

Acteoside counteracts interleukin-1 β -induced catabolic processes through the modulation of mitogen-activated protein kinases and the NF κ B cellular signaling pathway

Hyangl Lim

Chosun University

Do Kyung Kim

Chosun University

Tae-Hyeon Kim

Chosun University

Kyeong-Rok Kang

Chosun University

Jeong-Yeon Seo

Chosun University

Sung Sik Cho

Mokpo National University

Younghée Yun

Chung-Yeon Medical Institute

Ye-yong Choi

Chung-Yeon Medical Institute

Jungtae Leem

Chung-Yeon Medical Institute

Hyoun-Woo Kim

Jeollanamdo Forest Resources Institute

Geon-Ung Cho

Jeollanamdo Forest Resources Institute

Chan-Jin Oh

Jeollanamdo Forest Resources Institute

Deuk-Sil Oh

Jeollanamdo Forest Resources Institute

Hong-Sung Chun

Chosun University

jae-sung kim (✉ js_kim@chosun.ac.kr)

Chosun University

Research article

Keywords: Acteoside, Osteoarthritis, Chondrocytes, Inflammation, Anti-catabolism

Posted Date: May 23rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-30429/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Objectives

To verify the anti-catabolic effects of acteoside [α -L-rhamnosyl-(1 \rightarrow 3)- β -D-glucoside] against osteoarthritis and its anti-catabolic signaling pathway.

Methods

Primary rat chondrocytes were isolated enzymatically from the articular cartilage of rat knee joint. Cytotoxicity of acteoside was assessed by MTT and Cell Live/Dead assay. Proteoglycan content was measured by dimethylmethylene blue assay. The proteoglycan loss was assessed by histological analysis using safranin-O & fast green staining after *ex vivo* organ culture of articular cartilage. The alteration of catabolic factors such as cartilage degrading enzymes, pro-inflammation cytokines, and inflammatory mediators were assessed by qPCR, qRT-PCR, gelatin zymography, western blot, and cytokine array. Cellular signaling pathways were investigated by western blot and nucleus translocation. Acteoside was orally administrated to osteoarthritic animals generated by the destabilization of medial meniscus at the knee joint of mice for 8 weeks. Thereafter, proteoglycan loss was assessed by safranin-O & fast green staining.

Results

Acteoside did not decrease the viabilities of mouse fibroblast L929 cells used as a normal cells and primary rat chondrocytes. Acteoside counteracted the IL-1 β -induced proteoglycan loss in the chondrocytes and articular cartilage through suppressing the expression and activation of cartilage-degrading enzyme such as matrix metalloproteinase (MMP)-13, MMP-1, and MMP-3. Furthermore, acteoside suppressed the expression of inflammatory mediators such as inducible nitric oxide synthase, cyclooxygenase-2, nitric oxide, and prostaglandin E₂ in the primary rat chondrocytes treated with IL-1 β . Subsequently, the expression of pro-inflammatory cytokines was decreased by acteoside in the primary rat chondrocytes treated with IL-1 β . Moreover, acteoside suppressed not only the phosphorylation of mitogen-activated protein kinases in primary rat chondrocytes treated with IL-1 β but also the translocation of NF κ B from the cytosol to the nucleus through suppression of its phosphorylation. Oral administration of 5 and 10 mg/kg acteoside attenuated the progressive degeneration of articular cartilage in the osteoarthritic mouse model generated by destabilization of the medial meniscus.

Conclusion

Our findings indicate that acteoside is a promising potential anti-catabolic agent or supplement to attenuate or prevent progressive degeneration of articular cartilage.

Introduction

Osteoarthritis (OA) is the most common degenerative joint disease with chronic joint pain caused by progressive degeneration of articular cartilage at synovial joints [1]. Due to the increase in life expectancy, the prevalence of OA with loss of mobility and chronic joint pain caused by progressive degeneration of articular cartilage at synovial joints is estimated to be 18% and 9.6% in women after menopause and in men, respectively [2]. Although the worldwide prevalence of OA increases annually, the pathophysiological etiology of OA is still unknown. It may be caused by very complex and multifactorial risk factors such as aging, gender, genetic inheritance, traumatic joint injury, and severe mechanical joint load. Furthermore, the neuropathological relationships between progressive degeneration of articular cartilage and development of chronic joint pain are unknown [3]. Hence, the goal of clinical management for patients with OA is the maintenance of body mobility and mechanical joint function through relief from chronic joint pain, using pharmacological and non-pharmacological approaches and joint replacement surgery. The demand for development of effective intervention or supplementation, with long-term biological safety, to prevent or attenuate OA to maintain life quality through maintenance of mechanical joint function in the elderly population is increasing.

