Carboxymethylated chitosan protects rat chondrocytes from NO-induced apoptosis via inhibition of the p38/MAPK signaling pathway

BIN HE, HAIYING TAO, SHIQING LIU, AILIN WEI, FENG PAN, REN CHEN and XIAOHAI LI

Department of Orthopaedics, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Abstract. In the present study, the effect of carboxymethylated chitosan (CMCS) on nitric oxide (NO)-induced apoptosis, and activation of the p38/MAPK signaling pathway in chondrocytes were investigated. Cartilage was isolated from the knee joints of Sprague-Dawley rats, and was used to establish cultured primary chondrocytes. The chondrocytes were incubated with the NO donor, sodium nitroprusside (SNP), to induce apoptosis, and were treated with CMCS and the p38 inhibitor, SB203580. Cell viability was assessed using a Cell Counting Kit-8 assay. Apoptosis of the chondrocytes was detected using Annexin V-fluorescein isothiocyanate/propidium iodide staining. The activation of p38 was detected using Western blot analysis, and caspase-3 activity was detected using a caspase-3 detection kit. The results indicated that, in chondrocytes treated with SNP, the optical density values of the experimental groups were significantly lower, compared with the control group (P<0.05). The exposure of the cells to CMCS significantly prevented apoptosis (P<0.05), and a dose-dependent effect was demonstrated using fluorescence-activated cell sorting analysis (P<0.05). Examination of the expression and activity of p38 and caspase-3, respectively, showed that SNP increased the expression of p38 and activity of caspase-3, and this trend was reversed following the addition of CMCS and SB203580. Taken together, these findings indicated that CMCS prevented NO-induced apoptosis of chondrocytes via inhibition of the p38/mitogen-activated protein kinase signaling pathway in vitro.

Introduction

Osteoarthritis (OA) is a common, predominantly age-related joint disorder, which is characterized by the loss of chondrocytes, degradation of the extracellular matrix (ECM), subchondral bone remodeling and synovial inflammation (1). It has been demonstrated that chondrocyte apoptosis is associated with the initiation and severity of articular cartilage degradation (2), and chondrocyte apoptosis increases in human OA cartilage (3). Chondrocyte apoptosis is predominantly responsible for the progressive cartilage degradation, which occurs in osteoarthritis (OA) and has gradually become one of the potential therapeutic targets for OA (4).

Mitogen-activated protein kinase (MAPK) is a serine/threonine kinase, the signaling cascades of which control complex programs, including proliferation, differentiation and apoptosis (5,6). There are three major classes of MAPKs in mammals, the extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinase and p38 (7). Chondrocyte apoptosis in experimental OA is regulated by the p38/MAPK signaling pathway (8), and the p38/MAPK signal transduction pathway has also been implicated as a critical factor in nitric oxide (NO)-induced articular chondrocyte apoptosis in rabbits (9). The p38/MAPK cascades can be activated or phosphorylated by a series of inflammatory factors and stimuli from the environment, and phosphorylated (p)-p38 is then shuttled into the nucleus, where it activates transcription factors, initiates the expression of upstream apoptosis-associated genes and promotes the apoptosis of chondrocytes (10,11). Caspase-3 is a key member of the caspase family, which can cleave DNA repair proteins, including poly (ADP-ribose) polymerase, cytoskeletal proteins and inhibitor of caspase-activated deoxynucleosidase, thereby leading to the fragmentation of DNA and eventual cell apoptosis (12). NO is an important physiological and pathological signaling molecule, which can be produced by inflammatory stimuli, including sodium nitroprusside (SNP). It can inhibit the proliferation of chondrocytes and induce chondrocyte apoptosis, thereby damaging cartilage and impairing joint cartilage repair (13). NO can induce chondrocyte apoptosis through multiple pathways, including the p38/MAPK pathway. Inhibiting p38 signaling may effectively inhibit these negative effects, prevent chondrocyte apoptosis and promote cartilage repair.

