Gaining Insights To Understand The Anti-Inflammatory Functions of Mesenchymal Stem Cell-Derived Conditioned Medium In Combination With Stigmasterol In IL-1β-Stimulated Rat Articular Chondrocytes – An In-Vitro Approach

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Research Article

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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 per se treatments in alleviating interleukin-1beta (IL-1β)-induced inflammation in rat chondrocytes. Stigmasterol is a phytosterol exhibiting anti-inflammatory effects. IL-1β (10ng/ml) was used to induce inflammation and mimic OA in-vitro in primary rat articular chondrocytes. The IL-1β-stimulated chondrocytes were treated with MSC-CM, stigmasterol, and a combination of MSC-CM and stigmasterol for 24 hours. 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-κB 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-1β-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-1β-induced NF-κB activation evidenced by the negligible phosphorylation of p65 and IκBα subunits, thereby emphasizing the benefit of the combination therapy over the per se treatments.

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 vis-à-vis 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-1β, 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-κB), mitogen-activated protein kinases (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 (Ding et al., 2020). Till now there is no single therapeutic agent that has been deemed safe and effective for treating OA. Strategies aimed at targeting the IL-1β-induced catabolic metabolism and inflammatory responses have proven to offer immense promise(s) in curtailling OA progression.

Nevertheless, limitations in clinical stratification of OA patients as well as the choice of available therapeutics have opened up newer avenues in addressing OA management. In recent years, advances in regenerative medicine/stem cell research have led to promising stem cell-based therapies for cartilage repair in OA. Notably, adult mesenchymal stem cells (MSCs) have emerged as attractive candidates for application in laboratories and clinical studies for cartilage repair/regeneration in OA owning 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). MSCs have been demonstrated to offer their therapeutic potential towards regenerative medicine vis-à-vis 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). Of late, 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 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). 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). Accruing evidence(s) from several studies have shown therapeutic effects of MSC-CM/secretome in treating several diseases (Sagaradze et al., 2019).

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 efficient cartilage repair/regeneration compared to the per se treatments in a monosodium-iodoacetate induced rat model of OA (Pragasam et al., 2021). Hence, in the present study we aimed to assess the potential of a combining rat bone marrow mesenchymal stem cells-derived conditioned medium (MSC-CM) with
stigmasterol in ameliorating the IL-1β-induced inflammatory responses in rat primary chondrocytes. We hypothesize that combining MSC-CM with stigmasterol will elicit a superior anti-inflammatory vis-à-vis beneficial effect compared to the per se 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-weeks 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-weeks 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 hours 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 x 10³ cells/cm². The culture medium was changed every 2 days and upon reaching confluency, the cells were trypsinized using 0.25% trypin-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 weeks 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 x 10³ cells/cm². The culture medium was changed every 2 days 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. The BM-MSCs were also characterized for their expression of MSC specific markers CD29 (1:100, BD Biosciences), CD73 (1:100, BD Biosciences), CD90 (1:100, BD Biosciences) and CD106 (1:100, BD Biosciences) by immunofluorescence using a Leica SP5 confocal laser scanning microscope equipped with Leica Advanced Fluorescence software (Mannheim, Germany) as per established protocols.

**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 x 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 48h 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 stigmasterol on cell viability in the isolated rat primary chondrocytes was measured using the MTT assay. The chondrocytes were seeded at a density of 5x10^3 cells/well in a 96-well plate. After allowing the cells to adhere for 24h, the cells were treated with different concentrations of stigmasterol (0-100 μM) in culture medium. After 24 hours of the treatment, the media was removed, and the cells were incubated with MTT solution (5mg/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.

**Experimental approach**

The chondrocytes were grown in 6-well plates at a seeding density of 2 x10^5 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 24h (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 stigmasterol in plain medium for 24 h (IL-1β + S), (5) Chondrocytes treated with IL-1β (10 ng/ml) + MSC-CM + 50 μM stigmasterol for 24 h. 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 slight modifications.

