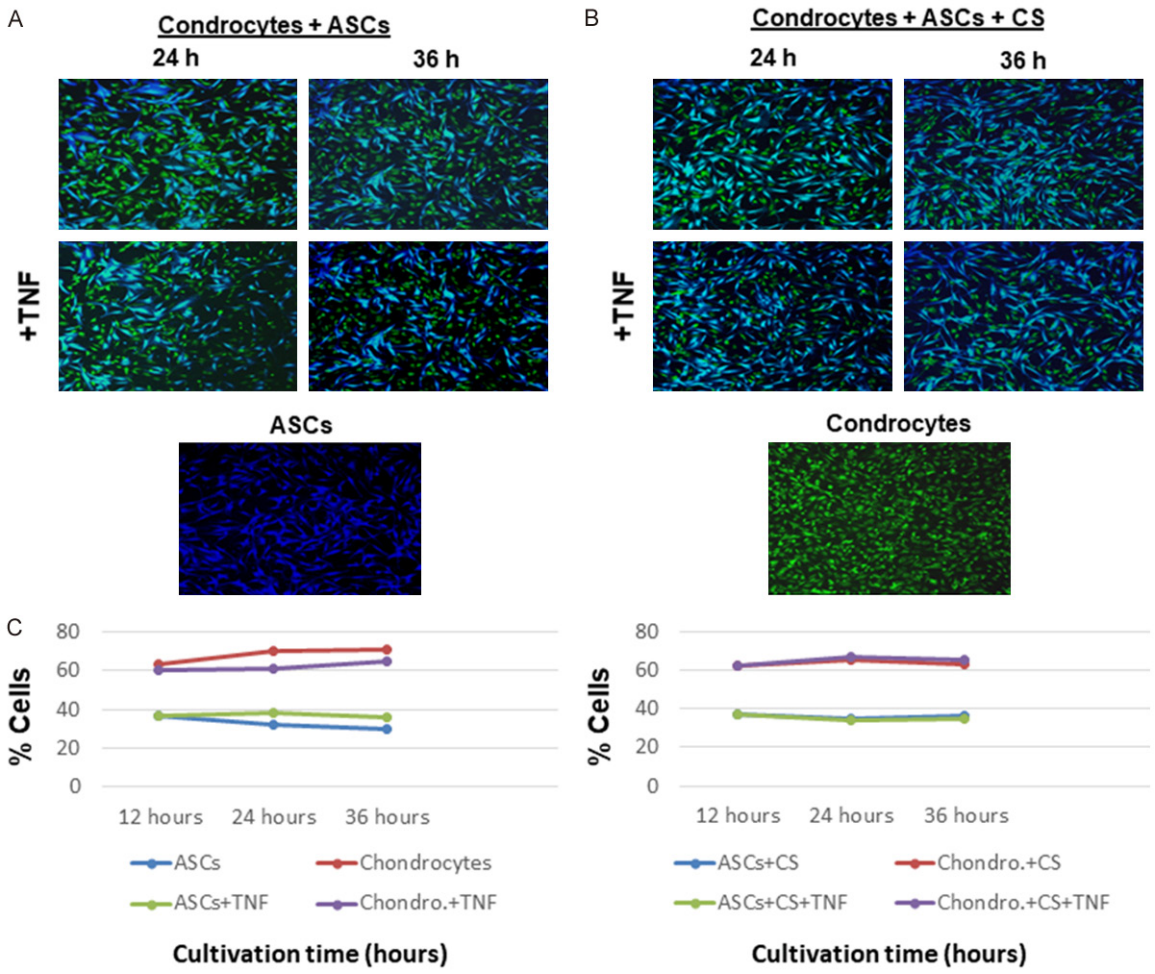

Figure 2. Monitoring of co-cultures proliferation using fluorescence microscopy (magnification 20×). A. ASCs (violet stained) and chondrocytes (green stained) in co-cultures at 24 and 36 h in culture with and without TNF. B. Co-cultures of ASCs (violet stained) and chondrocytes (green stained) at 24 and 36 hours in culture with and without TNF and CS. C. Flow cytometry quantification of cell proliferation in co-cultures at 12, 24 and 36 hours after induction of inflammation (left) and with addition of CS (right). The viability percentages are maintained for the two cell types, with a slightly reduction in the case of inflamed chondrocytes incubated in the absence of CS.

