

Impact of chitosan membrane culture on the expression of pro- and anti-inflammatory cytokines in mesenchymal stem cells

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Abstract. Osteoarthritis (OA) is a chronic inflammatory joint condition caused by various inflammatory cytokines. The pro-inflammatory cytokines controlling OA include interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , IL-6 and IL-18. The anti-inflammatory cytokines include IL-4, IL-10, IL-13, leukemia inhibitory factor (LIF), glycoprotein 130 (IL6ST), TNF- α -stimulated gene 6 and transforming growth factor (TGF)- β 1. Mesenchymal stem cells (MSCs) serve an anti-inflammatory role in the treatment of OA by secreting various cytokines. Previous studies demonstrated that the anti-inflammatory ability of MSCs decreased rapidly in a traditional plate culture. Maintaining the anti-inflammatory properties of MSCs *in vitro* remains challenging. Therefore, it is necessary to develop a more stable and efficient method to culture MSCs *in vitro*. Chitosan is a deacetylated derivative of chitin and is the second most abundant natural polysaccharide worldwide. The present study demonstrated that MSCs cultured on chitosan membranes (CM) spontaneously formed multicellular spheroids. Compared with the control group without CM, the formation of multicellular spheres in the CM enhanced the anti-inflammatory properties of MSCs. Expression levels of pro- and anti-inflammatory genes mRNA and their proteins in MSCs were detected by reverse transcription-quantitative PCR, western blot analysis and immunofluorescence assay. Protein and mRNA expression levels of pro-inflammatory cytokines IL-1 β , TNF- α , IL-6 and IL-18 were significantly decreased in CM-cultured MSCs compared with the control group ($P < 0.05$). Furthermore, mRNA and protein expression levels of anti-inflammatory cytokines TGF- β 1 in CM-cultured MSCs were significantly

increased compared with the control group ($P < 0.01$). These results indicated that the formation of multicellular spheroids by CM-cultured MSCs aided in maintaining anti-inflammatory effects.

Introduction

Osteoarthritis (OA) is a disease that causes articular cartilage degeneration, and there is currently no effective drug to prevent disease progression (1). Medical care is mainly based on alleviating painful symptoms (2). The pathological changes in OA involve all tissues that form joints. During the development of OA, intra-articular tissues exhibit inflammation of varying severity, and joint inflammation may cause joint destruction. It is hypothesized that the immune system is one of the factors involved in the pathogenesis, occurrence and development of OA (3). During OA development, the production and function of cytokines vary according to the duration and severity of the disease (4). Cytokines can be divided into pro- and anti-inflammatory according to their roles in OA (3). Cytokines interfere with the process of catabolism and anabolism, particularly in tissues that are often subjected to high mechanical loads, such as the human joints. The imbalance between synthesis and catabolism causes progressive degeneration of the articular cartilage, causing gradual development of the disease (5).

Mesenchymal stem cells (MSCs) are adult stem cells derived from the multi-directional differentiation potential of tissues and organs and can differentiate into osteogenic (6), cartilage (7) and liver cells (8). Previous studies have demonstrated that MSCs can be transformed into chondrocytes and induced into articular cartilage to repair damage, and have therapeutic effects on experimental OA (9,10). Currently, two-dimensional cultures cannot maintain the stability of MSCs. This is primarily due to the senescence of MSCs during the process of a traditional plate culture, self-differentiation of osteoblasts, decrease in anti-inflammatory ability and decline in proliferation ability which severely affect the function of MSCs as a cell therapy (11). Previous studies have reported that aggregation of MSCs into 3-dimensional (3D) spheroids could markedly enhance their trophic and anti-inflammatory properties (11,12).

Chitosan (CS) is a deacetylated derivative of chitin and is the second most abundant natural polysaccharide in the world (13). The biocompatibility of CS has been attributed to

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its structural and functional similarity to glycosaminoglycans, making it a biomaterial candidate for cartilage engineering (14). In the present study, MSCs were cultured on CS membranes (CMs) to form 3D multicellular spheres. The formation of multicellular spheres had a significant effect on the expression of anti-inflammatory genes in MSCs. The aim of the present study was to investigate the impact of a CM cultures on the expression of pro- and anti-inflammatory cytokines in human umbilical cord MSCs and to establish a foundation for further study on the role of CM-cultured MSCs in OA cartilage repair.

