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Chrysin protects human osteoarthritis chondrocytes by inhibiting inflammatory mediator expression via HMGB1 suppression

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Abstract. High-mobility group box chromosomal protein (HMGB-1) contributes to osteoarthritis (OA) by modulating various oxidative, inflammatory and apoptotic signaling pathways. The effect of chrysin (CH), a natural plant flavonoid, and its functional interaction with HMGB-1, was investigated in a chondrocyte model of OA. Human chondrocytes were pre-treated with CH, and then subsequently treated with IL-1β to induce the formation of chondrocytes similar to those found in OA joints. Next, the expression level of HMGB-1 was determined by immunofluorescence and western blot analysis. Additionally, inflammatory factor expression was measured by ELISA, and cell apoptosis was analyzed with flow cytometry. To further explore the effects of CH, HMGB-1 expression was silenced following CH treatment with small interfering (si)RNA. The results demonstrated that CH inhibited cell apoptosis, dose-dependently reduced matrix metalloproteinase (MMP) 13, collagenase and IL-6 expression, and increased collagen α-1 (II) chain (COL2A1) expression in human osteoarthritis chondrocytes. These effects of CH were accompanied by decreased HMGB-1 expression. Additionally, the expression of MMP13, collagenase, IL-6 and COL2A1, as well as apoptosis, was significantly reduced by HMGB-1 siRNA. These results demonstrated that HMGB-1 is critical for the protective effect of CH on human osteoarthritis chondrocytes, including cell apoptosis and inflammatory factor inhibition, which suggests that CH may have potential therapeutic effect in treating OA by protecting human osteoarthritis chondrocytes via HMGB1 suppression.

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Introduction

As the most common type of human arthritis and musculoskeletal disease, osteoarthritis (OA) is a degenerative and chronic joint disorder caused by the deterioration of hyaline cartilage, accompanied by chondrocyte hypertrophy, angiogenesis, chondrogenesis, and variable degrees of inflammation without systemic effects (1,2). Several important inflammatory factors, including interleukin (IL)-1β, tumor necrosis factor α, nitric oxide, matrix metalloproteinases (MMPs) and eicosanoids, are actively synthesized during dysfunctional cartilage homeostasis, which result in increased nuclear factor (NF)-κB and catabolic activity (3-5). Although numerous studies have revealed the contribution of genetic factors to OA, inflammatory factors and altered chondrocyte responses also contribute to OA progression (6-8). The etiology of OA is thought to be influenced by aging, genetics, trauma and obesity. Furthermore, a molecular target for treating OA has yet to be identified (9,10). As a result, the current treatment options predominantly consist of pain management, and no disease-modifying agent to effectively treat OA is currently available.

High-mobility group box chromosomal protein (HMGB-1) is a ubiquitous nuclear DNA-binding protein with a mass of ~27 kDa, which contains an amino acid sequence that is highly conserved between rodents and humans (11). Activation of HMGB-1 is typically triggered by necrotic cells, macrophages or other myeloid cells in response to an inflammatory stimulus (12). Previous studies have detected high levels of HMGB-1 in the synovial fluid of patients with

rheumatoid arthritis and collagen-induced arthritis animal models (11,13). These high HMGB-1 levels were shown to induce MMP and cytokine production, as well as angiogenesis, by enhancing oxidative stress *in vitro* (14). In addition, *in vitro* studies suggest that the chondrocyte hypertrophy and increased synthesis of type X collagen caused by OA may be driven by the HMGB-1 receptor, the receptor for advanced glycated end-products (RAGE) (15). As an important pro-inflammatory mediator, HMGB1, along with its receptor, have been associated with the onset and progression of cancers and arthritis (16,17); however, a limited number of studies have investigated HMGB1 and its various downstream genes as possible therapeutic targets in OA (18,19).

Chrysin (CH; 5,7-dihydroxyflavone), an important natural plant flavonoid, has been demonstrated to exert antioxidative, anti-allergic, anti-inflammatory, antifibrotic and anti-apoptotic effects in the central nervous and immune systems (20,21). However, few studies have investigated the potential use of CH for treating OA, despite the recently demonstrated ability of CH to inhibit inflammatory factor stimulation, and to produce therapeutic effects in human OA chondrocytes *in vitro* (22).

