



Beneficial effects of secretome derived from mesenchymal stem cells with stigmasterol to negate IL-1 β -induced inflammation in-vitro using rat chondrocytes—OA management

Samuel Joshua Pragasam Sampath¹ · Subha Narayan Rath² · Nagasuryaprasad Kotikalapudi¹ · Vijayalakshmi Venkatesan¹

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Abstract

Osteoarthritis (OA) is the most prevalent joint disease predominantly characterized by inflammation which drives cartilage destruction. Mesenchymal stem cells-condition medium (MSC-CM) or the secretome is enriched with bioactive factors and possesses anti-inflammatory and regenerative effects. The present study aimed at evaluating the effects of combining MSC-conditioned medium with stigmasterol compared with the individual treatments in alleviating interleukin-1 beta (IL-1 β)-induced inflammation in rat chondrocytes. Stigmasterol is a phytosterol exhibiting anti-inflammatory effects. IL-1 β (10 ng/ml) was used to induce inflammation and mimic OA *in-vitro* in primary rat articular chondrocytes. The IL-1 β -stimulated chondrocytes were treated with MSC-CM, stigmasterol, and a combination of MSC-CM and stigmasterol for 24 h. Cell viability was measured using MTT assay. Protein expression of inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), collagen II (COL2A1) and matrix metalloproteinase (MMP)-13 were evaluated by immunofluorescence. Gene expression levels of MMP-3, MMP-13 and A Disintegrin-like and Metalloproteinases with Thrombospondin Motifs (ADAMTS)-5 were measured using qRT-PCR. NF- κ B signaling pathway was studied using western blotting. A significant reduction in the expression of iNOS, IL-6, MMP-3, MMP-13 and ADAMTS-5, and a significant increase in COL2A1 expression was observed in the rat chondrocytes across all the treatment groups. However, the combination treatment of MSC-CM and stigmasterol remarkably reversed the IL-1 β -induced pro-inflammatory/pro-catabolic responses to near normal levels comparable to the control group. The combination treatment (MSC-CM + stigmasterol) elicited a superior anti-inflammatory/anti-catabolic effect by inhibiting the IL-1 β -induced NF- κ B activation evidenced by the negligible phosphorylation of p65 and I κ B α subunits, thereby emphasizing the benefit of the combination therapy over the individual treatments.

Keywords Osteoarthritis · MSC conditioned medium · Stigmasterol · Chondrocytes · Inflammation

Introduction

Osteoarthritis (OA) is the common joint disorder primarily affecting the diarthrodial joints and advances into severe degenerative arthritis. OA forms the leading cause

of disability and joint pain impacting the socio-economic status across the globe with a prevalence of 16% (Cui et al. 2020). With a multifactorial etiology including age, obesity, trauma, sports injuries and altered biomechanics, OA progresses as an irreversible, often heterogeneous disease commonly ending up with complete joint destruction (Chen et al. 2017). Inflammation triggered by mechanical trauma leading to initial cartilage breakdown has been identified to be a critical determinant in initiating and accelerating OA development (van der Kraan and van den Berg 2012). Evidence(s) suggest for a prominent role for IL-1 β , a key early-stage pro-inflammatory cytokine, in exacerbating the pathogenesis of OA by activating key signaling pathways including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), mitogen-activated protein kinases

✉ Vijayalakshmi Venkatesan
v.venkateshan@gmail.com

¹ Stem Cell Research Laboratory, Department of Cell and Molecular Biology, National Institute of Nutrition, Indian Council of Medical Research, Tarnaka, Hyderabad 500007, Telangana, India

² Regenerative Medicine and Stem Cells Laboratory, Department of Biomedical Engineering, Indian Institute of Technology Hyderabad, Kandi 502285, Telangana, India

(MAPK), and transcription factors (Daheshia and Yao 2008). Consequently, this triggers a sequelae of intracellular events that culminate in the activation of cartilage-degrading proteinases (MMPs and ADAMTS), production of inflammatory molecules (iNOS), pro-inflammatory cytokines (IL-6), causing a decrease in the synthesis of cartilage extracellular matrix ultimately leading to cartilage degradation and joint damage (Jenei-Lanzl et al. 2019; Kobayashi et al. 2005). Till now there is no single therapeutic agent that has been deemed safe and effective for treating OA. Strategies aimed at targeting the IL-1 β -induced catabolic metabolism and inflammatory responses have proven to offer immense promise(s) in curtailing OA progression (Ding et al. 2020).

In recent years, advances in regenerative medicine/stem cell research have led to promising stem cell-based therapies for cartilage repair in OA. Notably, mesenchymal stem cells (MSCs) have emerged as attractive candidates for application in laboratories and clinical studies for cartilage repair/regeneration in OA owing to their self-renewal, multipotent, anti-inflammatory and immunomodulatory functions (Jo et al. 2014; Lee et al. 2019a; Satué et al. 2019; Song et al. 2018; Xing et al. 2020). The MSCs are isolated from a variety of tissues such as bone marrow, adipose tissue, fetal liver, umbilical cord, muscle, endometrial polyps, dental tissue, synovial fluid, skin, foreskin, Wharton's jelly, placenta, dental pulp, breast milk, gingiva, amnion and menstrual blood, and characterized as plastic adherent cells with fibroblastic morphology in culture (Kangari et al. 2020). MSCs have been demonstrated to offer their therapeutic potential towards regenerative medicine and tissue repair by their intrinsic potential to migrate and home into the damaged tissue, differentiate and integrate into the cells of the host tissue (Fan et al. 2020). However, in recent times, there is increasing evidence to suggest that the efficacy of many MSC-based therapies could be attributed to their paracrine secretion of a broad spectrum of lipids, proteins, nucleic acid, and biologically active trophic factors which include anti-apoptotic factors, growth factors, anti-inflammatory cytokines, chemokines and extracellular vesicles generally referred to as the secretome or the MSC-conditioned medium (MSC-CM) (Ahangar et al. 2020; Chen et al. 2008; Kim et al. 2008; Walter et al. 2010; Zhou et al. 2013). Regarding the composition, the secretome has been demonstrated to contain an array of growth/differentiation factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor, insulin-like growth factor I and II (IGF-I, IGF-II), hepatocyte growth factor (HGF), fibroblast growth factor 2/basic fibroblast growth factor (FGF-2/bFGF), keratinocyte growth factor/fibroblast growth factor-7 (KGF/FGF-7), platelet-derived endothelial cell growth factor, heparin-binding epidermal growth factor, neural growth factor (NGF), and brain-derived neurotrophic factor (BDNF), in

addition to anti-inflammatory cytokines such as transforming growth factor- (TGF) β 1 and interleukins (IL), including IL-6, IL-10, IL-27, IL-17, and IL-13 (El Moshly et al. 2020). The secretome/MSC-CM can modulate the injured tissue environment and orchestrate subsequent regenerative processes including cell migration, proliferation, differentiation, and matrix synthesis (González-González et al. 2020). Findings from a large number of experimental studies have also shown that MSC-derived secretome demonstrated similar therapeutic effects to those observed after transplantation of MSCs whilst circumventing the side effects of MSC-based therapy, such as unwanted differentiation of engrafted MSCs (Harrell et al. 2019). The MSC secretome offers distinctive biological and logistical advantages over the transplanted MSCs in tissue engineering/regenerative medicine including the ease of economical mass production from commercial cell lines under controlled laboratory conditions, better storage without toxic cryopreservative agents for longer periods without loss of potency, immediate availability for practical application and content evaluation similar to pharmaceutical agents (Vizoso et al. 2017). MSC-CM can also be manipulated, stored and characterized more easily than cells and sterilization is possible without loss of efficacy (Benavides-Castellanos et al. 2020). Accruing evidence(s) from several studies have also demonstrated the therapeutic effects of MSC-CM/secretome in treating many diseases (Sagaradze et al. 2019).

Nevertheless, limitations in clinical stratification of OA patients as well as the choice of available therapeutics have opened up newer avenues to look out for combination therapies which could offer a newer paradigm of treatment to realize better treatment outcomes. Several pre-clinical and clinical studies have documented credible evidence(s) for the synergistic effects of combination therapies resulting in enhanced treatment outcomes with better tolerability and less adverse side effects especially in the case of musculoskeletal disorders (Haleagrahara et al. 2018; Kaur et al. 2011; Lee et al. 2019b; Usha and Naidu 2004; Yu et al. 2018). Indeed the impetus obtained from our earlier study re-instates the efficacy of combination line of approach in OA management (Pragasam et al. 2021). Phytosterols or plant sterols are a group of naturally occurring plant compounds that have been shown to elicit immense health benefits across a wide range of disease conditions. Stigmasterol, also known as anti-stiffness factor, is the most common phytosterol found in several nuts, seeds, legumes, banana, cabbage, and medicinal herbs including *Akebia quinata*, *Gypsophila oldhamiana*, *Emilia sonchifolia*, *Eucalyptus globules*, *Aralia cordata*, *Emilia sonchifolia*, *Theobroma cacao L* (Yadav et al. 2018). In addition to its proven anti-osteoarthritic effects (Chen et al. 2012; Gabay et al. 2010), stigmasterol possesses anti-nociceptive (Walker et al. 2017), anti-tumor (Ghosh et al. 2011), anti-psychotic (Yadav et al.