**Immunofluorescence**

At the end of the experimental period (24h), 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 hour 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 ± 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 ADAMTS5 in the treated chondrocytes. Total RNA was isolated from the chondrocytes cultured in 6-well plates using TRIzol reagent according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 1 μg 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^−ΔΔCT 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'-AATCCCCCTGATGTCCTCGTGGTA-3', (R): 5'-GGTCCTGAGAGATTTTCGCCAA-3'; MMP-13 (F): 5'-TCGCATTGTGAGAGTCATGCCAA-3', (R): 5'-TGTGGTTCCAGCCACGCATAGTCA-3'; ADAMTS5 (F): 5'-GGGGTGCACTGTTCTCGCTTT-3', (R): 5'-GCCGTTAGTGGGCAGGTAT-3'.

**Western Blot analysis**

The total protein was extracted from the chondrocytes cultured in 6-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 12000 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 μg) 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 2h, the membranes were incubated with the primary antibodies against NF-κB p65 (1:1000, Cell Signaling Technology), phospho-NF-κB p65 (1:1000, Cell Signaling Technology), IκBα (1:1000, Novus Biologicals), phospho-IκBα (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 (1:10000, Southern Biotech) at room temperature for 1 hr. 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 ± standard deviation (SD). Significant difference between the groups was measured by 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 0-100 µM for 24h. 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 µM was used for all the further experiments of the study. This dosage was selected also considering a previous study by Gabay et al. (2010).

**Characterization of rat BM-MSCs**

The cultured rat BM-MSCs were identified by 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 differentiated into the chondrogenic lineage as identified by the Alcian Blue staining for proteoglycans (Fig. 2B). The osteogenic differentiation potential of the BM-MSCs was confirmed by the Alizarin Red staining for calcium phosphate deposits (Fig. 2C) and the adipogenic differentiation potential of the BM-MSCs was confirmed by the Oil-Red-O staining of the lipid droplets (Fig. 2D). The BM-MSCs also 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.
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-1β stimulated rat chondrocytes by immunofluorescence

IL-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 per se 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-1β 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-1β stimulated chondrocytes treated with the MSC-CM (IL-1β + CM) or stigmasterol (IL-1β + S) per se 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-1β treated groups. However, the combination treatment of MSC-CM and stigmasterol (IL-1β + CM + S) to IL-1β 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 per se treatments. Figure 3, Figure 4, Figure 5, Figure 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-1β stimulated rat chondrocytes by qRT-PCR

The mRNA expression levels of MMP-3, MMP-13 and ADAMTS5 genes in IL-β-stimulated rat chondrocytes treated with MSC-CM and/or stigmasterol was 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 ADAMTS5 (Fig. 7C). Per se treatments with MSC-CM (IL-β +CM) or stigmasterol (IL-β +S) resulted in a significant reduction in the gene expression levels of MMP-3, MMP-13 and ADAMTS5 compared to the IL-β group. Co-treatment of MSC-CM and stigmasterol (IL-1β + CM + S) also resulted in a significant reduction in the expression of MMP-3, MMP-13 and ADAMTS5 genes when compared to the IL-β group. However, the combination therapy (IL-1β + 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 per se treatments. Figure 7