Results of previous studies have suggested an upregulation of HMGB-1 and inflammatory cytokine expression, including IL-6 or IL-8, in OA cartilage (23,24). Accordingly, the present study was designed to determine whether treatment with CH improved the characteristics of human OA chondrocytes by activating HMGB1, and thereby altering the production of inflammatory factors. The alterations in cellular function and inflammatory factors which occurred following HMGB-1 silencing were also examined. To the best our knowledge, this is the first study to evaluate the protective effects of CH, and to investigate the involvement of HMGB-1 in OA *in vitro*. The results of the present study may assist in the discovery of novel treatments for OA.

Materials and methods

Cell culture and treatment. Human chondrocytes (HC-a) were obtained from Shanghai CAFA Biological Technology (Shanghai, China). Cells were cultured for 24 h in high glucose-Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin/streptomycin (Corning Incorporation, Corning, NY, USA) in a humidified atmosphere with 5% CO_2 at 37˚C.

Following culture, the cells were diluted to single cell suspensions and seeded into 6-well plates ($1x10^4$ cells/well). Next, an OA model was induced by incubating the cells with IL-1β (200 μM) for 24 h at 37˚C. For CH treatment, the cells were incubated with 0, 0.4, and 4 μM CH (cat. no. C80105, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h at 37˚C, respectively. For transfection, the 50 nM HMGB1 siRNA (siRNA) and 50 nM negative control (NC) oligonucleotides were synthesized from Shanghai GenePharma, Co., Ltd. (Shanghai, China). The sequences were si-HMGB1, 5'-CCC GUUAUGAAAGAGAAAUUU-3' (sense), 5'-AUUUCUCUU UCAUAAUGGGUU-3' (antisense); si-NC, 5'-UUCGUCUGU ACUCCACAUATT-3' (sense), 5'-GAUGUCUUCUACAGU CCGATT-3' (antisense). The cells were transfected with si-NC or si-HMGB1 by using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 48 h at 37˚C following the manufacturer's protocols. Non-treated cells were used as a blank group.

Thus, the initial experimental groups were as follows: i) Blank (non-treated cells); ii) OA model (treated with 200 μM IL-1β); and iii) CH (treated with 200 μM IL-1β and the indicated concentration of CH). The transfection experimental groups were: i) Blank (non-treated cells); ii) OA model (treated with 200 μM IL-1β); iii) NC (treated with 200 μM IL-1β, si-NC); iv) siRNA (treated with 200 μM IL-1β and si-HMGB1); and v) CH + siRNA (treated with 200 μM IL-1β, si-HMGB1 and CH).

Apoptosis assay. Apoptotic cells were quantified using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Merck KGaA). Cells were collected by 0.25% trypsin digestion, washed with PBS and re-suspended in 200 μl binding buffer containing 5 μl Annexin V (10 μg/ml) in DMEM with FBS at 37˚C for 10 min in the dark. The cells were subsequently incubated with 10 μl PI (20 μg/ml) for 15 min at room temperature and analyzed with the EPICS® XL™ flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). Data acquisition and analyses were performed using CellQuest™ software version 5.1 (BD Biosciences, Franklin Lakes, NJ, USA). Early and late apoptotic cells were detected in the lower and upper right quadrants of the flow cytometry plots presented late apoptosis, and lower right represented early apoptosis. The percentage of apoptotic cells was presented for both early and late apoptotic cells.

Immunofluorescence staining. Cells were plated onto coverslips and incubated in RPMI-1640 medium (cat. no. 11875-093; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS for 24 h at 37˚C. Following treatment, cells were fixed with 4% paraformaldehyde for 20 min at 4˚C, incubated in 0.3% Triton X-100-PBS for 10 min at room temperature and subsequently blocked with 5% goat serum at 37˚C for 30 min. The cells were incubated with anti-human HMGB-1 (1:2,000; cat no. M-1702-100; Biosensis Pty Ltd., Thebarton, Australia) at 4˚C overnight, followed by incubation with goat anti-human immunoglobulin G conjugated to Cy3 (1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 37˚C for 1 h. Nuclei were counter stained with DAPI (1:1,000; Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. Images were obtained using an inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan) at x400 magnification.

