

Original Article

Sclareol exerts anti-osteoarthritic activities in interleukin-1 β -induced rabbit chondrocytes and a rabbit osteoarthritis model

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Abstract: Sclareol is a natural product initially isolated from *Salvia sclarea* which possesses immune-regulation and anti-inflammatory activities. However, the anti-osteoarthritic properties of sclareol have not been investigated. The present study is aimed at evaluating the potential effects of sclareol in interleukin-1 β (IL-1 β)-induced rabbit chondrocytes as well as an experimental rabbit knee osteoarthritis model induced by anterior cruciate ligament transection (ACLT). Cultured rabbit chondrocytes were pretreated with 1, 5 and 10 $\mu\text{g}/\text{mL}$ sclareol for 1 h and followed by stimulation of IL-1 β (10 ng/mL) for 24 h. Gene expression of matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-13, tissue inhibitors of metalloproteinase-1 (TIMP-1), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 was determined by quantitative real-time polymerase chain reaction (qRT-PCR). MMP-3, TIMP-1, iNOS and COX-2 proteins were measured by Western blotting. Enzyme-linked immunosorbent assay (ELISA) was applied for nitric oxide (NO) and prostaglandin E2 (PGE2) assessment. For the *in vivo* study, rabbits received six weekly 0.3 mL sclareol (10 $\mu\text{g}/\text{mL}$) intra-articular injections in the knees four weeks after ACLT surgery. Cartilage was harvested for measurement of MMP-1, MMP-3, MMP-13, TIMP-1, iNOS and COX-2 by qRT-PCR, while femoral condyles were used for histological evaluation. The *in vitro* results we obtained showed that sclareol inhibited the MMPs, iNOS and COX-2 expression on mRNA and protein levels, while increased the TIMP-1 expression. And over-production of NO and PGE2 was also suppressed. For the *in vivo* study, both qRT-PCR results and histological evaluation confirmed that sclareol ameliorated cartilage degradation. Hence, we speculated that sclareol may be an ideal approach for treating osteoarthritis.

Keywords: Osteoarthritis, sclareol, matrix metalloproteinase, nitric oxide, ACLT

Introduction

Osteoarthritis (OA) is a common degenerative disease of joints. The pathologic nature of OA is the impairment of cartilage self-repair capability, caused by biochemical and biomechanical changes in joints [1]. According to the current knowledge, inflammatory cytokines are the key elements participating in OA pathogenesis [2]. Interleukin-1 β (IL-1 β) is thought to be a representative cytokine involved [3]. Activation of IL-1 β intracellular signaling pathway will result in expression of other cytokines, enzymes and inflammatory mediators. The expression of matrix metalloproteinases (MMPs) up-regulates, mainly MMP-1 (interstitial collagenase), MMP-3 (stromelysin-1), MMP-13 (collagenase 3), which have destructive efficacy on extracellular matrix (ECM) [4, 5]. IL-1 β also blocks the

metabolism of ECM via interfering in aggrecan and type-2 collagen synthesis [6, 7]. In addition, nitric oxide (NO) and prostaglandin E2 (PGE2) overproduction can be observed due to the IL-1 β -induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 [8, 9]. Compounds blocking the IL-1 β may be novel approaches in the treatment of OA, given the inflammatory and catabolic effect of IL-1 β .

Recently, natural products have attracted researchers' attention in treatment of OA. Sclareol (labd-14-ene-8, 13-diol), as a phytochemical labdane diterpene, is mainly isolated and purified from the leaves and flowers of *Salvia Sclarea*. Several previous studies have showed the anti-tumor and immune-regulation activity of sclareol both *in vitro* and *in vivo* [10-

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Table 1. Primers of targeted genes

Targeted genes	Accession number	Primer sequences (5' to 3')	Size (bp)	Annealing (°C)
Rabbit MMP-1	NM_001082037	F: CAGATGGGCATATCCCTCTAAGAA R: CCATGACCAAATCTACAGTCCTCAC	88	63
Rabbit MMP-3	NM_001082280	F: ACACCGGATCTGCCAAGAGA R: CTGGAGAACGTGAGTGGAGTCA	89	63
Rabbit MMP-13	NM_001082037	F: CATGCCAACAAATCCCTGCTGTGGT R: TCTCTCCCTGCACCTCCAGATTT	115	63
Rabbit TIMP-1	AY829730	F: CAACTGCGGAACGGGCTCTTG R: CGGCAGCGTAGGTCTTGGTGAA	102	63
Rabbit COX-2	AF247705	F: CACGCAGGTGGAGATGATCTAC R: ACTTCCTGGCCACAGCAAAC	69	62
Rabbit iNOS	AF469048	F: CTGTGACGTCCAGCGCTACAA R: CACGGCGATGTTGATCTCTGTGA	118	62
Rabbit 18S	EU236696	F: GACGGACCAGAGCGAAAGC R: CGCCAGTCGGCATCGTTTATG	119	63

F = forward; R = reverse.