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

IL-1β exerts its downstream catabolic/inflammatory effects chiefly mediated through the NF-κB signaling pathway. The effects of MSC-CM, stigmasterol, and the combination of MSC-CM and stigmasterol on the IL-1β-induced NF-κB activation in rat primary chondrocytes was evaluated using western blot analysis. As shown in Fig. 8, the results indicated that IL-1β treatment triggered NF-κB activation as seen by increased expression of phosphorylated-p65 (Fig. 8B) and phosphorylated-IκBα (Fig. 8C). In the presence of MSC-CM (IL-1β + CM) or stigmasterol (IL-1β + S), the phosphorylation of both p65 (Fig. 8B)
and IkBα (Fig. 8C) decreased significantly. Nonetheless, the results revealed that the inhibition of IL-1β-induced NF-κB activation was the greatest in combination therapy group (MSC-CM + stigmasterol) evidenced by the negligible levels of phosphorylation of both p65 and IkBα comparable to control levels. The effects of the treatments in inhibiting IL-1β-induced NF-κB activation was found to be as follows: (IL-1β + CM + S) > (IL-1β + CM) > (IL-1β + S) when compared to the IL-1β group. Figure 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. In the current study, we evaluated for the first time the beneficial effects of combining MSC-CM with stigmasterol compared with the *per se* treatments in negating the IL-1β-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-1β exerts its detrimental effects on the chondrocytes by compromising the chondrocyte viability, inhibiting the anabolic processes critical to cartilage homeostasis vis-à-vis 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-1β 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-1β stimulation on the expression of inflammation-associated markers including iNOS, IL-6, and the cartilage turn-over markers COL2A1, MMP-3, MMP-13 and ADAMTS5 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-1β (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 a 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 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-1β 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-1β 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 per se 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 ADAMTS5) 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-κB) 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-1β-mediated production of the downstream inflammatory/catabolic mediators such as the iNOS, IL-6 and MMPs is essentially regulated by the NF-κB pathway (Chow and Chin, 2020) and hence strategies interfering with the activation of the NF-κB pathway hold immense potential in curtailing OA. Under normal conditions, the p65 subunit of NF-κB is sequestered into the cytoplasm and kept blocked by the IκBα proteins. However, during inflammation, NF-κB is activated where IκBα proteins are phosphorylated, followed by the subsequent phosphorylation and translocation of the NF-κB 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-1β stimulation resulted in NF-κB activation which was evidenced by a significant increase in phosphorylation of p65 and IκBα. Our results also indicated that the combination therapy (MSC-CM + stigmasterol) was the most effective in negating the IL-1β induced-NF-κB activation demonstrated by the negligible phosphorylation of p65 and IκBα which was comparable to that of the control group. These results reiterate the superiority of the combination therapy over the per se treatments in inhibiting iNOS, IL-6, MMP-3, MMP-13, ADAMTS5 and improved collagen-II synthesis which could apparently be attributed to the upstream suppression of NF-κB 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-κB 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-1β-stimulated human cartilage, and the MSC-CM also inhibited the gene expression of IL-1β, MMP-13, ADAMTS5 and decreasing glycosaminoglycan release. Another recent study by Chen et al. (2019) had shown the ability
of 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 per se treatments in IL-1β-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 per se 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 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 vis-à-vis stigmasterol for its anti-inflammatory and antioxidant functions. Accumulating evidence(s) from several studies merit the distinctive advantages of employing MSC-CM/secretome over the use of stem cells in regenerative research/tissue engineering (Vizoso et al., 2017). The MSC-CM offers 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, in addition to its ability as cell-free system to enhance the shelf life (Pawitan, 2014).

**Conclusion**

Taken together, the findings of our study clearly demonstrate the advantage of employing a combination therapy of MSC-CM and stigmasterol over the per se treatments in mitigating the IL-1β induced inflammatory response in a rat chondrocyte model of OA in vitro. The combination therapy significantly suppressed the IL-1β induced iNOS, IL-6, MMP3, MMP13 and ADAMTS5 expression chiefly by inhibiting the NF-κB 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.

**Declarations**

**Acknowledgement**

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**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).