2018), anti-oxidant (Panda et al. 2009), anti-inflammatory (García et al. 1999), anti-diabetic (Ramu et al. 2016), anti-allergic (Antwi et al. 2018), chemopreventive (Sofi et al. 2018) and neuroprotective effects (Haque et al. 2021). Very recently, we demonstrated that intra-articular transplantation of mesenchymal stem cells combined with stigmasterol resulted in better cartilage repair/regeneration compared to the individual treatments in a monosodium-iodoacetate induced rat model of OA (Pragasam et al. 2021). Considering the benefits of MSC-secretome in regenerative therapy, we anticipate that the MSC-derived secretome could represent a newer, cell-free MSC-based therapeutic approach for the attenuation of pro-inflammatory/catabolic responses in OA. Hence, in the present study, we aimed to assess the potential of combining rat bone marrow mesenchymal stem cells-derived conditioned medium (MSC-CM) with stigmasterol in ameliorating the IL-1 β -induced inflammatory responses in rat primary chondrocytes. We hypothesize that combining MSC-CM with stigmasterol would elicit a superior anti-inflammatory/anti-catabolic effect compared to the individual treatments *in-vitro*.

Materials and methods

Animals

The animal study was approved by the Institutional Animal Ethical Committee, National Institute of Nutrition (NIN), Hyderabad (P29F/III-IAEC/NIN/12/2016/SSJ/WNIN(CG)-6F/WNIN-Gr-Ob-42F). Four-week-old female Wistar rats were obtained from the Animal Facility, NIN and were housed in standard polypropylene cages, maintained at 22 ± 1 °C with 12-h dark/light cycles, and humidity of 50–60%, and were fed standard laboratory rat chow prepared at our animal facility with free access to water. All the experiments were performed in accordance with the regulations and guidelines of the *Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)*.

Isolation of rat primary chondrocytes

Primary articular chondrocytes were isolated from four-week-old WNIN Wistar rats according to a previously published method (Oseni et al. 2013) with slight modifications (Pragasam and Venkatesan 2020). Briefly, the rats were euthanized by CO₂ asphyxiation and the hind limbs were collected in sterile phosphate buffered saline (PBS, pH 7.4). The femur and tibia were separated from the hind limbs under sterile conditions. The cartilage at the ends of the femur and tibia were harvested using sterile scalpels, washed in sterile PBS, digested in 0.15% collagenase-II for 4 h at 37 °C, 5% CO₂, followed by the addition of culture

medium (Dulbecco's Modified Eagle Medium/Ham's F12 (1:1) (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), P/S (penicillin (100 IU/ml) and streptomycin (100 IU/ml)). The digested cell suspension was then centrifuged at 300xg for 10 min using a Sigma 3-18KS centrifuge. The cell pellet was washed twice in culture medium and seeded in a T25 culture flask at a density of 5×10^3 cells/cm². The culture medium was changed every 2 days and upon reaching confluency, the cells were trypsinized using 0.25% trypsin-EDTA and sub-cultured subsequently. The cells at passage 2 were used for all the further experiments.

Isolation of rat bone marrow mesenchymal stem cells (BM-MSCs)

Post euthanization by CO₂ asphyxiation, the femur bones were harvested from four-week-old female WNIN Wistar rats under aseptic conditions in sterile PBS containing antibiotics. The BM-MSCs were isolated according to an earlier published protocol (Madhira et al. 2012). Briefly, the femurs were cut open at the metaphyseal ends and the bone marrow was flushed out using a 2-ml syringe containing DMEM/F12 containing 10% FBS and P/S. The flushed out bone marrow was adequately dispensed to get a uniform cell suspension which was washed thrice at 1800 rpm for 10 min. The resulting cell pellet was resuspended in the cell culture medium (DMEM/F12 containing 10% FBS and P/S) and seeded in T25 flasks at a seeding density of 5×10^3 cells/cm². 48–72 h upon seeding, spindle-shaped adherent cells were observed and the non-adherent cells were removed during subsequent media change which was done every 2 days. The adherent cells formed a monolayer and upon reaching confluency, the cells were trypsinized using 0.25% trypsin-EDTA. The BM-MSCs at passage 3 to 5 were used for the subsequent experiments.

Characterization of rat BM-MSCs

BM-MSCs were characterized for their multi-lineage differentiation potential and phenotypic markers. The BM-MSCs were differentiated to chondrogenic, osteogenic and adipogenic lineages using commercially available kits (Gibco, Life Technologies, USA) as per the manufacturer's instructions. For chondrogenic and osteogenic differentiation, BM-MSCs (P3) were grown in 35-mm culture dishes in DMEM/F12 containing 10% FBS and P/S. When the cells reached 80% confluency, the culture medium was replaced with the chondrogenic and osteogenic differentiation media (basal medium with supplement) for 14 days and 21 days, respectively, with the medium being changed every 2 days. At the end of 14 days, the BM-MSCs differentiating into the chondrogenic lineage were washed with PBS, fixed with 4% paraformaldehyde and stained with 1% Alcian Blue solution

prepared in 0.1 N HCL for 30 min. The cells were rinsed with 0.1 N HCL, distilled water was added to neutralize the acidity, visualized under a light microscope and the images were captured. At the end of 21 days, the BM-MSCs differentiating into the osteogenic lineage were rinsed with PBS, fixed with 4% paraformaldehyde and stained with 2% Alizarin Red solution for 2–3 min. The cells were rinsed with distilled water, visualized under a light microscope and the images were captured. For adipogenic differentiation, monolayer cultures of BM-MSCs were grown in culture medium in 35-mm dishes until 80% confluency and replaced with adipogenic differentiation medium for 10 days with the medium being changed every 2 days. At the end of 10 days, the cells were rinsed with DPBS and fixed with 4% paraformaldehyde. The cells were rinsed with PBS and stained with Oil-Red-O stain, washed with PBS, visualized under a light microscope and images were captured. The BM-MSCs were also characterized for their expression of MSC specific markers CD29, CD73, CD90 and CD106 by immunofluorescence. Briefly, the BM-MSCs (P4) grown on coverslips in six-well culture dishes were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with 50% chilled methanol, serum-blocked with 4% horse serum and incubated overnight at 4 °C with primary antibodies specific to CD29 (1:100, BD Biosciences), CD73 (1:100, BD Biosciences), CD90 (1:100, BD Biosciences) and CD106 (1:100, BD Biosciences). The cells were washed with PBS, incubated with a fluorescent labeled (Cy3) secondary antibody (1:200 dilution) (Jackson's Laboratories, USA) for 1 h at room temperature, washed with PBS and mounted using DAPI (Vectashield, Vector Laboratories, USA). All images were captured using Leica Advanced Fluorescence software in a Leica TCS SP5 Confocal Microscope (Mannheim, Germany).

Preparation of MSC-CM or secretome

MSC-CM was obtained from the cultured rat BM-MSCs as per the published method (Kay et al. 2017). Briefly, the BM-MSCs were seeded in T75 flasks at a density of 1.5×10^6 cells/flask in DMEM/F12 containing 10% FBS and P/S. Upon reaching 80–90% confluency, the cells were washed with PBS and added with serum free DMEM/F12 and maintained at 37 °C, 5% CO₂. The flasks were incubated for 48 h after which the medium was removed and centrifuged at 1500 rpm for 5 min at 4 °C to remove any cell debris. The resulting supernatant was termed the MSC-CM and used for the subsequent experiments.

Cell viability assay

The effect of stigmaterol on cell viability in the isolated rat primary chondrocytes was measured using the MTT assay. The chondrocytes were seeded at a density of 5×10^3 cells/well in a 96-well plate. After allowing the cells to adhere for 24 h, the cells were treated with different concentrations of stigmaterol (0–100 μM) in culture medium. After 24 h of the treatment, the media were removed, and the cells were incubated with MTT solution (5 mg/ml) for 4 h at 37 °C. The supernatant was removed and the formazan crystals were dissolved by adding DMSO to the wells. The absorbance was measured at 570 nm using a microplate reader (BioTek, US). The number of viable cells in the treatment groups was expressed as a percentage of the number in the control group. The chondrocytes without any stigmaterol treatment (0 μM) were considered as the control group.