Materials and methods

Experimental materials. CS powder (Sinopharm Group Chemical Reagent Co., Ltd.), FBS (Gibco; Thermo Fisher Scientific, Inc.), DMEM (Gibco; Thermo Fisher Scientific, Inc.), a SDS-PAGE gel preparation kit (cat. no. AS1012; Aspen), RIPA total protein lysis and extraction buffer (cat. no. AS1004; Aspen), a BCA protein concentration assay kit (cat. no. AS1086; Aspen), protein markers (Thermo Fisher Scientific, Inc.), a fluorescence quantitative PCR instrument (Thermo Fisher Scientific, Inc.), TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), SYBR[®] Premix Ex Taq[™] (Takara Biomedical Technology, Co., Ltd.), PrimeScript[™] RT reagent kit with gDNA Eraser (Takara Biomedical Technology, Co., Ltd.), PCR primers (Wuhan Google Biotechnology Company Co., Ltd.), trypsin EDTA (Gino Biomedical Technology Co., Ltd.) and PBS (Gino Biomedical Technology Co., Ltd) were used in the present study.

Preparation of the CM. CS powder was dissolved in 1% glacial acetic acid solution (Sinopharm Group Chemical Reagent Co., Ltd.) to obtain a 1% CS solution, which was spread evenly at the bottom of a 6-well culture plate at 1.2 ml/well. The liquid was dried in an oven at 65°C for 24 h to prepare a CM substrate. The CM substrate was exposed to ultraviolet light overnight, neutralized using a 0.5 mol/l NaOH solution for 10 min at room temperature. Lye residue was thoroughly washed with sterile water three times. The CM substrate was subsequently washed with PBS three times.

Cultivation of primary MSCs. The present study was approved by the Ethics Committee of the Renmin Hospital of Wuhan University (Wuhan, China) and written informed consent. Umbilical cords were obtained from three healthy post-partum women (age, Patient 1, 28 years; Patient 2, 28 years; and Patient 3, 30 years) in May 2017. Umbilical cords were collected and washed thoroughly with PBS containing 100 µg/ml streptomycin and 100 U/ml penicillin. Wharton's jelly tissue was separated from the umbilical cords with forceps, cut into smaller pieces with scissors and digested with 0.1% type I collagenase (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 16 h to release MSCs. MSCs were obtained following by centrifugation for 5 min at 400 x g at room temperature and cultured in high glucose MEM/F12 (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Hyclone; GE Healthcare Life Sciences) and 10 ng/ml basic fibroblast growth factor (Peprotech EC Ltd.). The medium was replaced every 3 days with fresh medium.

MSCs were identified by morphological and flow cytometric analyses using an inverted microscope at a magnification of x40 and x100 (Olympus Corporation). When their proliferation reached 80%, the P1 generation was used as seed cells.

Experimental grouping and cell culture. The following groups were used in the present study: i) The experimental group, in which CM covered the bottom of the 6-well plate; and ii) the control group, in which the 6-well plate did not undergo treatment. MSCs P1 cells were inoculated into the groups (seeding density, 3x10⁵/well) in a low-sugar DMEM medium containing 10% FBS in a CO₂ incubator at 37°C. At 72 h post-inoculation, cell morphologies were observed using an inverted microscope at a magnification of x40 and x100 (Olympus Corporation) and cells were collected for subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Expression levels of pro- and anti-inflammatory genes in MSCs cultured in the experimental and control groups were detected using RT-qPCR. Total RNA was extracted from the two groups of cells cultured for 72 h and transcribed into cDNA using a reverse transcriptase kit. A total of 2 µl cDNA was used as a PCR amplification template. β-actin was used as the internal reference gene. All qPCR reactions were performed using the following thermocycling conditions: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 58°C for 30 sec and 72°C for 30 sec. SYBR[®] Premix Ex Taq[™] (Takara Biomedical Technology, Co., Ltd.) was used for the quantitative analysis using a PCR instrument. The 2^{-ΔΔC_q} method was used to analyze results (15). Primer sequences are presented in Table I.