Carboxymethylated chitosan (CMCS) is a soluble derivative of chitosan, which possesses several desirable physiochemical and biological features. Our previous studies showed that CMCS significantly suppresses the degeneration of cartilage in OA and protects chondrocytes from interleukin-1β-induced...
apoptosis (14, 15). It has been found that CMCS can stimulate the proliferation and synthesis of nerve growth factor in Schwann cells (SCs) by activation of the MAPK kinase (MEK)/ERK, phosphatidylinositol 3-kinase/Akt and Wnt/β-catenin signaling pathways (16,17), and protects SCs and nucleus pulposus cells from hydrogen peroxide-induced apoptosis (18,19). However, whether CMCS can inhibit chondrocyte apoptosis through inhibiting p38/MAPK signaling remains to be elucidated.

The aim of the present study was to investigate whether CMCS can inhibit chondrocyte apoptosis using an in vitro model of rat articular chondrocyte apoptosis. Numerous studies have indicated that the p38/MAPK signaling pathway is one of the most important signaling pathways in NO-induced cell apoptosis, including chondrocytes (9). Therefore, the present study also aimed to elucidate the mechanism underlying the protective role of CMCS on chondrocytes by examining activation of the p38/MAPK signaling pathway.

Materials and methods

Animals and reagents. A total of 24 healthy Sprague-Dawley (SD) rats (3 weeks old) with an average body weight of 362±35 g were obtained from the Experimental Animal Center of Wuhan University (Wuhan, China). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA), fetal bovine serum (FBS) was obtained from GE Healthcare Life Sciences (Logan, UT, USA). CMCS (purity >99%) was supplied by the Institute of Chemistry and Environmental Science of Wuhan University (Wuhan, China). Primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). Anti-p38 and anti-p-p38 antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-β-actin antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A Caspase-3 Activity Assay kit (cat. no. C1115) was purchased from the Beyotime Institute of Biotechnology (Haimen, China). A Cell Counting Kit-8 (CCK-8; cat. no. CK04) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). SB203580 (cat. no. S3807), type-2 collagenase (cat. no. C6885) and SNP (cat. no. C1115) were obtained from Sigma-Aldrich (St. Louis, MO, USA). An Annexin V-Fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit (cat. no. 556547) was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Other commonly used reagents were of high purity and commercially available.

Cell isolation and culture. Rat articular chondrocytes were isolated and cultured, as previously described (20). The utilization of rat articular cartilage was approved by the Animal Ethics Committee of Wuhan University. Rats were anesthetized with a single intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg; purchased from Sigma-Aldrich; cat. no. 1507002). The articular cartilage tissue was removed from the knee joints of the 3-week-old rats using a scalpel. The extracted cartilage was cut into 1x1x1 mm³ sections. Animals were sacrificed by an overdose of sodium pentobarbital. Subsequently, chondrocytes were isolated from the extracted cartilage by enzymatic digestion with 0.25% trypsin (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 1 h, and 0.2% type-2 collagenase (381 U/mg; Sigma-Aldrich) in DMEM for 4 h. Following collection of individual cells by brief centrifugation, the chondrocytes were resuspended in DMEM supplemented with 10% FBS, 100 U/ml streptomycin and 100 U/ml penicillin (HyClone, Logan, UT, USA). The culture medium was replaced every other day, and the second and third passage of chondrocytes were used in the subsequent experiments.

Cell treatment and grouping. To establish the apoptotic model of cultured chondrocytes, SNP was used, which generates NO (21). Briefly, the chondrocytes (5x10⁴ cells/cm²) were seeded and cultured at 37°C for 24 h, following which the cells were randomly divided into five groups, in which the medium was replaced with complete medium (DMEM with 10% FBS and antibiotics) containing 0, 0.5, 1, 2 or 3 mM SNP, cultured at 37°C for different durations (3, 6, 12 and 24 h). CMCS was added into the culture medium at different concentrations (50, 100 and 200 µg/ml). Subsequently, in order to determine the activation of the p38/MAPK signaling pathway, the p38/MAPK specific inhibitor, SB203580 was used (22).