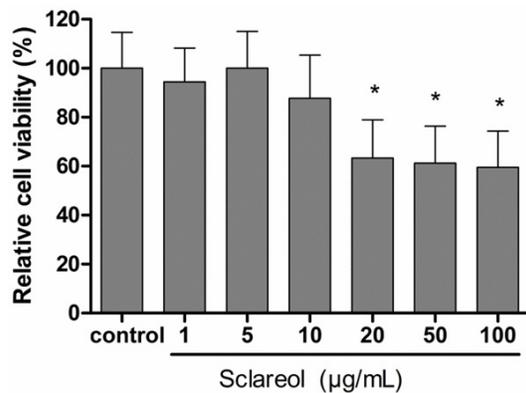


Figure 1. Effects of sclareol on cell viability. Chondrocytes were treated with sclareol for 24 h and measured by MTT assay. Sclareol showed no adverse effects on cell viability with concentrations of 1, 5, and 10 µg/mL. * $P < 0.05$ compared with control.

12]. Huang et al. noted that sclareol regulates the inflammatory response through inhibiting the expression of COX-2 and iNOS [13]. These findings indicate that sclareol has the potential to treat arthritis. In this study, we investigated the anti-OA activities of sclareol in IL-1 β -induced chondrocytes and an experimental rabbit OA model induced by anterior cruciate ligament transection (ACLT).

Materials and methods

Reagents

Reagents were obtained from different sources, mainly from Sigma-Aldrich in St. Louis, MO,

USA and Gibco BRL in Grand Island, NY, USA, in which the former provided recombinant human IL-1 β , 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazoliumbromide (MTT), sclareol and collagenase II and the latter provided Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS), 0.25% trypsin, and collagenase II. Sclareol, as the main object in this experiment, was dissolved in dimethyl sulfoxide (DMSO).

Isolation and culture of chondrocytes

The knee articular cartilage was harvested from 4-week-old New Zealand white rabbits (Animal Center of Zhejiang University) and this experimental study was approved by the Zhejiang University Institutional Animal Care and Use Committee, Hangzhou, China. First process was to isolate chondrocytes by digesting the thin slices of cartilage in 0.25% Trypsin for 30 minutes followed by 0.1% collagenase II in DMEM mixed with streptomycin (100 mg/mL) and penicillin (100 U/mL) at 37°C for 4 h. Next process was to culture the extracted cells in 25 cm² culture flasks in complete DMEM with 10% FBS, streptomycin (100 mg/mL) and penicillin (100 U/mL) in 5% CO₂ at 37°C. And the final process was to passage the confluent chondrocytes into a ratio of 1:3. The third-generation chondrocytes were used for this study.

Assessment of cell viability

We applied the MTT assay to evaluate the cytotoxicity of sclareol. Firstly, Chondrocytes were