The effect of ASCs and CS on NF-κB translocation

It is known that NF-κB, is involved in the effects of TNF on inflammatory and catabolic mediators. TNF quickly induces translocation of NF-κB into the nucleus and the binding of DNA to trigger gene transcription. We studied the possible regulation of NF-κB by ASCs and CS. **Figures 3** and **4** show the results of the NF-κB activity assay for all experimental conditions. Referring to **Figure 3**, the lower level of fluorescence observed in image B, compared to image A, proves that stimulating ASCs with TNF reduced the DNA binding of p65-NF-κB. However, when chondrocytes where co-cultured with ASCs or CS was added to the culture medium, NF-κB translocation was blocked (**Figure 4**).

Observation of the action of CS in reducing PGE_2 concentration in inflamed cells

We assessed the effects of CS on the secretion of PGE_2 using ELISA. As can be seen in **Figure 5**, PGE_2 was present in small concentrations in our control samples and the presence of CS in the absence of any inflammatory stimulus had little effect on this. When TNF was used to produce an inflammation response in cells, PGE_2 production increased considerably, however, for both isolated ASCs and chondrocytes as well as co-cultures, the presence of CS acted to suppress PGE_2 production.

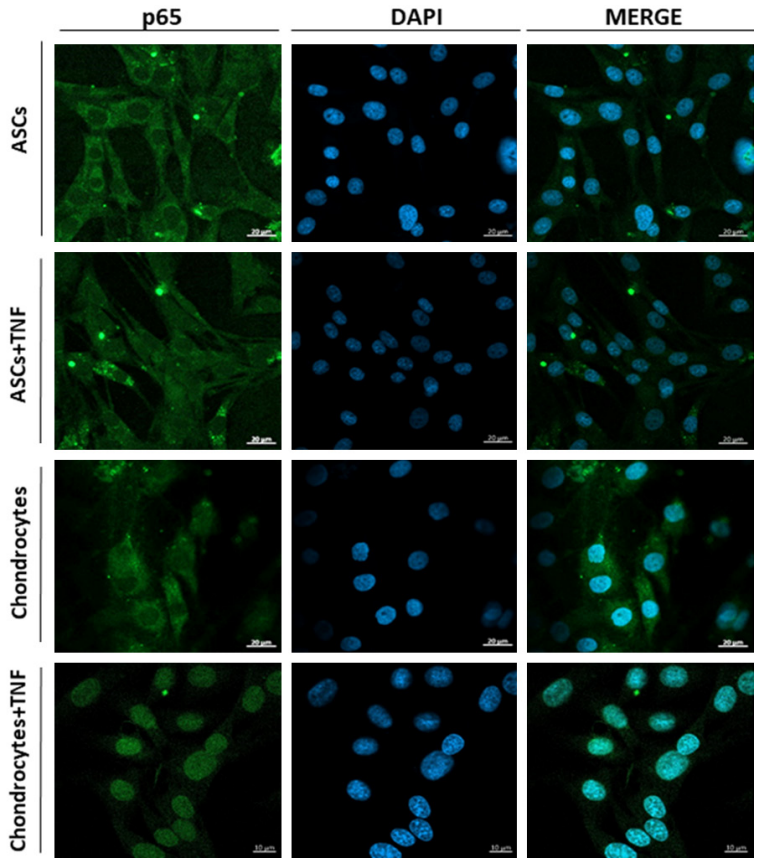

Figure 3. Effect of TNF on NF-κB activation in ASCs and chondrocytes. Cells were stimulated with TNF for 12 hours and p65-NF-κB binding to DNA was determined by immunofluorescence.

Detailed analysis of our data shows that the effects of CS are most pronounced for the ASC-chondrocyte cocultures. For ASC and chondrocyte monocultures, CS resulted in a 11.3% and 10.8% production in PGE_2 respectively while for the ASC-chondrocyte co-culture combined with CS the rate was 7.3%. This is a significant result and shows that the most promising OA treatment option would involve a combination of ASCs and CS.

IL-6, iNOS, TNF, MMP-13, IDO and TGF-β expression in ASCs-chondrocyte co-cultures

We analyzed the expression of IL-6, iNOS, TNF and MMP-13 using qPCR and the results for all experimental conditions are shown in **Figure 6**. The expression of IL-6 and iNOS significantly increased ($P \leq 0.005$) in chondrocyte cultures stimulated with TNF. However, in the presence of CS, IL-6 expression was significantly reduced. IL-6 expression also increased in ASC cultures stimulated with TNF but less so than in chondrocyte cultures. Its expression reduced most significantly for ASC-chondrocyte co-cultures treated with CS.

When iNOS expression was examined, it was found to be extremely elevated in chondrocytes stimulated with TNF. As can be seen in **Figure 6**, the presence of CS reduced levels of iNOS expression in general. It is clear, however, that chondrocytes saw the greatest reductions in iNOS levels in the presence of CS. Nevertheless, although the presence of CS had less effect on iNOS expression in ASC-chondrocyte co-cultures, these samples had lower overall levels.