References

1. Antwi AO, Obiri DD, Osafo N, Essel LB, Forkuo AD, Atobiga C (2018) Stigmasterol Alleviates Cutaneous Allergic Responses in Rodents. BioMed research international 2018:3984068. https://doi.org/10.1155/2018/3984068
2. Charlier E, Relic B, Deroyer C, Malaise O, Neuville S, Collée J, Malaise MG, and D. De Seny. 2016. Insights on Molecular Mechanisms of Chondrocytes Death in Osteoarthritis. *Int J Mol Sci* 17:2146. https://doi.org/10.3390/ijms17122146
3. Chen D, Shen J, Zhao W, Wang T, Han L, Hamilton JL, Im HJ (2017) Osteoarthritis: toward a comprehensive understanding of pathological mechanism. Bone research 5:16044. https://doi.org/10.1038/boneres.2016.44
4. Chen W-P, Yu C, Hu P-F, Bao J-P, Tang J-L, Wu L-D (2012) Stigmasterol blocks cartilage degradation in rabbit model of osteoarthritis. Acta Biochim Pol 59: https://doi.org/10.18388/abp.2012_2088
5. Chen W, Sun Y, Gu X, Hao Y, Liu X, Lin J, Chen J, Chen S (2019) Conditioned medium of mesenchymal stem cells delays osteoarthritis progression in a rat model by protecting subchondral bone, maintaining matrix homeostasis, and enhancing autophagy. J Tissue Eng Regen Med 13:1618–1628. https://doi.org/10.1002/term.2916
6. Choi MC, Jo J, Park J, Kang HK, Park Y (2019) NF-κB Signaling Pathways in Osteoarthritic Cartilage Destruction. Cells 8: https://doi.org/10.3390/cells8070734
7. Chow YY, Chin K-Y (2020) The Role of Inflammation in the Pathogenesis of Osteoarthritis. Mediators Inflamm 2020:8293921–8293921. https://doi.org/10.1155/2020/8293921
8. Cui A, Li H, Wang D, Zhong J, Chen Y, Lu H (2020) Global, regional prevalence, incidence and risk factors of knee osteoarthritis in population-based studies. EClinicalMedicine 29: https://doi.org/10.1016/j.eclinm.2020.100587
9. Daheshia M, Yao JQ (2008) The interleukin 1beta pathway in the pathogenesis of osteoarthritis. J Rheumatol 35:2306–2312. https://doi.org/10.3899/jrheum.080346
10. Ding S-I, Pang Z-y, Chen X-m, Li Z, Liu X-x, Zhai Q-I, Huang J-m, and Z.-y. Ruan (2020) Urolithin a attenuates IL-1β-induced inflammatory responses and cartilage degradation via inhibiting the MAPK/NF-κB signaling pathways in rat articular chondrocytes. *Journal of Inflammation* 17:13. https://doi.org/10.1186/s12950-020-00242-8
11. Fan XL, Zhang Y, Li X, Fu QL (2020) Mechanisms underlying the protective effects of mesenchymal stem cell-based therapy. Cell Mol Life Sci 77:2771–2794. https://doi.org/10.1007/s00018-020-
12. Flannery CR, Little CB, Hughes CE, Curtis CL, Caterson B, Jones SA (2000) IL-6 and its soluble receptor augment aggrecanase-mediated proteoglycan catabolism in articular cartilage. Matrix biology: journal of the International Society for Matrix Biology 19:549–553. https://doi.org/10.1016/S0945-053X(00)00111-6

13. Gabay O, Sanchez C, Salvat C, Chevy F, Breton M, Nourissat G, Wolf C, Jacques C, Berenbaum F (2010) Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. Osteoarthritis cartilage 18:106–116. https://doi.org/10.1016/j.joca.2009.08.019

14. Garcia, M.D., M.T. Sáenz, M.A. Gómez, and M.A. Fernández. 1999. Topical antiinflammatory activity of phytosterols isolated from Eryngium foetidum on chronic and acute inflammation models. Phytotherapy research: PTR 13:78–80. https://doi.org/10.1002/(SICI)1099-1573(199902)13:1<78::AID-PTR384>3.0.CO;2-F

15. Ghosh T, Maity TK, Singh J (2011) Evaluation of antitumor activity of stigmasterol, a constituent isolated from Bacopa monnieri Linn aerial parts against Ehrlich Ascites Carcinoma in mice. Oriental Pharmacy Experimental Medicine 11:41–49. https://doi.org/10.1007/s13596-011-0001-y

16. Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma H-L, Flannery CR, Peluso D, Kanki K, Yang Z, Majumdar MK, Morris EA (2005) Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature 434:644–648. https://doi.org/10.1038/nature03369

17. Goldring MB, Marcu KB (2009) Cartilage homeostasis in health and rheumatic diseases. Arthritis research therapy 11:224. https://doi.org/10.1186/ar2592

18. González-González A, García-Sánchez D, Dotta M, Rodríguez-Rey JC, Pérez-Campo FM (2020) Mesenchymal stem cells secretome: The cornerstone of cell-free regenerative medicine. World journal of stem cells 12:1529–1552. https://dx.doi.org/10.4252/wjsc.v12.i12.1529