Cell viability assay. The chondrocytes were cultured in 96-well microplates at a density of 2x10⁴ cells per well with DMEM containing 0.1% FBS at 37°C for 24 h, and then exposed to different concentrations of SNP (0.5, 1, 2 and 3 mM), CMCS (50, 100 and 200 µg/ml) or SB203580 (10 µM) for 24 h, and for different durations (0, 3, 6, 12 or 24 h). For the quantitative analysis of the cell viability, 10 µl CCK-8 solution was added to each well and incubated at 37°C for 1 h. The optical densities were measured at 450 nm using an ELx800 Absorbance Microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Cell viability was determined as a percentage of the number of control (untreated) cells. Viability in the control group was designated as 100%, and cell viability in the treatment groups was determined as follows:

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\text{Viability} = \left( \frac{Ae - Ab}{Ac - Ab} \right) \times 100\%
\]

Where Ae and Ac represent the A450 of the experimental, blank and control groups, respectively. All experiments were performed in triplicate in three independent experiments.

Flow cytometric analysis. The apoptotic rates of the chondrocytes was measured using flow cytometry (FCM), according to the manufacturer’s protocol of the Annexin V-FITC/PI Apoptosis Detection kit. In brief, the chondrocytes were treated with SNP (0.5, 1, 2 and 3 mM), CMCS (50, 100 and 200 µg/ml) or SB203580 (10 µM). Following culture of the chondrocytes for the indicated durations (0, 3, 6, 12 or 24 h), trypsinization was performed with 0.25% trypsin without ethylene diamine tetraacetic acid for analysis of apoptosis using the Annexin V-FITC/PI Apoptosis Detection kit. Cell apoptosis was analyzed using a BD FACSVerse™ flow cytometer (Becton Dickinson, Heidelberg, Germany) at 488 nm. The data were analyzed using CellQuest™ software (version 4.01; Becton Dickinson).

Caspase-3 activity assay. Caspase-3 activity was determined using a colorimetric assay kit (cat. no. C1115; Beyotime Institute of Biotechnology). The chondrocytes were first cultured in
a mixture of DMEM supplemented with 5% FBS, SNP and CMCS. Following incubation at 37°C for 24 h, the cells were treated, according to the manufacturer's protocol. The cell lysates were prepared, and assays were performed in 96-well plates (at a density of 2x10^4 cells/well) by incubating 10 μl cell lysate per sample in 80 μl reaction buffer (1% NP-40, 20 mM Tris-HCl, 137 mM NaCl and 10% glycerol) containing 10 μl caspase-3 substrate (Ac-DEVD-pNA; Beyotime Institute of Biotechnology). The cell lysates were incubated at 37°C for 4 h. The absorbance at 405 nm was read using an ELx800 Absorbance Microplate reader (BioTek Instruments, Inc.). Caspase-3 activities were determined as the percentage of enzyme activity, compared with the control. All experiments were performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The mRNA of the treated chondrocytes was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. An ABI Prism 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to detect the mRNA expression levels of the target genes. The sequences of the primers used, for B cell lymphoma (Bcl)-2, Bcl-2-associated X protein (Bax) and GAPDH, are shown in Table I.

For RT reaction, 5 μl mRNA was reverse transcribed into cDNA and amplified using specific reverse primers of the target genes using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). The PCR reaction mixture included 1 μl forward and reverse primers (10 μM), 1 μl SYBR Green I fluorescent dye and 1 μl cDNA. The PCR conditions were as follows: Initial step at 95°C for 2 min, followed by 40 cycles at 95°C for 20 sec and 60°C for 40 sec. The relative expression levels of target genes were analyzed using the comparative cycle threshold method (23). Data were standardized by GAPDH. The results were obtained from three independent experiments.