Western blotting. Expression levels of pro- and anti-inflammatory cytokines in MSCs were detected using western blotting. MSCs in the experimental and control groups were lysed with pre-cooled RIPA buffer for 30 min and centrifuged at 13,000 x g for 5 min at 4°C. The supernatant was collected and total protein amounts were measured using a BCA kit (Beyotime Institute of Biotechnology). A total of 20 µg total protein/lane was electrophoresed on SDS-PAGE (10% gel) and transferred to 0.22-µm PVDF membranes. Membranes were blocked with 5% skim milk TBST (10 mmol/l Tri-HCl; 150 mmol/l NaCl; 0.25% Tween-20; pH 7.5) for 1 h at room temperature. Membranes were then incubated with polyclonal rabbit anti-human β-actin (1:10,000; cat. no. TDY051; BEIJING TDY BIOTECH CO.,LTD.), interleukin (IL)-1β (1:1000; cat. no. ab2105; Abcam), tumor necrosis factor-α (TNF-α; 1:500; cat. no. ab66579; Abcam), IL-6 (1:1000; cat. no. 21865-1-AP; Proteintech Group, Inc), IL-18 (1:1000; cat. no. 10663-1-AP; Proteintech Group, Inc), IL-4 (1:1000; cat. no. ab9622; Abcam), IL-10 (1:1000; cat. no. DF6894; affbiotech), IL-13 (1:500; cat. no. ab106732; Abcam), leukemia inhibitory factor (LIF; 1:1000; cat. no. ab113262; Abcam), glycoprotein 130 (IL6ST; 1:500; cat. no. ab202850; Abcam), TNF-α-stimulated gene 6 (TSG6; 1:500; cat. no. ab128266; Abcam), transforming growth factor (TGF) β1 (1:1000; cat. no. AF1027; affbiotech) overnight at 4°C. Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (1:10000; cat. no. AS1107; Wuhan Aspen

Table I. Primer sequences of the genes used for RT-qPCR.

Target gene	Primer sequence (5'-3')	
	Forward	Reverse
β -actin	GTCCACCGCAAATGCTTCTA	TGCTGTACCTTCACCGTTC
IL-1 β	ACGATGCACCTGTACGATCACT	GAGAACACCACTTGTTGCTCCA
TNF- α	CTCTTCTCCTTCTGATCGTGG	CTTGTCACCTCGGGGTTGAG
IL-6	TCAGCCCTGAGAAAGGAGACAT	GCTCTGGCTTGTTCCTCACTACT
IL-18	TGCATCAACTTTGTGGCAATG	CTTCAAATAGAGGCCGATTTCC
IL-10	AACCTGCCTAACATGCTTCG	GAGTTCACATGCGCCTTGAT
LIF	AGGTCTTGGCGGCAGTACAC	CCAAGGTACACGACTATGCGG
IL6ST	ACTTGGAGCCAGATTCTCTCT	CCCCTTGTCTTCTCACTCCA
IL-4	GCAGTTCTACAGCCACCATGAG	TCTCTCTCATGATCGTCTTTAGCC
IL-13	CAACATCACCCAGAACCAGAAG	GCATCCTCTGGGTCTTCTCG
TSG6	GATGGATGGCTAAGGGCAGAG	CGTGTGGGTTGTAGCAATAGGC
TGF- β 1	CAGCAACAATTCCTGGCGATA	GCTAAGGCGAAAGCCCTCAAT

IL, interleukin; TNF- α , tumor necrosis factor α ; LIF, leukemia inhibitory factor; IL6ST, glycoprotein 130; TSG6, TNF- α -stimulated gene 6; TGF- β 1, transforming growth factor β 1.

Biotechnology, Co., Ltd.) for 1 h at room temperature. Proteins were visualized using ECL Western blotting kit (Invitrogen; Thermo Fisher Scientific, Inc.) and densitometric values were analyzed using AlphaEaseFC software (AlphaInnotech, Inc. version no. 4.0).

Immunofluorescence detection. Sterile coverslips were placed on 6-well plates. Suspended cell culture solutions were added to the coverslip and incubated with 5% CO₂ for 2 h at 37°C in an incubator until cells were fixed. Cell cultures were then fixed with 4% paraformaldehyde (cat. no. AS1018; Wuhan Aspen Biotechnology, Co., Ltd.) for 30 min at room temperature. After washing three times with phosphate-buffered saline (cat. no. AS1025; Wuhan Aspen Biotechnology, Co., Ltd.), the cells were incubated for 1 h at room temperature in PBS containing 5% bovine serum albumin (cat. no. 10735078001; Roche Diagnostics (Shanghai), Co., Ltd.). Cells were incubated with polyclonal rabbit anti-human COX2 (1:200; cat. no. ab52237; Abcam), IL-4 (1:100; cat. no. bs-20685R; bioss), IL-10 (1:100; cat. no. ab34843; Abcam), leukemia inhibitory factor (LIF; 1:200; cat. no. ab113262; Abcam) overnight at 4°C followed by incubation with corresponding secondary antibodies of FITC-labeled Goat Anti-Rabbit (cat. no. AS-1110), FITC-labeled Goat Anti-Mouse (cat. no. AS-1112), CY3-labeled Goat Anti-Rabbit (cat. no. AS-1109), CY3-labeled Goat Anti-Mouse (cat. no. AS-1111), CY3-labeled Donkey Anti-Goat (cat. no. AS-1113) for 50 min at room temperature. All secondary antibodies were purchased from Wuhan Aspen Biotechnology, Co., Ltd. and were used at a dilution of 1:50. Cells were then washed three times with PBS for 5 min each time. DAPI staining solution was added to each well, incubated for 5 min at room temperature and washed three times with PBS for 5 min each time. Following this, an anti-fluorescence quencher (cat. no. AS1089; Wuhan Aspen Biotechnology, Co., Ltd.) was added to the cells and samples