19. Haleagrahara N, Hodgson K, Miranda-Hernandez S, Hughes S, Kulur AB, Ketheesan N (2018) Flavonoid quercetin–methotrexate combination inhibits inflammatory mediators and matrix metalloproteinase expression, providing protection to joints in collagen-induced arthritis. Inflammopharmacology 26:1219–1232. https://doi.org/10.1007/s10787-018-0464-2

20. Haque MN, Hannan MA, Dash R, Choi SM, Moon IS (2021) The potential LXRβ agonist stigmasterol protects against hypoxia/reoxygenation injury by modulating mitophagy in primary hippocampal neurons. Phytomedicine: international journal of phytotherapy phytopharmacology 81:153415. https://doi.org/10.1016/j.phymed.2020.153415

21. Huang X, Xi Y, Pan Q, Mao Z, Zhang R, Ma X, You H (2018) Caffeic acid protects against IL-1β-induced inflammatory responses and cartilage degradation in articular chondrocytes. Biomed Pharmacother 107:433–439. https://doi.org/10.1016/j.biopharma.2018.07.161

22. Jo CH, Lee YG, Shin WH, Kim H, Chai JW, Jeong EC, Kim JE, Shim H, Shin JS, Shin IS, Ra JC, Oh S, Yoon KS (2014) Intra-articular injection of mesenchymal stem cells for the treatment of osteoarthritis of the knee: a proof-of-concept clinical trial. Stem Cells 32:1254–1266. https://doi.org/10.1177/0363546517716641
23. Kaur S, Bijjem KRV, Sharma PL (2011) Anti-inflammatory and antihyperalgesic effects of the combination of ibuprofen and hemin in adjuvant-induced arthritis in the Wistar rat. Inflammopharmacology 19:265. https://doi.org/10.1007/s10787-011-0090-8

24. Kay AG, Long G, Tyler G, Stefan A, Broadfoot SJ, Piccinini AM, Middleton J, Kehoe O (2017) Mesenchymal Stem Cell-Conditioned Medium Reduces Disease Severity and Immune Responses in Inflammatory Arthritis. Sci Rep 7:18019. https://doi.org/10.1038/s41598-017-18144-w

25. Lee WS, Kim HJ, Kim Ki, Kim GB, and W. Jin (2019a) Intra-Articular Injection of Autologous Adipose Tissue-Derived Mesenchymal Stem Cells for the Treatment of Knee Osteoarthritis: A Phase IIb, Randomized, Placebo-Controlled Clinical Trial. Stem cells translational medicine 8:504–511. https://doi.org/10.1002/sctm.18-0122

26. Lee YS, Lee SY, Park SY, Lee SW, Hong KW, Kim CD (2019b) Cilostazol add-on therapy for celecoxib synergistically inhibits proinflammatory cytokines by activating IL-10 and SOCS3 in the synovial fibroblasts of patients with rheumatoid arthritis. Inflammopharmacology 27:1205–1216. https://doi.org/10.1007/s10787-019-00605-5

27. Li X, He P, Hou Y, Chen S, Xiao Z, Zhan J, Luo D, Gu M, Lin D (2019) Berberine inhibits the interleukin-1 beta-induced inflammatory response via MAPK downregulation in rat articular chondrocytes. 80:637–645. https://doi.org/10.1002/ddr.21541

28. Liu CC, Zhang Y, Dai BL, Ma YJ, Zhang Q, Wang Y, Yang H (2017) Chlorogenic acid prevents inflammatory responses in IL–1β–stimulated human SW–1353 chondrocytes, a model for osteoarthritis. Mol Med Rep 16:1369–1375. https://doi.org/10.3892/mmr.2017.6698

29. Madhira SL, Challa SS, Chalasani M, Nappanveethl G, Bhonde RR, Ajumeera R, Venkatesan V (2012) Promise(s) of Mesenchymal Stem Cells as an In Vitro Model System to Depict Pre-Diabetic/Diabetic Milieu in WNIN/GR-Ob Mutant Rats. PLOS ONE 7:e48061. https://doi.org/10.1371/journal.pone.0048061