Protein isolation and western blot analysis. The total protein was extracted from cells by incubation with lysis buffer (12.5 ml Tris HCL, 2 g SDS, 10 ml glycerol and 67.5 ml distilled water). Nuclear protein was extracted with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocols. The concentrations were measured by the protein assay kit (Qcbio Science Technologies Co., Ltd., Shanghai, China). The protein (30 μg) were separated by electrophoresis on Novex® 4-20% Tris-Glycine 12-well polyacrylamide gradient gels (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, the separated proteins were transferred onto a nitrocellulose membrane by using a Protean Mini Cell system

(Bio-Rad Laboratories, Inc., Hercules, CA, USA). The gel was blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST; Merck KGaA) for 2 h at room temperature. The membrane was incubated with anti-human HMGB-1 (1:10,000, ab77302; Abcam, Cambridge, UK), anti-GAPDH as a loading control (1:2,000, sc-47724; Santa Cruz Biotechnology, Inc., Dallas, CA, USA), and lamin B (1:2,000, ab122919; Abcam) overnight at 4˚C, followed by blotting with horseradish peroxidase-conjugated secondary antibodies (1:2,000, anti-mouse, cat. no. SC-2005 and anti-rabbit, cat. no. SC-2004) for 1 h at room temperature; following which, it was washed again with TBST. Finally, the blots were analyzed by the enhanced chemiluminescence (ECL) substrate kit an ECL system (both from GE Healthcare, Chicago, IL, USA).

ELISA. The concentrations of MMP13 (1:5,000, ab9128), collagenase (1:5,000, ab182881), IL-6 (1:5,000, ab7737) and collagen α-1 (II) chain (COL2A1, 1:5,000, ab34712) (all from Abcam) were quantified with commercial human ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's protocols. All samples were assayed in duplicate. The mean concentration was determined for each sample. Stop solution was then added to each well, and its optical density at 450 nm (OD_{450}) was immediately measured on an Infinite M200 microtiter plate reader (Tecan Group, Ltd., Maennedorf, Switzerland).

Statistical analysis. All statistical analyses were performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Data were presented as the mean ± standard deviation. Student's t-test was used to analyze differences between two groups, and one-way analysis of variance followed by Tukey's post-hoc test was used to determine the significance of differences among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were independently repeated three times.

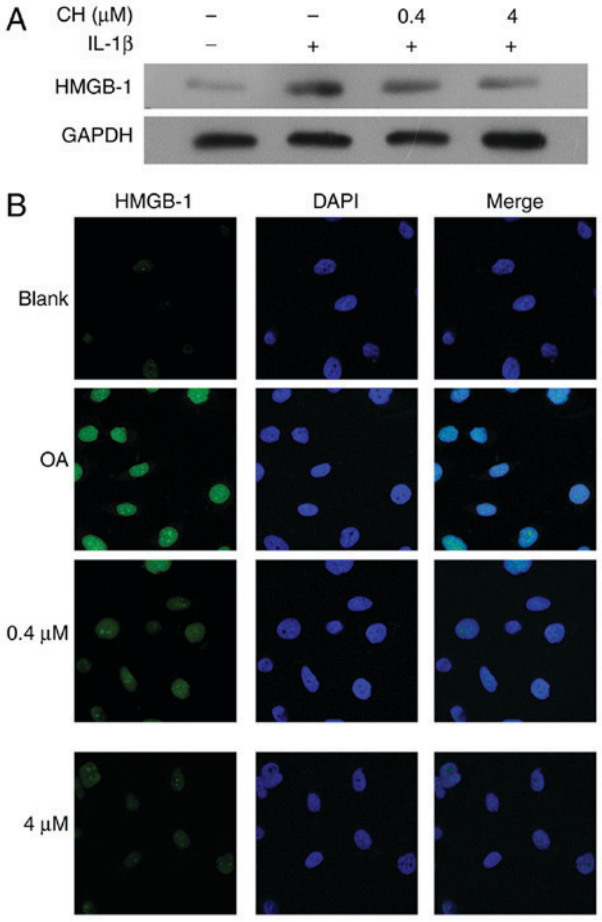

Figure 1. Differential expression of HMGB-1 in human chondrocytes in response to IL-1β and CH treatments. (A) HMGB-1 expression was detected by western blotting. (B) HMGB-1 expression and localization was investigated by immunofluorescence and observed with a laser scanning confocal microscope. Regions with HMGB-1 are green; cell nuclei were stained with DAPI. Magnification, x400, scale bar=20 μm. CH, chrysin; IL-1β, interleukin-1β; HMGB-1, high-mobility group box chromosomal protein-1; OA, osteoarthritis model.

