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Basic Science

Extracellular vesicle and soluble fractions of adipose tissue-derived mesenchymal stem cells secretome induce inflammatory cytokines modulation in an *in vitro* model of discogenic pain

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ABSTRACT

BACKGROUND CONTEXT: Mesenchymal Stem Cells (MSCs) secretome or conditioned medium (CM) is a complex cocktail of different molecules, some of which, particularly those contained in extracellular vesicles, already have proven therapeutic applications.

PURPOSE: CM may well represent promising therapy for discogenic pain and the intention of this work is to assess its therapeutic potential using an *in vitro* model of this condition.

STUDY DESIGN: This is an experimental study.

METHODS: Our *in vitro* model comprised Nucleus Pulposus (NP) and Annulus Fibrosus (AF) cells inflamed with TNF. To assess the potential therapeutic value of CM and its components, extracellular vesicles (EVs) and soluble culture fraction (SF), cell inflammation took place under 3 different conditions: either in the presence of whole CM, isolated EVs or SF, and concentrations of pro-inflammatory cytokines, metalloproteinases (MMPs) and neurotrophic factors produced in all 3 cases were compared.

RESULTS: In the presence of whole CM, both *in vitro* gene expression by the NP and AF test cells and analysis of their protein content showed high modulatory effects on inflammation and MMP inhibition. The presence of EVs and SF showed similar but much smaller effects, and this was particularly marked in the case of NP cells.

CONCLUSIONS: Our results show that, compared to EVs and SF, the presence of whole CM has the greatest positive effect on the modulation of pro-inflammatory and catabolic factors. These observations suggest that CM could protect against inflammation and the resulting intervertebral disc (IVD) degeneration that leads to discogenic pain.

CLINICAL SIGNIFICANCE: Many patients' expectations are not met by current non-operative and surgical treatments for discogenic low back pain. We propose the use of the MSCs secretome

FDA device/drug status: Not Applicable

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Abbreviations: ADAMTs, Metalloproteinases with thrombospondin motifs; AF, Annulus Fibrosus; ASCs, Adipose tissue-derived mesenchymal stem cells; BSA, Bovine serum albumin; CM, Conditioned medium; DMEM, Dulbecco's Modified Eagle's Medium; ECM, Extracellular Matrix; EV, Extracellular vesicle; FBS, Fetal bovine serum; IL, Interleukin; IVD, Intervertebral Disc; MMPs, Metalloproteinases; MSCs, Mesenchymal stromal cells; NP, Nucleus Pulposus; PBS, Phosphate buffered saline; PFA, Paraformaldehyde; PG, Proteoglycans; PGE_2, Prostaglandin E_2; Qpcr, Quantitative real-time PCR; SD, Standard deviation; SF, Soluble fraction; TEM, Transmission electron microscopy; TNF, Tumor necrosis factor; TIMPs, Tissue inhibitors of metalloproteinases

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for assessing its potential as cell-free therapy to treat degenerative disc disease modulating the inflammatory response.

Keywords: Adipose tissue mesenchymal stem cells; Secretome; Extracellular vesicles; Soluble fraction; Inflammation; Cytokines; Intervertebral disc

Introduction

Back pain is one of the most prevalent pathologies in the world, and is associated with significant personal and social costs [1,2]. Although the exact causes of back pain are not fully determined, it has been related strongly to degeneration and herniation of the intervertebral disc (IVD) [3].

The structure of the IVD is regulated by a balance between anabolic and catabolic processes, during disc degeneration the rate of the latter is increased, leading numerous structural changes [3–6]. These changes mainly cause NP degeneration, specifically, the proteoglycan (PG) content of the NP decreases drastically and with it the capacity of the extracellular matrix (ECM) to recruit water. This results in the NP becoming less gel-like in character, and more fibrous which in turn leads to a loss of structural distinction between the NP and the AF [7]. In this way, progressive degeneration causes structural defects in the IVD resulting in increasing loss of their mechanical strength and function [3].

The degenerative changes described lead to an increase in expression of growth factors and proinflammatory cytokines by NP cells and infiltrating immune cells like mast cells or lymphocytes [8]. The proinflammatory mediators particularly associated with IVD degeneration include tumor necrosis factor (TNF), interleukins IL-1 α/β, IL-6, IL-17, IL-8, IL-2, IL-4, IL-10, interferon-γ (IFN-γ), chemokines, and prostaglandin (PGE_2), of which TNF and IL-1β appear to be the most important [9]. It has been shown that TNF and IL-1β have a role in disc degeneration through various processes including matrix destruction through up-regulation of the matrix metalloproteinases (MMPs), and a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS) [10,11].

Pain in IVD degeneration has been linked to levels of cytokines and changes in vascularization that occur during disc degradation [12]. In addition, discogenic pain is associated with elevated levels of 2 neurotrophins, brain derived neurotrophin (BDNF) and nerve growth factor (NGF) and these factors are thought to be related to hyperinnervation [13,14].

Current therapies for discogenic pain resulting from IVD degeneration include conservative strategies that cannot address its underlying cause, or surgical procedures that are likely either to intensify the degenerative processes of adjacent anatomical structures [15–17] or do not last in the long term [18]. Hence, there is a significant need for alternative therapies.

Cell therapies, especially those involving mesenchymal stem (stromal) cells (MSCs), have been studied extensively in the context of IVD degeneration [19]. As discussed elsewhere [20] MSCs can potentiate tissue regeneration and also modulate immune responses in several pathological conditions. In the case of IVD degeneration specifically, MSCs have been proved to repair matrix degradation and inhibit the catabolic processes implicated in this condition [21,22].

Bone marrow is the most commonly used source of MSCs, however they are not exclusive to this tissue, and can be isolated from adipose tissue, brain, liver, spleen and other sources. Adipose tissue is one of the best alternatives as a source for MSCs due to the accessibility and abundance of this tissue, they can be harvested with minimum morbidity and ease using minimally invasive procedures [23]. ASCs have a large therapeutic potential focus on terms of their differentiation potential into distinct lineages [24].

Recent studies have shown that the regenerative ability of MSCs derives from the secretion of certain soluble molecules [25,26]. MSC conditioned medium (CM) contains a cocktail of MSC-secreted molecules including numerous soluble factors and extracellular vesicles (EVs). These MSC products are involved in several different processes including cell differentiation, tissue repair [27], and inflammation reduction [27]. Although the mechanisms are not fully understood, in recent years EVs have emerged as being a particularly promising component of CM [25]. EVs are lipid bilayer-delimited particles that are naturally released from cells [28] and studies have demonstrated the capabilities of MSC-derived EVs in several areas: tissue repair and regeneration, including cardiac remodeling, and as anti-apoptotic and anti-inflammatory agents [29–34].

In a previous study concerning an *in vitro* model of IVD degeneration we showed that adipose tissue derived-MSCs (ASCs) produce immunomodulation IVDs [35]. Bearing in mind the above discussion, it seems clear that the modulatory effects observed are mediated by components in the CM and, further, we hypothesize that the isolated EVs and SF comprising the ASC secretome might each exert different effects. In this work, we test this hypothesis we used a pro-inflammatory *in vitro* model of IVD degeneration to comparing the effects of the whole secretome (CM), isolated EVs and SF. In this way we hope to be able to demonstrate which fraction of ASC secretome has the greatest therapeutic potential.

