

Isoliquiritigenin suppresses IL-1 β induced apoptosis and inflammation in chondrocyte-like ATDC5 cells by inhibiting NF- κ B and exerts chondroprotective effects on a mouse model of anterior cruciate ligament transection

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Abstract. Isoliquiritigenin (ISL), a natural flavonoid extracted from licorice, has been demonstrated to exert attenuation of the nuclear factor- κ B (NF- κ B) signaling pathway and anti-inflammatory activity in a wide variety of cells. In the present study, the authors first evaluated the effects of ISL on cartilage degeneration in interleukin-1 β (IL-1 β)-stimulated chondrocyte-like ATDC5 cells and in a mouse model of osteoarthritis (OA). The data of a cell counting kit-8 and flow cytometry assay indicated that ISL suppressed the inhibitory effect of IL-1 β on cell viability. The mRNA and protein expression levels of cyclooxygenase-2 and matrix metalloproteinase-13 were significantly decreased, while the expression of collagen II was increased, as indicated by RT-qPCR and western blot analysis following the chondrocyte-like ATDC5 cells were co-intervened with IL-1 β and ISL for 48 h. Also, ISL attenuated protein expressions level of pro-apoptotic Bax, cleaved-caspase-3 and cleaved-caspase-9 and promoted expression of anti-apoptotic Bcl-2. Moreover, ISL inhibited NF- κ B p65 phosphorylation induced by IL-1 β . In addition, ISL also increased improved the thickness of hyaline cartilage and the production of proteoglycans in the cartilage matrix in a mouse OA model. These results indicated that ISL exerted anti-inflammatory and anti-apoptotic effects on IL-1 β -stimulated chondrocyte-like ATDC5 cells, which may be associated with the downregulation of the NF- κ B signaling pathway. In this way, the data supported the conclusion that

ISL may be a novel potential preventive agent suitable for use in OA therapy.

Introduction

Osteoarthritis (OA) is the most common age-related degenerative disease. It is a metabolically dynamic process that involves all joint structures (loss of cartilage matrix, inflammation of synovium, formation of osteophytes, sclerosis of subchondral bone) (1,2). These pathologies lead to chronic joint pain, limited movement and eventually disability. Osteoarthritis to varying degrees happened in an approximate 10-15% of adults >60 all around the world (3). The cost of OA is estimated to make up of 0.50% of a country's gross domestic product (4). Currently, no agent has been approved for OA management by the FDA or any other agencies worldwide. Available drugs provide only temporarily symptomatic relief but most involve numerous side effects. Therefore, there is a dire need for effective and widely available approaches in OA management, which is partly hindered by our limited understanding of the pathomechanisms of OA.

In diarthrodial joints, articular cartilage serves a critical weight-bearing role. Chondrocytes, the unique cells in articular cartilage, account for maintaining the balance of degradation and synthesis of extracellular matrix (ECM) (5). In progression of articular cartilage degradation in OA, an increased synthesis of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), matrix metalloproteinases (MMPs) and cyclooxygenase-2 (COX-2) results in loss of the major components of the ECM (6). Chondrocyte death, as a consequence of direct damage as well as apoptotic cell death, may be a result of the lack of certain ECM components bounding to the cell surface, which in turn implicate cartilage matrix degradation and further joint inflammation (7). It is known that IL-1 β is one of the major cytokines in the pathogenesis of OA (8), which inhibits the synthesis of ECM proteins and induces the releases of MMPs in chondrocytes (9). In particular, IL-1 β also has been shown to induce the nuclear factor- κ B (NF- κ B) signaling pathway in chondrocytes, which was considered

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as the key molecular pathway in progression of cartilage degradation in OA (10). The NF- κ B molecules not only could enhance the articular damage through promote secretion of many degradative enzymes, including MMP-1, MMP-2 and MMP-3, but also promote synthesis of catabolic factors, such as COX2, nitric oxide (NO) and NO synthase (NOS), which aggravate apoptosis of OA chondrocytes and cartilage inflammation (11).

Traditional Chinese medicine is widely used in Asian societies. Many herbs were employed for the treatment of arthritis and other inflammatory diseases for centuries (12,13). Isoliquiritigenin (ISL) (Fig. 1), a natural flavonoid extracted from licorice, has drawn wide attention due to its lots of biological activities, including vasorelaxant, anti-viral, anti-oxidant, anti-platelet aggregation as well as anti-inflammatory properties and its proven pharmacologic safety (14,15). The licorice is also used in Western countries widely for culinary purpose. Although Zhu *et al* (16) reported that licorice be used in inhibition of osteoclast differentiation, which is a significant physiopathological mechanism of OA (17), a search of Medline, PubMed (carried out at in February, 2017) revealed no article on the subject of licorice be used in treatment of OA. Conversely, it has been previously reported that ISL could prevent the progression of psoriasis-like symptoms in mice and inhibit LPS-stimulated COX-2 expression in RAW 264.7 macrophages, which both as a result of attenuation of the NF- κ B signaling pathway (18), which is a central regulator of the inflammatory cytokine-induced catabolic actions in chondrocytes (19). A further literature review indicated that the effects of ISL on chondrocyte-like ATDC5 cells have not been investigated at the cellular or molecular levels yet. Accordingly, the aim of the present study was to access whether ISL could inhibit IL-1 β -stimulated inflammation and apoptosis by decreasing NF- κ B activation in chondrocyte-like ATDC5 cells. In addition, the authors determined whether ISL had potential protective effects on cartilage of anterior cruciate ligament transaction models in mice.

Materials and methods

Ethics approval. The experimental schemes were approved by the Institutional Animal Care and Use Committee of First Affiliated Hospital of Xinjiang Medical University (protocol no. IACUC20160616-08).

Materials and methods. ISL (purity >98%) was purchased from Aladdin® (Shanghai, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's minimum essential medium/Ham's F12 medium (DMEM/F12), penicillin, streptomycin, insulin, transferrin, selenium (ITS) and Trypsin were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Alcian Blue 8GX was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The primary antibodies against GADPH, Bax, Bcl-2, NF- κ B p65, phospho-p65, caspase-3, cleaved-caspase-3, caspase-9 and cleaved-caspase-9 were bought from Cell Signaling Technology, Inc. (Danvers, MA, USA); COL II, MMP-13, COX-2 were purchased from Abcam (Cambridge, MA, USA) and C57BL/6 male mice (n=80) of 3 months old were purchased from Vital River Laboratories (Beijing, China).