Experimental approach

The chondrocytes were grown in six-well plates at a seeding density of 2×10^5 cells/well and cultured in DMEM/F12 containing 10% FBS and P/S until confluency. The study comprised of the following five groups: (1) chondrocytes maintained in DMEM/F12 medium for 24 h (Control), (2) chondrocytes treated with IL-1β (10 ng/ml) for 24 h (IL-1β), (3) chondrocytes treated with IL-1β (10 ng/ml) + MSC-CM for 24 h (IL-1β + CM), (4) chondrocytes treated with IL-1β (10 ng/ml) + 50 μM stigmaterol in plain medium for 24 h (IL-1β + S), (5) chondrocytes treated with IL-1β (10 ng/ml) + MSC-CM + 50 μM stigmaterol for 24 h (IL-1β + CM + S). The final volume across the different groups was kept constant at 2 ml. All the experiments were carried out under identical conditions and the experiment was designed according to a previous study by Huang et al. (2018) with the treatments given for 24 h.

Immunofluorescence

At the end of the experimental period (24 h), the chondrocytes grown on coverslips (all five groups) were washed with PBS and fixed with 4% paraformaldehyde. The cells were rinsed with PBS again and permeabilized with 50% chilled methanol, serum-blocked with 4% horse serum and incubated overnight at 4 °C with primary antibodies specific to collagen II alpha 1 (COL2A1) (1:25; Developmental Studies Hybridoma Bank, IA USA), inducible nitric oxide synthase (iNOS) (1:100, Abcam, MA, USA), Matrix

metalloproteinase (MMP-13) (1:100; Abcam, MA, USA) and interleukin-6 (IL-6) (1:100, Invitrogen, MA, USA). The cells were washed with PBS, incubated with a Cy-3-labeled secondary antibody (1:200 dilution) (Jackson Laboratories, USA) for 1 h at room temperature, washed with PBS and mounted using DAPI (Vectashield, Vector Laboratories, USA). All images were captured using Leica Advanced Fluorescence software in a Leica TCS SP5 Confocal Microscope (Mannheim, Germany). The fluorescence intensities were calculated as relative fluorescent units (RFU) using the LAF software and represented as RFU per unit area. Values are represented as mean \pm SD from three independent experiments performed in duplicate.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

qRT-PCR analysis was carried out to quantify the gene expression levels of OA-specific markers: MMP-3, MMP-13 and ADAMTS-5 in the treated chondrocytes. Total RNA was isolated from the chondrocytes cultured in six-well plates using TRIzol reagent according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 1 μ g of total RNA using the OneScript cDNA Synthesis kit (Applied Biological Materials, Canada). qRT-PCR was performed using a TB Green *Premix Ex Taq* II real-time PCR kit (Takara Bio, CA, USA) employing an Applied Biosystems StepOnePlus real-time PCR system (ThermoFisher Scientific, MA, USA). The level of target mRNA was normalized to the level of GAPDH and compared with control. Data were analyzed using the $2^{-\Delta\Delta CT}$ method. The primer sequences of the target genes used for the qRT-PCR were designed using the NCBI Primer-Blast tool and are listed as follows: MMP-3 (F): 5'-AATCCCTGATGTCCTCGTGGTA-3', (R): 5'-GGTCCTGAGAGATTTTCGCCAA-3'; MMP-13 (F): 5'-TCGCATTGTGAGAGTCATGCCAACA-3', (R): 5'-TGTGGTT-CCAGCCACGCATAGTCA-3'; ADAMTS-5 (F): 5'-GGGGTCAGTGTTCTCGCTCTTG-3', (R): 5'-GCCGTTAGGTGGGCAGGGTAT-3'.

Western Blot analysis

The total protein was extracted from the chondrocytes cultured in six-well plates using ice-cold radio immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors. The lysates were sonicated, kept on ice for 10 min followed by centrifugation at 12,000 rpm for 15 min at 4 °C. The protein concentration in the supernatants was

measured using the bicinchoninic acid (BCA) protein assay kit (G-Biosciences, MO, USA). Equal amounts of protein (40 μ g) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, USA). After blocking with 5% nonfat milk for 2 h, the membranes were incubated with the primary antibodies against NF- κ B p65 (1:1000, Cell Signaling Technology), phospho-NF- κ B p65 (1:1000, Cell Signaling Technology), I κ B α (1:1000, Novus Biologicals), phospho-I κ B α (1:1000, Novus Biologicals) and β -actin (1:1000, Cell Signaling Technology) overnight at 4 °C with gentle rocking. The membranes were washed with TBST and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000, Southern Biotech) at room temperature for 1 h. After washing with TBST, the membranes were visualized with an enhanced chemiluminescence reagent (G-Biosciences, MO, USA) and the images were captured using an iBrightFL1500 Imaging System (ThermoFisher Scientific, USA).

Statistical analysis

The values given represent average of three independent experiments, carried out in duplicates. All data have been expressed as mean \pm standard deviation (SD). Significant difference between the groups was measured using one-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism 8.0.2 software. $p < 0.05$ implied significance.

Results

Effect of stigmasterol on cell viability

The rat primary articular chondrocytes were isolated and cultured in DMEM/F12 containing 10% FBS and P/S. The cultured chondrocytes were identified by their typical polygonal shape when viewed under a bright field inverted microscope (Fig. 1A). The effect of stigmasterol on the cell viability/cytotoxicity of the cultured rat articular chondrocytes was evaluated by the MTT assay. The cells were treated with stigmasterol at varying concentrations ranging from 0 to 100 μ M for 24 h. There was no significant cytotoxicity in the chondrocytes upon treatment with stigmasterol across all the tested concentrations (Fig. 1B). Stigmasterol at a concentration of 50 μ M was used for all the further experiments of the study. This dosage was selected also considering a previous study by Gabay et al. (2010) Fig. 1.

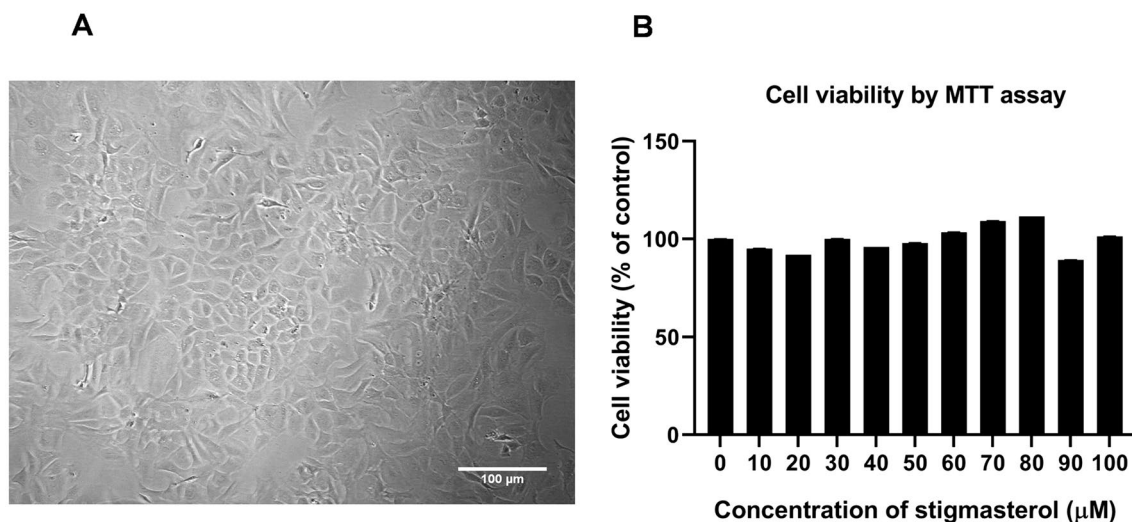


Fig. 1 **A** Bright field micrograph of rat articular chondrocytes. **B** Cell viability of the rat chondrocytes treated with stigmasterol (0–100 μM) for 24 h measured using MTT assay. No significant change in cell viability was observed

Characterization of rat BM-MSCs

The cultured rat BM-MSCs were identified by their physical adherence to the plastic cell culture dish and their typical fibroblast-like spindle shaped morphology when viewed under the microscope (Fig. 2A). The ability of the isolated BM-MSCs to differentiate into the chondrogenic, osteogenic and adipogenic lineages was assessed using commercially available kits following the manufacturer's instructions. The BM-MSCs were subjected to chondrogenic differentiation by maintaining the cells in chondrogenic differentiation medium for 14 days which was identified by the Alcian Blue staining for proteoglycans (Fig. 2B). The osteogenic differentiation potential of the BM-MSCs was carried out by maintaining the BM-MSCs in the osteogenic differentiation medium for 21 days and was confirmed by the Alizarin Red staining for calcium phosphate deposits (Fig. 2C). The adipogenic differentiation potential of the BM-MSCs was performed by maintaining the BM-MSCs in the adipogenic differentiation medium for 10 days and confirmed by the Oil-Red-O staining of the lipid droplets (Fig. 2D). The BM-MSCs (P4) grown on coverslips were paraformaldehyde fixed and processed for the expression of MSC-specific markers assessed by immunofluorescence. The BM-MSCs stained positive for the expression of MSC-specific markers CD29 (Fig. 2E), CD73 (Fig. 2F), CD90 (Fig. 2G) and CD106 (Fig. 2H) as confirmed by immunofluorescence using a Leica SP5 confocal laser scanning microscope using the LAS software Fig. 2.