were viewed under a fluorescence microscope at a magnification of x200.

Statistical analysis. Data were analyzed using SPSS software (version 22.0; IBM Corp.). Data are presented as the mean \pm standard deviation and paired Student's t-test were used for comparisons between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Morphological observation of control and experimental MSCs. As presented in Fig. 1, MSCs in the control group were adherent, fibroblast-like cells, which aggregated into clusters following proliferation. MSCs cultured with CM shown suspended growth. Initially, MSCs were suspended on the surface of the CM as a single sphere; however, after ~24 h, cells aggregated spontaneously into multicellular spheroids on the surface of the CM and increased progressively (data not shown).

Effects of CM on pro- and anti-inflammatory cytokine mRNA expression in MSCs. The mRNA expression levels of pro-inflammatory genes IL-1 β , TNF- α , IL-6 and IL-18 were significantly decreased in the experimental group compared with the control group (* $P < 0.05$, ** $P < 0.01$; Fig. 2A). By contrast, the mRNA expression levels of anti-inflammatory genes TGF- β 1 were significantly increased in the experimental group compared with the control group ($P < 0.01$; Fig. 2B). The relative expression levels of IL-4 and IL-13 in the experimental group were 10-fold higher compared with the control group.

Effects of CM on pro- and anti-inflammatory cytokine protein expression in MSCs. The protein expression levels of pro-inflammatory genes IL-1 β , TNF- α , IL-6 and IL-18 in the