Results

HMGB-1 expression in the human chondrocyte OA model. To validate the OA cell model used in this study, HMGB-1 expression was detected in human chondrocytes following pre-treatment with IL-1β, followed by treatment with CH. The results demonstrated that the HMGB1 expression levels were increased in response to IL-1 treatment, but was notably decreased in the CH treated groups, compared with the OA group (Fig. 1A). The results from immunofluorescence assays revealed that the increase in HMGB-1 expression in response to IL-1β, followed by a dose-dependent decrease in HMGB-1 expression in response to CH (Fig. 1B).

CH treatment alters the expression of inflammatory mediators and reduces apoptosis. The results of the ELISAs are presented in Fig. 2. Compared with the blank group, the levels of MMP13, collagenase and IL-6 were significantly increased in the OA model group; however, that of COL2A1 were significantly decreased. Compared with the OA model group, the levels of MMP13, collagenase and IL-6 were significantly decreased in the CH treatment groups; however that of COL2A1 were significantly increased in the CH (4 μM) group. The results suggested that CH treatment inhibited the levels of inflammatory mediators in IL-1β-induced HC-a cells ($P<0.01$; Fig. 2).

Additionally, cell apoptosis was analyzed by flow cytometry following treatment with IL-1β and CH. The results demonstrated that the number of apoptotic cells was significantly increased in OA model group compared with the blank group, while the number of apoptotic cells was significantly decreased in the CH treatment groups compared with the OA model group ($P<0.01$; Fig. 3). The results indicated that CH treatment suppressed the apoptotic ability of IL-1β-induced HC-a cells.

Validation of HMGB-1 knockdown in the human chondrocyte OA model. Following treatment with IL-1β for 24 h, OA model chondrocytes were transfected with si-HMGB-1 and/or treated with CH (4 μM). As presented in Fig. 4A and B, HMGB-1 expression in the siRNA-transfected cells was significantly inhibited when compared with HMGB-1 expression in cells transfected with the si-HMGB-1-NC control (NC group). Immunofluorescence analyses were performed to demonstrate that in the nucleus, HMGB-1 silenced cells

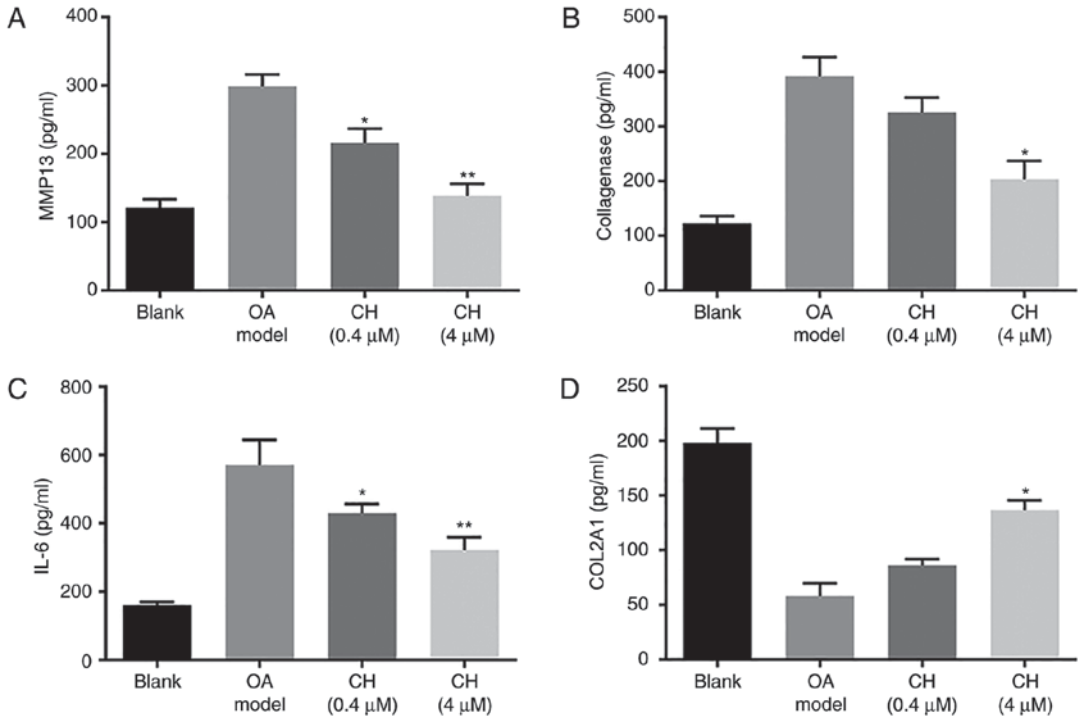

Figure 2. MMP13, collagenase, IL-6 and COL2A1 expression levels. (A) MMP13, (B) collagenase, (C) IL-6 and (D) COL2A1 expression in medium 24 h after IL-1β and CH treatments. Expression levels were detected by ELISA. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01, vs. OA model group. CH, chrysin; OA, osteoarthritis model; IL-6, interleukin-6; MMP13, matrix metalloproteinase 13; COL2A1, collagen α-1 (II) chain.