As shown in Fig. 1, acteoside (CAS No. 61276-17-3; C₂₉H₃₆O₁₅) is a caffeoylphenylethanoid glycoside isolated from several herbal plants such as *Verbascum phlomoides* [4], *Buddleja globosa* [5], and *Plantago australis* [6]. Acteoside has various biological activities such as antimicrobial [5], anti-inflammatory, [7] anticancer [8], antioxidative [9], cytoprotective [9], and neuroprotective effect [10]. Further, oral administration of acteoside at high dosage does not cause genotoxicity [11].

Hence, we hypothesized that acteoside with anti-inflammatory biological safety has anti-catabolic effects associated with the protection of articular cartilage against progressive degeneration of articular cartilage through suppression of catabolic factors such as the pro-inflammatory cytokines, inflammatory mediators, and cartilage-degrading enzymes in synovial joints. Therefore, the aim of this study was to investigate the acteoside-induced anti-catabolic effects and its cellular signaling pathway both *in vitro*, using primary chondrocytes isolated from the articular cartilage of rat knee joint, and *in vivo*, using an OA-animal model generated by surgical destabilization of the median meniscus in the knee joint of mice.

Materials And Methods

Isolation and culture of primary rat chondrocytes

Primary rat chondrocytes were isolated from the articular cartilage of rat (5-day-old; Sprague–Dawley) knee joints, in accordance with the protocol (CIACUC2019-A0027) approved by the Institutional Animal Care and Use Committee of Chosun University, Gwangju, Republic of Korea. Isolated primary rat chondrocytes were maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) (Thermo Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS), antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin), and 50 µg/mL ascorbic acid.

Cell Viability Assay

The dimethyl thiazolyl diphenyl tetrazolium salt (MTT) assay was performed to assess the viabilities of mouse fibroblast cell line L929 cells used as a normal cell and primary rat chondrocytes treated with acteoside. Briefly, L929 cells and primary rat chondrocytes were cultured at a cell density of 8×10^5 cells/ml in culture plates for 24 h, and then treated with 2.5, 5, 10, 25, 50, and 100 μM acteoside for 24 h. After treatment with MTT solution, both L929 cells and chondrocytes were further cultured for 4 h. After incubation, the formed MTT crystals were suspended completely in dimethyl sulfoxide and measured for absorbance at 570 nm using a spectrometer (Epoch microplate spectrophotometer, BioTek®, Winooski, VT, USA) to assess cell viability.

Cell Live/Dead Assay

Cell survival was performed using Cell Live/Dead assay kit (Molecular Probes, Carlsbad, CA, USA), which composed of green calcein AM for labeling live cells (with green fluorescence) and ethidium homodimer-1 for labeling dead cells (with red fluorescence). Briefly, both L929 cells and primary rat chondrocytes were cultured at a cell density of 8×10^5 cells/ml on chamber slides (Nunc® Lab-Tek® Chamber Slide™ system; Sigma-Aldrich; Merck KGaA) for 24 h, and then treated with 50 and 100 μM acteoside for 24 h. After cultivation, cell survival assay was performed according to the manufacturer's instruction. Thereafter, stained cells were imaged using a fluorescence microscope (Eclipse TE200; Nikon Instruments, Melville, NY).

Dimethylmethylene Blue (DMMB) Assay

DMMB assay was performed to assess the alteration of proteoglycan content in primary rat chondrocytes treated with acteoside for 21 days in the presence or absence of IL-1 β . To maintain the characteristics of primary rat chondrocytes for 21 days, primary rat chondrocytes (2×10^6 cells) were suspended in 1 ml of 1.2% alginate and then encapsulated by dripping the cell/alginate suspension to a solution of 105 mM CaCl₂. The primary rat chondrocytes encapsulated in alginate were cultured for 24 h in DMEM/F12 (containing 10% FBS, 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, and 50 $\mu\text{g}/\text{mL}$ ascorbic acid), and then adapted for 24 h in DMEM/F12 containing 1% mini-insulin–transferrin–selenium (mini-ITS) and 50 $\mu\text{g}/\text{mL}$ ascorbic acid. Subsequently, the chondrocytes were treated with 50 or 100 μM acteoside in the presence or absence of 1 ng/mL IL-1 β for 21 days. At day 21, the primary rat chondrocytes were collected for assessment of proteoglycan content using the DMMB assay, as described previously [12]. In addition, to quantify proteoglycan content per cells and assess the proliferation of primary rat chondrocytes, cell numbers were measured by DNA assay using PicoGreen (Molecular Probes, Carlsbad, CA), according to manufacturer's instructions.

Ex vivo organ culture of rat articular cartilage tissues

Articular cartilage tissues were isolated from the knee joints of 5-day-old Sprague–Dawley rats and then cultured in DMEM/F12 supplemented with 10% FBS. Next, the articular cartilage samples were treated with 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 7 days. At the end of the culture period, the samples were collected and fixed in 4% paraformaldehyde for 72 h for histological analysis.