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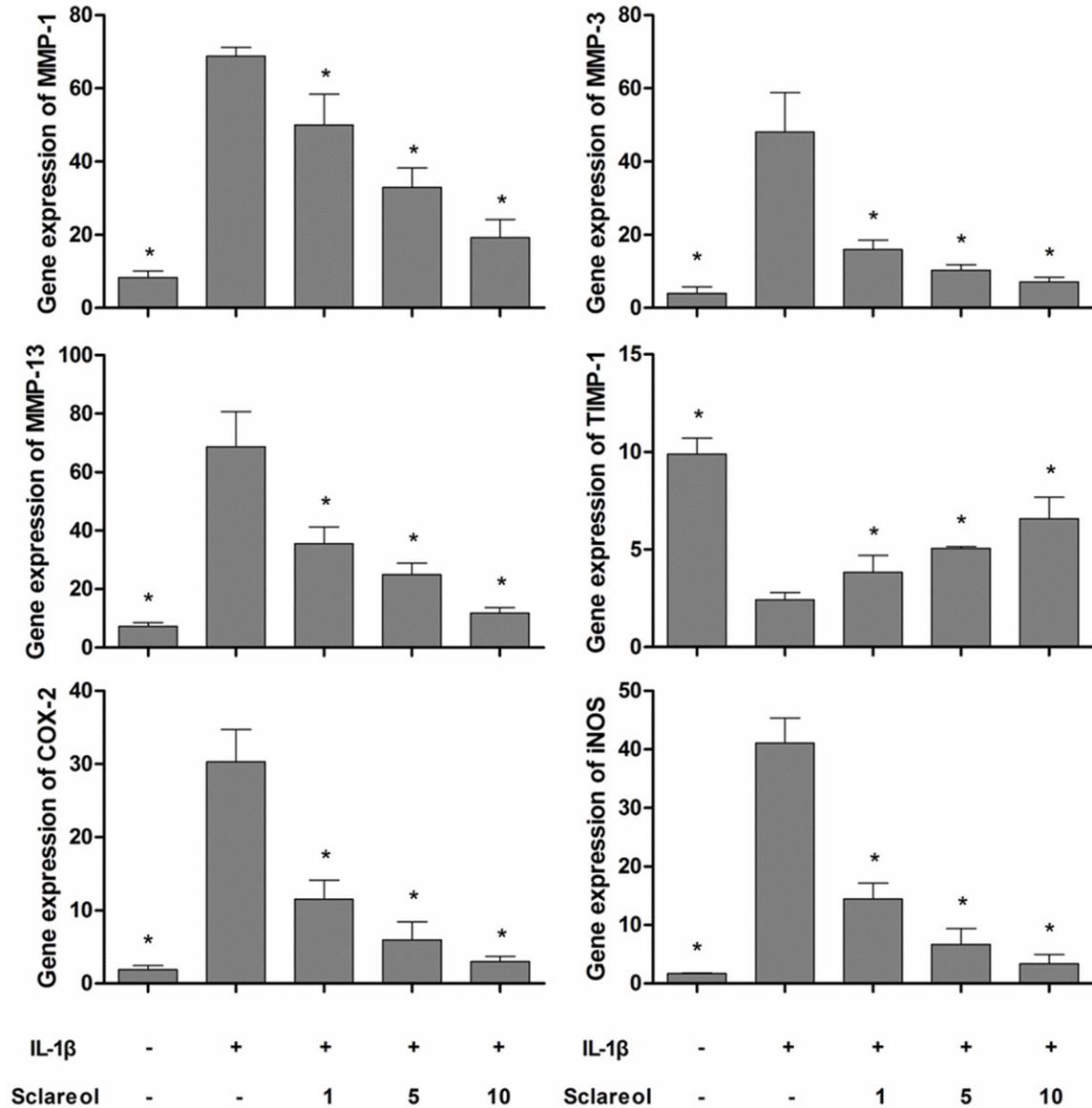


Figure 2. Effects of sclareol on MMP-1, -3, -13, TIMP-1, iNOS and COX-2 gene expression in IL-1 β -induced rabbit chondrocytes. Chondrocytes were pre-treated with sclareol (1, 5, or 10 μ g/mL) for 1 h, followed by stimulation with 10 ng/mL IL-1 β for 24 h. Levels of gene expression were determined by qRT-PCR. Sclareol significantly inhibited the expression of MMP-1, -3, -13, COX-2 and iNOS, but promoted the TIMP-1 expression. * $P < 0.05$ compared with chondrocytes stimulated with IL-1 β alone.

seeded in 96-well plates at a density of 5×10^3 per well and tested with six different concentrations of sclareol for 24 h. Then, cells were incubated with 20 μ L of MTT solution (5 mg/mL in phosphate buffered saline) for 4 h at 37°C. Added each well with 150 μ L DMSO after aspirating the supernatant. Finally, we used a micro-plate reader (Bio-Rad, Hercules, CA, USA) to measure the absorbance at 570 nm. The culture medium was used as a blank.

Chondrocytes treatments

Chondrocytes seeded in six-well plates (1×10^5 /well) were serum-starved overnight. Pre-treated chondrocytes with different concentrations of sclareol for 1 h, and then stimulated chondrocytes with IL-1 β (10 ng/mL) for 24 h. After these processes, conditioned medium was collected for measurement of NO and PGE2 by enzyme-linked immunosorbent assay