Western blot analysis. The chondrocytes were treated with SNP, with or without CMCS, following which the chondrocytes were lysed in lysis buffer at 4°C for 30 min. The lysates were centrifuged at 13,000 g or 15 min at 4°C. The supernatants were collected and stored at -80°C. Total protein (50 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology) and electroblotted onto polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). After being blocked with 5% non-fat milk in Tris-buffered saline, the membrane was incubated overnight at 4°C with primary antibodies against p38 (rabbit polyclonal IgG, cat. no.9212; Cell Signaling Technology, Inc.), p-p38 (rabbit polyclonal IgG, cat. no. 9211; Cell Signaling Technology, Inc.) and β-actin (rabbit polyclonal IgG, cat. no. sc-10731; Santa Cruz Biotechnology, Inc.) at dilutions of 1:200 for 2 h at room temperature. Subsequently, the membrane was incubated with a peroxidase-conjugated secondary antibody (goat anti-rabbit horseradish peroxidase-conjugated polyclonal IgG, cat. no. sc-45101; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Signals were detected by enhanced chemiluminescence. Optical band density was quantified using a Geliance 200 Imaging system (PerkinElmer, Waltham, MA, USA) and Gene Snap software (version 6.08.04; Syngene, Cambridge, UK). The values for p38 and p-p38 were normalized to that of β-actin.

Statistical analysis. The data are presented as the mean ± standard deviation. Statistical significance was analyzed by one-way analysis of variance using SPSS, version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

CMCS increases cell viability in SNP-induced chondrocytes.

To determine the protective effects of CMCS on chondrocytes, a model of apoptosis was established in the present study by exposure of chondrocytes to SNP, an inorganic compound with the formula Na[Fe(CN)]_6·2H_2O. As shown in Fig. 1A, SNP induced a dose-dependent cytotoxic effect on the chondrocytes, and treatment with 3 mM SNP for 24 h induced a decrease in cell viability by >90%. In addition, treatment with 3 mM SNP for different durations induced a time-dependent cytotoxic effect (Fig. 1B). Therefore, the results indicated that SNP induced dose- and time-dependent cytotoxic effects on the chondrocytes. Based on these data, chondrocytes treated with 3 mM SNP for 24 h were used as the in vitro apoptosis model for subsequent experiments. To investigate the protective effects of CMCS on chondrocytes, the chondrocytes were pretreated with different concentrations of CMCS for 24 h prior to SNP induction, following which cell viability was measured. As shown in Fig. 1C, CMCS caused an increase in SNP-induced cytotoxicity, indicating that CMCS protected the chondrocytes from the SNP-induced decrease in viability, and may offer potential as a potent drug to treat OA.

CMCS inhibits SNP-induced apoptosis in rat chondrocytes.

To confirm the apoptosis-inducing effects of SNP, Annexin V-FITC/PI staining, followed by FCM was performed. As shown in Fig. 2, the numbers of normal cells in the SNP-induced groups were significantly lower, compared with the number in the control group, whereas the numbers of early apoptotic,

| Table I. Primer sequences of target genes. |
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| Gene | Primer sequence | Product size (bp) |
| Bcl-2 | F: 5'-GGCCGTCACAGGGAGATGTCA-3' | 225 |
| | R: 5'-GGATGTCACCCAGAGTATG-3' | 209 |
| Bax | F: 5'-GGCGGAGATTGAGATGAACTG-3' | 256 |
| | R: 5'-GATGTCACCTGGGCACTTTAG-3' | 256 |
| GAPDH | F: 5'-TGTCTTCCGACCTAACCAG-3' | 256 |
| | R: 5'-GAGGCCCATGTAGGCCATGAG-3' | 256 |

Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein; F, forward; R, reverse.
late apoptotic and dead cells were significantly higher in number, compared with those in the control group. Increasing concentrations and durations increased the apoptotic rate, and the maximum pro-apoptotic effect was observed following treatment with 3 mM SNP for 24 h. These results are consistent with previous studies, demonstrating that SNP treatment leads to chondrocyte apoptosis (24,25). Following treatment with different concentrations of CMCS, the proportion of early apoptotic, late apoptotic and dead cells decreased, and there was a dose-dependent effect of CMCS treatment, with 200 µg/ml CMCS exerting the most marked inhibitory effect on SNP-induced apoptosis.