30. Oseni AO, Butler PE, Seifalian AM (2013) Optimization of chondrocyte isolation and characterization for large-scale cartilage tissue engineering. J Surg Res 181:41–48. https://doi.org/10.1016/j.joca.2013.03.018

31. Panda S, Jafri M, Kar A, Meheta BK (2009) Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from Butea monosperma. Fitoterapia 80:123–126. https://doi.org/10.1016/j.fitote.2008.12.002

32. Pawitan JA (2014) Prospect of Stem Cell Conditioned Medium in Regenerative Medicine. BioMed research international 2014:965849. https://doi.org/10.1155/2014/965849

33. Pragasam SSJ, Venkatesan V (2020) Metabolic Syndrome Predisposes to Osteoarthritis: Lessons from Model System. Cartilage 1947603520980161. https://doi.org/10.1177/1947603520980161

34. Ramu R, Shirahatti PS, Nayakavadi S, R V, Zameer F, Dhananjaya BL, Prasad Mn N (2016) The effect of a plant extract enriched in stigmasterol and β-sitosterol on glycaemic status and glucose metabolism in alloxan-induced diabetic rats. Food function 7:3999–4011. https://doi.org/10.1039/C6FO00343E
35. Robinson WH, Lepus CM, Wang Q, Raghu H, Mao R, Lindstrom TM, Sokolove J (2016) Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. Nat Rev Rheumatol 12:580–592. https://doi.org/10.1038/nrrheum.2016.136

36. Sagaradze G, Grigorieva O, Nimiritsky P, Basalova N, Kalinina N, Akopyan Z, Efimenko A (2019) Conditioned Medium from Human Mesenchymal Stromal Cells: Towards the Clinical Translation. Int J Mol Sci 20:1656. https://doi.org/10.3390/ijms20071656

37. Sampath SJP, Kotikalapudi N, Venkatesan V (2021) A novel therapeutic combination of mesenchymal stem cells and stigmasterol to attenuate osteoarthritis in rodent model system—a proof of concept study. Stem cell investigation 8:5. https://doi.org/10.21037/sci-2020-048

38. Sasaki K, Hattori T, Fujisawa T, Takahashi K, Inoue H, Takigawa M (1998) Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit articular chondrocytes. J BioChem 123:431–439. https://doi.org/10.1210/endo.137.9.8756539

39. Satué M, Schüler C, Ginner N, Erben RG (2019) Intra-articularly injected mesenchymal stem cells promote cartilage regeneration, but do not permanently engraft in distant organs. Sci Rep 9:10153. https://doi.org/10.1038/s41598-019-46554-5

40. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S (2011) The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 1813:878–888. https://doi.org/10.1016/j.bbamcr.2011.01.034

41. Shiomi T, Lemaître V, D’Armiento J, Okada Y (2010) Matrix metalloproteinases, a disintegrin and metalloproteinases, and a disintegrin and metalloproteinases with thrombospondin motifs in non-neoplastic diseases. 60:477–496. https://doi.org/10.1111/j.1440-1827.2010.02547.x

42. Simental-Mendía M, Lozano-Sepúlveda SA, Pérez-Silos V, Fuentes-Mera L, Martínez-Rodríguez HG, Acosta-Olivo CA, Peña-Martínez VM, Vilchez-Cavazos F (2020) Anti-inflammatory and anti-catabolic effect of non-animal stabilized hyaluronic acid and mesenchymal stem cell-conditioned medium in an osteoarthritis coculture model. Mol Med Rep 21:2243–2250. https://doi.org/10.3892/mmr.2020.11004

43. Sofi MS, Sateesh MK, Bashir M, Ganie MA, Nabi S (2018) Chemopreventive and anti-breast cancer activity of compounds isolated from leaves of Abrus precatorius L. 3 Biotech 8:371. https://doi.org/10.1007/s13205-018-1395-8

44. Song Y, Du H, Dai C, Zhang L, Li S, Hunter DJ, Lu L, Bao C (2018) Human adipose-derived mesenchymal stem cells for osteoarthritis: a pilot study with long-term follow-up and repeated injections. Regenerative medicine 13:295–307. https://doi.org/10.2217/rme-2017-0152