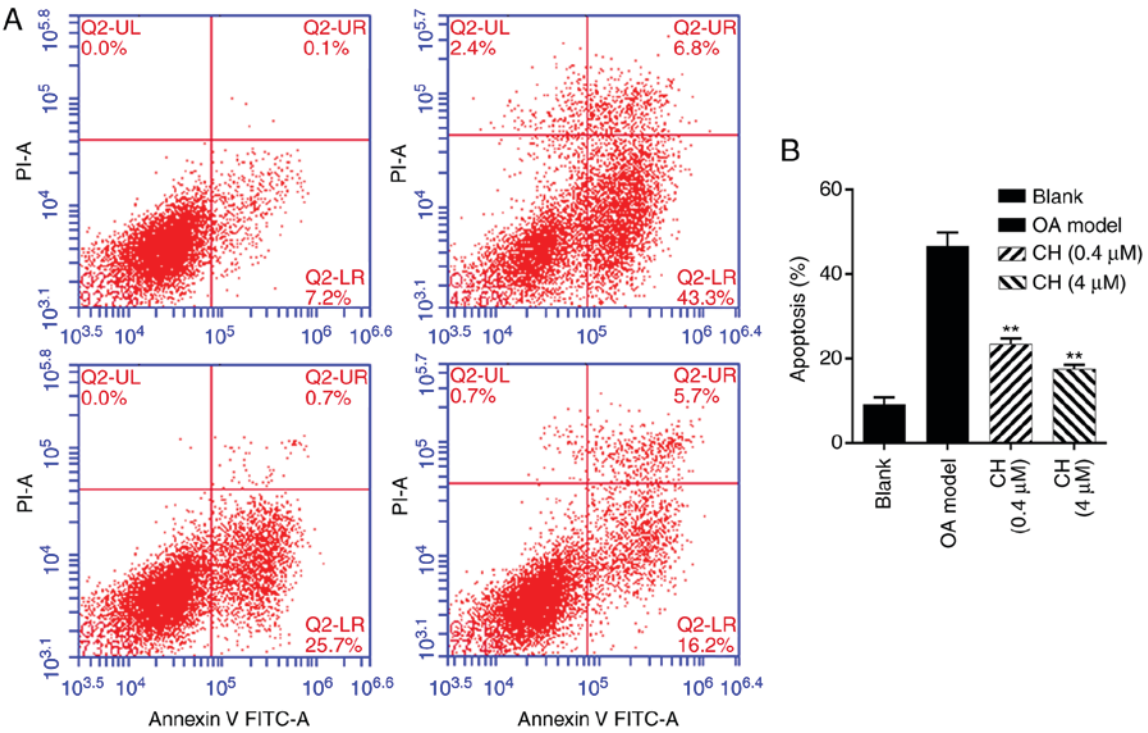

Figure 3. CH reduced the number of apoptotic cells in aliquots of IL-1β-treated cells. (A) Flow cytometry results. (B) The percentage of apoptotic cells was statistically analyzed. Data are presented as the mean ± standard deviation. Experiments were independently repeated three times. **P<0.01 vs. the model group. IL-1β, interleukin-1β; CH, chrysin; OA, osteoarthritis model; FITC, fluorescein isothiocyanate; PI, propidium iodide.

emitted less florescence compared with cells in the NC group (Fig. 4B and C). The results suggested that silencing of HMGB1 and CH treatment downregulated the expression levels of the total and nuclear HMGB-1 protein in OA model cells.