Materials and methods

Biological material

The primary cells used in the experimental procedures described here were all sourced from Innoprot: human adipose-derived mesenchymal stem cells (HAdMSC, Ref. P10763), human annulus fibrosus cells (HAFC, Ref. P10974) and human nucleus pulposus cells (HNPC, Ref: P10973). Human Annulus Fibrosus and Human Nucleus Pulposus Cells provided by Innoprot are isolated from human healthy intervertebral disc. Human Adipose-derived Mesenchymal Stem Cells are isolated from healthy human adipose tissue. Cells are cryopreserved at passage one and delivered frozen.

ASC cultivation

Cells were resuspended and proliferated in T150 flasks with DMEMc (DMEM, Hyclone) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone) and 1% (v/v) penicillin/streptomycin (Hyclone) at 37˚C in a humid atmosphere containing 5% CO_2.

Conditioned medium (CM) collection

ASCs ($\sim 1\times10^6$ cells) were maintained in DMEMc to approximately 80% confluency with 2 passages. To avoid possible contamination by factors, present in the FBS Cell culture supernatants (CM) were collected 24 h after the cells were supplemented with serum-free DMEM (Hyclone) and with 1% penicillin/streptomycin (Hyclone).

EV isolation

EVs were isolated from the CM using a precipitation method performed using a commercial isolation kit (Total EV Isolation (from cell culture media) kit, Invitrogen) and following the manufactures' instructions. In brief, 5 ml of the EV precipitation solution was added to 10 ml of the supernatants and the mixture was incubated at 4˚C overnight. Samples were then centrifuged at 10,000 x g for 1 h at 4˚C. The pellet, containing the isolated EVs, was resuspended in PBS.

Soluble fraction (SF) isolation

Obtaining EV-free soluble fraction of the conditioned medium was carried out following the procedure described according to Théry et al., 2006 [36] which allows the isolation of EV-free SF. CM was centrifuged at 300 x g for 10 min at 4˚C and then at 2,000 x g for 10 min at 4˚C to remove all cellular material. The supernatant was then centrifuged at 10,000 x g for 30 min at 4˚C to remove large particles. Finally, EVs were removed by ultracentrifugation at 100,000 x g for 70 min at 4˚C twice. 15 mL of this supernatant obtained were transferred to an Amicon Ultra-15 10K device and concentrated further to 500 μL using an Allegra X-15R centrifuge at 4,000 g at 4˚C for 30 minutes. The final supernatant collected comprised the soluble fraction.

EV validation

Transmission electron microscopy

The presence of EVs in our samples was also verified using TEM following a standard protocol as previously described by other authors [36]. Briefly, 25 μl of EVs and SF samples were fixed with 2% paraformaldehyde (PFA). Five μl of each sample were placed on Formvar-carbon-coated EM grids and left to adsorb for 20 minutes at room temperature. Samples were fixed with 1 % glutaraldehyde, contrasted in an uranyl oxalate solution pH 7 and then embedded in a mixture of 4% uranyl acetate and 2% methylcellulose in a ratio of 1:9 on ice. Grids were removed with stainless steel loops and the excess fluid was blotted with filter paper. After drying, samples were visualized using a Transmission Electron Microscope (JEM 1010, JEOL).

Western blot

EV markers, such as ALIX and TSG101 were measured by Western blot. Soluble fraction proteins were used as control. Protein concentration was quantified using the Bicinchoninic acid (BCA) protein assay (Bio-Rad) in a SpectraMax microplate reader (bioNova científica). Samples containing 10 μg total protein were combined with 5X loading buffer [250 mM Tris-HCl (Sigma) pH 6.8, 500 mM β-mercaptoethanol (Sigma), 50 % glycerol (Sigma), 10 % SDS (Sigma), and bromophenol blue (Sigma) in H_2O and denatured by boiling. Proteins were resolved by SDS-PAGE acrylamide gels, using Mini-PROTEAN Electrophoresis System (Bio-Rad). Fractionated proteins were transferred onto nitrocellulose membranes by electroblotting using Mini Trans-Blot cell (Bio-Rad). Membranes were blocked with 5 % nonfat dry milk in 1X Tris Buffer Saline (TBS) [50 mM Tris, 150 mM NaCl (Sigma), pH 8.0) containing 0.1 % Tween-20 (Sigma) (TBST-0.1 %)] and incubated with primary antibodies overnight at 4˚C. The primary antibodies used for each marker were: TSG101 (0.11 μg/ml, Abcam) and ALIX (0.8 μg/ml, Abcam). Horseradish peroxidase-conjugated antibodies were used as secondary antibodies to detect immunoreactive protein bands by Western Lightning Enhanced Chemiluminiscence (ECL) Reagent (PerkinElmer) and exposed to X-ray films (Fujifilm) in a Curix 60 Developer (Agfa).

EV internalization assay

In order to estimate the internalization capacity of EVs into NP and AF cells, ExoGlow-Membrane EV labeling kit was used following the manufacturer's instructions. In brief, 12 μl of reaction buffer and 2 μl of labeling dye were mixed with 100 μg of EV fraction suspended in 1 ml PBS. Excess protein binding was saturated using 1% BSA. PBS 1X was added to the mixture which was centrifuged at

100,000×g for 1 h at 4°C. Labeled EVs were suspended in DMEMc supplemented with 10% (v/v) exosome-depleted FBS and 1% (v/v) penicillin/streptomycin and added to either NP cells or AF cultures (10,000 cells / 500 μl of DMEM) on an 8-well Nunc Lab-Tek Chamber Slide System. After 12 hours of incubation, the cells were fixed with 2% PFA, permeabilized with 0.1% Triton X-100 and blocked with 3% BSA in PBS. Nuclei were stained with DAPI (1:1500). Fluorescence confocal microscopy was performed using a Zeiss LSM 800 laser scanning confocal microscope. Cells cultured with PBS (EVs free) stained with dye solution was used as a negative control.

In addition, flow cytometry was performed on NP and AF cells cultured with labeled EVs using the MACSQuant analyzer 10 flow cytometry equipment. This method allowed us to quantify the EVs which were able to enter into the NP and AF cells. Data analysis, based on the fluorescence of the B2-A channel, was analyzed by MACSQuantify software, with a minimum of 10,000 events per acquisition and excluding doublets and higher aggregates by gating, following the manufacturer's instructions. Negative controls were cells cultured with PBS (EVs free) and stained with the dye solution.