Effects of MSC-CM, stigmasterol, and combination of MSC-CM and stigmasterol on the expression of iNOS, IL-6, MMP-13 and COL2A1 in IL-1 β stimulated rat chondrocytes by immunofluorescence

IL-1 β is the most critical pro-inflammatory cytokine implicated in OA pathogenesis which upregulates several inflammatory/catabolic mediators involved in cartilage degradation. The effects of MSC-CM and stigmasterol alone or in combination on the expression of key OA-related inflammatory markers such as iNOS (Fig. 3), IL-6 (Fig. 4) and MMP-13 (Fig. 5), and cartilage turnover marker COL2A1 (Fig. 6) in the rat chondrocytes upon were evaluated by immunofluorescence using confocal microscopy. The IL-1 β stimulated chondrocytes showed a significant increase in the expression of iNOS, IL-6 and MMP-13 and a significant reduction in the expression of COL2A1 in the rat chondrocytes compared to the control. The IL-1 β -stimulated chondrocytes treated with the MSC-CM (IL-1 β +CM) or stigmasterol (IL-1 β +S) alone resulted in a significant reduction in the expression of iNOS, IL-6 and MMP-13 and a significant increase in the expression of COL2A1 when compared to the IL-1 β -treated groups. However, the combination treatment of MSC-CM and stigmasterol (IL-1 β +CM+S) to IL-1 β stimulated chondrocytes significantly reversed the levels of iNOS (Fig. 3), IL-6 (Fig. 4), MMP-13 (Fig. 5) and COL2A1 (Fig. 6) expression to near normal levels as comparable to the control group indicative of the beneficial effect of the combination therapy over the individual treatments Figs. 3, 4, 5 and 6.

Effects of MSC-CM, stigmasterol, and combination of MSC-CM and stigmasterol on mRNA expression levels of MMP-3, MMP-13 and ADAMTS-5 in IL-1 β stimulated rat chondrocytes by qRT-PCR

The mRNA expression levels of MMP-3, MMP-13 and ADAMTS-5 genes in IL- β -stimulated rat chondrocytes treated with MSC-CM and/or stigmasterol were evaluated using qRT-PCR. As depicted in Fig. 7, IL- β stimulation in chondrocytes (IL- β group) resulted in a significant increase in the mRNA expression levels specific for MMP-3 (Fig. 7A), MMP-13 (Fig. 7B) and ADAMTS-5 (Fig. 7C). Treatments with either MSC-CM (IL- β + CM) or stigmasterol (IL- β + S) alone resulted in a significant reduction in the gene expression levels of MMP-3, MMP-13 and ADAMTS-5 compared to the IL- β group. Co-treatment of MSC-CM and stigmasterol (IL-1 β + CM + S) also resulted in a significant reduction in the expression of MMP-3, MMP-13 and ADAMTS-5 genes when compared to the IL- β group. However, the combination therapy (IL-1 β + CM + S) markedly reduced the gene expression of these OA-specific tissue degrading proteases to near normal levels as comparable to that of the control group underscoring the benefit of the combination therapy when compared to the individual treatments Fig. 7

Effects of MSC-CM, stigmasterol, and combination of MSC-CM and stigmasterol on the inhibition of IL-1 β -mediated activation of NF- κ B in rat chondrocytes by western blot analysis

IL-1 β exerts its downstream catabolic/inflammatory effects chiefly mediated through the NF- κ B signaling pathway. The effects of MSC-CM, stigmasterol, and the combination of MSC-CM and stigmasterol on the IL-1 β -induced NF- κ B activation in rat primary chondrocytes was evaluated using western blot analysis. As shown in Fig. 8, the results indicated that IL-1 β treatment triggered NF- κ B activation as seen by an increased expression of phosphorylated-p65 (Fig. 8B) and phosphorylated-I κ B α (Fig. 8C). In the presence of MSC-CM (IL-1 β + CM) or stigmasterol (IL-1 β + S), the phosphorylation of both p65 (Fig. 8B) and I κ B α (Fig. 8C) decreased significantly. Nonetheless, the results revealed that the inhibition of IL-1 β -induced NF- κ B activation was the greatest in combination therapy group (MSC-CM + stigmasterol) evidenced by the negligible levels of phosphorylation of both p65 and I κ B α comparable to control levels. The effects of the treatments in inhibiting IL-1 β -induced NF- κ B activation was found to be as follows: (IL-1 β + CM + S) > (IL-1 β + CM) > (IL-1 β + S) when compared to the IL-1 β group Fig. 8.

Discussion

Chronic low-grade inflammation driven by pro-inflammatory mediators has been identified to have a pivotal role in propelling OA pathogenesis characterized by cartilage destruction, subchondral bone remodeling, pain, joint failure and deformity (Robinson et al. 2016). Apparently, therapeutic strategies targeting the inflammatory responses in OA could hold immense clinical value in treating OA (Conaghan et al. 2019; Pelletier et al. 2001; Philp et al. 2016). In the current study, we evaluated for the first time the beneficial effects of MSC-CM and stigmasterol alone and compared with a combination of MSC-CM with stigmasterol in negating the IL-1 β -induced inflammatory responses in rat primary chondrocytes *in-vitro*.

Chondrocytes form the sole cellular components found in the cartilage tissue; they synthesize the extracellular matrix components and provide matrix turnover which are indispensable for maintaining the functional and structural integrity of the cartilage (Goldring and Marcu 2009). However, under OA, this dynamic balance between the cartilage matrix synthesis and degradation is disrupted by the induction of stress-induced inflammatory mediators. Notably, IL-1 β exerts its detrimental effects on the chondrocytes by compromising the chondrocyte viability, inhibiting the anabolic processes critical to cartilage homeostasis including production of ECM components, impairing their fate of differentiation and dysregulating their functions to induce pro-inflammatory and pro-catabolic responses (Charlier et al. 2016). Hence IL-1 β induction in chondrocytes has been employed as a conventional way to generate an OA model *in-vitro* (Li et al. 2019).

In our present study, we assessed the effects of IL-1 β stimulation on the expression of inflammation-associated markers including iNOS, IL-6, and the cartilage turn-over markers COL2A1, MMP-3, MMP-13 and ADAMTS-5 in rat chondrocytes. The inducible nitric oxide synthase (iNOS) contributes to OA pathogenesis by increasing the production of nitric oxide which in turn inhibits the synthesis of collagen type II and proteoglycan (Sasaki et al. 1998). IL-6 is another key pro-inflammatory cytokine and a well-known player in amplifying OA by its inherent ability to work in synergy with IL-1 β (Flannery et al. 2000) and through its role in transsignaling (Scheller et al. 2011). The cartilage matrix degrading enzymes including those that belong to the matrix metalloproteinase (MMP) family and the disintegrin and metalloproteinase with thrombospondin type-1 motifs (ADAMTS) family have been identified as potent diagnostic/therapeutic targets for early OA. Of the many MMPs involved in OA pathogenesis, MMP-13 has been identified as the central