The CS response patterns for MMP-13 and TNF were similar to that seen for iNOS expression. As expected, high levels of MMP-13 were observed in response to exposure to inflammatory stimulus, but chondrocytes treated with CS showed significantly reduced levels compared to untreated cells. The effect was also observed for ASC-chondrocyte co-cultures but to a lesser degree. In both instances, the presence of CS reduced levels of MMP-13 and TNF to those seen for non-inflamed cells.

Both ASCs and chondrocytes stimulated with TNF showed increased levels of TGF-β production but at levels close to those seen for the un-stimulated chondrocyte control group. In ASC-chondrocyte co-cultures stimulated with TNF, the presence of CS significantly reduced, TGF-β levels.

IDO was over-expressed in chondrocytes, ASCs and co-cultures stimulated with TNF and the presence of CS increased expression levels. However, this increase was not significant and varied greatly between experimental runs, as indicated by the large error bar shown. This being the case, no reliable conclusions can be drawn with respect to the effect of CS on the expression of this gene.

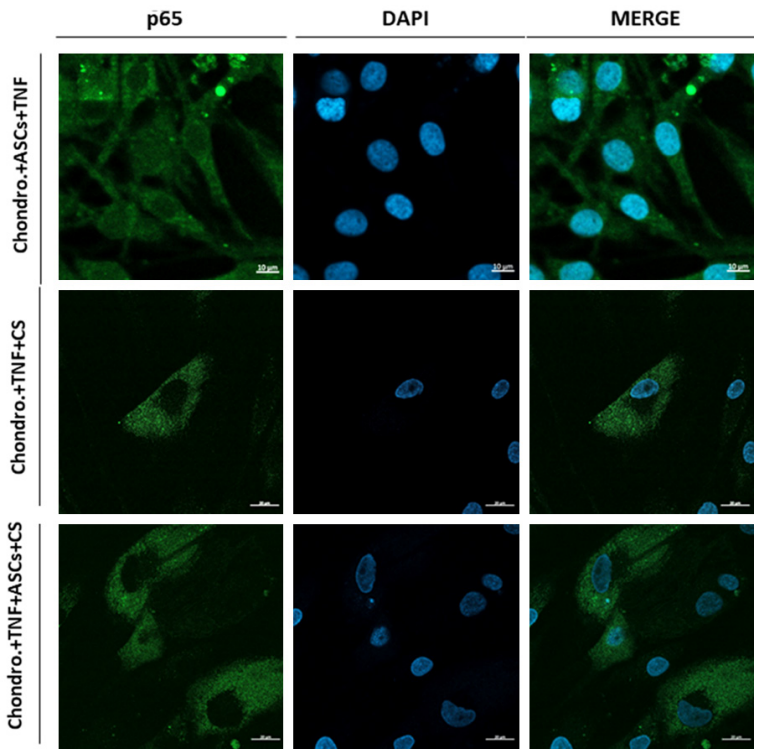

Figure 4. Effect of ASCs and CS on NF-κB activation in inflamed chondrocytes. Cells were stimulated with TNF for 12 hours and p65-NF-κB binding to DNA was determined by immunofluorescence.

Evolution of specific chondrogenic gene expression

We used qPCR to analyse the expression of specific genes associated with chondrogenesis, such as SOX-9, aggrecan (ACAN), and collagen type II (Col2a1). The results of this analysis for all experimental conditions are shown in **Figure 7**. We observed that, without TNF stimulation, the presence of CS significantly increased levels of Col2a1 and ACAN in chondrocytes. Where chondrocytes were stimulated with TNF, the expression of all three of these chondrogenesis-specific genes, was largely unaffected and the presence of CS had no appreciable additional effect. In the case of ASC-chondrocyte co-cultures we saw some similar patterns in that the expression of 2 genes (SOX-9 and ACAN) increased in the presence of CS, however, this effect was not significant.

Discussion

MSCs are an attractive alternative to conventional regenerative therapies [19]. They have been suggested as a new source for stem cells for use in the treatment of OA due to their capacity to differentiate into chondrocytes, the paracrine effects of the bioactive substances which they secrete, as well as their immunomodulatory effects [20]. CS is the major GAG component of native cartilage tissue and has a key role in the processes that stimulate cells to proliferate, migrate, differentiate and produce ECM compounds [21]. CS has also been shown to have anti-inflammatory effects in that it reduces the concentration of pro-inflammatory cytokines such as TNF and IL-1β [22]. We hypothesized that the combination of ASCs with CS should be capable of enhancing the cartilage regeneration and diminishing the inflammation in an *in vitro* model of OA. We evaluated the immunomodulatory effect of CS combined with ASCs in co-culture with inflamed chondrocytes. The expression of specific cartilage genes was also analysed.