**CMCS inhibits the activation of p38 and decreases caspase-3 activity.** To further examine the mechanism underlying the effects of CMCS on signaling cascades, the present study analyzed the activation of p38/MAPK. As shown in Fig. 3, quantification of the Western blot bands showed that the protein levels of p-p38 were significantly higher in the SNP-treated groups, compared with the control group. Following treatment with CMCS or the p38 inhibitor, SB203580, the expression of p-p38 was decreased. As shown in Fig. 4, caspase-3 activity was significantly increased, to 63% of the control, in the SNP-induced chondrocytes, however, pretreatment with 50, 100 or 200 µg/ml CMCS attenuated the SNP-induced increase in caspase-3 activity by ~41, 54 and 56%, respectively.

**CMCS increases the expression of Bcl-2 and decreases the expression of Bax in SNP-induced chondrocytes.** In order to further investigate the mechanisms underlying the effects of CMCS on SNP-induced chondrocyte apoptosis, the mRNA expression levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 were detected using RT-qPCR analysis. As shown in Fig. 5A, the mRNA expression levels of Bcl-2 were markedly elevated in the CMCS-treated chondrocytes, compared with the 3 mM SNP-treated group without CMCS, at CMCS concentrations of 50 µg/ml (1.68±0.16-fold), 100 µg/ml (2.23±0.21-fold) and 200 µg/ml (4.12±0.23-fold). As shown in Fig. 5B, 3 mM SNP significantly increased the mRNA level of Bax (2.14±0.17-fold), compared with the control group. This increase was reversed by the addition of CMCS. These findings indicated that CMCS inhibited the mRNA expression of Bax and promoted the mRNA expression of Bcl-2 in the SNP-induced chondrocytes.

**CMCS prevents SNP-induced apoptosis via p38/MAPK.** As p38/MAPK was found to be activated in response to SNP, the present study proceeded to address the role of p38/MAPK in the CMCS-induced inhibition of chondrocyte apoptosis, by treating the chondrocytes with SB203580, a specific inhibitor of p38 activation. The chondrocytes were pretreated with 10 µM SB203580 for 1 h and then stimulated with CMCS for 24 h. A subsequent CCK-8 assay showed that pretreatment with CMCS and/or SB203580 decreased the inhibitory effect of SNP on viability (Fig. 6A). In addition, FCM showed that pretreatment with CMCS or SB203580 prevented SNP-induced apoptosis (Fig. 6B). These findings suggested that CMCS prevented NO-induced apoptosis, possibly through the inhibition of p38/MAPK activation.

**Discussion**

CMCS has shown therapeutic effects on non-alcoholic fatty liver disease and hydrophobic drug delivery (26,27). Our previous study indicated that CMCS inhibits chondrocyte, SC and nucleus pulposus cell apoptosis *in vitro* (15,18,19). However, the mechanism or signaling pathway underlying the effect of CMCS in OA remains to be fully elucidated. The results of the present study indicated that pre-treatment with CMCS in SNP-induced chondrocytes promoted the expression of Bcl-2, inhibited the expression of Bax and reduced caspase-3 activation. These effects by CMCS are partially mediated via the inhibition of p38/MAPK signaling.
OA is a degenerative joint disease with multiple underlying pathogenic mechanisms, caused by various risk factors, including NO (28), polychlorinated biphenyl 126 (29), bupivacaine and levobupivacaine (30), and C/EBP homologous protein (31) has been reported to induce apoptosis in chondrocytes. As these apoptosis-inducing substances may be involved in the pathogenesis of OA, manipulation of the mechanism mediated by these stimuli has the potential for substantial therapeutic effects.