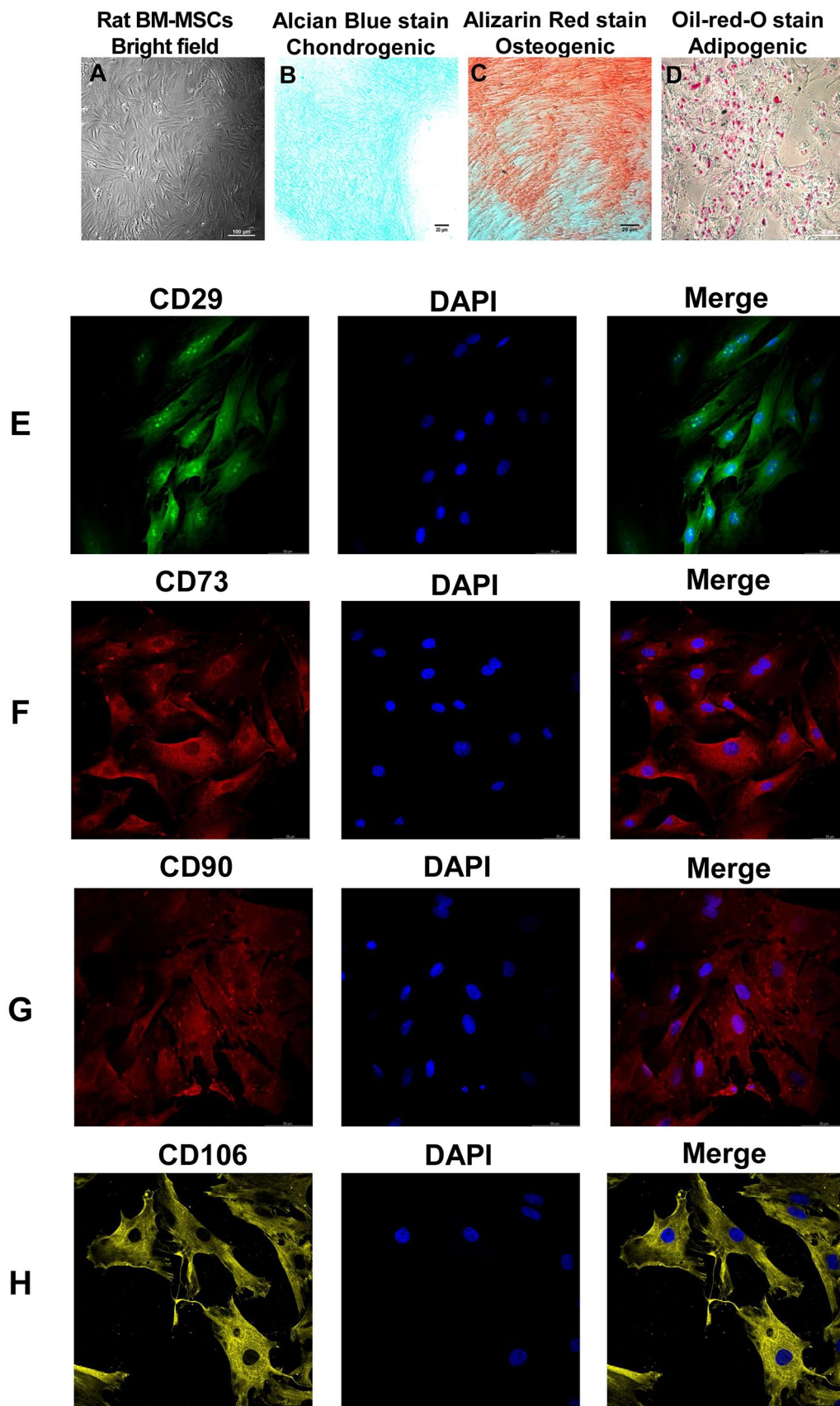


Fig. 2 Characterization of rat bone-marrow mesenchymal stem cells (BM-MSCs). **A** Bright field micrograph of the rat BM-MSCs. **B** Alcian Blue Staining for chondrogenic differentiation of BM-MSCs. **C** Alizarin Red staining for osteogenic differentiation of BM-MSCs. **D** Oil-Red-O staining for adipogenic differentiation of BM-MSCs. **E** Immunofluorescence staining of BM-MSCs for the expression of CD29, **F** CD73, **G** CD90, and **H** CD106 by confocal microscopy

node in the cartilage degradation network in OA and is highly efficient in degrading collagen II, in addition to degrading proteoglycan, types IV and type IX collagen, osteonectin and perlecan in cartilage (Shiomi et al. 2010). MMP-3 also plays a crucial role in cartilage degradation capable of degrading a broad range of substrates including collagen types II, III, and IV, laminin, proteoglycans, and fibronectin, and also activates other MMPs including MMP-1, MMP-2, MMP-9 and MMP-13 (Tong et al. 2017). ADAMTS-5 is the primary aggrecanase responsible for aggrecan degradation in OA with evidence demonstrating for ADAMTS-5 knockout mice to exhibit significant resistance to cartilage erosion in a surgical model of OA (Glasson et al. 2005; Stanton et al. 2005). In our current study, we found that IL-1 β stimulation significantly up-regulated the protein expression of iNOS, IL-6, MMP-13 with a concomitant down-regulated protein expression of COL2A1 in the rat chondrocytes. IL-1 β treatment also significantly increased the gene expression levels of MMP-3 and ADAMTS-5 in the chondrocytes. These altered gene/protein expression levels of key OA-specific markers were significantly reversed by the MSC-CM/stigmasterol individual treatments. However, the combination therapy of MSC-CM and stigmasterol demonstrated a remarkably better anti-inflammatory and anti-catabolic effect by significantly restoring the levels of these OA-specific markers (iNOS, IL-6, COL2A1, MMP-3, MMP-13 and ADAMTS-5) to near normal levels comparable to that of the control group accentuating the benefits of the combination therapy over the individual treatments.

In OA, the overexpression of pro-inflammatory and catabolic factors in response to inflammatory stimuli is regulated by a set of pro-inflammatory signaling pathways. Chiefly, the transcription factor nuclear-factor kappa B (NF- κ B)-mediated signaling is a critical and master regulator orchestrating the expression of various genes involved in inflammation, immune response, adhesion, tissue degradation and apoptosis implicated in OA pathophysiology (Choi et al. 2019). In chondrocytes, the IL-1 β -mediated production of the downstream inflammatory/catabolic mediators such as the iNOS, IL-6 and MMPs is

essentially regulated by the NF- κ B pathway (Chow and Chin 2020) and hence strategies interfering with the activation of the NF- κ B pathway hold immense potential in curtailing OA. Under normal conditions, the p65 subunit of NF- κ B is sequestered into the cytoplasm and kept blocked by the I κ B α proteins. However, during inflammation, NF- κ B is activated where I κ B α proteins are phosphorylated, followed by the subsequent phosphorylation and translocation of the NF- κ B p65 subunit into the nucleus, where it binds to the promoter regions of the DNA and activates the transcription of target genes (Liu et al. 2017). In our present study, western blot studies revealed that IL-1 β stimulation resulted in NF- κ B activation which was evidenced by a significant increase in phosphorylation of p65 and I κ B α . Our results also indicated that the combination therapy (MSC-CM + stigmasterol) was the most effective in negating the IL-1 β induced-NF- κ B activation demonstrated by the negligible phosphorylation of p65 and I κ B α which was comparable to that of the control group. These results reiterate the superiority of the combination therapy over the individual treatments in inhibiting iNOS, IL-6, MMP-3, MMP-13, ADAMTS-5 and improved collagen-II synthesis which could apparently be attributed to the upstream suppression of NF- κ B activation.

Studies by Gabay et al. (2010) had demonstrated for the anti-inflammatory effect of stigmasterol in inhibiting key pro-inflammatory and matrix degradation mediators involved in OA-induced cartilage degradation, partly by inhibiting the NF- κ B pathway. In similar lines, Simental-Mendía et al. (2020) had reported for an anti-inflammatory effect by MSC-CM treatment in an explant culture of IL-1 β -stimulated human cartilage, and the MSC-CM also inhibited the gene expression of IL-1 β , MMP-13, ADAMTS-5 and decreasing glycosaminoglycan release. Another recent study by Chen et al. (2019) had shown the ability of bone marrow derived MSC-CM to alleviate OA in a rodent model surgically induced with OA by maintaining the extracellular matrix homeostasis in cartilage tissue in-vivo. The findings from our current study using stigmasterol or MSC-CM treatments in IL-1 β -stimulated rat chondrocytes are in line with the evidences from these earlier published reports. However, it is noteworthy that the anti-inflammatory/anti-catabolic effects exhibited by the combination therapy (MSC-CM and stigmasterol) clearly shows potent promise(s) as compared to the individual treatments, underscoring for possible synergistic effect augmented by the combination treatments.