An in vitro *model of discogenic pain*

Our *in vitro* model of discogenic pain comprised 2 types of IVD cell, NP and AF cells (P2) inflamed with TNF. The model was subjected to 5 different experimental conditions in order to examine the potential therapeutic benefits of whole CM, and isolated EVs and SF. The protein concentration of the conditioned medium, EVs and soluble fraction were measured using a Micro BCA Protein Assay Kit, using BSA as a standard following the manufacturer's instructions. Equal amounts of protein (50 ng/ml) were added to perform all the experiments. For this purpose, cells of each type were seeded separately on 6-well plates, with 1×10^6 cells/well. When 80% cell confluence was reached, samples were then divided into 5 groups and treated as follows:

Group 1 (NP and AF control samples) was treated with serum-free DMEM (Hyclone) and 1% penicillin/streptomycin (Hyclone);

Group 2 (untreated TNF inflamed samples) was treated with serum-free DMEM (Hyclone) and 1% penicillin/streptomycin (Hyclone) plus 25 ng/ml recombinant TNF (Cusabio Technology).

Group 3 was treated with 25 ng/ml recombinant TNF (Cusabio Technology) and exposed to CM (TNF+CM samples);

Group 4 was treated with 25 ng/ml recombinant TNF (Cusabio Technology) and exposed to ASC-derived EVs (TNF+EV samples);

Group 5 was treated with 25 ng/ml recombinant TNF (Cusabio Technology) and exposed to ASCs-derived SF (TNF+SF samples).

After 12 hours of incubation, cell samples were collected and analyzed using real-time quantitative PCR (qPCR).

qPCR

Total RNA extraction from cell culture was performed using the GeneMATRIX universal RNA purification kit (EURx) following the manufacturer's instructions. RNA concentration was determined with NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific).

1000 ng of total RNA was used to synthesize cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's instructions. Gene expression of IL-1α, IL-1β, IL-6, IL-8, IL-17, NGF, BDNF, IFN-γ and TNF were determined using qPCR. Primer sequences for detecting differentiation-related genes are listed in Table 1. ACT-β was used as a control for the input RNA level.

The qPCR reactions were performed using Power SYBR Green PCR Master Mix 2 × (Applied Biosystems) in a total volume of 20 μl and on StepOne real-time PCR system (Applied Biosystems), following the manufacturer's instructions. Target gene expression was calculated by the $2-\Delta\Delta Ct$ method and normalized to the control gene, ACT-β.

Enzyme-linked immunosorbent assay (ELISA)

Commercial ELISA kits (Elabscience Biotechnology) were used to measure the concentrations of 5 secreted proteins in the cell culture supernatants: specific human MMP-1, MMP-2, MMP-3, MMP-13 and ADAMTS-5. These proteins were tested under all 5 experimental conditions outlined on Table 1 and the manufacturer's instructions were followed in all cases. Absorbance at 450 nm was measured for all samples using an ELISA plate reader (model 680; Bio-Rad).

NF-κB activity assay

AF and NP cells were seeded at 3×10^5 cells/well in 2 Nunc Lab-Tek chamber slide systems (Thermo Fisher Scientific) and stimulated for 12 hours with TNF (25 ng/ml). Cells were fixed with 2% formaldehyde in PBS for 15 minutes at room temperature and then incubated overnight with human antip65-NFκB pS529-FITC antibody (Miltenyi Biotech) at 4°C. Finally, using Vectashield mounting medium containing DAPI, chamber slides were assembled and examined under a Zeiss LSM 800 laser scanning confocal microscope. Images of fluorescent cell nuclei obtained were used to estimate the total number of positive translocated cells on each sample using the NIH Image J Software. All the experiments were developed using 3 replicates.

Statistical analysis

To obtain the quantitative data used in this study each experiment was repeated, independently, 3 times.

Table 1
Primer sequences and conditions used for qPCR

Gen	NCBI RefSeq	Primer sequence (5'-3') (Forward/Reverse)	Tª melting C	Product size (bp)
ACT-β	NM_001101	GACGACATGGAGAAAATCTG	59,7	131
		ATGATCTGGGTCATCTTCTC	58,0	
GAPDH	NM_002046	ACAGTTGCCATGTAGACC	55,7	157
		TTTTTGGTTGAGCACAGG	59,9	
TNF	NM_000594	CCCTGAAAACAACCCTCAGACGC	77,9	155
		CTCCTCGGCCAGCTCCACGTCCC	79,3	
IL-1α	NM_000575	AGAGGGAAGAAATCATCAAGC	57,5	123
		TTATACTTTGATTGAGGGCG	59,2	
IL-1β	NM_000576	TGGCCCTAAACAGATGAAGTGCTCC GAACC	71,7	158
		AGCATCTTCCTCAGCTTGTCCA	74,4	
IL-6	NM_000600	GCCCCACAGACAGCCACTCAC	74,4	169
		TTTCAGCATCTTTGGAAGGTTCAGGT	72,5	
IL-8	NM_000584	CAGCAGAGCACACAAGCTTCTAGGACA	72,1	161
		GCACTCCTTGGCAAAACTGCACCTTC	74,6	
NGF	NM_002506	GGTGCATAGCGTAATGTC TGAAGTTTAGTC	56,5	135
		CAGTGGG	57,7	
IFN-γ	NM_000619	GGTAATGACTTGAATGTCC	56,0	93
		TTTTCGCTTCCCTGTTTTAG	60,8	
BDNF	NM_170731	CAAAAGTGGAGAACATTTGC	59,8	173
		AACTCCAGTCAATAGGTCAG	55,7	
IL-17	NM_002190	GGTCAACCTGAACATCCATAACCGGAA	73,3	187
		GTAGTCCACGTTCCCATCAGCGTT	70,9	

All results quoted are the mean ± SD of the 3 values obtained from these separate experiments. Statistical analysis was performed using IBM SPSS Statistics 17, the normal distribution of the data was analyzed using the Shapiro-Wilk test. Significant differences among groups were determined using ANOVA followed by Tukey's post-hoc analysis. Results with p≤.05 were considered statistically significant.

Results

EVs validation

In a previous work [37] exosomes from ASC-derived conditioned medium were characterized. EVs detection in both fractions were confirmed using TEM. This analysis showed the presence in EV fraction of EVs (Fig. 1A, white arrows) which were no present in the soluble fraction