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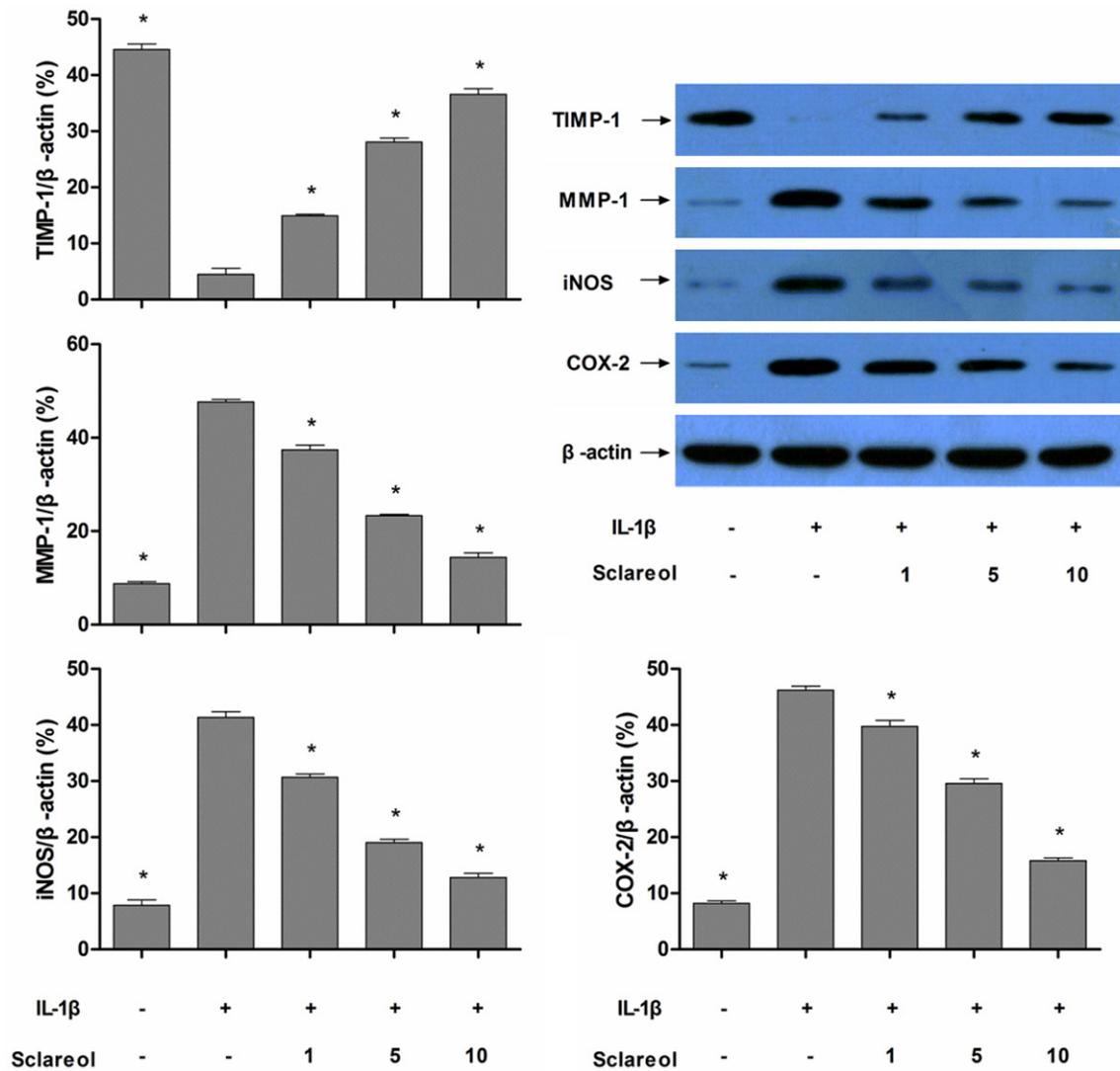


Figure 3. Effects of sclareol on TIMP-1, MMP-1, iNOS and COX-2 protein synthesis in IL-1 β -induced rabbit chondrocytes. Chondrocytes were pre-treated with sclareol followed by stimulation with IL-1 β . Protein levels were assessed by Western blotting analysis. Protein levels of MMP-1, iNOS, and COX-2 were suppressed, as TIMP-1 protein level increased. * $P < 0.05$ compared with chondrocytes stimulated with IL-1 β alone.

(ELISA) whereas chondrocytes were harvested for quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting.

NO and PGE2 measurements

According to the manufacturer's protocol (R&D Systems, Minneapolis, MN, SA), levels of NO and PGE2 were investigated using commercially available ELISA kits. All assays were performed in duplicate.

Gene expression analysis

According to the manufacturer's protocol, Total RNAs were extracted from chondrocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA).

Total RNA (600 μ g), 1 μ L dNTPs (10 mM) and 1 μ L primer mix were added into a 200 μ L RNase-free centrifuge tube. After adding 15 μ L DEPC-treated water, the centrifuged tubes were incubated on ice. Then, the tubes were incubated for 5 minutes at 70°C. The next step was adding 4 μ L 5 \times first-strand buffer, 2 μ L 0.1 M DTT, 25 units RNase inhibitor and 200 units Superscript II reverse transcriptase (Invitrogen). Finally, RNA was reverse-transcribed into cDNA.