Substantiating these findings, we had recently reported (Pragasam et al. 2021) in-vivo that intra-articularly transplanted MSCs in combination with stigmasterol facilitated

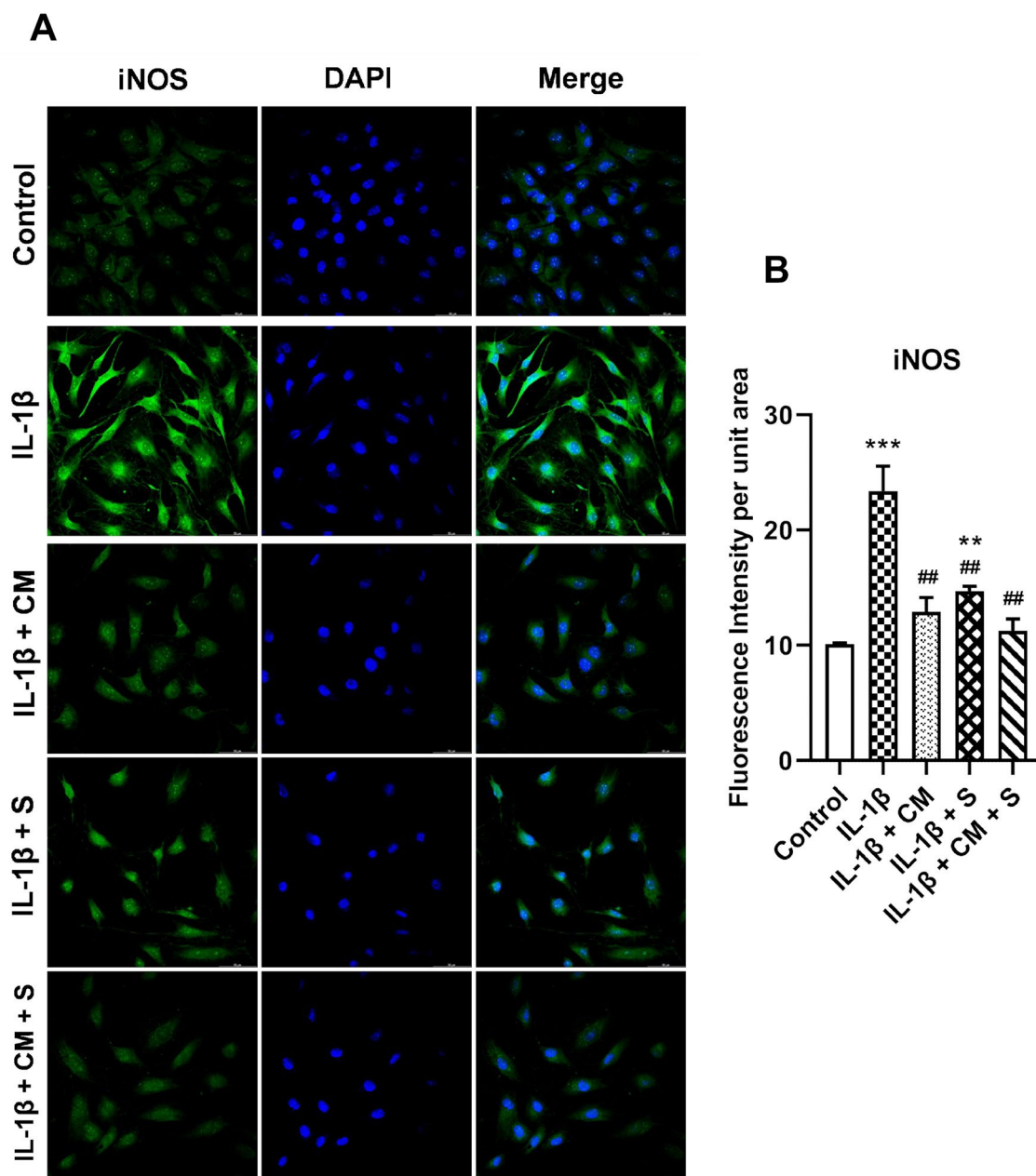


Fig. 3 **A** Immunofluorescence studies for iNOS expression in rat chondrocytes. MSC-CM, stigmasterol, and combination of MSC-CM and stigmasterol treatment reduced iNOS expression in IL-1 β -stimulated rat chondrocytes. Combination of MSC-CM and stigmasterol treatment greatly reduced iNOS protein expression to near

normal levels. **B** Quantitative fluorescence measurements for iNOS expression in the chondrocytes obtained from three independent experiments and values represented as mean \pm SD. Significant differences between the groups defined as: ** p < 0.01; *** p < 0.001 compared with control group; # p < 0.01 compared with IL-1 β group

for significant cartilage repair and regeneration in a monosodium-iodoacetate-induced rat model of OA. We attribute the beneficial effects to the paracrine, autocrine and immunomodulatory effects of MSCs in synergy with the anti-inflammatory and antioxidant functions of stigmasterol. Several studies have demonstrated the distinctive

advantages of employing MSC-CM/secretome in regenerative research/tissue engineering (Vizoso et al. 2017). The MSC-CM offers an optimal platform for therapy in retaining all the bioactive factors, can be freeze-dried, packaged, and transported whilst avoiding the operational and logistic challenges observed with stem cells,

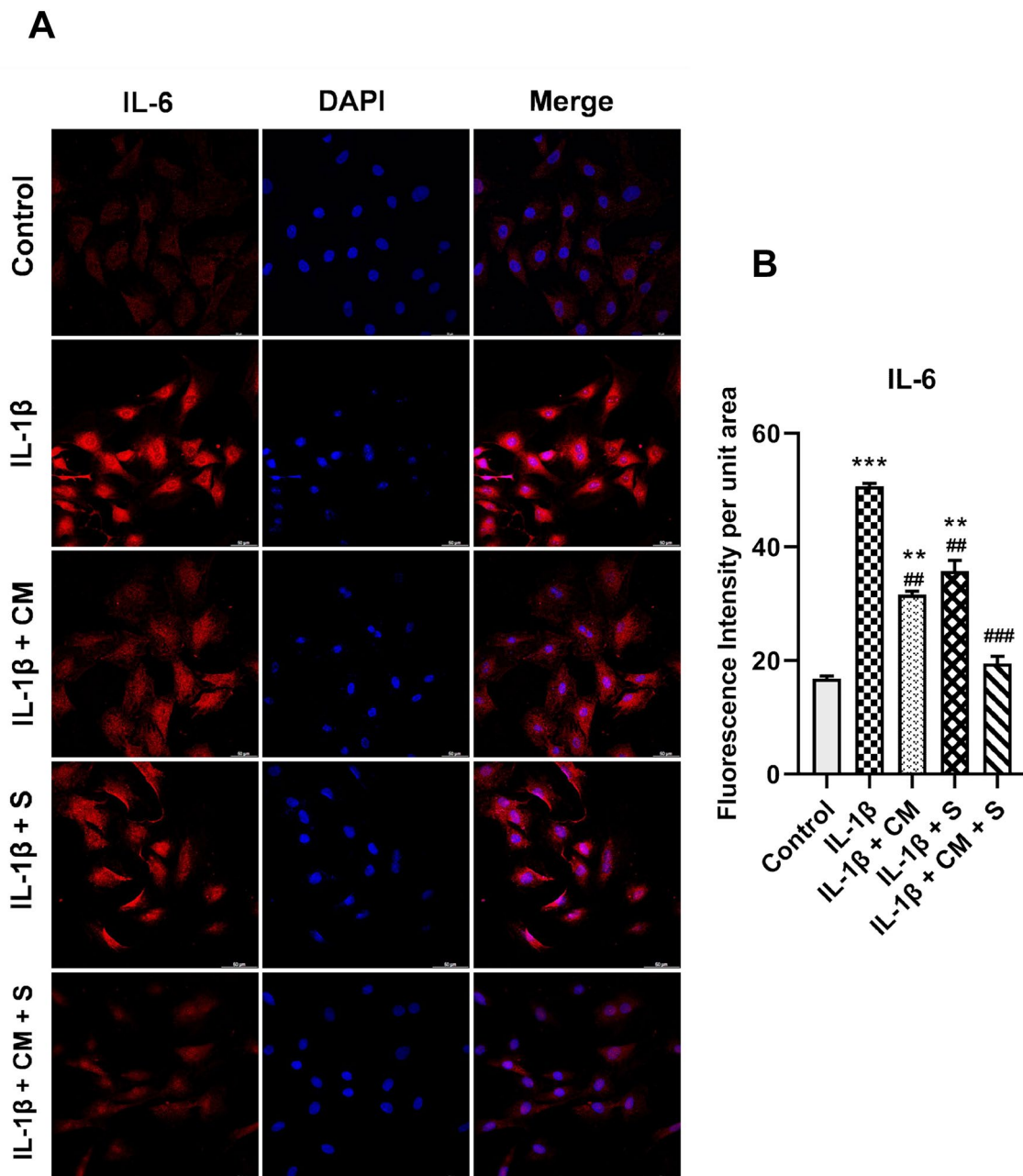


Fig. 4 **A** Immunofluorescence studies for IL-6 expression in rat chondrocytes. MSC-CM, stigmasterol, and combination of MSC-CM and stigmasterol treatment attenuated IL-6 expression in IL-1 β stimulated rat chondrocytes. IL-6 protein expression was significantly reduced to near normal levels in chondrocytes treated with the combination of MSC-CM and stigmasterol. **B** Quantitative fluorescence measure-