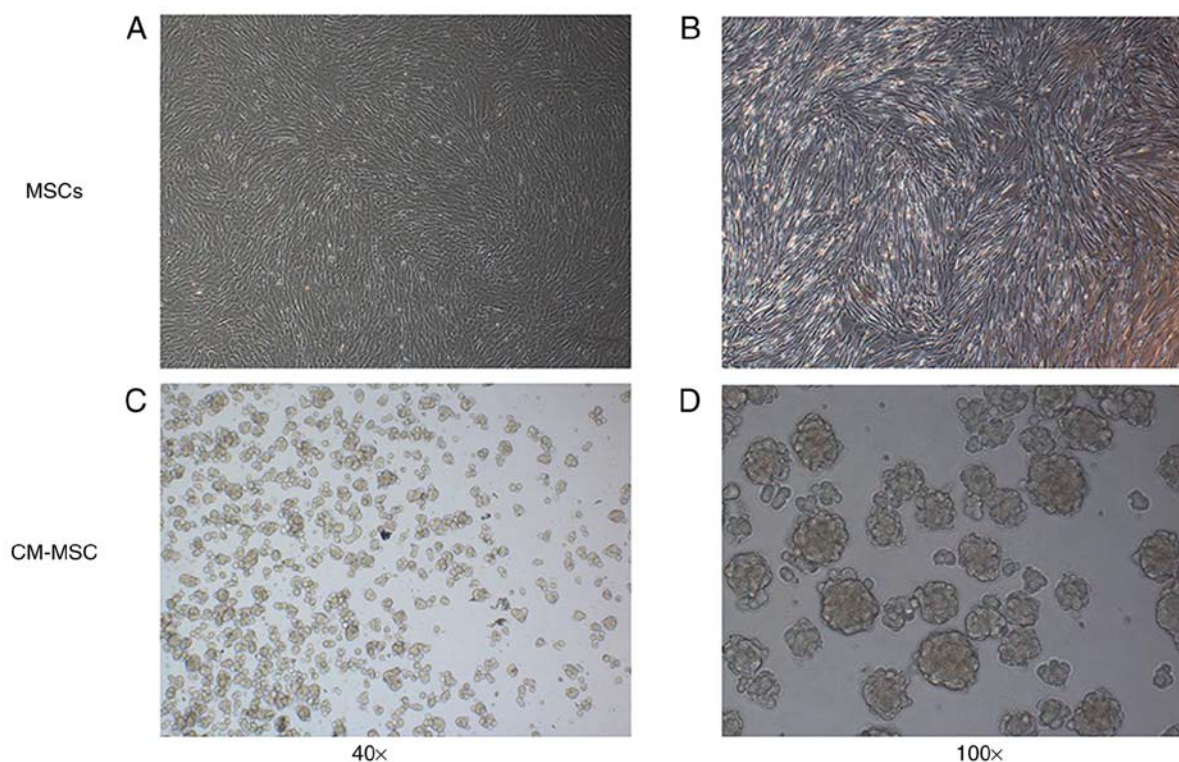


Figure 1. Morphological observation in control and experimental MSCs. Following 72 h of culturing, cell morphology in the control group at a magnification of (A) x40 and (B) x100, and the experimental group at a magnification of (C) x40 and (D) x100 was visualized. MSCs, mesenchymal stem cells; CM, chitosan membrane.

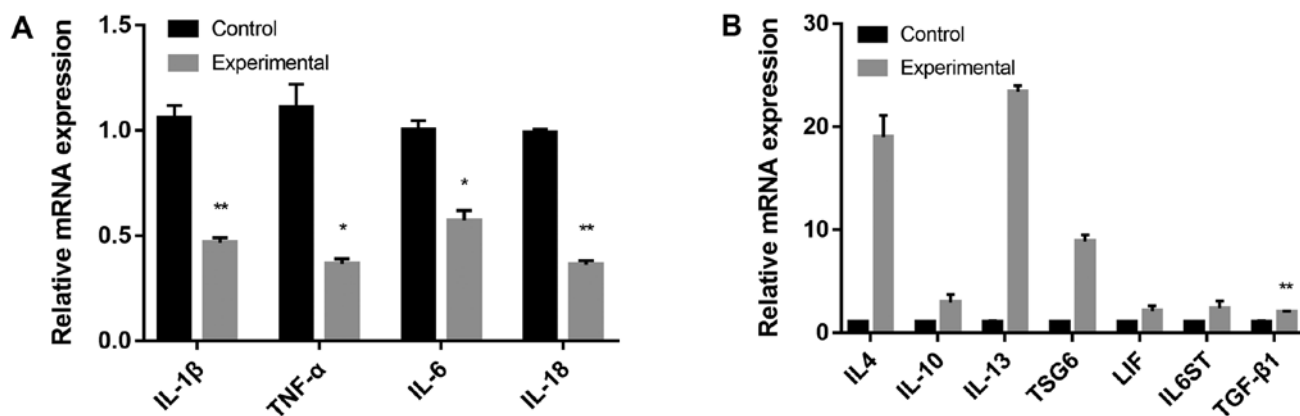


Figure 2. (A) mRNA expression levels of IL-1 β , TNF- α , IL-6 and IL-18 in the control and experimental groups. (B) The mRNA expression levels of IL-4, IL-10, IL-13, TSG6, LIF, IL6ST and TGF- β 1 in the control and experimental groups. The control group was cultured using a traditional plate culture, while the experimental group was cultured in a plate with a CM. IL, interleukin; TNF- α , tumor necrosis factor α ; TSG6, TNF- α -stimulated gene 6; LIF, leukemia inhibitory factor; IL6ST, glycoprotein 130; TGF- β 1, transforming growth factor β 1; CM, chitosan membrane. * P <0.05, ** P <0.01.

experimental group were significantly decreased compared with the control group (* P <0.05, ** P <0.01, *** P <0.001; Fig. 3). Furthermore, the protein expression levels of TGF- β 1 were significantly increased in the experimental group compared with the control group (P <0.01; Fig. 3).

Immunofluorescence observations. Proteins expressed by prostaglandin synthase-2 (COX-2), IL-4, IL-10 and LIF in the experimental and control groups were located in cells and belonged to endogenous proteins (Fig. 4). The protein content of COX-2, IL-4, IL-10 and LIF in the experimental group was higher compared to the control group, indicating that the CM

increased the expression of COX-2, IL-4, IL-10 and LIF in MSCs.