Cell differentiation and treatment. Cultures of undifferentiated ATDC5 cells (Riken Cell Bank, Tsukuba, Japan) were maintained in DMEM/F12 supplemented with 5% (v/v) FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified incubator with 5% CO₂ at 37°C. Once the culture was confluent 70-80%, the medium (as described above) supplemented with 1% ITS additionally. The differentiation medium was changed every two days for 3 weeks to induce differentiation into chondrocyte-like cells. Differentiation was confirmed by the expression level of mRNA for COL II and COL X. The production of glycosaminoglycan was visualized by 1% Alcian Blue staining. The cells were used for the experimental procedures after differentiation for 14 days. The serum-starved chondrocyte-like cells (cultivated in 1% FBS) were incubated alone for 24 h and pretreated with ISL (2.5, 5, 10, 20 and 40 μ mol/l) for 1 h, then co-treated with IL-1 β (10 ng/ml) for a further 48 h in monolayer cultures. The cells treated with IL-1 β only were used as the model group and the chondrocyte-like cells which only exposed to serum-starved medium served as the normal control.

Cell viability assay. Chondrocyte-like ATDC5 cells were seeded in 96-well plates with 4×10^3 cells/well and cultured with 100 μ l maintenance medium for 24 h. The following day, the cells pretreated with ISL (2.5, 5, 10, 20 and 40 μ mol/l) for 1 h, then co-incubated with IL-1 β (10 ng/ml) for a further 24, 48 and 72 h, respectively and Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to monitor cell viability. The number of viable cells was assessed by measurement of absorbance at 450 nm after additional 2-h incubation with CCK-8 by a microplate reader Multiskan Go (Thermo Fisher Scientific, Inc.).

Quantitative analysis of apoptosis cells. PE-Annexin V/7-amino-actinomycin (7-ADD) double-fluorescence labeling and flow cytometry were performed to quantify changes of cell apoptosis. Following co-treatment with IL-1 β (10 ng/ml) and ISL (2.5, 5 and 10 μ mol/l) for 48 h, 1×10^6 cells were harvested and washed 3 times with pre-cold phosphate-buffered saline (PBS), then re-suspended in binding buffer followed by apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA). The cells were also treated with only ISL (5, 10, 20 and 40 μ mol/l) for 48 h and received the same pretreatment. The samples were analyzed using FACS Aria™ II flow cytometer (BD Biosciences) after PE-Annexin V and 7-ADD marking at indoor temperature for 15 min in the dark.

Gene expression analysis. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's protocols to extract total RNA from chondrocyte-like ATDC5 cells. A spectrophotometer at 260 nm was selected to quantify total RNA and purity was evaluated by determining the ratio of A260/A280. All samples had ratios above 1.90. cDNA was synthesized using $\sim 2 \mu$ g RNA with an PrimeScript™ RT Master Mix (Takara Bio, Inc., Otsu, Japan) and was subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR) reactions with SYBR® Fast qPCR Mix (Takara Bio, Inc.) using a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, MA, USA) under the condition of 94°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, 60°C

Table I. Primer sequences for different genes.

COL II	F: 5'-ACGAAGCGGCTGGCAACCTCA-3' R: 5'-CCCTCGGCCCTCATCTCTACATCA-3'
COX-2	F: 5'-TTCCAATCCATGTCAAAACCGT-3' R: 5'-AGTCCGGGTACAGTCACACTT-3'
MMP-13	F: 5'-TGTTTGCAGAGCACTACTTGAA-3' R: 5'-CAGTCACCTCTAAGCCAAAGAAA-3'
GAPDH	F: 5'-AGGTCGGTGTGAACGGATTG-3' R: 5'-GGGGTCGTTGATGGCAACA-3'

COL, collagen; COX, cyclooxygenase; MMP, matrix metalloproteinase.

for 10 sec, and finally the dissociation curve of each primer pair was analyzed to determine the primer specificity. The primers were synthesized and supplied by Sangon Biotech Co., Ltd. (Shanghai, China). Sequences of the primers used are listed in Table I. All of the PCR reactions were performed 3 times for each gene. The data were analyzed by the $2^{-\Delta\Delta C_q}$ method (20) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control.

Western blot analysis. Radioimmunoprecipitation assay lysis buffer with protease inhibitors (Sigma-Aldrich; Merck KGaA) were used to lyse treated cells on ice for 30 min according to the manufacturer's protocols. The protein concentration was determined by the bicinchoninic acid protein assay kit (Sangon Biotech Co., Ltd.). Equal amounts (20 μ g/load) of protein samples were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Inc.) electrophoresis and transferred on to polyvinylidene fluoride difluoride membranes (Merck KGaA). 5% bovine serum albumin (BSA) were selected to block the membranes for 1 h at room temperature and then incubated with the primary antibodies COL II (1:5,000, cat. no. ab34712), MMP-13 (1:3,000, cat. no. ab39012), COX-2 (1:1,000, cat. no. ab62331) (all from Abcam), GAPDH (1:1,000, cat. no. 2118S), Bcl-2 (1:1,000, cat. no. 3498), Bax (1:1,000, cat. no. 14796), caspase-3 (1:1,000, cat. no. 14220), cleaved caspase-3 (1:1,000, cat. no. 9654), caspase-9 (1:1,000, cat. no. 9508), cleaved caspase-9 (1:1,000, cat. no. 7237), NF- κ B p65 (Cell Signaling Technology Inc., 1:1,000, cat. no. 6956), phospho-NF- κ B p65 (1:1,000, cat. no. 3036) (all from Cell Signaling Technology, Inc.) at 4°C overnight with gentle rocking. The membranes were then washed 3 times, and then cultured with the secondary antibody (peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L), 1:5,000, cat. no. ZB-2301, OriGene Technologies, Inc., Beijing, China) for 2 h, and immunoreactivity was detected with the ECL substrate (Thermo Fisher Scientific, Inc.). GAPDH was used as an internal control in all western blot analyses.

Anterior cruciate ligament transection (ACLT) model in mice. The experimental schemes were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Xinjiang Medical University. The animals were kept

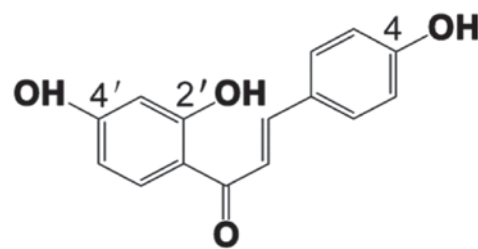


Figure 1. Molecular structure of isoliquiritigenin (ISL, $C_{15}H_{12}O_4$, MW=256.25).

in a controlled environment (temperature, 22–25°C; light/dark cycle, 12 h; relative humidity, 60%) and were allowed free access to drinking water and fodder. The 3-month-old male C57BL/6J mice were anesthetized with intravenous injection of 1% pentobarbitone (40 mg/kg). After a routine sterilize, the patella was laterally dislocated through medially parapatellar arthrotomy. The ACL was visualized and transected with a scissors. The articular cavity was irrigated and sutured layer-by-layer, then the mice underwent penicillin treatment (400,000 IU/Kg) for one week. Sham operation was conducted by opening the joint capsule and suturing the incision in the right knee of independent rodents.