Based on Sequence Information (**Table 1**), MMP-1, MMP-3, MMP-13, TIMP-1, COX-2 and iNOS expression levels were quantified by qRT-PCR, using iQ SYBR Green Supermix PCR kit and the iCycler system (Bio-Rad). Rabbit 18S

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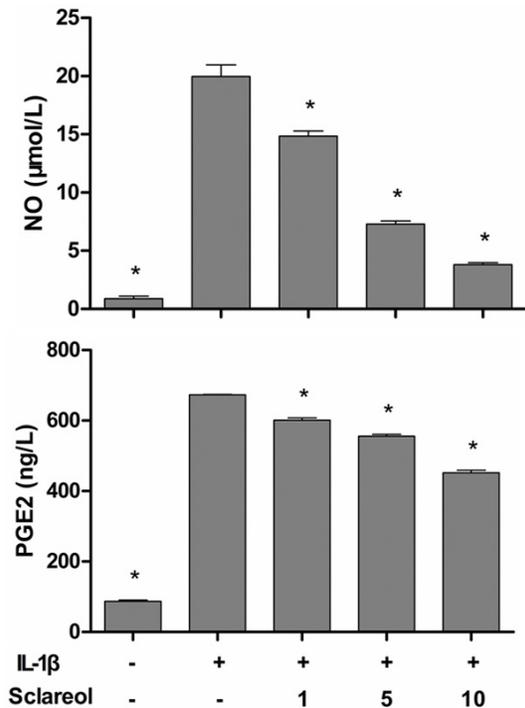


Figure 4. Effects of sclareol on NO and PGE2 production in IL-1 β -induced rabbit chondrocytes. Chondrocytes were pre-treated with sclareol followed by stimulation with IL-1 β . Conditioned medium was collected for NO and PGE2 assessment by ELISA. Production of NO and PGE2 was suppressed by sclareol. * $P < 0.05$ compared with chondrocytes stimulated with IL-1 β alone.

was amplified as an internal control. And finally, calculated the relative levels of targeted gene expression with the following formula: $2^{-(\Delta\text{ct target gene} - \Delta\text{ct 18s rRNA})}$.

Western blotting analysis

Cytoplasmic proteins were isolated with an extraction kit (Beyotime, Jiangsu, China) after the stimulated chondrocytes were washed twice with ice-cold phosphate buffered saline. Next, cytoplasmic proteins were resolved by SDS-PAGE and transferred to PVDF membranes. After blocking with 5% milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h, the membranes were incubated with antibodies for β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MMP-1 (ab12-6847, Abcam, Cambridge, MA, USA), TIMP-1 (SC-377097, Santa Cruz Biotechnology), iNOS (ab21775, Abcam), and COX-2 (ab15191, Abcam) overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for

1 h at room temperature, and signals were detected by X-ray film exposure (Kodak, China) using an Enhanced Chemiluminescence (ECL) kit.

Animal studies

Sixteen New Zealand Rabbits weighing 2.0 kg (Animal Center of Zhejiang University) were used in the animal study with the approval from the Zhejiang University Institutional Animal Care and Use Committee. Twelve rabbits received bilateral ACLT surgeries to induce OA in knee joints, as previously described [14]. The other 4 rabbits received sham operations as controls. The OA rabbits received weekly intra-articular injections of 0.3 mL sclareol (10 $\mu\text{g}/\text{mL}$) in the right knee and vehicle (DMSO) in the left knee for 6 weeks at 4 weeks after surgery. All rabbits were sacrificed 7 days after the last intra-articular injection. Femoral condyles were obtained for morphology, histology and gene expression evaluation.

Histologic evaluations

Femoral condyles were fixed in 4% paraformaldehyde and decalcified in 10% formic acid. Decalcified specimens were dehydrated and embedded in paraffin, and sliced into 5- μm thick sections. The specimens were stained with safranin O-fast green, and evaluated for the degree of histological changes in a blinded manner according to the Mankin scoring system [15].

Statistical analysis

All data were expressed in mean \pm standard deviation (SD). The ELISA and MTT assays data were performed using the unpaired t -test while histological and gene expression data were analyzed by the paired t -test. A P -value < 0.05 was considered statistical significance.

Results

Effects of sclareol on cell viability

The MTT assay was applied to measure the toxicity of 1, 5, 10, 20, 50 and 100 $\mu\text{g}/\text{mL}$ sclareol in normal chondrocytes. In chondrocytes stimulated with 1, 5 and 10 $\mu\text{g}/\text{mL}$ sclareol, there is no significant difference in relative cell viability compared with the control. But concentrations ≥ 20 $\mu\text{g}/\text{mL}$ were toxic (**Figure 1**). Thus, we