Discussion

The incidence and development of OA is characterized by the inflammatory catabolism process, which damages tissues, and the anti-inflammatory anabolism process, which protects tissues (5). Various pro- and anti-inflammatory factors mediate these processes. In OA, inflammatory cytokines interact and restrict each other to maintain the healthy metabolism of articular cartilage (1). An imbalance of inflammatory cytokines causes

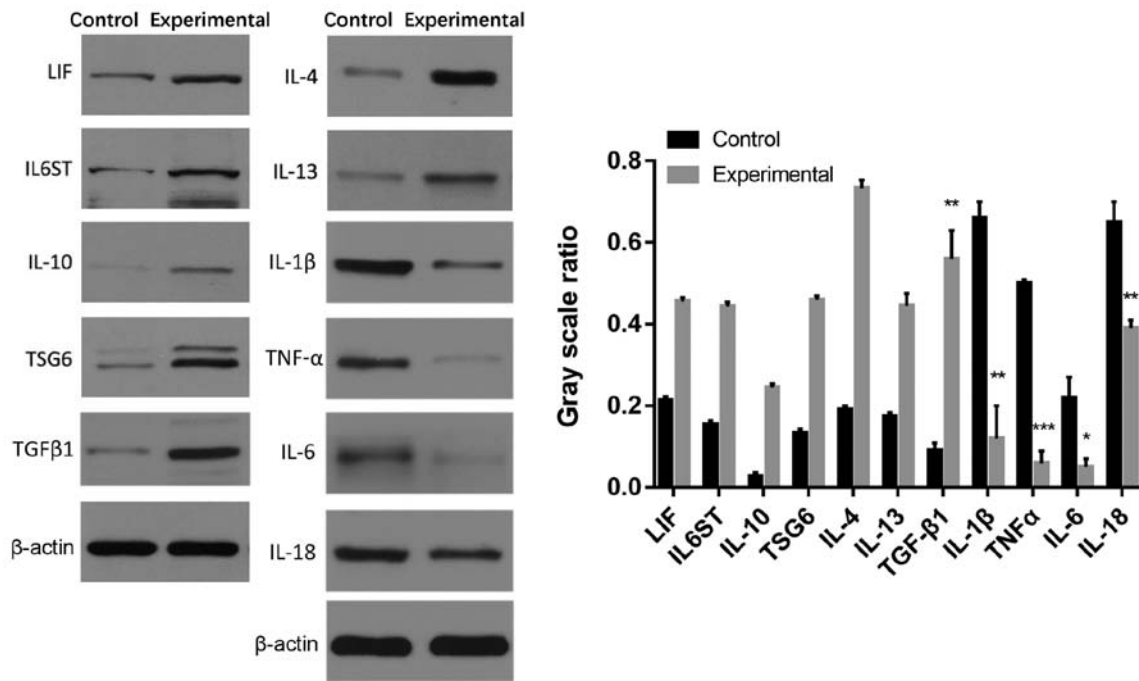


Figure 3. Protein expression of pro- and anti-inflammatory cytokines LIF, IL6ST, IL-10, TSG6, TGF-β1, IL-4, IL-13, IL-1β, TNF-α, IL-6 and IL-18 in the control and experimental groups. The control group was a traditional plate culture, while the experimental group was cultured in a plate with a CM. LIF, leukemia inhibitory factor; IL6ST, glycoprotein 130; IL, interleukin; TSG6, TNF-α-stimulated gene 6; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor α; CM, chitosan membrane. *P<0.05, **P<0.01, ***P<0.001.