Preliminary experiment was performed firstly, the optimal dose (40 mg/kg) was identified using multiple concentrations of ISL (10, 20 and 40 mg/kg) injected every other day for 2 months post-operation (Fig. 8). Lower concentration (10 or 20 mg/kg) had minimal effects on chondroprotection. The authors did not continue to increase the dose because nearly half of mice died in higher concentration (80 mg/kg group) during the 2 months post-operation. Therefore, in the formal experiment, the mice were randomized to sham group, ACLT + vehicle group, or ACLT + ISL (40 mg/kg) group. Beginning the second day after surgery, ISL or equivalent volume of vehicle (10% Tween-80) was injected intraperitoneally every other day for 60 days. Mice were sacrificed by CO_2 inhalation at 60 days after surgery. Knee joints of mice were dissected, fixed in 10% buffered formalin for 48 h, and decalcified in 10% EDTA (pH 7.4) for 3 weeks. Specimens were embedded in paraffin and cut for hematoxylin and eosin (H&E) and Safranin O and fast green staining. Osteoarthritis Research Society International-modified Mankin criteria (OARSI) scores were calculated for evaluating the state of articular cartilage in each group.

Statistical analysis. If the data met homogeneity of variance, statistical significance between different groups was evaluated by one-way analysis of variance followed by Tukey's post hoc test. If the data did not meet homogeneity of variance, Dunnett's T3 post hoc test was used. Student's t-test were performed to compare mean values from different samples. Statistical tests were carried out using SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as mean \pm standard deviation from three separate experiments.

Results

Differentiation of ATDC5 cells. The authors evaluated whether treatment with ITS induces formation of cartilage nodules in ATDC5 cells. The cells were stimulated with ITS

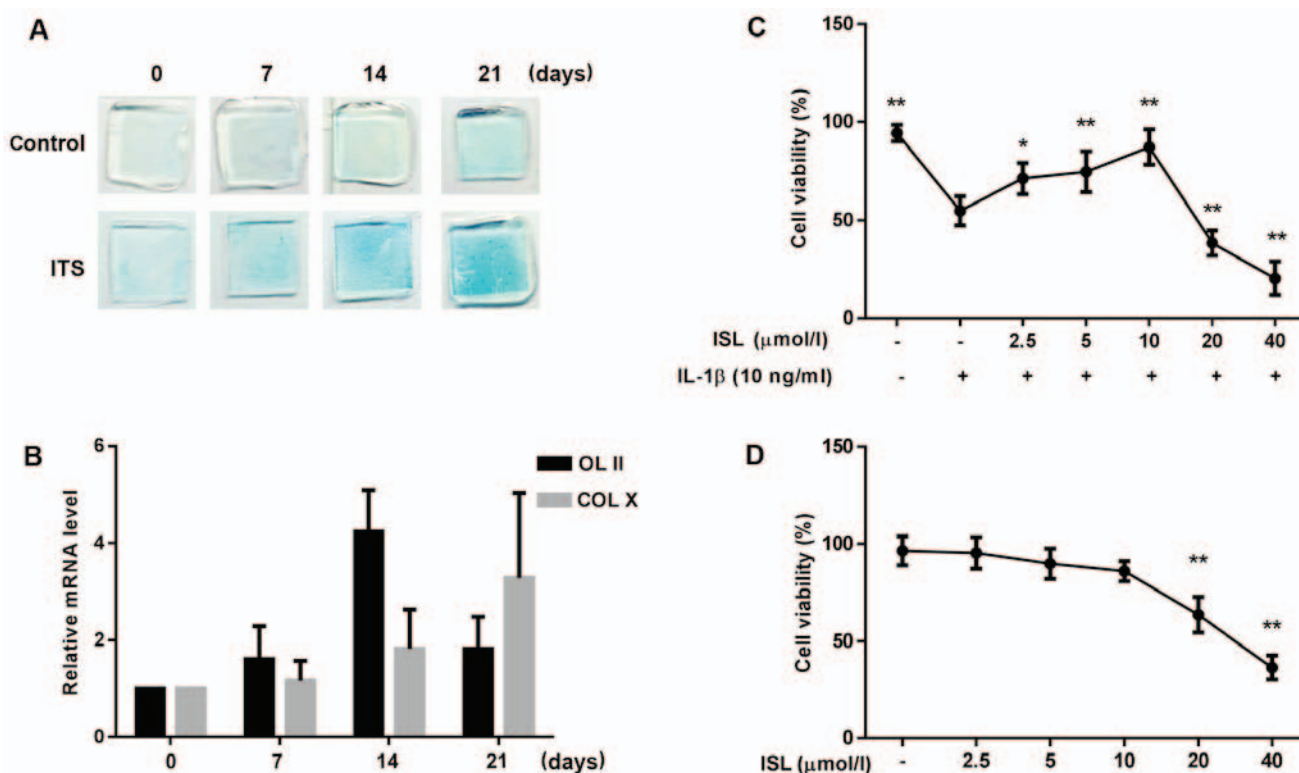


Figure 2. (A) Differentiation of ATDC5 cells was determined by 1% Alcian blue staining at 0, 7, 14 and 21 days. (B) Relative expression of collagen II, X mRNA was determined by reverse transcription-quantitative polymerase chain reaction. (C) The cells were treated with 10 ng/ml IL-1 β plus ISL at different concentrations for 48 h. ISL promoted chondrocyte-like ATDC5 cells viability by CCK-8 assay. (D) The cells were treated with only ISL at different concentrations for 48 h and the viability was evaluated by CCK-8 assay. Data was expressed as mean \pm standard deviation of triplicates. * $P < 0.05$ and ** $P < 0.01$ vs. (C) the model group which treated with only IL-1 β or (D) control group. IL-1 β , interleukin-1 β ; ISL, isoliquiritigenin; CCK-8, cell counting kit.

for 21 days and 1% Alcian blue staining were performed at 0, 7, 14 and 21 days. Fig. 2A presents that staining intensities gradually increased in a time-dependent manner from 0 to 21 days in ATDC5 cells cultured with ITS. The authors then assessed the expression of chondrogenic differentiation markers including COL II and COL X by using RT-qPCR. As Fig. 2B indicates, COL II mRNA increased significantly after 7 days of chondrogenic induction and further increased with a maximum elevation at 14 days, which indicates early-stage differentiation of chondrocytes. mRNA expression of collagen X gradually increased and exceeded COL II at 21 days, indicating late-stage differentiation of chondrocytes. These results show that undifferentiated ATDC5 cells differentiate in culture from proliferative chondrocytes to hypertrophic chondrocytes. Therefore, ATDC5 cells with differentiation of 14 days were selected as following experimental cells.