The loss of chondrocyte function, ECM degradation and apoptosis are crucial in the progression of OA, thus inhibiting the development of these factors is important for the treatment of OA. To the best of our knowledge, the signaling pathways involved in the effects of CMCS on OA remain to be fully elucidated. In the present study, the anti-apoptotic effect of CMCS was evaluated using a classical apoptosis model induced by NO.

Chondrocyte apoptosis is key in the degeneration and degradation of articular cartilage in cases of OA. Reduced cellularity is a characteristic feature of OA cartilage, and apoptosis has been suggested as an underlying cause (32).

In the present study, the results of a CCK-8 assay revealed that cell viability in the articular chondrocytes following...
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SNP induction were markedly decreased, compared with those in normal chondrocytes, and cell viability correlated positively with the percentage of apoptotic chondrocytes. This suggested that reduced cellularity in OA cartilage may, at least partially, be attributed to cell death by apoptosis. The results from the FCM showed that CMCS suppressed apoptosis of the SNP-induced chondrocytes. These results were confirmed in the in vitro experiments in the present study, which revealed that CMCS inhibited the increase in the proportion of early and late apoptotic cells in the NO-induced chondrocytes, suggesting that CMCS prevented the degeneration and degradation of articular cartilage by inhibiting chondrocyte apoptosis.

Apoptosis is an autonomous process of programmed cell death, regulated by multiple signaling pathways. One of the important pathways during the apoptotic process involves the activation of caspase-3, and induction of the hydrolysis of nucleic acids and cytoskeletal proteins (33). In vitro studies have confirmed that caspase-3 activity is increased significantly in NO-induced chondrocyte apoptosis (34,35). Notably, caspase-3 is activated by cytochrome c (Cyt c), which is induced by apoptotic signals. These signaling events are set in motion by the pro-apoptotic protein, Bax, a member of the Bcl-2 family, which migrates to the mitochondrial membrane and induces the release of Cyt c (36,37). Caspase-3 is a member of the downstream Cyt c signaling pathway, and is the most important executor of cell apoptosis. A previous study reported that millimeter wave treatment inhibited p38/MAPK signaling and inhibited caspase-3 activation and chondrocyte...
apoptosis (20). Therefore, inhibiting the activation of caspase-3 may be the key to decreasing chondrocyte apoptosis. The results of the present study indicated that CMCS inhibited the activation of caspase-3 induced by NO, and this may be one of the most important anti-apoptotic mechanisms for CMCS.

The present study demonstrated that CMCS treatment inhibited NO-induced effects on chondrocytes in vitro, including apoptosis and the expression of caspase-3, and effects involving inhibition of the p38 MAPK signaling pathway. CMCS (50, 100 and 200 µg/ml) protected chondrocytes from SNP-induced apoptosis, and inhibited the apoptotic rates of NO-induced rat chondrocytes, with effects on the expression levels of Bcl-2 and Bax. CMCS also reduced caspase-3 activity in the SNP-induced chondrocytes, and suppressed p38 phosphorylation, suggesting that the p38/MAPK signaling pathway may represent a target for the effects of CMCS, which may offer potential in the treatment of OA.

There were several limitations to the present study. Notably, all experiments were performed in a cell-based in vitro system, and whether equivalent effects are be observed consistently in an in vivo model and in patients remains to be elucidated. Furthermore, all data were acquired with CMCS-pretreated cells, which appeared to be essential for the protective effects of the compound in the present study and in previous reports (15-19). Further investigations, to examine whether the pre-treated cells have altered signaling pathways in SNP and other biological processes, may assist in improving the preparation of cells against apoptotic treatment.

In conclusion, the present study demonstrated that CMCS inhibited SNP-induced chondrocyte apoptosis, promoted the expression of Bcl-2, and inhibited the expression of Bax and caspase-3 activity via its effects on the p38/MAPK signaling pathway. Collectively, these results indicated that CMCS may have a potential therapeutic role for the treatment of OA.

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References