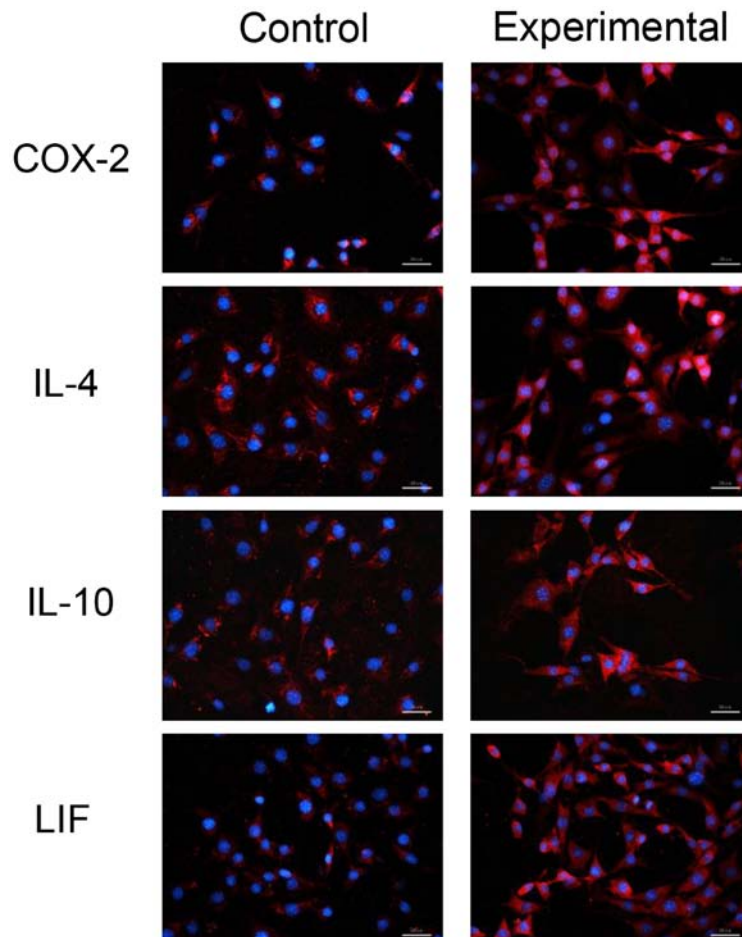


Figure 4. Distribution of COX-2, IL-4, IL-10 and LIF in MSCs in the control and experimental groups. Blue fluorescence represents nuclei. Red fluorescence represents the cytoplasm. The expression level of COX-2, IL-4, IL-10 and LIF in the experimental group was higher compared with that in the control group. Scale bars: 50 μm. COX-2, prostaglandin synthase-2; IL, interleukin; LIF, leukemia inhibitory factor.

the abnormal metabolism of articular cartilage, which leads to deformation, loss and degradation of articular cartilage (3).

In the pathogenesis of OA, pro-inflammatory cytokines IL-1 β and TNF- α induce a release of inflammatory mediators via the regulation of NF- κ B and microtubule-associated protein kinase (MAPK) signaling pathways, causing articular cartilage degeneration, destruction and degradation. The pro-inflammatory effects of IL-1 β and TNF- α in the pathogenesis of OA have been confirmed (16-18). Previous studies have demonstrated that IL-1 β and TNF- α promote the absorption and degradation of articular cartilage, causing the occurrence of OA (19,20). In the pathogenesis of OA, IL-1 β and TNF- α initially bind to their respective receptors and regulate the release of pro-inflammatory cytokines, including IL-1 β , TNF- α , IL-6 and IL-18 via the NF- κ B and MAPK signaling pathways (21). The expression levels of a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4), ADAMTS-5, nitric oxide and prostaglandin E₂ are upregulated and the contents of matrix metalloproteinase (MMP)-1, MMP-3 and MMP-13 are increased in chondrocytes and synovial fibroblasts, thereby enhancing the inflammatory reaction, promoting extracellular matrix (ECM) degradation, inhibiting extracellular matrix synthesis and, ultimately, leading to articular cartilage degeneration, destruction and degradation (3).

Furthermore, anti-inflammatory cytokines serve important roles in the pathogenesis of OA by inhibiting pro-inflammatory cytokines, downregulating the expression of MMPs, hindering the inflammatory response, destroying articular chondrocytes, promoting the synthesis of proteoglycans and collagen in chondrocytes, and inhibiting the progression of OA. Anti-inflammatory cytokines in OA primarily include IL-4, IL-10, IL-13, LIF, IL6ST, TSG6 and TGF- β 1 (22).

Previous studies have reported that IL-4 inhibits IL-1 and induces the release of MMP-13, thereby preventing the degradation of cartilage ECM, protecting cartilage integrity and delaying the process of OA (23,24). Furthermore, IL-4 has been revealed to inhibit the expression of ADAMTS-4 and ADAMTS-5, delay the degradation of articular cartilage ECM, and promote the synthesis of proteoglycans and collagen in chondrocytes (23). Reportedly, IL-10 inhibits the synthesis and secretion of IL-6 and other related pro-inflammatory cytokines, thus serving a role in the regulation of inflammatory immunity (25). Additionally, IL-10 inhibits the release of MMP-1, MMP-3, MMP-13 and nitric oxide in cartilage tissues, inhibits the inflammatory reaction and destruction of articular chondrocytes, and promotes the synthesis of proteoglycan and collagen in chondrocytes (23). TGF- β is a cytokine secreted by various cells and has a regulatory effect on cell growth, differentiation and immune function (26). Previous studies have demonstrated that TGF- β promoted the expression of metalloproteinase inhibitor-4 RNA, inhibited the activity of MMPs, reduced the formation of MMPs and protected cartilage, which delayed the development of OA (27,28). Furthermore, TGF- β promotes chondrocyte DNA synthesis, increases the number of chondrocytes and repairs inflammation-induced cartilage damage (29).