Effects of ISL on cell viability. CCK-8 analysis was performed to study effects of ISL on viability of chondrocyte-like ATDC5 cells incubated with IL-1 β . The results demonstrated a significant decrease of IL-1 β -induced chondrocytes viability compared with the normal control, which was reversed by ISL at low concentrations (2.5, 5 and 10 μ mol/l) in a dose-dependent manner (Fig. 2C). However, higher doses of ISL (20 and 40 μ mol/l), did not reverse the viability of chondrocyte-like ATDC5 cells. Correspondingly, the cells were treated with only ISL at different concentrations indicated that the viability of the cells was not remarkable change within the

scope of 2.5-10 μ mol/l, while significant decrease at high concentrations (20 and 40 μ mol/l) (Fig. 2D). As a result, 2.5, 5 and 10 μ mol/l ISL were selected as the low, medium and high concentrations, respectively. The criterion was selected in subsequent experiments.

ISL decreased the apoptotic rate of chondrocyte-like ATDC5 cells. The effects of ISL on cell apoptosis was determined by flow cytometry using PE-Annexin V/7-ADD double-fluorescence labeling. The percentage of apoptotic cells in IL-1 β treated group was prominently increased compared with the normal control. Chondrocyte-like ATDC5 cells co-treated with ISL and IL-1 β showed a decrease apoptotic rate, in a dose-independent manner (Fig. 3). Additionally, ISL induced only mild cytotoxicity at lower concentrations (5 and 10 μ mol/l), but it exhibited apparent cytotoxicity at higher concentrations (20 and 40 μ mol/l) (Fig. 4).

Protective effects of ISL against catabolic responses on mRNA and protein expression. Because IL-1 β contributes to the synthesis of more pro-inflammatory factors and matrix-degraded enzymes such as MMPs and COX-2. The authors further examined the anti-inflammatory effect of ISL on chondrocyte-like ATDC5 cells. The mRNA expressions of COL, COX-2 and MMP-13 gene were assessed by RT-qPCR (Fig. 5A). The results showed that in the IL-1 β -induced ATDC5 cells treated by ISL, the mRNA levels of COX-2 and MMP-13 were evidently decreased dose-dependently, whereas the level of COL was increased significantly. The results of western

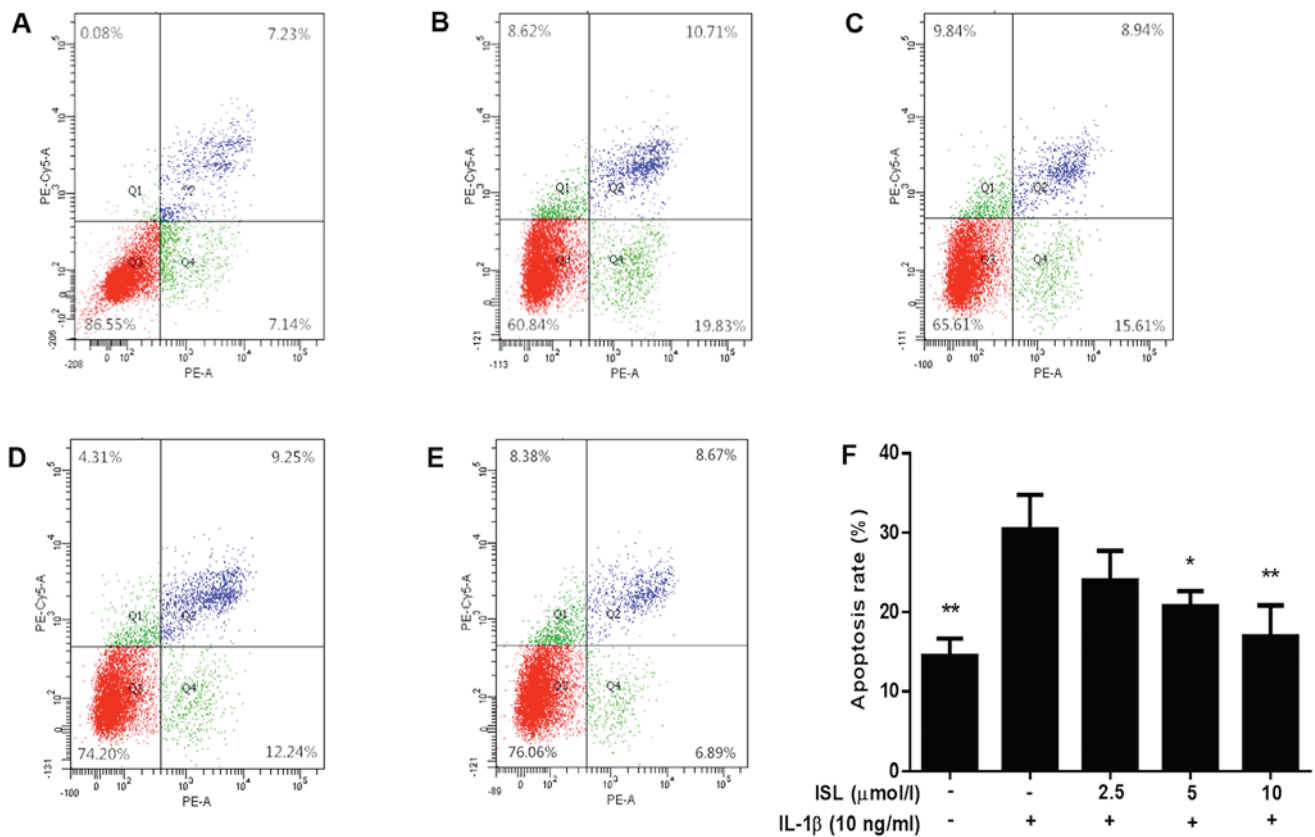


Figure 3. (A) Suppression of apoptosis by ISL was determined by using flow cytometry analysis of PE-Annexin V/7-amino-actinomycin double-fluorescence labeling. Chondrocyte-like ATDC5 cells in the control group were incubated without additional treatment. (B) The cells incubated with only IL-1 β as model group. Treatment groups were incubated with IL-1 β and (C) 2.5, (D) 5 and (E) 10 μ g/ml ISL, respectively. (F) The apoptotic rate for each group is presented as the means \pm standard deviation of 5 replicates. * P <0.05 and ** P <0.01 vs. the model group. ISL, isoliquiritigenin; IL, interleukin; PE, phycoerythrin.