CH and siRNA cotreatment further reduces apoptosis. ELISA assays was performed to the detect the concentrations of MMP13, collagenase, IL-6 and COL2A1. The results proved that compared with the OA group, the expression of MMP13,

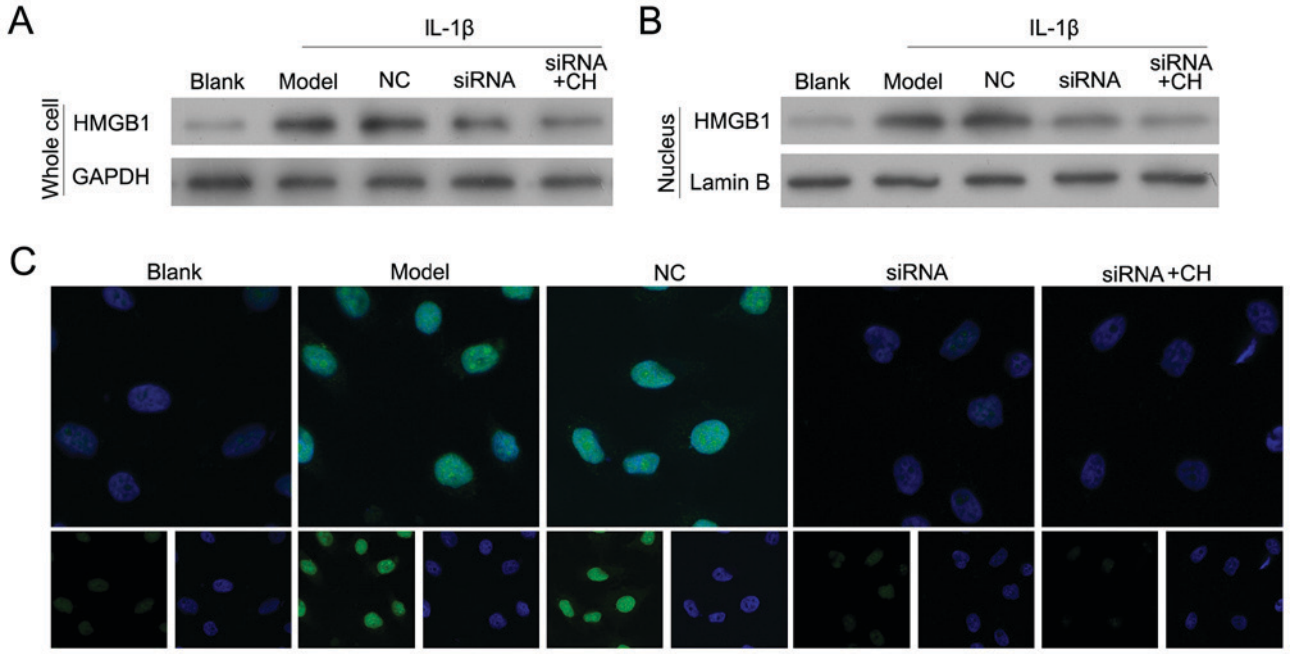

Figure 4. Validation of HMGB-1 levels in OA human chondrocyte model cells. Following CH treatment, HMGB-1 was silenced by HMGB-1-specific siRNA or transfected with NC siRNA. (A) HMGB1 expression in whole cells from each group. (B) HMGB1 expression in the nucleus of each group. (C) HMGB1 immunofluorescence was observed in cells under a laser scanning confocal microscope. Regions with HMGB-1 are green; cell nuclei were stained with DAPI. Magnification, x400, scale bar=20 μm. CH, chrysin; HMGB-1, high-mobility group box chromosomal protein-1; siRNA, small interfering RNA; NC, negative control.