MSCs are multipotent, undifferentiated cells with extensive differentiation potential and self-replication abilities. MSCs have become a focus of interest in the treatment of OA as they can be easily isolated and exhibit *in vitro* proliferative abilities, plasticity, immunosuppressive characteristics,

autocrine- and paracrine-mediated effects, migration ability to injured sites, and phenotypic stability (9). MSCs involved in tissue regeneration and can migrate to injured sites. In the local microenvironment, the directional differentiation of MSCs into specific types of functional cells directly participates in the process of tissue repair (30). Furthermore, MSCs secrete various bioactive substances, including cytokines and growth factors, which can improve microenvironment regeneration of the injured sites and inhibit local inflammation (31). Previous studies have confirmed that intra-articular injection of MSCs can be used to treat early arthritis, protect articular cartilage and increase the expression of certain anti-inflammatory-related genes and cartilage-protective factors (32,33).

The survival rate of MSCs amplified by traditional adherent cultures in clinical and animal experiments is low and biological activities and therapeutic effects are poor (32). The division and differentiation of MSCs are closely associated with their microenvironment, and cytokines and proteins in the microenvironment all influence the differentiation of MSCs (34). Previous studies have demonstrated that 3D cell cultures can produce multicellular spheres and reproduce the microenvironment and associated physiological activities *in vivo* (35,36). Therefore, 3D MSCs exhibit stronger biological functions and therapeutic effects compared with traditional adherent MSCs (37). The results of the present study revealed that a 3D CM culture was more effective in maintaining the anti-inflammatory properties of MSCs compared with a 2D traditional culture.

CS has high biocompatibility, no immunogenicity and low toxicity (38). The present study demonstrated that CM-cultured MSCs overcame the shortcomings of plate cultures. Similarly to the 3D culture model of hanging drop culture, CM-cultured MSCs form spheroids and exhibit enhanced transformation efficiency (39). Previous studies have reported that MSCs adhering to the CM can self-assemble to form 3D spherical cells (14). During this process, MSCs adhere and diffuse on the CM and reduce the number of pseudopods to form multicellular spheres. This aggregation process is different from that of suspensions or suspension cultures (13).

It has been reported that the formation of spheroids in the CM is associated with the involvement of various genes and proteins, including cadherin (40,41), the Rho/Rho-associated protein kinase pathway (12) and Wnt (41). Although certain changes in gene and protein expression levels have been observed, the exact mechanism of spheroid formation in the CM remains unclear.

3D suspension cultures of MSCs form multicellular spheroids, affect the epigenetic status of MSCs and improve the anti-inflammatory properties of MSCs (12). The formation of multicellular spheres affects the expression of inflammatory cytokines in MSCs. The results of the present study demonstrated that, in the CM group, the expression levels of pro-inflammatory genes IL-1 β , TNF- α , IL-6 and IL-18 were significantly lower compared with the control group, and the expression levels of anti-inflammatory genes TGF- β 1 were significantly higher compared with the control group, indicating that the formation of multicellular spheres in CM-cultured MSCs was more conducive to maintaining the anti-inflammatory characteristics of MSCs.

The present study had certain limitations. Firstly, the amount of cytokines secreted by CM-cultured MSCs compared with MSCs was not examined. Secondly, cells were identified

as MSCs by cell morphology. Further cell type identification assays would have been beneficial.

In conclusion, compared with the control group, CM-cultured MSCs formed multicellular spheres, significantly decreased the expression of pro-inflammatory cytokines and significantly increased the expression of anti-inflammatory-related genes, indicating that CM cultures could enhance the anti-inflammatory properties of MSCs. Although the role of pro- and anti-inflammatory cytokines in the pathogenesis of OA remains unclear, the present study provided a novel approach that may be beneficial for the OA research.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XX and PY reviewed literature, researched data and drafted the manuscript. PY was involved in revising the manuscript and participated in the interpretation of data. HL made contributions to the acquisition of data. BQ was responsible for the conception, design of the study and revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Renmin Hospital of Wuhan University, Wuhan, China.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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