Most previous studies examining chondrocyte depletion during OA progression show that a variety of factors including the presence of cytokines, such as TNF, are implicated in the progressive degeneration of joint cartilage seen in OA [23]. Chondrocytes incubated with TNF showed reduced cell viability compared to those in the control sample (no TNF). The viability of chondrocytes was, however, unaffected when cells were incubated with TNF in the presence of CS. The addition of CS to uninflamed cell cultures had no effect on cell viability.

Other studies have reported that MSCs can be induced to express enhanced levels of IDO and PGE_2. ASCs are known to secrete PGE_2 and that production significantly increases in co-cultures [24]. In this work, however, co-cultured ASCs showed no measurable increase in PGE_2 concentration where no inflammatory stimulus was present. Indeed, for all non-inflamed cell lines PGE_2 concentration remained at very low base levels throughout the experiment. According to other literature, CS itself has no effect

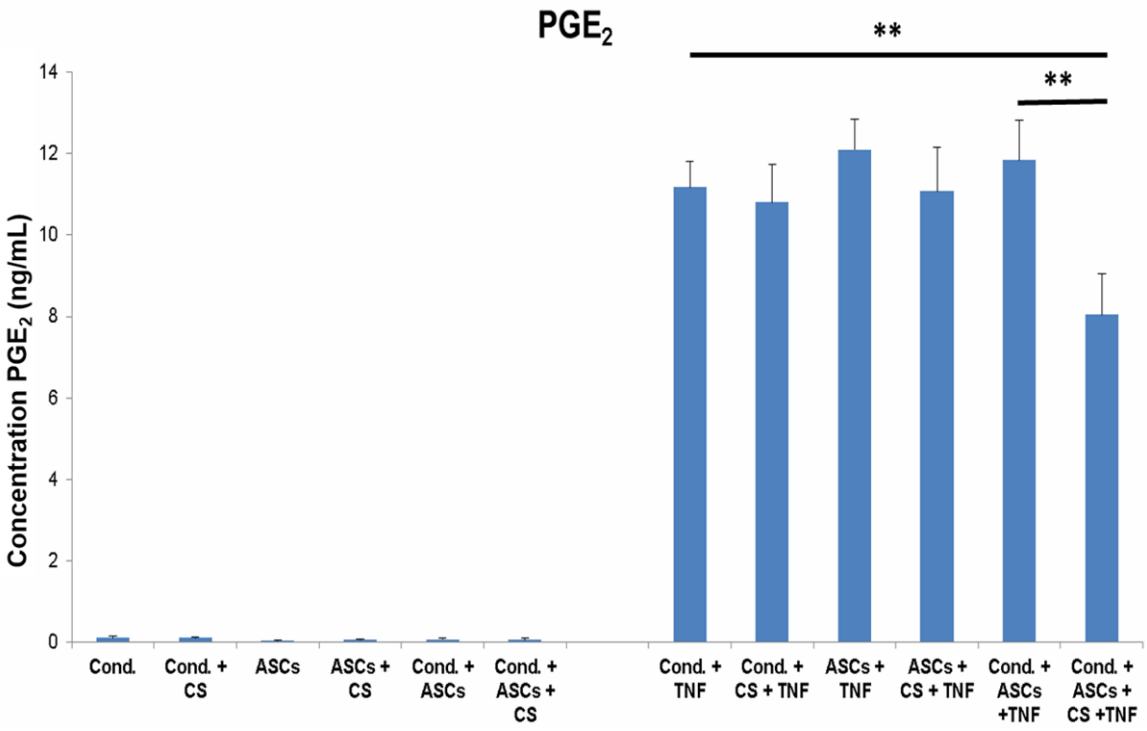

Figure 5. Effect of CS on TNF-stimulated cells PGE_2 production, both in chondrocyte and ASC monocultures and co-cultures. In an inflammatory microenvironment, the combination of CS and ASCs reduced the production of PGE_2. **P≤0.01 with regard to the corresponding group without CS.