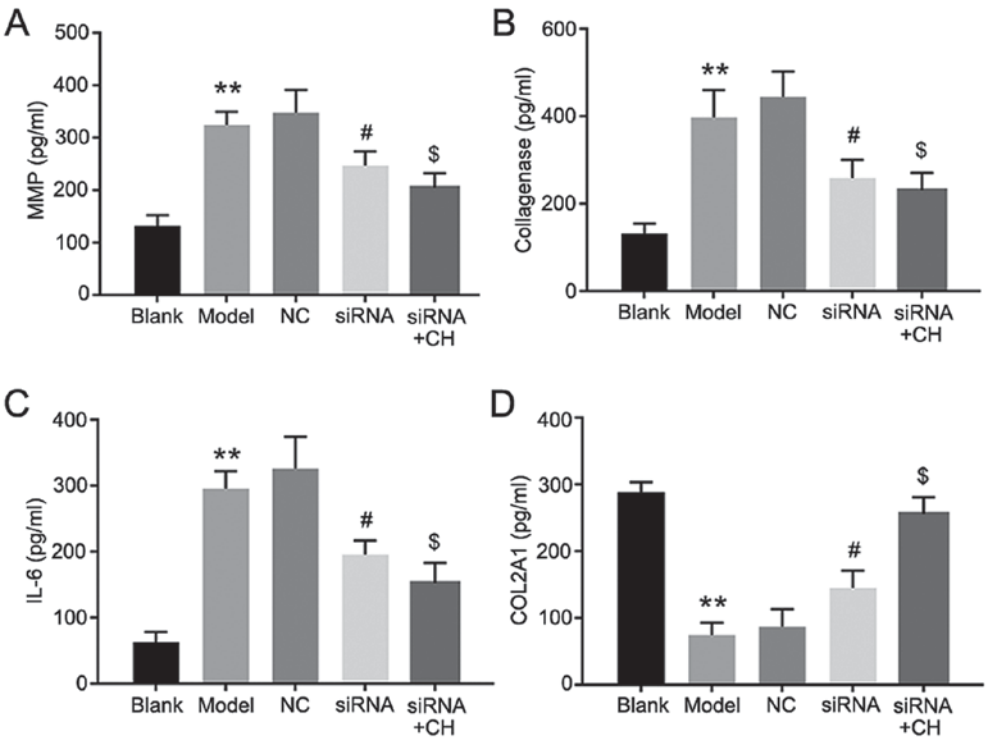

Figure 5. MMP13, collagenase, IL-6, and COL2A1 expressions levels following the silencing of HMGB-1. (A) MMP13, (B) collagenase, (C) IL-6 and (D) COL2A1 expression in medium. Data are presented as the mean ± standard deviation. Experiments were independently repeated three times. **P<0.01 vs. blank group; $^{\#}$P<0.05 vs. NC group; $^{\$}$P<0.05 vs. siRNA group. CH, chrysin; IL-6, interleukin-6; MMP13, matrix metalloproteinase 13; COL2A1, collagen α-1 (II) chain; siRNA, small interfering RNA; NC, negative control.

collagenase and IL-6 was decreased following HMGB-1 knockdown, while COL2A1 expression was increased. Additionally, MMP13, collagenase and IL-6 expression was further reduced in CH and si-HMGB-1 treated cells, compared with cells transfected with si-HMGB-1 alone. Furthermore, COL2A1 expression was significantly increased in the CH and si-HMGB treatment group, compared with the siRNA group (P<0.05, P<0.01; Fig. 5). Flow cytometry revealed that the number of