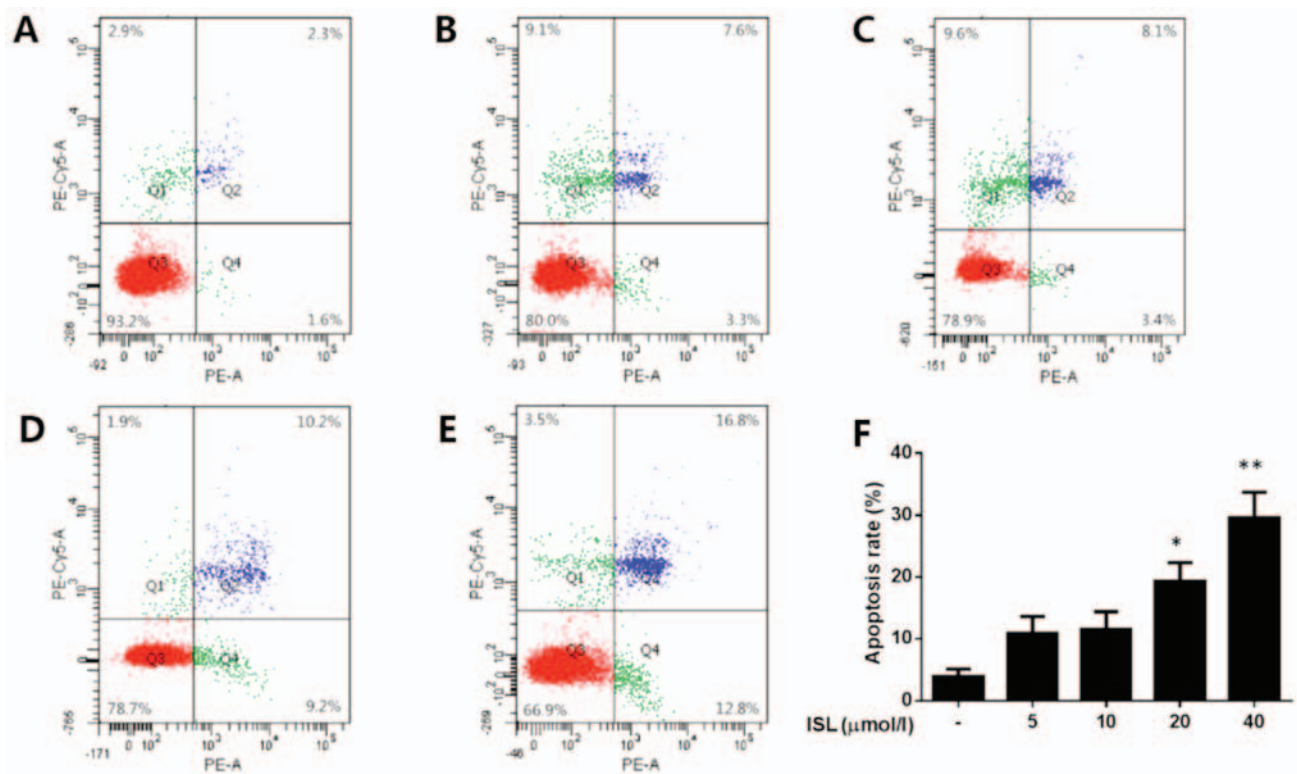


Figure 4. The cytotoxicity of ISL was examined by flow cytometry using PE-Annexin V/7-ADD double-fluorescence labeling. The chondrocyte-like ATDC5 cells were treated with only ISL (5, 10, 20 and 40 μ mol/l) for 48 h. (A) The cells in the control group were incubated without any treatment. Treatment groups were incubated with ISL at (B) 5, (C) 10, (D) 20 and (E) 40 μ mol/l, respectively. (F) The apoptotic rate for each group were presented by mean \pm standard deviation of five replicates. * P <0.05 and ** P <0.01 vs. control group. ISL, isoliquiritigenin; PE, phycoerythrin; 7-ADD, 7-amino-actinomycin.

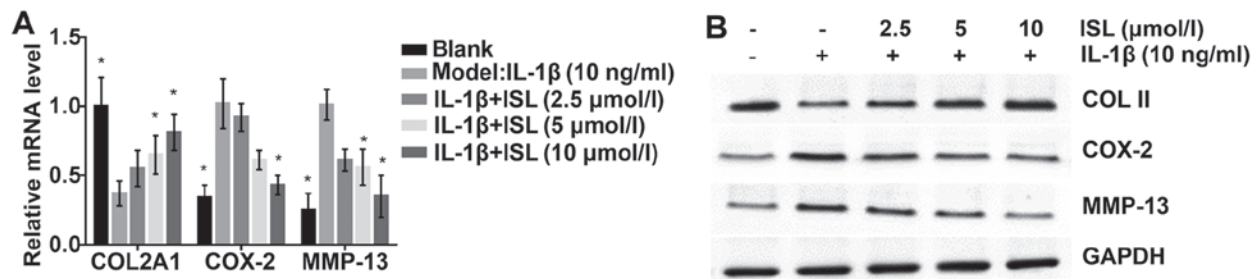


Figure 5. The chondrocyte-like ATDC5 cells were co-treated with IL-1 β (10 ng/ml) and different concentrations of ISL (2.5, 5 and 10 μ mol/l) for 48 h. The COL II, COX-2 and MMP-13 mRNA and protein expression was detected by (A) reverse transcription-quantitative polymerase chain reaction and western blotting (B), respectively. GAPDH was used as internal control. The results represent the mean \pm standard deviation of three independent experiments. * P <0.05 vs. model group treated with only IL-1 β . IL, interleukin; ISL, isoliquiritigenin; COL, collagen; COX, cyclooxygenase; MMP, matrix metalloproteinase.