ments for IL-6 expression in the chondrocytes from three independent experiments and values represented as mean \pm SD. Significant differences between the groups defined as: ** p < 0.01; *** p < 0.001 compared with control group; ## p < 0.01 ### p < 0.001 compared with IL-1 β group

in addition to its ability as cell-free system to enhance the shelf life (Pawitan 2014). Furthermore, there is greater potential to further enhance the MSC-CM by identifying their components such as anti-inflammatory factors, extracellular vesicles (EV)/exosomes which could

be purified and employed in isolation or in combination with complementing agents to maximize the therapeutic response. Several studies have also demonstrated the efficacy as well as safety of MSC-CM in treating a broad spectrum of diseases administered through various

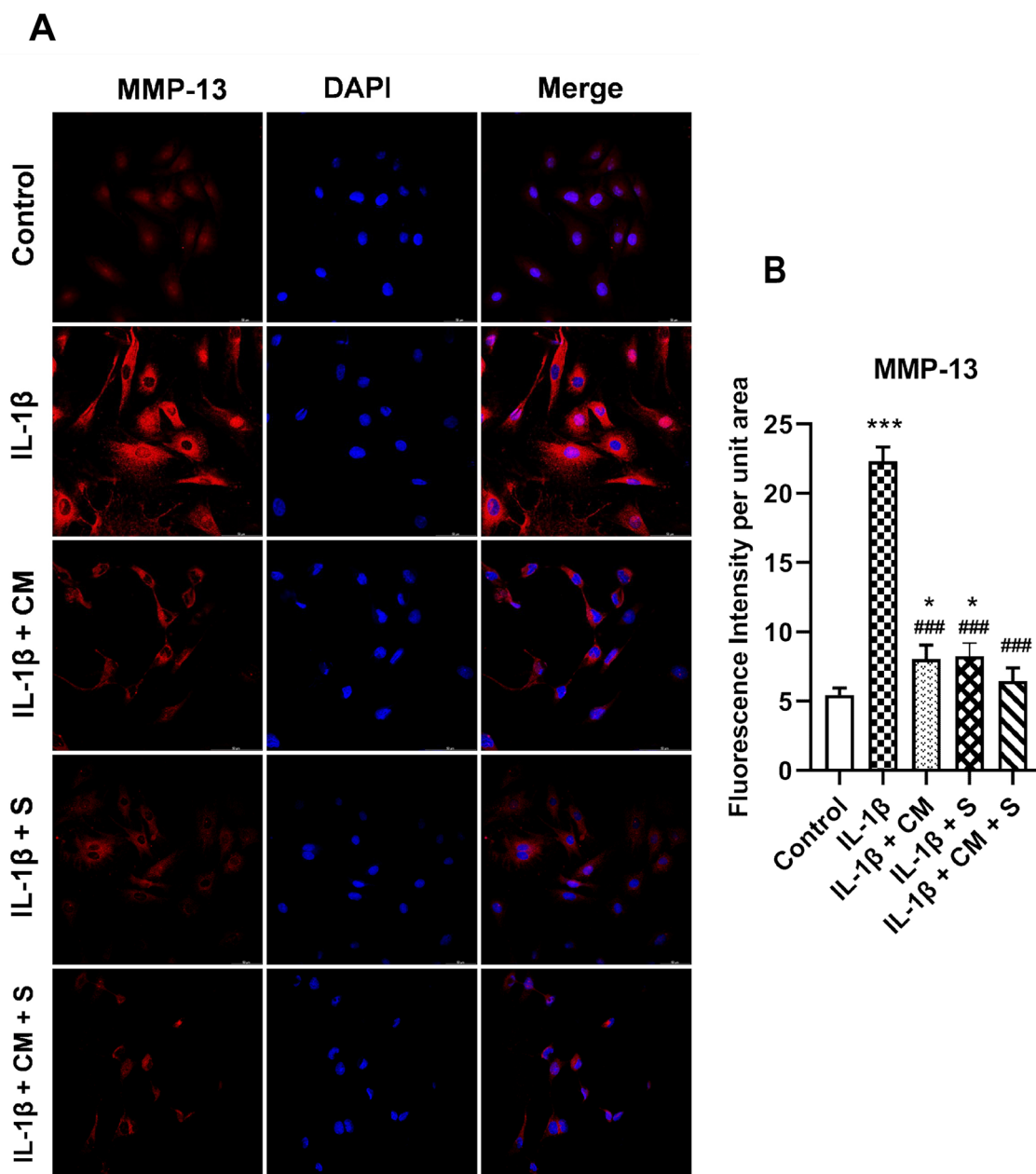


Fig. 5 **A** Immunofluorescence studies for MMP-13 expression in rat chondrocytes. MSC-CM, stigmasterol, and the combination of MSC-CM and stigmasterol treatment significantly attenuated MMP-13 expression in IL-1 β -stimulated rat chondrocytes. MMP-13 expression was significantly reduced to near normal levels in chondrocytes treated with the combination of MSC-CM and stigmasterol. **B** Quan-

titative fluorescence measurements for MMP-13 expression in the chondrocytes from three independent experiments and values represented as mean \pm SD. Significant differences between the groups defined as: ** $p < 0.01$; *** $p < 0.001$ compared with control group; ### $p < 0.001$ compared with IL-1 β group

routes including topical (Joseph et al. 2020), intra-dermal (Cho et al. 2010), subcutaneous (Zhou et al. 2020), intravenous, intra-peritoneal (Hashemi et al. 2020), intra-nasal (Kojima et al. 2020), intra-muscular (Shabbir et al. 2009), intra-articular delivery (Chen et al. 2019) with no reports

of adverse events in either pre-clinical or clinical trials (Montero-Vilchez et al. 2021). Thus, the MSC-CM represents an attractive therapeutic option to be used alone/ in combination with agents with complementing mechanisms of action in treating OA.