on basal PGE_2 release [25] and our work confirms this. TNF and IL-1, are known to increase PGE_2 expression in both chondrocytes [26] and in MSCs [27]. Thus PGE_2 is considered to be a pro-inflammatory cytokine, although different theories exist with regards to its beneficial or detrimental role in OA [28]. As a treatment for OA, Ronca *et al.,* 1998 [29], showed that the effects of CS are due to the various ways in which it acts to reduce PGE_2 concentration in the joint [30]. However, we observed that in TNF-stimulated cells the level of PGE_2 production increased considerably under all experimental conditions. Nevertheless, although overall PGE_2 production was high for all TNF stimulated cell lines, it is worth noting that the lowest concentrations of PGE_2 were seen for ASC-chondrocyte co-cultures in the presence of CS. Current research suggests that PGE_2 seems to be involved in the up-regulation of the anti-inflammatory cytokine interleukin (IL)10 while reducing the secretion of TNF [31]. Therefore, we would speculate that the inhibition of PGE_2 production by CS could reduce the degenerative effects of OA in cartilage. Lastly, CS has been shown to inhibit the expression of enzymes involved in PGE_2 synthesis, COX-2 and mPGES-1 [32].

As we discuss later, ASCs reduce levels of certain pro-inflammatory cytokines whose production is associated with PGE_2. In this way, it seems likely that a combination of treatments involving both CS and ASCs would reduce OA processes each acting via several different routes. Our results agree with those obtained by other authors. Research has concluded that, as the main inducer of inflammation, the cytokine IL-6 is responsible for the pain experienced by OA sufferers [32]. Decreasing levels of pro-inflammatory cytokines, including IL-6, is then a major goal for any potential OA treatment and MCSs have been proved to do just this [25]. Although it has been shown that IL-6 is one of the main interleukins which induce inflammation, there is currently some debate as to its precise role in OA due to evidence that, in fact, this interleukin could have an anti-inflammatory effect [35]. With regard to PGE_2, while it is known to accelerate the expression of pain-associated molecules such as IL-6 and iNOS [32], MSC-derived PGE_2 always acts indepen-