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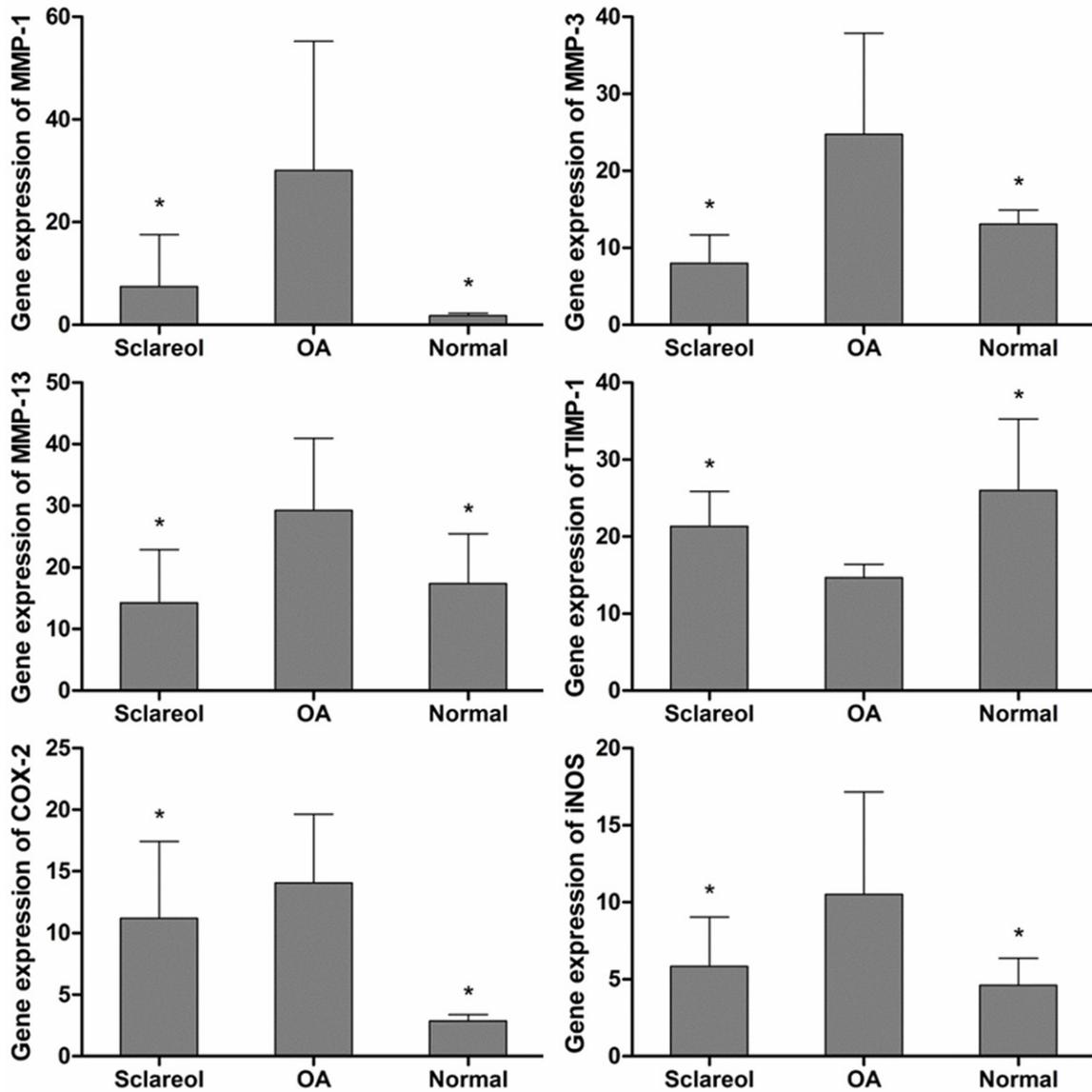


Figure 5. Effects of sclareol on gene expression in cartilage. Gene expression levels in cartilage were analyzed by qRT-PCR. Compared with the normal group, expression of MMP-1, -3, -13, COX-2 and iNOS increased significantly, whereas expression of TIMP-1 decreased in the OA group. Sclareol inhibited expression of MMP-1, -3, -13, COX-2, and iNOS and promoted the TIMP-1 expression. Sclareol Group: underwent ACLT and was treated with sclareol via intra-articular injections; OA Group: underwent ACLT and was injected with vehicle (DMSO); Normal Group: received sham operations. * $P < 0.05$, compared with the OA group.

chose 1, 5, and 10 $\mu\text{g}/\text{mL}$ as the optimal concentrations for further study.

Effects of sclareol on the expression of MMPs, TIMP-1, iNOS and COX-2 in rabbit chondrocytes

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess the gene expression in IL-1 β -induced chondrocytes. The gene expression of MMP-1, MMP-3, MMP-

13, iNOS and COX-2 was up-regulated. On the contrary, the gene expression of TIMP-1 was down-regulated. We found that pre-incubation with 1, 5 and 10 $\mu\text{g}/\text{mL}$ sclareol significantly suppressed the IL-1 β -mediated high expression of MMP-1, MMP-3, MMP-13, iNOS and COX-2 but up-regulated TIMP-1 expression (Figure 2).