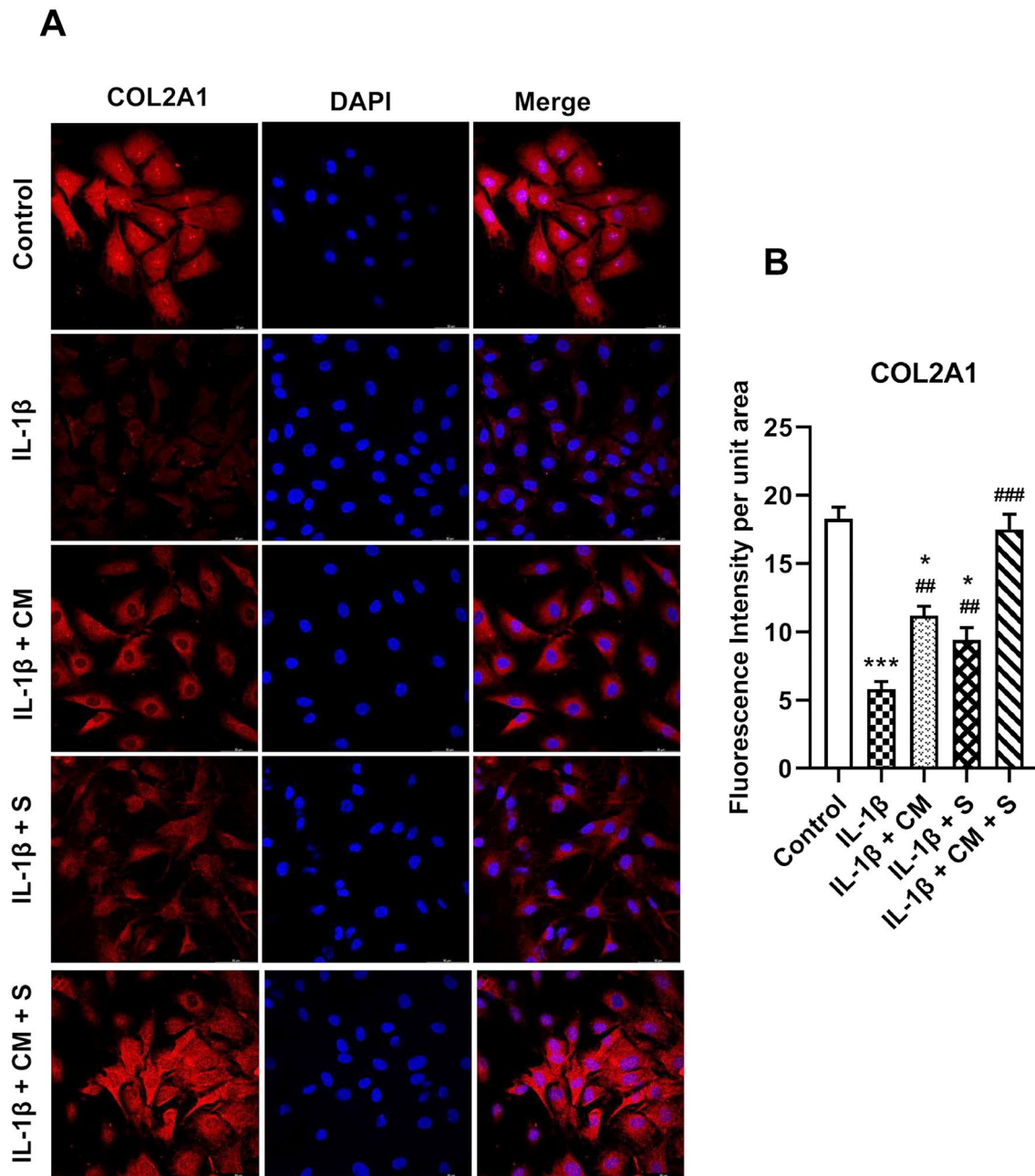


Fig. 6 **A** Immunofluorescence studies for COL2A1 expression in rat chondrocytes. MSC-CM, stigmasterol, and combination of MSC-CM and stigmasterol treatment significantly increased COL2A1 expression in IL-1 β stimulated rat chondrocytes. The combination of MSC-CM and stigmasterol treatment significantly restored COL2A1 expression to near normal levels comparable to the control group. **B**

Quantitative fluorescence measurements for COL2A1 expression in the chondrocytes from three independent experiments and values represented as mean \pm SD. Significant differences between the groups defined as: ** p < 0.01; *** p < 0.001 compared with control group; # p < 0.05; ## p < 0.01; ### p < 0.001 compared with IL-1 β group

Conclusion

Taken together, the findings of our study clearly demonstrate the advantage of employing a combination therapy of

MSC-CM and stigmasterol over the individual treatments in mitigating the IL-1 β -induced inflammatory response in a rat chondrocyte model of OA in-vitro. The combination therapy significantly suppressed the IL-1 β -induced iNOS,

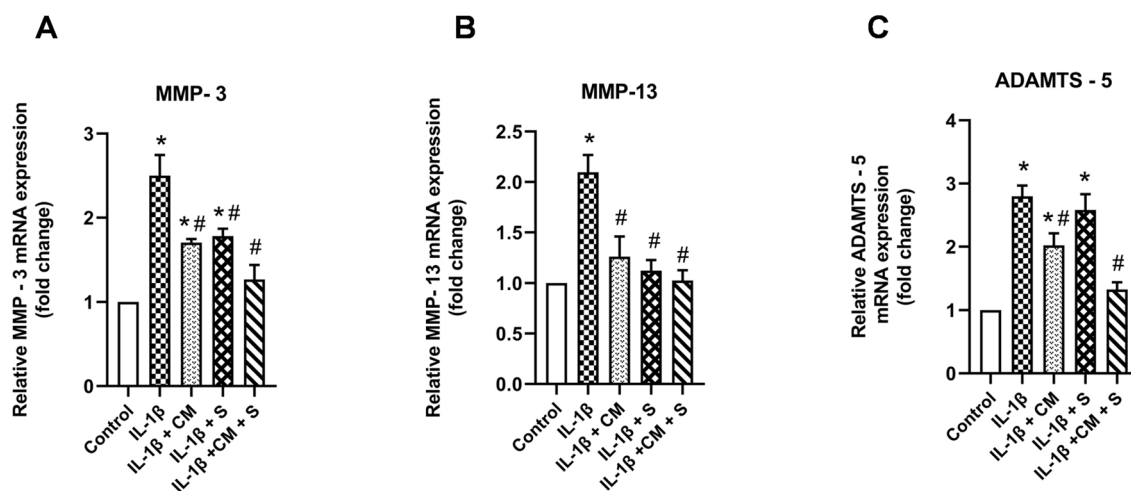


Fig. 7 Effects of MSC-CM, stigmasterol, and combination of MSC-CM and stigmasterol on the mRNA expression levels of **A** MMP-3, **B** MMP-13, and **C** ADAMTS-5 in IL-1 β -stimulated chondrocytes. Values are expressed as mean \pm SD with results obtained from three

independent experiments performed in duplicate. Significant differences between the groups defined as: * $p < 0.05$ compared with control; # $p < 0.05$ compared with IL-1 β group

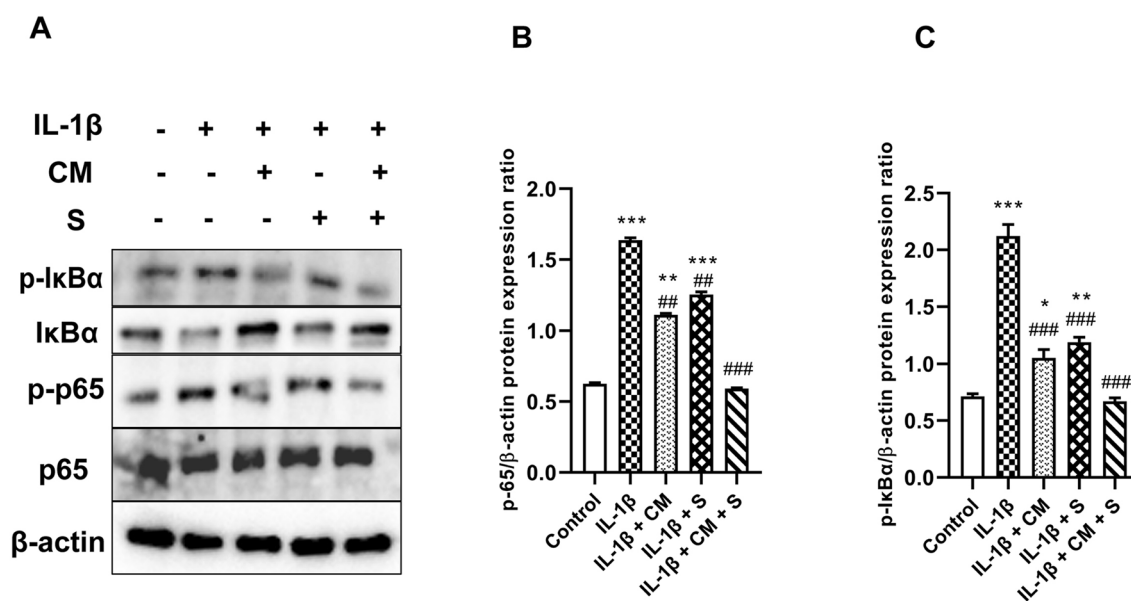


Fig. 8 **A** Western blot studies for the effects of MSC-CM, stigmasterol, and combination of MSC-CM and stigmasterol on the protein expression of p65, p-p65, I κ B α and p-I κ B α in IL-1 β -stimulated chondrocytes. **B** Quantification analysis for the expression of p-p65. **C** Quantification analysis for the expression of p-I κ B α . Values are

expressed as mean \pm SD with results obtained from three independent experiments performed in duplicate. Significant differences between the groups defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with control; # $p < 0.01$; ### $p < 0.001$ compared with IL-1 β group

IL-6, MMP-3, MMP-13 and ADAMTS-5 expression chiefly by inhibiting the NF- κ B signaling pathway. The leads obtained from our previous and current studies have shown promise for the possible application and evaluation of this combination therapy in clinical settings at least in OA patients presenting with early OA-like changes.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval The animal studies were reviewed and approved by the Institutional Animal Ethical Committee, National Institute of Nutrition (NIN), Hyderabad (P29F/III-IAEC/NIN/12/2016/SSJ/WNIN(CG)-6F/WNIN-Gr-Ob-42F) and carried out in accordance with the regulations and guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

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