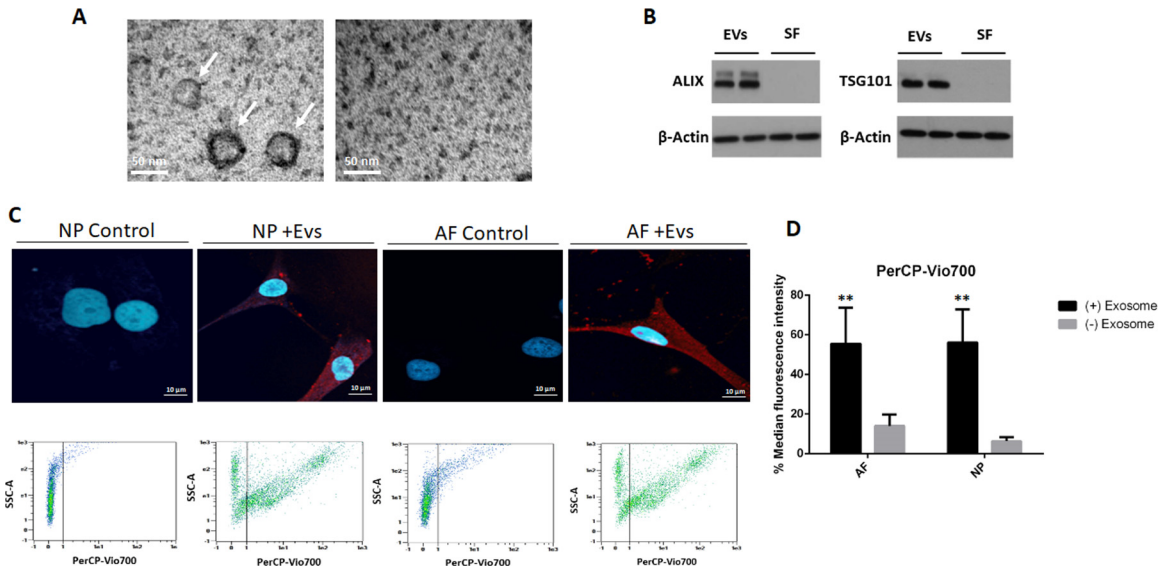

Fig. 1. (A) EVs validation. Representative images of transmission electron micrographs of EVs isolated (white arrows) in EV fraction (left) and soluble fraction (right) (magnification 30,000x, scale bar=50 nm). (B) Western blot bands from EVs released into the media from ASCs-conditioned medium corresponding to different EV markers (ALIX and TSG101). Soluble fraction was used as a reference, no EV markers were detected in this sample. (D) EVs uptake assay. Cellular internalization of ASC−derived EVs into annulus fibrosus (AF) and nucleus pulposus (NP) cells. Scale bar = 10 μm. Cell internalization was quantified using flow cytometry. Dot plots are shown beside the confocal image. (D) EVs uptake analysis in AF and NP cells. The results are expressed as the mean±SD of three independent experiments ** p≤.01.

samples (Fig. 1A right). In order to assess EVs presence in the corresponding EV fraction and not in the SF, we have developed a Western blot assay to detect EV markers, such as ALIX and TSG101 in the EV fraction isolated. Thus, Western blot assay revealed an enrichment of the EV markers ALIX and TSG101 in the EV fraction compared to those obtained from soluble fraction (Fig. 1B).

EVs are able to enter NP and AF cells

Isolated EVs obtained from the conditioned cell culture supernatants were used to assess cellular extracellular vesicles uptake Fig. 1.C shows the positive immune-staining of both AF and NP cells exposed to isolated EVs, so confirming EV uptake. Furthermore, when internalized EVs were quantified using flow cytometry, major differences were observed when comparing NP and AF cells that had been exposed to isolated EVs to those that had not (control) (Fig. 1D). No significant differences were observed between NP and AF cells exposed to isolated EVs.

Comparing the effects of CM, EVs and SF on the production of MMPs and ADAMTS-5 enzymes on inflamed AF and NP cells

To compare the potential therapeutic effects of CM, EVs and SF on inflamed IVD cells we used ELISA to examine the production of MMPs (MMP-1, MMP-2, MMP-3 and MMP-13) and ADAMTS-5 in our 5 different groups of samples (see section 2.7). Results can be seen in Fig. 2. Both NP and AF cells express endogenous MMPs in basal conditions (NP and AF control samples), and inflammation with TNF increased the production of all MMPs in the case of AF cells. For NP cells, TNF inflammation caused increases only in the expression of MMP-1, MMP-13 and ADAMTS-5 over the control samples. Comparing the performance of CM, EVs and SF in reducing levels of MMPs below those seen in inflamed cells, it appears that for MMP-1, -3 and -13, CM reduces levels more than either EVs or SF. Concerning ADAMTS-5 production, here results suggest that CM is very effective in reducing protein levels beneath those seen in inflamed cells. Indeed, it appears to be more effective than EVs in both NP and AF cells.

Comparing the effects of CM, EVs and SF on mRNA expression of pain-related and pro-inflammatory cytokines by inflamed AF and NP cells

TNF, IL-1α, IL-1β, IL-6, IL-8, IL-17 and IFN-γ are all genes that have been related to inflammation and are therefore of interest in the context of discogenic pain. The expression of these genes was analyzed for all 5 sample groups using qPCR.

Results show the CM was able to trigger a greater modulatory response in both NP and AF cells compared to EVs and SF (see Figs. 3 and 4). The increase in TNF expression between non-inflamed (control) and inflamed (TNF) NP cell samples under all experimental conditions is shown in Fig. 3 (**p≤0.01). Inflamed NP cells exposed to CM showed significantly lower TNF expression compared to NP cells exposed to either EVs or SF. The expression of IL-1α and IL-1β in NP cells showed a similar pattern to that obtained in the case of TNF. that is, increased expression comparing inflamed cells to control cells and with inflamed cells exposed to CM, showing the greatest reduction in gene expression compared to those exposed to either EVs or SF. Analyzing the results for IL-6, IL-8 and IL-17, as expected, we observed that inflamed NP cells showed elevated levels of expression with respect to control samples. Inflamed cells exposed to either CM, EVs or SF all showed decreased levels of IL-6, IL-8 and IL-17 expression compared to untreated inflamed cells. This decrease was greatest for CM samples. Results for IFN-γ expression in NP cells (Fig. 3) showed considerable increases for inflamed cells compared to control cells. The most decrease in IFN-γ expression was observed in TNF+CM samples.

Referring to Fig. 4, results for AF cells show that although TNF expression was most reduced in the case of CM, as for NP cells, the differences between different samples were not statistically significant. In the case of AF cells, the expression of IL1-β, IL-6 and IL-17 in inflamed cells exposed to CM or EVs was significantly lower than in untreated inflamed cells or those exposed to SF, and this difference was statistically significant (*p≤0.05, **p≤0.01). No statistically significant differences were observed in IL-1α expression in AF cells under any of our experimental conditions. Conversely AF cells in the control group showed the highest level of IFN-γ expression compared to any other sample group (Fig. 4). As has been described for NP cells, AF cells exposed to TNF+CM showed the most reduced levels of IFN-γ expression, but effects were not significant

Comparing the effects of CM, EVs and SF on neurotrophin expression in inflamed AF and NP

The mRNA expression levels for 2 neurotrophins, NGF and BDNF, were analyzed using qPCR and this confirmed the presence of both in all samples. Referring to Fig. 5, expression levels of NGF exhibited similar trends in both NP and AF cells, specifically, a statistically significant increase in expression for inflamed cells exposed to CM compared to control and untreated inflamed samples. Similar but smaller increases was observed for inflamed cells treated with either EVs or SF. BDNF results showed a significant increase in NP and AF cells in TNF+CM samples. As reported in NGF results, a lower BDNF expression compared to NP and AF cells exposed to either EVs or SF was observed.