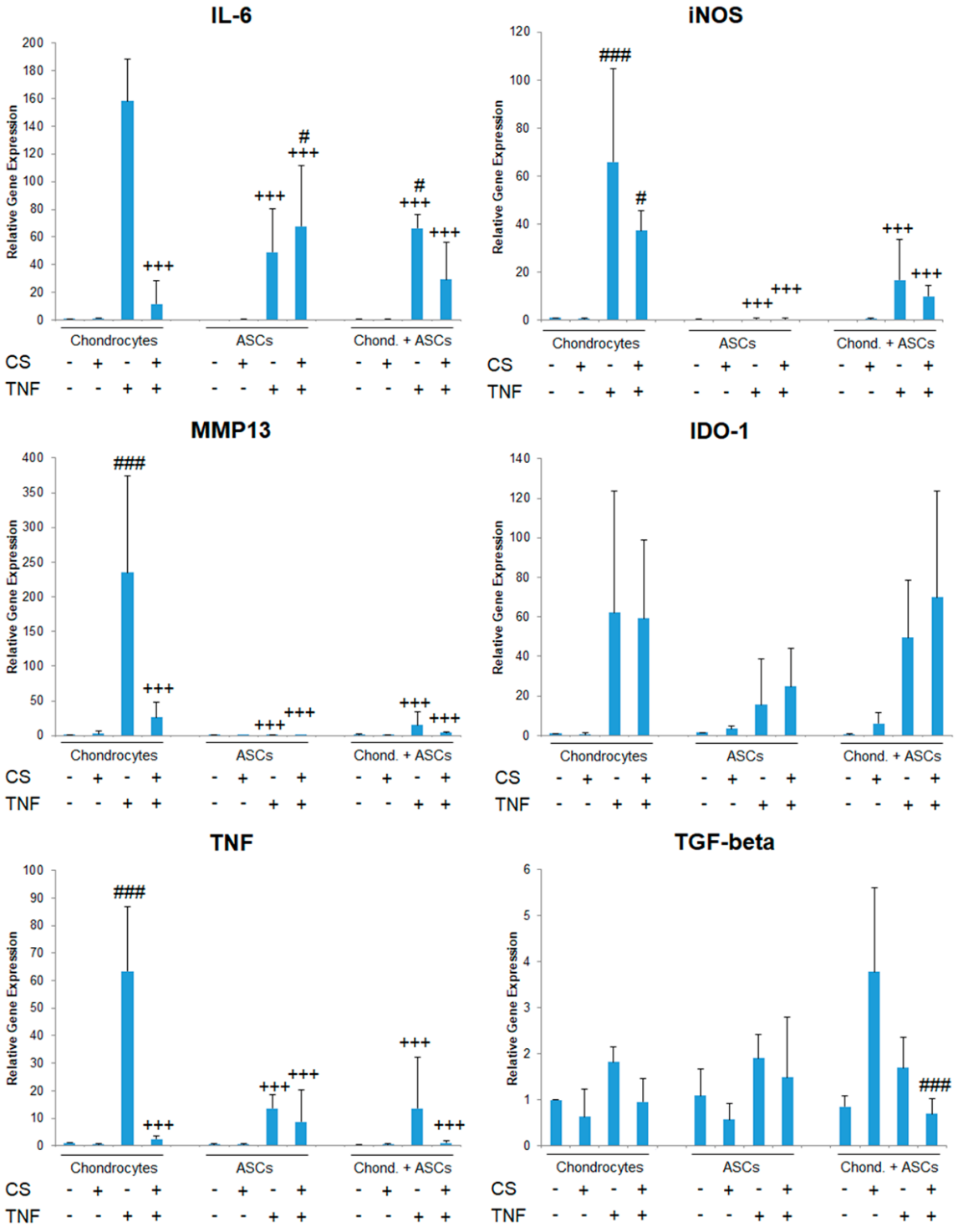

Figure 6. Relative expression of IL-6, iNOS, MMP-13, IDO, TNF and TGF-β in chondrocytes, ASCs and ASC-chondrocyte co-cultures. Cultures were incubated with TNF (to induce inflammation) and CS for 12 hours. $^{+++}P\leq0.005$ with regard to chondrocytes stimulated with TNF. $^{\#}(P\leq0.05)$ $^{\#\#\#}(P\leq0.005)$ with regard to chondrocytes exposed to TNF.

dently of IL-6 [33]. In our work, we observed that the expression of IL-6 in TNF stimulated ASC-chondrocyte co-cultures significantly decreased in the presence of CS. In fact, under these experimental conditions, levels of IL-6 were less than 15% those seen in TNF stimulated chondrocytes. This effect, where a combination of MSCs and CS has been found to