Next, we applied Western blotting to examine the protein synthesis in chondrocytes. In chon-

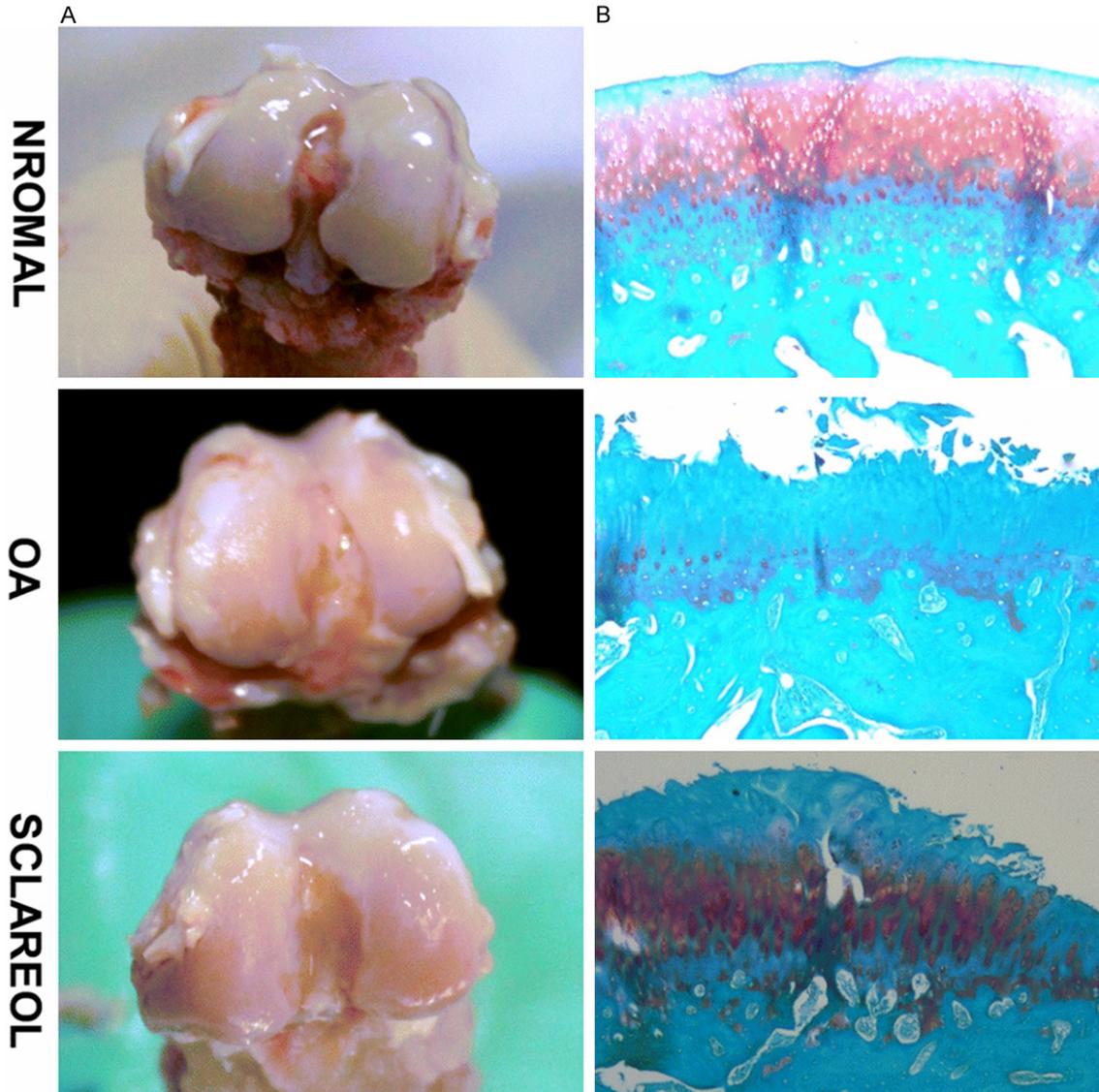


Figure 6. Macroscopic observations (A) and Safranin-O-Fast Green staining (B) of articular cartilage of different groups. Typical changes in cartilage lesions are seen.

drocytes induced by IL-1 β , protein concentrations of MMP-1, iNOS, and COX-2 increased, however, protein synthesis of TIMP-1 was inhibited. These effects mediated by IL-1 β were blocked by different concentrations of sclareol to varying degrees (**Figure 3**).

Effects of sclareol on the expression of NO and PGE2 in cell culture medium

ELISA was used to determine NO and PGE2 production. Our results showed that IL-1 β treatment resulted in over-production of NO and PGE2 in culture medium. Sclareol significantly inhibited the production of these two molecules (**Figure 4**).

Gene expression in cartilage

Gene expression levels of MMP-1, MMP-3, MMP-13, iNOS and COX-2 were down-regulated significantly in rabbit knee articular cartilage obtained from the sclareol group, whereas the TIMP-1 gene was up-regulated, compared with the OA group (joints underwent ACLT and were injected with vehicle) (**Figure 5**).