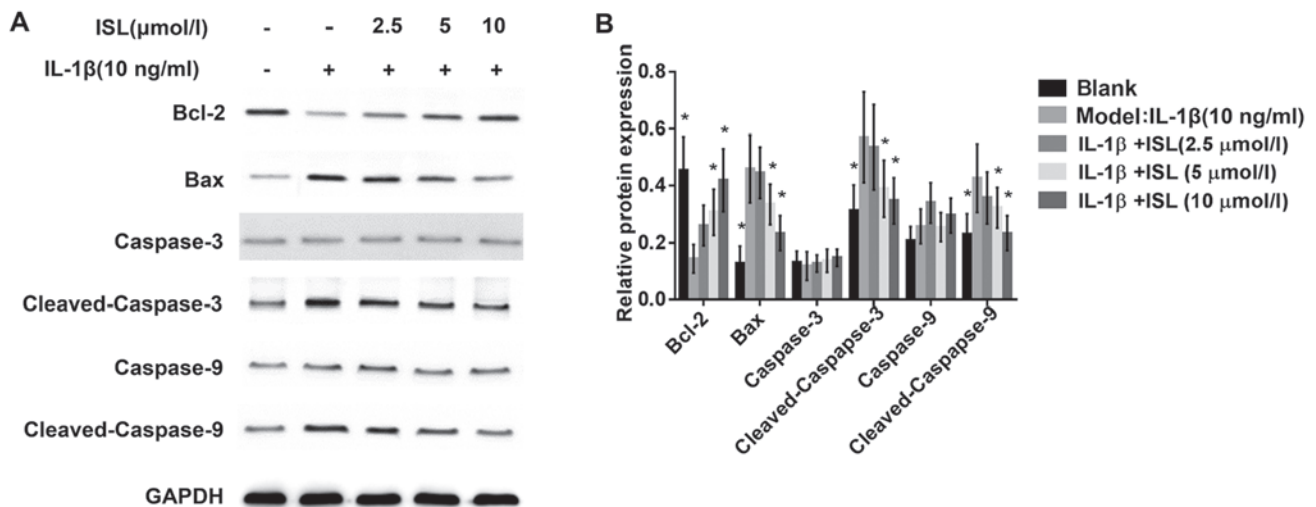


Figure 6. The effect of ISL on the levels of anti-apoptotic and pro-apoptotic proteins in IL-1 β -induced chondrocyte-like ATDC5 cells. (A) The IL-1 β stimulated the cells were treated with different doses of ISL (2.5, 5 and 10 μ mol/l) for 48 h. Then protein expressions Level of Bcl-2, Bax, caspase-3, cleaved-caspase-3, caspase-9 and cleaved-caspase-9 were detected by western blotting. (B) The protein analysis of quantitative fluorescence. GAPDH was used as internal control. The values represent the mean \pm standard deviation of three independent experiments. * P <0.05 vs. model group treated with only IL-1 β . ISL, isoliquiritigenin; IL, interleukin.

blotting consistently demonstrated the anti-inflammatory effect of ISL (Fig. 5B).

Anti-apoptotic effects of ISL on IL-1 β -stimulated chondrocyte-like ATDC5 cells. In order to evaluate whether ISL could modulate the expressions of anti-apoptotic and promoting-apoptotic genes, western blotting were performed and the results demonstrated that the expression of anti-apoptotic protein Bcl-2 dose-dependent increased with the increasing concentration of ISL while the expression level of pro-apoptotic protein Bax decreased in a concentration manner when ATDC5 cells were stimulated with IL-1 β (Fig. 6A). These results indicated ISL exerted anti-apoptotic effects in IL-1 β -induced chondrocyte-like ATDC5 cells.

To provide further evidence for anti-apoptotic effects of ISL on IL-1 β -stimulated chondrocyte-like ATDC5 cells, the downstream protein levels of caspase-3 cleavage and caspase-9 cleavage that are in the mitochondria apoptosis pathway were assessed. Western blot data revealed that ISL had few influence on expression of caspase-3 and caspase-9. However, the expression of cleaved-caspase-3 and cleaved-caspase-9 was prominent in chondrocyte-like ATDC5 cells

stimulated with IL-1 β and was blocked in the cells treated with ISL (Fig. 6B).

ISL inhibits NF- κ B p65 phosphorylation in IL-1 β -stimulated chondrocytes. Based on the data above, the authors speculated that the NF- κ B signaling pathway may be inhibited by ISL. To determine whether ISL inhibited IL-1 β -induced NF- κ B p65 phosphorylation, nuclear protein extracts from serum-starved chondrocyte-like ATDC5 cells were probed for the phosphorylated form of NF- κ B after pretreatment with ISL for 1 h followed by IL-1 β stimulation for a further 48 h. The western blot data indicated that pretreatment of ISL attenuated IL-1 β -stimulated NF- κ B phosphorylation in a dose-dependent manner (Fig. 7A).

ISL exerts chondroprotective effects on knee joint tissue of mice. At last, the *in vivo* therapeutic effects of ISL in OA were evaluated by using a mouse ACLT model. The medial of the tibia plateau from the operative hind leg was sectioned and observed 8 weeks after operation. H&E staining demonstrated decreased thickness of calcified cartilage zone in ISL (40 mg/kg)-treated ACLT mice relative to vehicle-treated

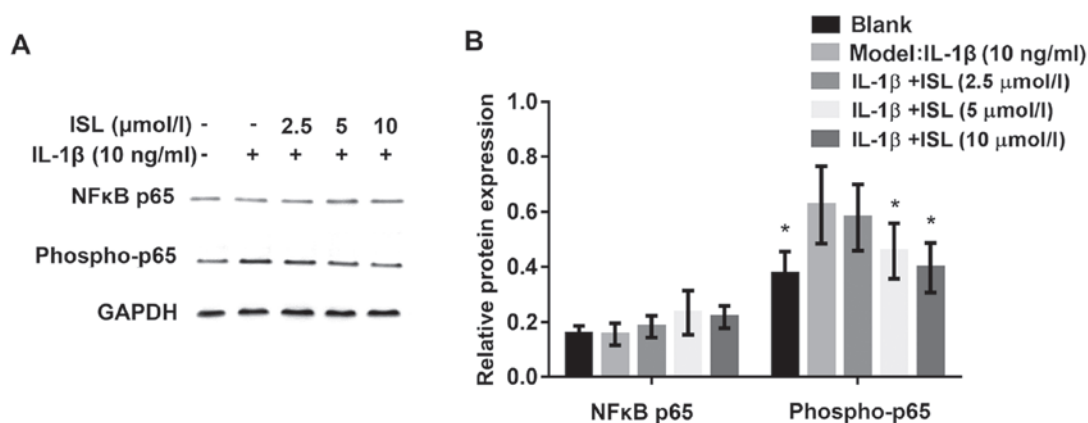


Figure 7. ISL blocks the IL-1 β -induced accumulation and phosphorylation of p65. (A) The IL-1 β (10 ng/ml) stimulated chondrocyte-like ATDC5 cells were treated with ISL for 48 h. Whole cell extracts and nuclear cells monolayers were harvested. Western blotting was performed with anti-p65 and anti-p-p65. (B) Relative protein expression of p65 and pp65 was quantitative analyzed. GAPDH was used as internal control. The values represent the mean \pm standard deviation of three independent experiments. * P <0.05 vs. model group treated with only IL-1 β . ISL, isoliquiritigenin; IL, interleukin; NF, nuclear factor.