Comparing the effects of CM, EVs and SF on NF-κB translocation

The effects of TNF on inflammatory and catabolic mediators are known to be regulated by NF-κB. TNF enables the

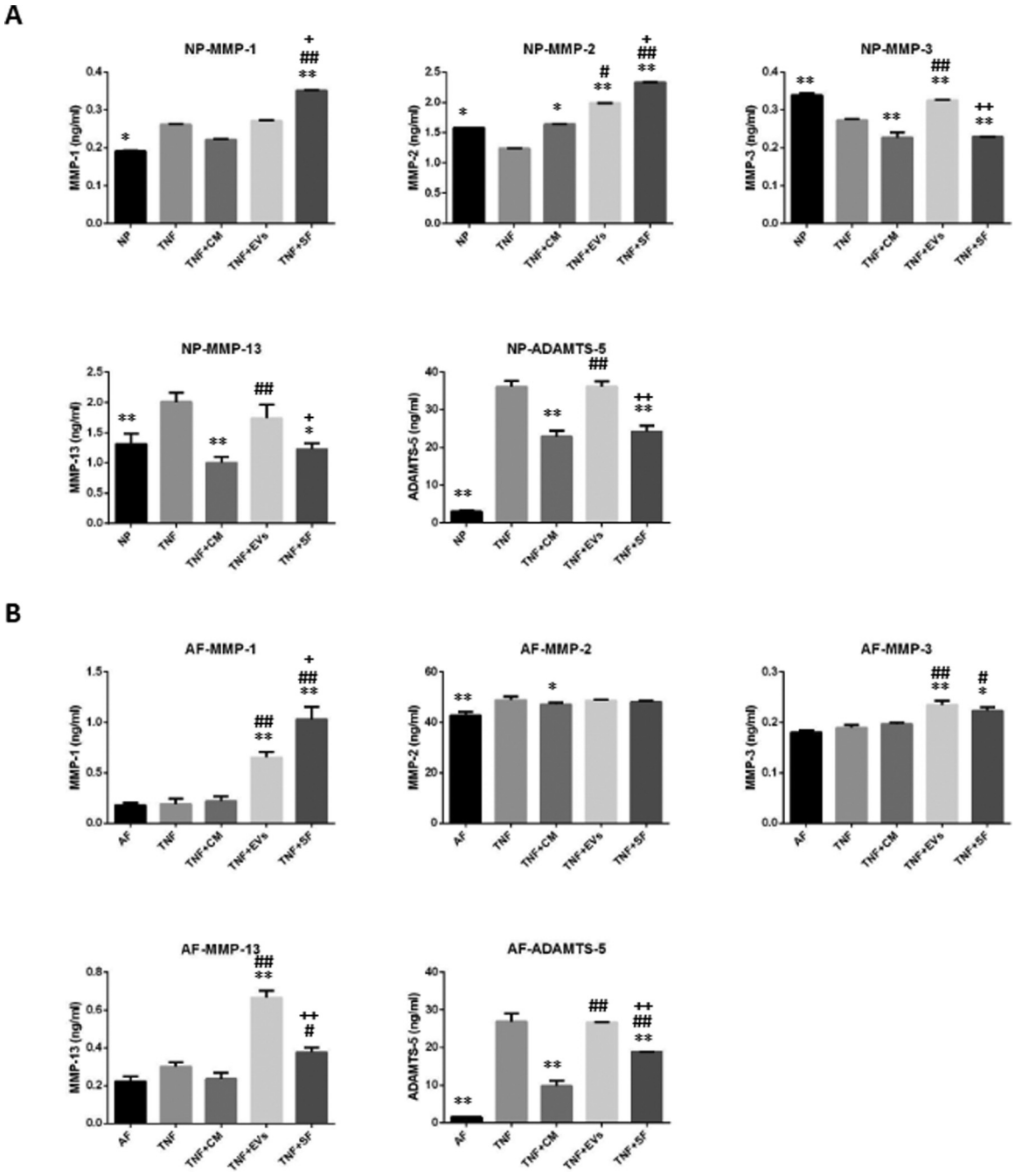

Fig. 2. MMP and ADAMTS-5 production in TNF inflamed NP (A) and AF cells (B). Control NP and AF cells compared with those exposed for 12 hrs to TNF (25 ng/ml), TNF+CM, TNF+EVs, or TNF+ SF. Culture medium was collected from samples and analyzed for protein levels of MMP-1, MMP-2, MMP-3, MMP-13 and ADAMTS-5. The results are expressed as the mean±SD of five independent experiments. * p<.05 ** p<.01 using ANOVA test comparing TNF treated samples with all other groups. # p<.05 ## p<.01 using ANOVA test comparing TNF+CM with TNF+ EVs and TNF+SF. + p<.05 ++ p<.01 using ANOVA test comparing TNF+ EVs with TNF+SF..

translocation of NF-κB into the nucleus such that it can bind to DNA and trigger gene transcription. We studied the possible suppression of NF-κB translocation in inflamed NP and AF cells due to exposure to either CM, EVs or SF Fig. 6. shows the results of the NF-κB activity assays completed under all experimental conditions. In NP and AF control samples green fluorescence, indicating the presence of NF-κB, was observed in the cytoplasm of these cells. However, in TNF inflamed NP and AF cells a marked green fluorescence was observed in the cellular nuclei (arrows). indicating TNF induced NF-κB translocation to the nucleus. Results show that in inflamed NP and AF cells exposed to