Macroscopic observation and histologic evaluation

Cartilage lesions were observed in femoral condyles from knee joints underwent ACLT. Com-

Table 2. Histological score of articular cartilage

Femoral condyle	Sclareol	OA
Structural changes	2.83 ± 0.69*	3.67 ± 0.47
Cellular changes	2.17 ± 0.69*	2.83 ± 0.37
Safranin staining	2.50 ± 0.96*	3.33 ± 0.47
Tidemark	0.33 ± 0.47*	1
Sum of score	7.83 ± 1.21*	10.83 ± 0.37

Values are the means ± SD. * $P < 0.05$ compared with OA group analyzed by paired-samples t-test.

pared with the sclareol group, cartilage lesions in OA group were much more severe (**Figure 6A**).

For the histologic evaluations, large amount of safranin O-fast green staining reduction was noted in OA group. Histologic changes in sclareol group indicated that intra-articular injection of sclareol partly reversed the reduction (**Figure 6B**). Cartilage of sclareol group showed lower Markin score than the OA group (**Table 2**).

Discussion

OA is a widespread disabling disease caused by irreversible cartilage destruction. Main pathological characteristics of OA are cartilage degradation, synovial inflammation and remodeling of subchondral bone including osteophyte formation, bone remodeling, subchondral sclerosis, and attrition [1]. Cartilage degradation is promoted by a network of various cytokines, and the catabolic cytokine IL-1 β plays a crucial role in particular [2]. It is well accepted that IL-1 β exerts its activities in OA by enhancing the expression of MMPs, COX-2 and iNOS and block the ECM structural compounds synthesis, as we mentioned previously.

MMPs, as a family of proteinases induced by a range of inflammatory cytokines, are able to degrade ECM components such as aggrecan and collagens [16]. MMP-1 (interstitial collagenase), MMP-3 (stromelysin-1) and MMP-13 (collagenase 3) are the most relevant enzymes in OA [17]. MMP-1 and MMP-13, the collagenases in the MMPs family, are regarded as rate-limiting enzymes in the degradation of type II collagen. Moreover, MMP-13 also has the ability to degrade aggrecan in ECM, giving it a multifunctional role in cartilage matrix breakdown [16]. MMP-3 acts as an activator of other MMPs

[18], and can degrade aggrecan, fibronectin, laminin in ECM directly, but not for type II collagen [19]. The activities of all known MMPs can be regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). Previous studies showed that the effect of MMPs is highly depended on the MMPs to TIMPs ration and an excess activity of MMPs over TIMPs will result in pathologic cartilage destruction [17].

Nitric oxide synthase (NOS) family includes three members: neuronal NOS (nNOS) and endothelial NOS (eNOS), as constitutive NOS, and inducible NOS (iNOS). Cytokines such as IL-1 and TNF- α can induce iNOS to product NO [20]. NO plays its role in pathogenesis of OA via inducing chondrocytes and synoviocytes death [21]. Elevated level of NO was observed in cartilage and serum of OA patients [22]. IL-1 β stimulates the expression of COX-2 to increase the synthesis of PGE2, which is responsible for bone resorption and joint pain in OA [23, 24]. Both of NO and PGE2 are capable of up-regulating the production of MMPs and other inflammatory cytokines [25, 26].

In our *in vitro* study, we mimicked the OA micro-environment by cultured rabbit chondrocytes stimulated with IL-1 β . In the chondrocytes stimulated with IL-1 β , up-regulation of the gene expression and production of MMPs, iNOS and COX-2, down-regulation of TIMP-1 and over-production of NO and PGE2 were observed. Pretreatment with 1, 5 or 10 $\mu\text{g}/\text{mL}$ sclareol significantly decreased the expression of MMPs on both mRNA and protein levels, while TIMP-1 expression level was elevated. Sclareol also suppressed the induction of iNOS and COX-2 by IL-1 β .

To investigate the *in vivo* effect of sclareol, we used ACLT to establish an experimental rabbit OA model. Macroscopic observations and Histological changes confirmed that intra-articular injection of sclareol ameliorated cartilage degradation in OA. Consistent with the *in vitro* findings, sclareol inhibited the expression of MMP-1, MMP-3, MMP-13, iNOS and COX-2, while TIMP-1 expression was up-regulated in rabbit cartilage.

In conclusion, our study firstly demonstrated that sclareol exerts chondroprotective effect by regulation the balance between MMPs and TIMPs and inhibiting iNOS and COX-2 expres-

sion *in vitro* and *in vivo*. The results we obtained indicate sclareol as a possible therapeutic agent to treat OA. However, the molecular mechanisms and involved signaling pathways by which sclareol regulated MMPs, TIMPs, iNOS and COX-2 remain unclear. Comparisons between sclareol and classical anti-OA agents in potency, toxicity and potential side effects are still needed in further researches.

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Disclosure of conflict of interest

None.

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