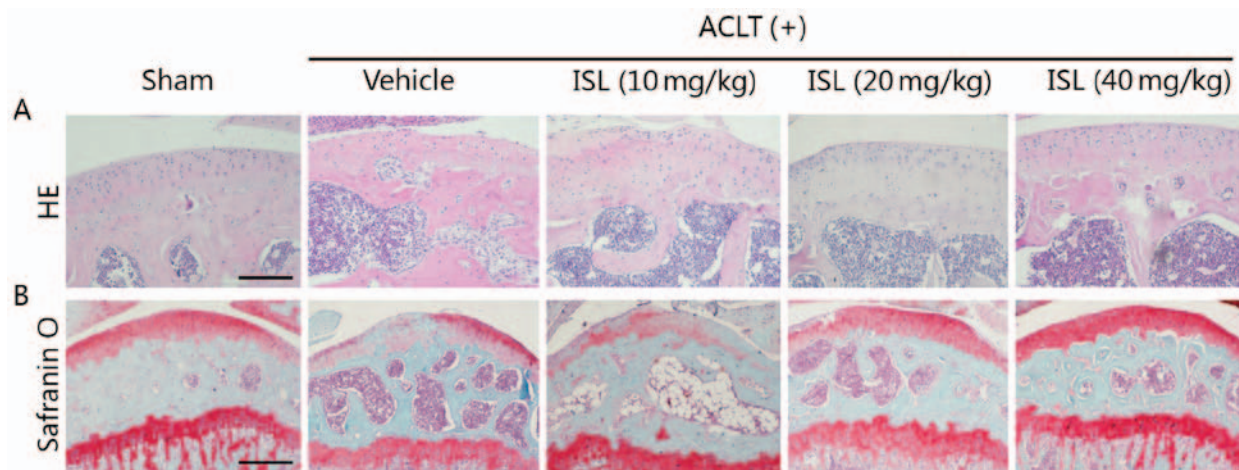


Figure 8. In preliminary experiment, ISL exhibited chondroprotective effects on a mice ACLT model in a concentration-dependent manner. Mice underwent ACLT operation and received intraperitoneal injections with 10% Tween-80 or ISL as described in Materials and methods. The mice were sacrificed and their knee joints were excised 8 weeks after ACLT operation. (A) Sagittal medial tibial condyle sections stained with H&E (upper) and (B) Safranin O and fast green (lower). Representative microscopic images of sham-operation, vehicle, and different concentration groups (10, 20 and 40 mg/kg) are shown. Scale bars, 400 μ m, n=6/group. ISL, isoliquiritigenin; ACLT, anterior cruciate ligament transection; H&E, hematoxylin and eosin.

ACLT controls (P <0.05) (Fig. 9A and B). Specifically, in the vehicle group, the surface of the articular cartilage was rough, and the intensity of Safranin O staining in the matrix was low. However, the superficial layer of the cartilage in ISL-treated ACLT mice was smooth. There was no disruption of surface integrity and strong staining with Safranin O was observed in these ACLT mice (Fig. 9A, lower right panel). OARS1 scores in ISL-treated ACLT mice were improved compared to the vehicle-treated ACLT controls, whereas no difference was noted in ISL versus sham controls (Fig. 9C).

Discussion

Historically, different types of therapeutic agents and drug leads have been extracted from natural products (21). A great deal of effort has recently been put toward finding bioactive small molecules from natural herbage that may be suitable for treatment of OA, especially those with minimal or no adverse side effects (22-24). ISL, a bioactive small molecules

isolated from licorice, which is one of the most commonly used herbs in traditional Chinese medicine, has been used to treat many kinds of ailments ranging from skin diseases to peptic ulcers and rheumatoid arthritis (23). In the present study, the anti-apoptotic and anti-inflammatory effects of ISL on chondrocyte-like ATDC5 cells were investigated. The present study is the first to demonstrate that ISL can prevent IL-1 β -induced chondrocyte-like ATDC5 cells apoptosis and inflammation by inhibiting NF- κ B activation. These chondro-protective effects were also observed in an ACLT model of osteoarthritis.

Pro-inflammatory cytokines have been identified to play a crucial role in the progression of OA, especially IL-1 β and TNF- α , which are considered as prominent cytokines for destruction of articular cartilage (8). One of the major effects of IL-1 β seems to be the induction of catabolic processes that affects cartilage matrices. It has been reported that IL-1 β not only induces the release of degenerative enzymes, such as MMPs, from synoviocytes and chondrocytes, but also inhibits

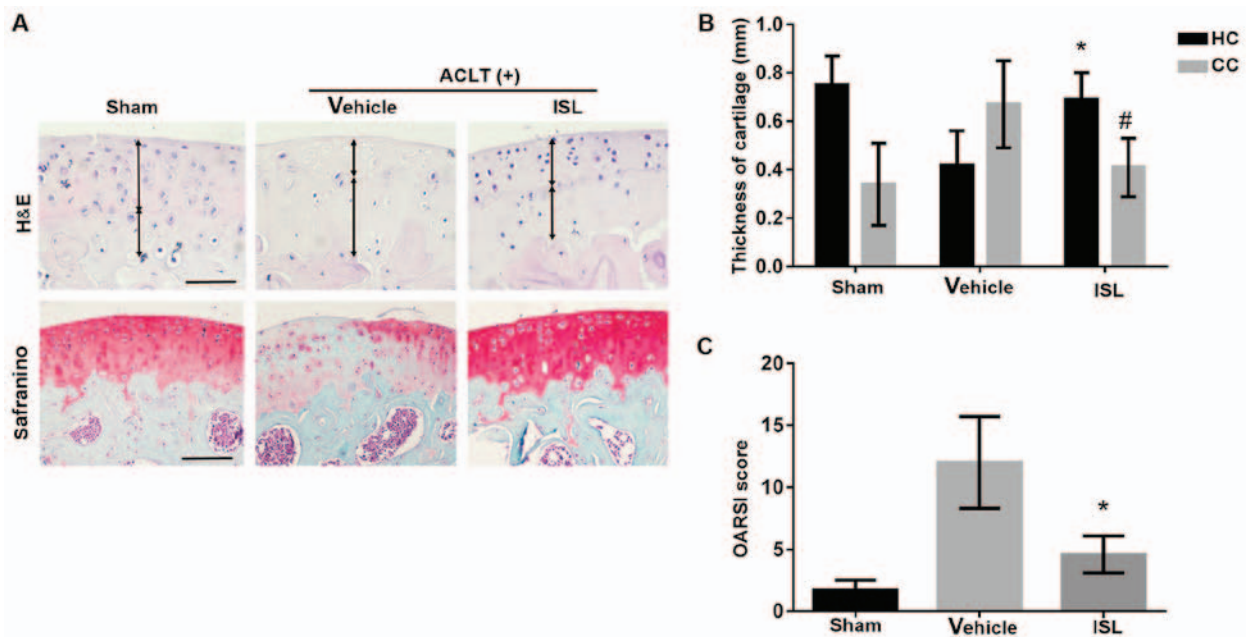


Figure 9. ISL exhibited chondroprotective effects on a mice ACLT model. Mice underwent ACLT operation and received intraperitoneal injections with 10% Tween-80 or ISL as described in Materials and methods. The mice were sacrificed and their knee joints were excised eight weeks after ACLT operation. (A) H&E staining (top) where the thickness of CC and HC in each group were measured (double-headed arrows) and (B) quantitative analyzed. Scale bars, 100 μ m. Safranin O and fast green staining (bottom): Solid arrows indicate proteoglycan loss and cartilage destruction. Scale bars, 100 μ m. (C) OARSI scores of articular cartilage at 8 weeks after surgery. Sham, sham-surgery; vehicle, ACLT-surgery treated with 10% Tween-80; ISL, ACLT-surgery treated with isoliquiritigenin. n=6/group. The values represent the mean \pm standard deviation. * $P<0.05$ and # $P<0.05$ vs. vehicle group. ISL, isoliquiritigenin; ACLT, anterior cruciate ligament transection; H&E, hematoxylin and eosin; CC, calcified cartilage zone; HC, hyaline cartilage zone; OARSI, Osteoarthritis Research Society International-modified Mankin.