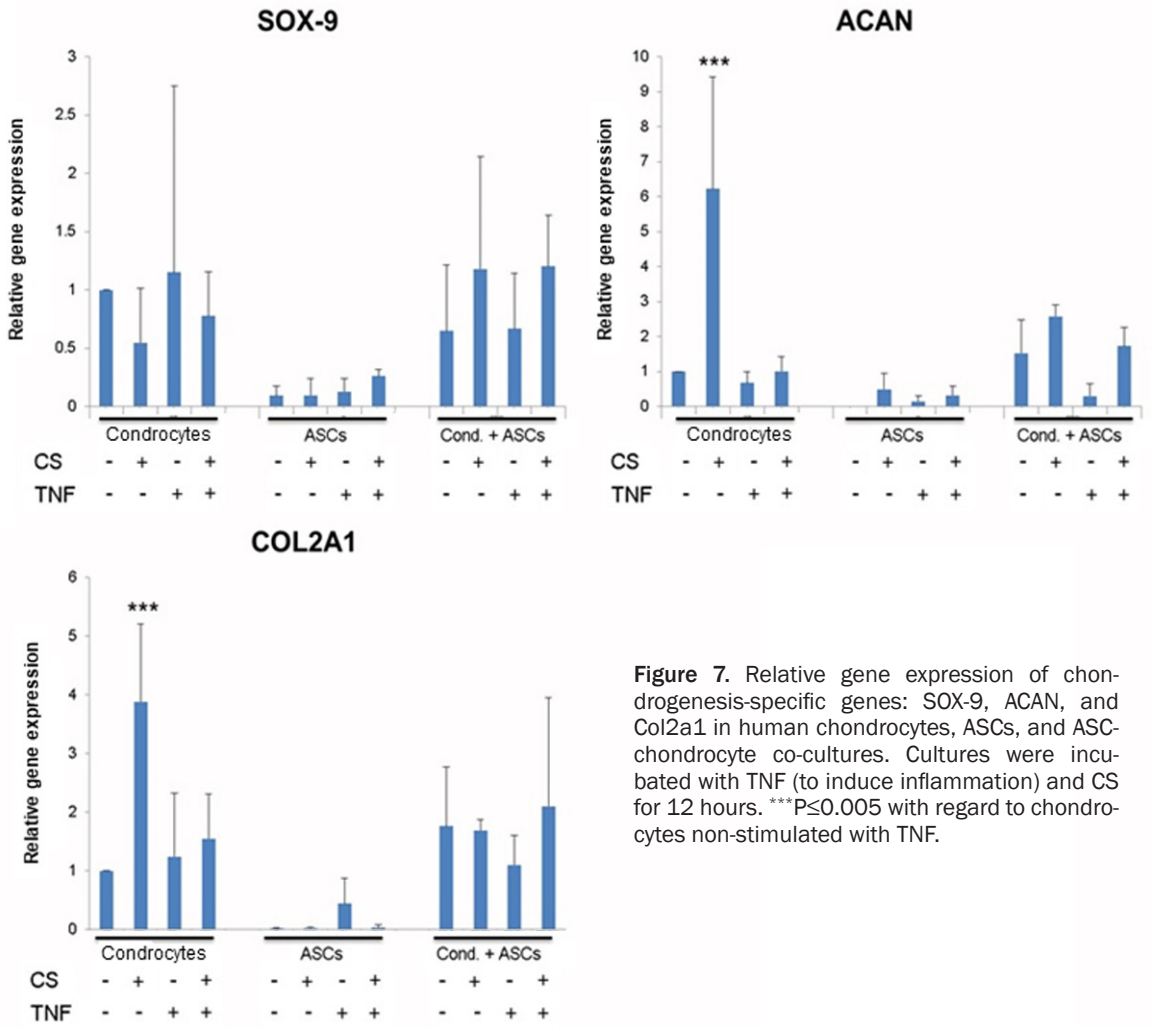

Figure 7. Relative gene expression of chondrogenesis-specific genes: SOX-9, ACAN, and Col2a1 in human chondrocytes, ASCs, and ASC-chondrocyte co-cultures. Cultures were incubated with TNF (to induce inflammation) and CS for 12 hours. ***P≤0.005 with regard to chondrocytes non-stimulated with TNF.

reduce the expression of pro-inflammatory cytokines, including IL-6 has been reported in other studies [34]. It is interesting to note, however, that the best results were seen for CS alone. Values for the relative expression of IL-6 in inflamed chondrocytes were 10 and 160 with and without CS respectively; for the ASC-chondrocyte co-culture with CS this value was somewhat higher at 30.

When iNOS expression was examined in TNF stimulated chondrocytes, it was found that these chondrocytes expressed extremely high iNOS levels, which is in agreement with Charles *et al*., 1993 [36]. However, the expression level in ASCs was minimal, as predicted by Ren *et al*., 2009 [37-39]. As with the expression of other genes investigated here, the treatment of chondrocytes with CS and ASCs dramatically reduced iNOS expression, with CS lowering it by more than 40 times and the combination of ASCs and CS by up to 50 times.

With respect to metalloproteinase, stimulated chondrocytes also produced MMP-13. Levels of this enzyme, were significantly reduced for inflamed chondrocytes co-cultured with ASCs and treated with CS. Increased expression of IL-6 is related to the production of enzymes from the MMPs group [31]. Nevertheless, their levels, particularly that of MMP-13, were reduced in inflamed chondrocytes treated with CS and ASCs. Deletion of the MMP-13 gene reduces articular cartilage degradation (targeting type II collagen), and it has been shown that it is a critical downstream target gene for TGF-β signaling during OA development [40]. It has recently been shown that global MMP-13 deletion could prevent articular cartilage erosion [41]. In this way, our results are very promising.

Both ASCs and inflamed chondrocytes showed high levels of TNF expression, which decreased in the presence of CS. The levels we saw

were not as high as might have been expected and we attribute this to the fact that our inflamed cells also produced elevated levels of PGE_2, as discussed previously, and this prevents TNF proliferation [15]. TNF, together with IL-1β, is considered to be a key inflammatory cytokine in the pathophysiological processes occurring in OA, and it blocks the synthesis of proteoglycan components and Col2a1 in chondrocytes. Moreover, TNF is responsible for the increased production of iNOS and IL-6 [31].

TGF-β levels slightly increased when ASCs and chondrocytes were stimulated with TNF, either separately or in co-culture. However, this was not a significant effect as values were very close to those obtained from the chondrocyte control group. In the presence of CS, levels were minimally reduced in all cases. Observations by Shen *et al.*, 2014 [40] showed that the inhibition of TGF-β signaling in chondrocytes leads to terminal differentiation and the development of OA due to the fact that this cytokine is responsible for stimulating the production of proteoglycans, Col2a1 and chondrogenesis. Other publications confirm that the amount of TGF-β is low or even undetectable in patients with OA [31].

Lee *et al.*, 2014 [24] demonstrated that tissue damage induces IDO expression in MSCs. Accordingly, tissues damaged or stimulated with TNF show increased production of IDO [15]. Our results agree with this, since they show increased expression of IDO in chondrocytes with induced inflammation and, indeed, it also increased in the presence of CS and ASCs. We therefore suggest, as do other authors [41], that IDO is probably expressed as a natural protector against inflammation [41].