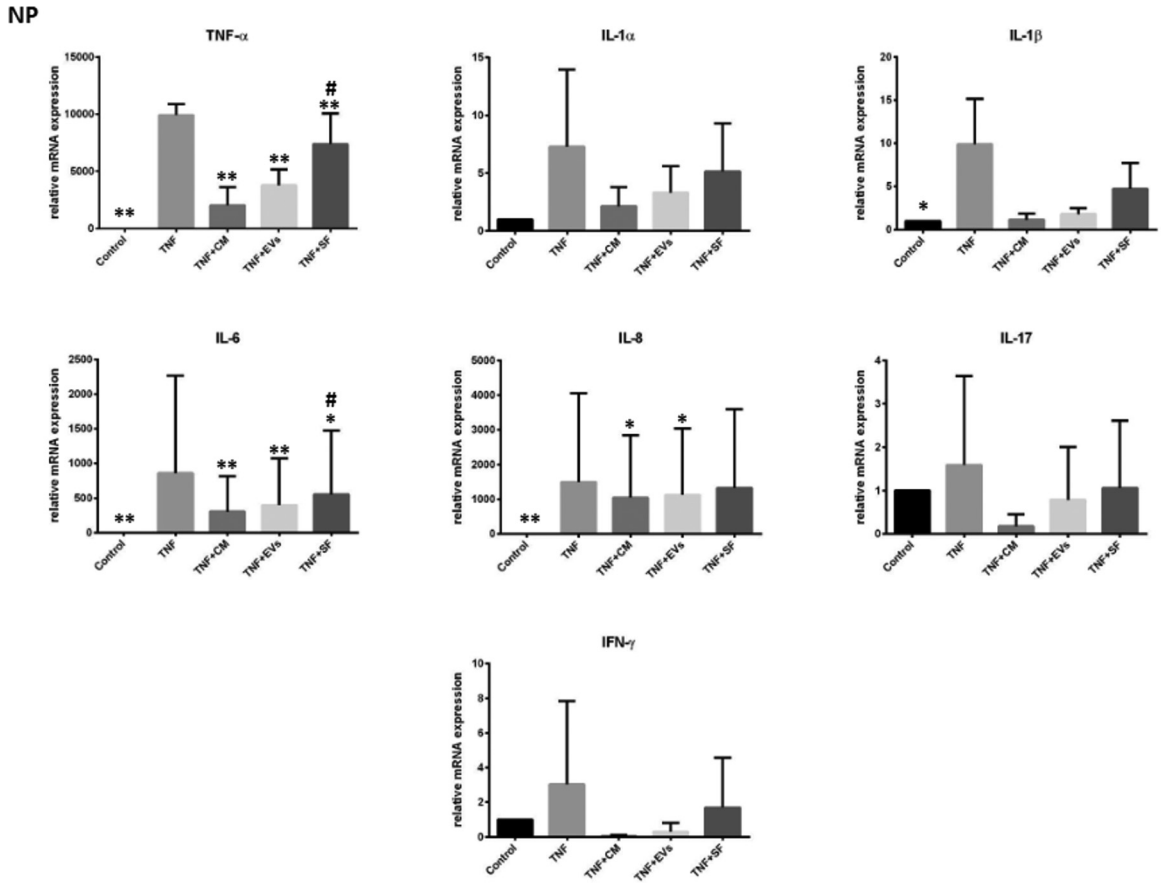

Fig. 3. NP cell gene expression analysis of TNF induced pro-inflamatory cytokines released by NP cells. Control NP cells compared with those exposed for 12 hrs to TNF (25 ng/ml), TNF+CM, TNF+EVs, or TNF+ SF. Gene expression analysis was developed using qPCR on TNF, IL-6, IL-1α, IL-1β, IL-8, IL-17 and IFN-γ genes. The results are expressed as the mean ± SD of five independent experiments. * p<.05 ** p<.01 using ANOVA test comparing TNF treated samples with all other groups. # p<.05 ## p<.01 using ANOVA test comparing TNF+CM with TNF+ EVs and TNF+SF. + p<.05 ++ p<.01 using ANOVA test comparing TNF+ EVs with TNF+SF..

CM NF-κB translocation was blocked. This blocking was also seen in inflamed NP cells exposed to EVs. In contrast, inflamed cells of either type exposed to SF demonstrated no blocking of NF-κB translocation compared to controls.

Discussion

In recent years, MSCs have been at the focus of research into therapies aimed at alleviating discogenic pain [30]. In line with several studies which have found that therapeutic effects of MSCs do not lie in the cells themselves but are principally mediated by paracrine mechanisms, especially the secretion of growth factors and EVs [38,39], we study the possible MSC-secretome modulatory effect capable of regulating pro-inflammatory cytokines and MMPs in IVD tissues [38,40].

This study was conceived with the intention of developing future clinical treatments for IVD degeneration. To this end, we investigated the effects of ASC-derived whole secretome (CM) in comparison to its isolated component parts, EVs and SF, in an *in vitro* model of IVD inflammation. The *in vitro* analysis of gene expression and protein content in TNF-inflamed NP and AF cells showed that CM gave the most promising results. In comparison to EVs and SF alone, CM produced higher overall modulatory effects on inflammation and MMP inhibition, and this was especially the case for NP cells.

It is thought that surface receptors and adhesion molecules retained from the MSC cytomembrane during EV formation are responsible for enabling the endocytosis of EVs [41,42]. We used an EV internationalization assay to confirm that the ASC-secreted EVs used in this study were able to enter both NP and AF cells.

In NP and AF cells, canonical activation of the nuclear translocation factor NF-kB by TNF [43], which is closely linked to the inflammatory cascade, plays a key role in the expression of proteases and inflammatory cytokines. In order to understand the possible mechanism by which CM might downregulate these inflammatory mediators, and explore whether there are any differences in regulatory capabilities between EVs and SF, we studied the effect of CM, EVs and SF on NF-κB translocation. Our findings suggest that the observed inhibitory effects of CM and EVs on the expression of