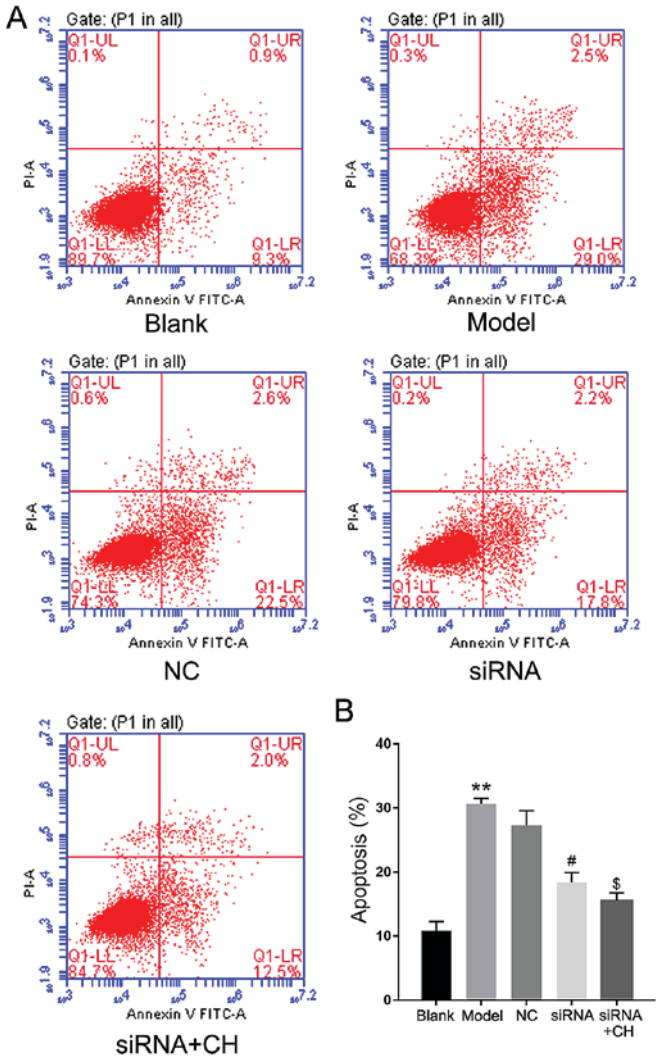

Figure 6. Apoptosis in each cell group following knockdown of HMGB-1. (A) Flow cytometry results. (B) The percentage of apoptotic cells was determined. **P<0.01 vs. blank group; $^{\#}$P<0.05 vs. NC group; $^{\$}$P<0.05 vs. siRNA group. CH, chrysin; HMGB-1, high-mobility group box chromosomal protein-1; siRNA, small interfering RNA; NC, negative control.

apoptotic cells was significantly increased in OA model group compared with the blank group. Silencing of HMGB1 significantly inhibited the apoptotic potential of IL-1β-induced HC-a cells compared with the NC group; treatment with CH enhanced the inhibition mediated by HMGB1 siRNA compared with the silencing group (P<0.05, P<0.01; Fig. 6).

Discussion

Complementary and alternative medical techniques, including certain traditional Chinese medicines (TCMs), including paeonol (25), isofraxidin (26) and Jingui external lotion (27), have been widely used for treating OA for centuries; these are primarily thought to produce chondroprotective effects and even repair cartilage (28,29). Consequently, there is growing interest in exploring TCMs as potential drugs that may aid in reducing inflammation, protecting cartilage against damage, improving joint function and restoring a patient's activity level (30). The present study was designed to evaluate whether CH may be effective against OA. This was investigated using an *in vitro* cartilage cell model to examine if CH exerted a positive effect on cartilage health through targeting HMGB-1.

As previously reported, HMGB-1 is one of several nuclear DNA-binding proteins that may be passively released in response to an inflammatory stimulus resulting from an OA injury (19). Abundant evidence indicates that certain complexes (cluster of differentiation 24, siglec-10 and tumor-infiltrating dentric cells) induce innate immune responses, and the production of inflammatory mediators is induced by the binding of HMGB1 to bacterial products (31,32). Extracellular HMGB1 has been reported to induce cell proliferation, migration and differentiation by interacting with RAGEs and toll-like receptors (TLRs), including TLR-2 and TLR-4 (33,34). Interactions between HMGB1 and phosphatidylserine on the cell surface inhibit the phagocytosis of apoptotic neutrophils by macrophages, which may lead to the activation of monocytes, macrophages and dendritic cells, which prevents the resolution of inflammation (2,35,36).

Chondrocyte apoptosis is a typical occurrence during OA progression. In response to structural changes in the cartilage matrix, chondrocytes serve a critical role in recreating the anabolic-catabolic balance required for tissue function and matrix maintenance. Therefore, inhibiting chondrocyte apoptosis while promoting the maintenance of healthy chondrocytes represents a potential strategy for preventing cartilage degeneration (37,38). As TCMs are increasingly being used to treat OA (39), scientists have suggested that certain TCMs, particularly those used in combination formulas, may produce therapeutic effects in a synergistic manner. For example, XuanHuSuo powder (XHSP), a conventional herbal formulation developed in China, has been extensively used in OA treatment (40). XHSP has shown reasonable efficacy as an anti-apoptotic and anti-inflammatory agent when applied following stimulation with a cytokine (IL-1β) or estrogen (40). As an active component of various Chinese herbs, berberine chloride has been demonstrated to benefit matrix synthesis and cell survival in IL-1β-stimulated chondrocytes, and displays great therapeutic potential as a promoter of cartilage repair in rat OA models (41). However, there is no effective way to target and promote cartilage protection at present. To the best of our knowledge, the present study was the first to demonstrate that the herbal extract CH may inhibit chondrocyte apoptosis by targeting HMGB-1.

Taken together, the data indicated that CH ameliorated OA *in vitro*. It does this, at least partially, by inhibiting various processes mediated by HMGB-1, including chondrocyte apoptosis, cellular inflammatory responses and inflammatory cytokine generation. Further studies in animal models are required to evaluate the safety and efficacy of this herbal extract.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CZ, WZY and ZQH designed the experiments. CZ, CBH and QHD performed most of the experiments. CZ, CHL, LW and ZYZ collected and analyzed the data. CZ drafted the manuscript. ZYZ provided the administrative support.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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