During the more advanced stages of OA levels of Col2a1 and ACAN decrease due to denaturation [42], this is influenced by an increase in the expression of MMPs. We observed that the presence of CS significantly raised levels of Col2a1 and ACAN in TNF stimulated chondrocyte co-cultures. This raises questions as to the connection between Col2a1 and ACAN, key components of ECM, and the increase of proteoglycans thanks to the CS. CS is widely distributed in the collagen matrix where it forms an essential component of proteoglycans by making covalent links with proteins [25], thus aiding regenerative processes. Our results confirm that CS boosts the expression of certain genes present in the extracellular matrix (ACAN, Col2a1). Moreover, the expression of these genes was always higher in co-cultures compared to the inflamed chondrocytes without CS. With respect to SOX9, a critical factor for chondrocyte differentiation that facilitates the expression Col2a1 [43], we found no significant differences in its expression under any experimental conditions.

Canonical activation of the nuclear translocation factor NFκB, which is closely related to the inflammatory cascade, plays a key role in the expression of MMPs ADAMTS and indeed, inflammatory cytokines, in chondrocytes. TNF is known to induce MMP expression in chondrocytes through several activation pathways, specifically, via mitogen-activated protein kinase (MAPK), kappa nuclear factor B (NF-κB), and protein activator 1 (AP-1) [44]. In order to understand the possible mechanism by which ASCs downregulate these mediators, we investigated the effect of ASCs and CS on NF-κB. Our findings suggest that the observed inhibitory effects of ASCs and CS on the expression of catabolic and pro-inflammatory molecules could be related to the reduction of NF-κB translocation in TNF-inflamed chondrocytes. Jomphe *et al.*, 2008 [45], used an *in vitro* study to show that CS inhibits the translocation of NFκB. The therapeutic efficacy of CS and ASCs could be due to their anti-inflammatory activity and the stimulation of proteoglycan synthesis. It may also lie in their ability to decrease the catabolic activity of chondrocytes which inhibits certain proteolytic enzymes such as metalloproteases and inflammatory mediators such as TNF, iNOS, IL-6, PGE_2, NFκB [11].

Conclusions

To sum up, we conclude that the combination of CS and ASCs appears to inhibit the synthesis of certain pro-inflammatory and degradative mediators known to exert a deleterious effect on cartilage. This is a promising result for the treatment of OA. The therapeutic benefits of CS and ASCs lie in at least three mechanisms that contribute to delaying the progression of osteoarthritis: inhibition of the synthesis of inflammatory mediators (TNF, IL-6, PGE_2 and NO) mediated by TNF and interleukin-1; inhibition of the synthesis of catabolic enzymes such as MMP-13; and stimulation of the

synthesis of extracellular matrix components such as collagen type II and aggrecan. Further research is needed regarding the molecular pathways which control the functional behavior of cartilage under both physiological and pathological conditions. This will enable the development of more effective strategies for the treatment of OA and other cartilage-related diseases.

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Disclosure of conflict of interest

None.

Abbreviations

ACT-β, Actin Beta; AT-MSCs, Adipose Tissue derived Mesenchymal Stem Cells; BM-MSCs, Bone Marrow derived Mesenchymal Stem Cells; Col2A1, Collagen type II; COX-2, cyclooxygenase-2; CS, Chondroitin Sulfate; DMEM, Dulbecco's Modified Eagle's Medium; ECM, Extracellular Matrix; ELISA, Enzyme-Linked Immunosorbent Assay; FBS, Fetal Bovine Serum; GAG, Glycosaminoglycans; IDO, Indoleamine-pyrrole 2,3-dioxygenase; IBMX, Isobutylmethylxanthine; IgG, Immunoglobulin G; IL-1β, Interleukin-1 beta; IL-6, Interleukin-6; iNOS, inducible Nitric Oxide Synthase; ISCT, International Society for Cellular Therapy; MMPs, Matrix Metalloproteinase; MMP13, Matrix Metalloproteinase-13; mPGES-1, microsomal Prostaglandin E Synthase-1; MSCs, Mesenchymal Stem or Stromal Cells; NO, Nitric Oxide; NOS, Nitric Oxide Synthase; OA, Osteoarthritis; PGE_2, Prostaglandin E_2; RA, Rheumatoid Arthritis; RT-PCR, Real-Time Reverse Transcriptase-Polymerase Chain Reaction; SD, standard deviation; S/DMOAD, Structure/Disease Modifying Anti-Osteoarthritis Drug; SySADOA, Symptomatic Slow-Acting Drug on OA; TNF, Tumor Necrosis Factor; TGF-β, Transforming Growth Factor Beta.

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