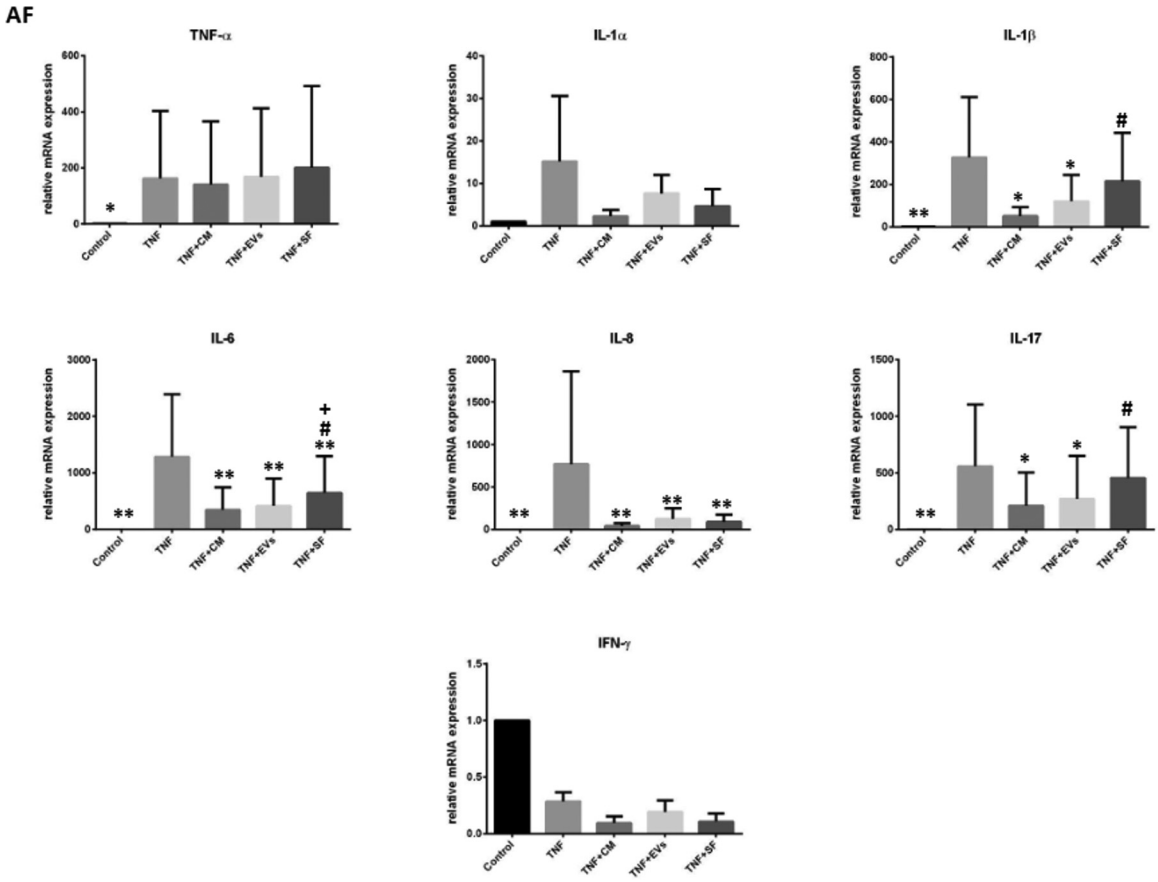

Fig. 4. AF cell gene expression analysis of TNF induced pro-inflamatory cytokines released by AF cells. Control AF cells compared with those exposed for 12 hrs to TNF (25 ng/ml), TNF+CM, TNF+EVs, or TNF+ SF. Gene expression analysis was developed using qPCR on TNF, IL-6, IL-1α, IL-1β, IL-8, IL-17 and IFN-γ genes. The results are expressed as the mean ± SD of five independent experiments. * p<.05 ** p<.01 using ANOVA test comparing TNF treated samples with all other groups. # p<.05 ## p<.01 using ANOVA test comparing TNF+CM with TNF+ EVs and TNF+SF. + p<.05 ++ p<.01 using ANOVA test comparing TNF+ EVs with TNF+SF.

catabolic and pro-inflammatory molecules could be related to their ability to reduce NF-κB translocation in TNF-inflamed NP cells. In AF cells the inhibition NF-κB translocation was observed only when CM was added. This implies that the anti-catabolic effects of the CM are mediated principally by the EV fraction of the secretome rather than SF [44]. This result is in line with recent studies that demonstrated the anti-inflammatory effects of MSC-derived EVs in different pathologies [34,45]. Indeed, EVs have been shown to inhibit metalloproteases and inflammatory mediators such as TNF, iNOS, IL-1, IL-6 or NFκB [46,47].

Interestingly, both gene expression evidence and the analysis of the protein content between our 3 experimental conditions suggest that whole CM had a higher anti-inflammatory potential than isolated EVs or SF. Specifically, reduction in pro-inflammatory cytokines and MMP enzymes was found, in general, to be greatest for inflamed cells exposed to CM. This result suggests that there is a further factor at work. A possible explanation might be found in the work of Giannasi et al [26]., who demonstrated that MMP activity was neutralized as a direct result of active TIMPs present in ASC secretome [48,49]. Furthermore, compared to the EV fraction, the whole secretome was found to be enriched in TIMP inhibitors [26]. In addition, results showed that CM was more effective than isolated EVs and SF in reducing ADAMTS-5 expression in inflamed NP and AF cells.

Our study has shown that the whole secretome increases modulation of pro-inflammatory and catabolic factors in NP and AF cells and therefore provides protection against inflammation. Thus, CM represents a valuable potential therapy to mitigate against IVD degeneration. Although CM usually produces a decrease in MMPs production, variable effects have been observed on the response of MMP-2 and MMP-3. This effect may be due to the fact that MMPs either shed or cleave proteins, thereby influencing the substrates' activity, localization and function [50]. Importantly, MMPs can have more than 1 substrate, providing 1 explanation why multiple distinct injury and cell-dependent

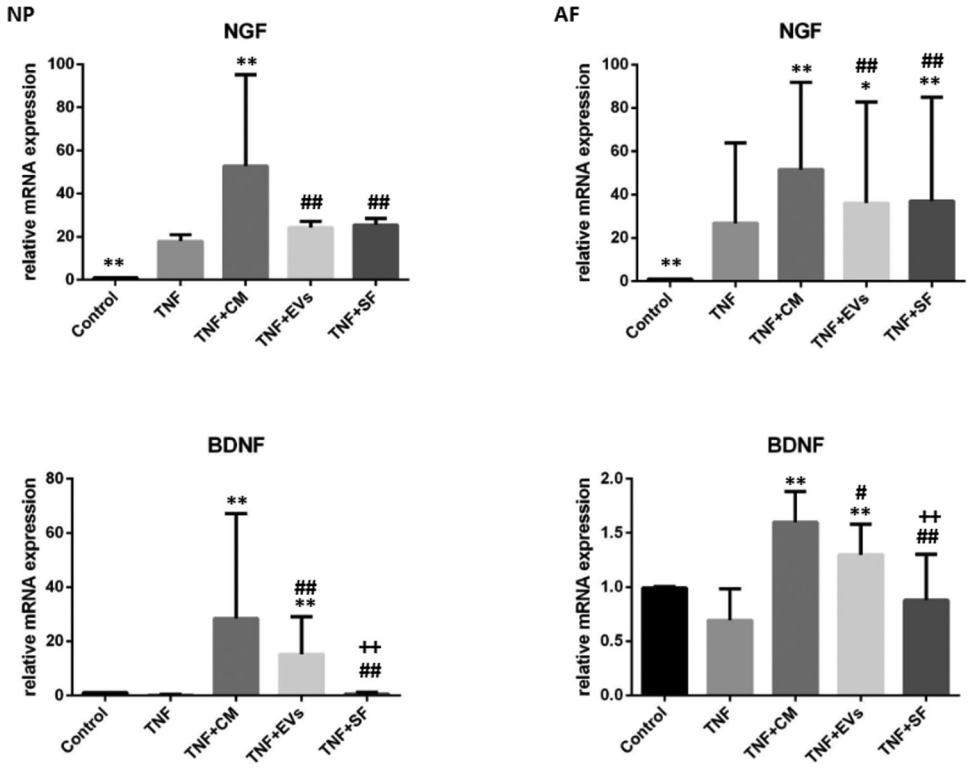

Fig. 5. Gene expression analysis of neurotrophins: comparing NP and AF cells. Control NP and AF cells compared with those exposed for 12 hrs to TNF (25 ng/ml), TNF+CM, TNF+EVs, or TNF+ SF. Gene expression of NGF and BDNF was analyzed using qPCR. The results are expressed as the mean ± SD of five independent experiments. * p<.05 ** p<.01 using ANOVA test comparing TNF treated samples with all other groups. # p<.05 ## p<.01 using ANOVA test comparing TNF+CM with TNF+ EVs and TNF+SF. + p<.05 ++ p<.01 using ANOVA test comparing TNF+ EVs with TNF+SF..

inflammatory phenotypes can be observed. Identification of MMP substrates techniques have shown that the hemopexin domain of MMP-2 binds to monocyte chemoattractant protein-3 (MCP-3), leading to its cleavage and converting it from a receptor agonist to a potent antagonist [51].

Numerous studies have suggested that neurotrophic factors are present in the degenerated intervertebral disc [52,53], and isolated IVD cells can actively secrete these bioactive mediators in culture [54,55]. The present study also showed that CM increased production of 2 neurotrophic factors, NGF and BDNF, in both NP and AF cells and although these results were not significant, they are in line with other studies. For instance, there are reports showing a low affinity of the TNF receptor superfamily for NGF [56] and recently, Martins et al [57]. observed that CM induced axonal outgrowth and confirmed that this effect was mediated by BDNF. In addition, other studies have reported nerve growth at sites of degenerated connective tissue repair [58] and there are descriptions of neurotrophin production in early stages of fracture repair in bone tissue [59]. Our results agree with these studies suggesting that the increase in neurotrophins production in NP and AF cells by CM could be due to a regenerative process in these cells.