the expression of a large number of genes related with the differentiated chondrocyte phenotypes such as COL II (8). IL-1 β was also found to activate cytokine receptors on surfaces of articular chondrocytes, which leads to induction of the NF- κ B signaling pathway (25). This pathway, acting either by itself, or in cooperation with bone morphogenetic protein, Wnt or other signaling cascades, interferes with the anabolic activity of chondrocytes (26) and leads to increases in expression of the inflammatory gene products COX-2 (25). Expression of inducible NOS, which is induced by a single stimulation of IL-1 β (27), was detected in synovial tissue and articular cartilage in joints affected by OA (28,29). In this way, IL-1 β may cause apoptotic death of chondrocytes via overproduction of NO. Moreover, increased levels of apoptosis were observed in human chondrocytes from normal and OA cartilage that had been incubated with human IL-1 β (30). For this reason, IL-1 β was selected to treat ATDC5 cells in cell viability assay (Fig. 2C) and following experiment, which made this *in vitro* study more reliable.

In the cell viability assay, it is noteworthy that instead of reverse the viability of chondrocyte-like ATDC5 cells, higher doses of ISL (20 and 40 μ mol/l) lead to significant decrease of IL-1 β -induced chondrocytes viability. Following that, cytotoxicity of ISL was examined by CCK-8 assay and flow cytometry using PE-Annexin V/7-ADD double-fluorescence labeling. The ATDC5 cells were treated with only ISL at different concentrations, and results indicated that ISL induced only mild cytotoxicity at lower concentrations (2.5, 5 and 10 μ mol/l), but it exhibited apparent cytotoxicity at higher concentrations (20 and 40 μ mol/l) (Figs. 2D and 4). These data indicated the stacking effect of toxicity in cells treated with 10 ng/ml IL-1 β plus ISL at different concentrations and help

explain the biphasic pattern in the viability of IL-1 β -stimulated chondrocytes depending on the concentration of ISL. Beyond that, ISL is one kind of phytoestrogen. Dual-directional regulation effect of phytoestrogen (31), which means that the contrary effect would be present depending on the concentration of treatment, may contribute to this complex mechanism. The results also indicated that IL-1 β promoted the levels of MMP-13 and COX-2, and decreased the levels of COL II in chondrocyte-like ATDC5 cells. This effect was also reversed by ISL in a dose-independent manner (Fig. 5).

Chondrocytes are the only cell type in articular cartilage, and they play a significant role in dynamic balance between anabolism and catabolism of cartilage ECM (5). A regular balance between tissue destruction and repair is essential to maintaining joint function. However, this balance is broken towards overall tissue destruction in pathological process of OA (19). This death of chondrocytes is the most prominent feature of arthritis. Natural chondrocytes survival factors have potential to be developed for therapeutic application. Our results from Annexin V/7-ADD double-fluorescence staining indicated that ISL inhibited the apoptotic rates of IL-1 β -induced chondrocyte-like ATDC5 cells in a dose-independent manner (Fig. 3).

Inflammatory cytokines simulate a series of downstream signaling pathways, including MAPK and NF- κ B pathways, which are aberrantly activated in chondrocytes of OA (18). The NF- κ B molecules are a family of widely expressed transcription factors involved in stress responses, immunity, cell proliferation, inflammatory diseases and cell apoptosis (32). The NF- κ B pathway is a central regulator of the inflammatory cytokine-induced catabolic actions in chondrocytes and triggers the secretion of several matrix-degrading proteinases,

including the MMPs and the aggrecanases, ADAMTS4 and ADAMTS5, leading to articular cartilage breakdown (33). Moreover, the NF- κ B pathway leads to upregulation of the inflammatory gene products COX-2, which rapidly induces the production of pro-inflammatory cytokines and MMPs during the early stage of OA, upregulates the expression of pro-apoptotic genes, and downregulates the expression of anti-apoptotic genes at the late stage of OA (30). Meanwhile, NF- κ B has been shown to regulate the expression of several members of the Bcl-2 gene family transcriptionally, which remains the prototypic anti-apoptotic protein (34). Phosphorylation of NF- κ B p65 is one of major signaling mechanisms associated with cell apoptosis. The present results indicated ISL had no obvious influence on the expression of p65, but it could inhibit phosphorylation of p65 (Fig. 6) and markedly alleviated the increase in cleaved-caspase-3 and cleaved-caspase-9 activity in IL-1 β -stimulated chondrocyte-like ATDC5 cells (Fig. 5).

In addition, the authors found that ISL exerted protective effects on cartilage in ACLT mice. Hyaline cartilage is the primary degenerative substance during OA, linking the calcified cartilage by tidemark. Calcified cartilage is a highly mineralized region in the articular cartilage, which is the end stage of hyaline cartilage (35). It is here reported that the activation of the NF- κ B signaling pathway can facilitate the transformation of articular chondrocytes from a pre-hypertrophic state to a terminal differentiation state, hypertrophy. The outcome of this hypertrophic transformation is the calcification of chondrocytes from hyaline cartilage zone and the formation of osteophytes at the joint periphery in the progression of OA (36). In mild OA, the thickness of calcified cartilage first increased at first and then decreased, but it increased progressively in cases of moderate OA, which suggests that the early pathological changes in calcified cartilage are reversible (37). The data collected here indicated that the calcified cartilage zone was significantly thicker 8 weeks after the ACLT operation, which was reversed by ISL. The OARSI scores as determined using Safranin O staining, demonstrated retention of proteoglycan in ISL-treated ACLT mice relative to vehicle-treated ACLT controls. These outcomes above suggest that ISL may work at the early pathological change of OA, which could serve as a potential preventive therapy for OA.

According to treatment of ISL in IL-1 β induced chondrocyte-like ATDC5 cells and mouse ACLT models, ISL was found to suppress IL-1 β -induced apoptosis and inflammation in chondrocyte-like ATDC5 cells by inhibiting the NF- κ B signaling pathway. These results also indicated that ISL exerts protective effects on articular cartilage *in vivo*, indicating that ISL may be a potential novel preventive therapy for OA. These results may in part explain the mechanisms by which ISL exerts its beneficial effects in OA, and they have certain limits. The mechanism underlying the effect of ISL on the NF- κ B pathway in chondrocytes has not yet been fully determined and requires further study.

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