45. Stanton H, Rogerson FM, East CJ, Golub SB, Lawlor KE, Meeker CT, Little CB, Last K, Farmer PJ, Campbell IK, Fourie AM, Fosang AJ (2005) ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. Nature 434:648–652. https://doi.org/10.1038/nature03417

46. Tong Z, Liu Y, Chen B, Yan L, Hao D (2017) Association between MMP3 and TIMP3 polymorphisms and risk of osteoarthritis. 8: https://doi.org/10.18632/oncotarget.18745
47. Usha PR, Naidu MUR (2004) Randomised, Double-Blind, Parallel, Placebo-Controlled Study of Oral Glucosamine, Methylsulfonylmethane and their Combination in Osteoarthritis. Clin Drug Investig 24:353–363. https://doi.org/10.2165/00044011-200424060-00005

48. van der Kraan PM, van den WB, Berg (2012) Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? Osteoarthritis cartilage 20:223–232. https://doi.org/10.1016/j.joca.2011.12.003

49. Vizoso FJ, Eiro N, Cid S, Schneider J, Perez-Fernandez R (2017) Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. 18:1852. https://doi.org/10.3390/ijms18091852

50. Walker CIB, Oliveira SM, Tonello R, Rossato MF, da Silva Brum E, Ferreira J, Trevisan G (2017) Antinociceptive effect of stigmasterol in mouse models of acute and chronic pain. Naunyn-Schmiedeberg's Archives of Pharmacology 390:1163–1172. https://doi.org/10.1007/s00210-017-1416-x

51. Xing D, Wu J, Wang B, Liu W, Liu W, Zhao Y, Wang L, Li JJ, Liu A, Zhou Q, Hao J, Lin J (2020) Intra-articular delivery of umbilical cord-derived mesenchymal stem cells temporarily retard the progression of osteoarthritis in a rat model. Int J Rheum Dis 23:778–787. https://doi.org/10.1111/1756-185X.13834

52. Yadav M, Parle M, Jindal DK, Dhingra S (2018) Protective effects of stigmasterol against ketamine-induced psychotic symptoms: Possible behavioral, biochemical and histopathological changes in mice. Pharmacological Reports 70:591–599. https://doi.org/10.1016/j.pharep.2018.01.001

53. Yu W, Xu P, Huang G, Liu L (2018) Clinical therapy of hyaluronic acid combined with platelet-rich plasma for the treatment of knee osteoarthritis. Exp Ther Med 16:2119–2125. https://doi.org/10.3892/etm.2018.6412

**Figures**
Figure 1

A. Bright field micrograph of rat articular chondrocytes. B. Cell viability of the rat chondrocytes treated with stigmasterol (0-100 μM) for 24h measured using MTT assay. No significant change in cell viability was observed.
Figure 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 CD29, F. CD90, and G. CD106 by confocal microscopy.
Figure 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-1β 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 ± SD.
Significant differences between the groups defined as: **p < 0.01; ***p < 0.001 compared with control group; ## p < 0.01 compared with IL-1β group.

Figure 4

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-1β 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 measurements for IL-6 expression in the chondrocytes from three independent experiments and values represented as mean ± 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-1β group.

Figure 5
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-1β 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. Quantitative fluorescence measurements for MMP-13 expression in the chondrocytes from three independent experiments and values represented as mean ± SD. Significant differences between the groups defined as: **p < 0.01; ***p < 0.001 compared with control group; ###p < 0.001 compared with IL-1β group.
Figure 6

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-1β 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 ± SD. Significant differences between the groups defined as: **p < 0.01; ***p < 0.001 compared with control group; ###p < 0.001 compared with IL-1β group.

Figure 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-1β-stimulated chondrocytes. Values are expressed as mean ± 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-1β group.
Figure 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, IκBα and p-IκBα in IL-1β-stimulated chondrocytes. B. Quantification analysis for the expression of p-p65. C. Quantification analysis for the expression of p-IκBα. Values are expressed as mean ± 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-1β group.