MSC-secretome therapy is developing as a potential treatment option for a variety of disorders, particularly those with an inflammatory component [60]. CM has been shown to be able to regenerate the IVD in some animal models [61,62]. In certain ways, secretome therapeutics might be superior to cell-based therapy in terms of safety, production, storage, product shelf life, and potential as a quickly available biological therapeutic agent. However, clinical trials including the use of MSC-secretome to restore the damaged disc are currently lacking [63]. In order to improve their therapeutic application, essential technological factors and the evaluation of probable adverse effects must be addressed [64,65].

Intravenous injection, local injection, intranasal delivery, and other methods of secretome administration are the most commonly used. The secretome's biodistribution and half-life in the human body need to be researched further. Currently, researchers have proposed a number of optimization approaches for MSC-secretome synthesis and purification as a medical delivery system, however the majority of these methods lack standardization and quality control [66].

The limitations of the present study include the proinflammatory degenerative microenvironment of the disc, which cannot be totally mimicked, and the short experimental time of the study, which makes simulation of a therapy in a long-term perspective very difficult. Moreover, AF and NP cells behave differently to inflammatory stimuli with

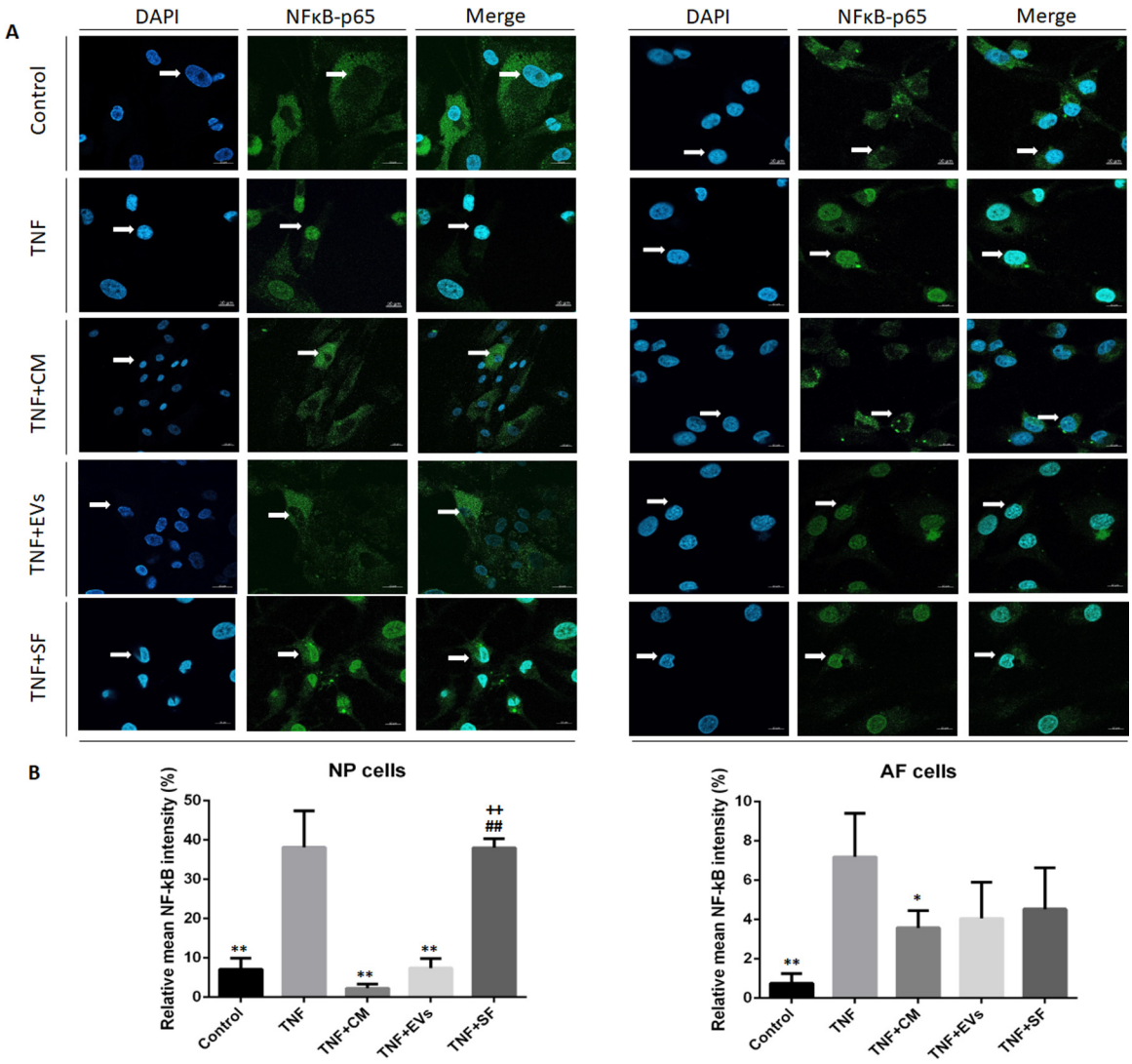

Fig. 6. Comparing the effects of CM, EVs and SF TNF-induced nuclear translocation of NF-κB p65 in NP (left panel) and AF cells (right panel). (A) Control NP and AF cells compared with those exposed for 12 hrs to TNF (25 ng/ml), TNF+CM, TNF+EVs or TNF+ SF. Localization of NF-κB p65 was visualized using confocal microscopy after immunofluorescence staining with NF-κB p65 antibody (green). Nuclei (white arrows) were stained with DAPI (blue). A representative sample of three independent experiments is shown. Scale bar: 10 μm. (B) A comparison of the green fluorescence in the cell nuclei in each experimental condition. The results are expressed as the mean ± SD of three independent experiments. * p<.05 ** p<.01 using ANOVA test comparing TNF treated samples with all other groups. # p<.05 ## p<.01 using ANOVA test comparing TNF+CM with TNF+ EVs and TNF+SF. + p<.05 ++ p<.01 using ANOVA test comparing TNF+ EVs with TNF+SF.

TNF. Additionally, the communication between secretome and cells of the immune system should be researched in an *in vivo* model.

Conclusions

Overall, our results suggest that the EVs and SF derived from CM promote *in vitro* anti-inflammatory modulation in IVD cells in a highly synergistic way. This is supported by findings displaying that CM contains a greater variety of bioactive factors, soluble, freely dissolved proteins, nucleic acids, and lipids [40] than either of its isolated fractions. Our findings also highlight that the use of whole secretome, rather than isolated EVs or SF, should be considered the more promising therapeutic strategy for IVD degeneration. The use of whole secretome also presents significant advantages over the use of EVs as it is easier, faster, and cheaper to produce. Further studies are needed, however, and these should focus on confirming the efficacy of CM as a treatment for IVD degeneration and discogenic pain.

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