Histological Analysis

Histological analysis using safranin-O and fast green staining was performed to verify proteoglycan loss in the articular cartilage treated with 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 7 days. Briefly, fixed articular cartilage samples were decalcified in ethylenediaminetetraacetic acid and then embedded in paraffin. Thereafter, the prepared paraffin blocks containing articular cartilage were serially sliced to 5 μ m thickness and placed on slides. Safranin-O and fast green staining was subsequently performed to assess proteoglycan loss in the articular cartilage ground substance. In addition, hematoxylin and eosin staining was performed to observe the general morphology of the articular cartilage.

Western Blotting

Western blotting was performed to investigate the expression of catabolic factors including MMP-13, MMP-1, MMP-3, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) and the alteration of cellular signaling molecules such as mitogen-activated protein kinases and nuclear factor-kappa B (NF κ B). Briefly, rat primary chondrocytes were treated with 50 or 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, rat primary chondrocytes were harvested by centrifugation and were lysed using lysis buffer (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. In addition, to verify the nuclear translocation of NF κ B, rat primary chondrocytes were treated with 50 or 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, cytosolic and nuclear fractions were extracted using NE-PER™ Nuclear and Cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. The concentration of total protein extracted from primary rat chondrocytes was determined using a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. In addition, the conditioned medium was collected to detect the levels of cartilage-degrading enzymes secreted from chondrocytes. Equal amounts of protein and conditioned medium were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes. Thereafter, western blotting was performed using targeted primary antibodies against MMP-13, MMP-1, MMP-3, iNOS, COX-2, phospho-ERK1/2, total-ERK1/2, phospho-p38, total-p38, phospho-JNK, total JNK, phospho-NF κ B, total NF κ B, β -actin, and lamin B. Immunoreactive bands were visualized using an enhanced chemiluminescence system (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instruction and then imaged by a Microchemi device (DNR Bioimaging Systems, Jerusalem, Israel).

Quantitative polymerase chain reaction (qPCR) and quantitative real time PCR (qRT-PCR)

Primary rat chondrocytes were treated with 50 or 100 µM acteoside in the presence or absence of 10 ng/mL IL-1β for 24 h. Thereafter, total RNA was isolated from the primary rat chondrocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA concentration was measured using a Nanodrop 2000 (Thermo Scientific, Rockford, IL, USA). To synthesize cDNA, 1 µg RNA was reverse transcribed using a ThermoScript reverse transcription-PCR system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qPCR of cDNA was performed using 2 × TOPsimple™ DyeMIX-nTaq (Enzyomics, Seoul, Republic of Korea) and specific primers on a TaKaRa PCR Thermal Cycler Device (TaKaRa Bio Inc., Shiga, Japan). Thereafter, the PCR products were electrophoresed on an agarose gel to determine the expression levels of target genes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. In addition, for qRT-PCR, cDNA was amplified using an Eco™ Real-Time PCR system (illumine Inc., San Diego, CA, USA). β-actin was used as an endogenous control. The sequences of the primers used in the qPCR and qRT-PCR are summarized in Tables 1 and 2, respectively.

Table 1
Quantitative PCR primer sequences used in this study.

Gene	Primer sequences	NCBI gene No.
MMP-13	Forward: 5'- GGCAAAAGCCATTCTATGCTCCC-3' Reverse: 5'- AGACAGCATCTACTTGTCGCCA-3'	NM_133530.1
MMP-1	Forward: 5'- CAACGCAGATTAGCCTCCGA-3' Reverse: 5'- GAGATGCCAGGACCACAGT-3'	NM_001134530.1
MMP-3	Forward: 5'- TCCTACCCATTGCATGGCAGTGAA-3' Reverse: 5'- GCATGAGCCAAGACCATTCCAGG-3'	NM_133523.3
iNOS	Forward: 5'- GCATCGGCAGGATTCACTGG-3' Reverse: 5'- TAGCCAGCGTACCGGATGAG-3'	NM_012611.3
COX-2	Forward: 5'- CCCTTCCTCCTGTGGCTGAT-3' Reverse: 5'- CCCAGGTCCCTCGCTTCTGAT-3'	NM_017232.3
GAPDH	Forward: 5'- TGATGCTGGTGTGAGTATG-3' Reverse: 5'- GGATGCAGGGATGATGTTCT-3'	NM_017008.4

Table 2
Quantitative real time PCR primer sequences used in this study.

Gene	Primer sequences	NCBI gene No.
MMP-13	Forward: 5'-TTGGCTTAGATGTGACTGGC-3' Reverse: 5'-CCCTCGAACACTCAAATGGT-3'	NM_133530.1
MMP-1	Forward: 5'-CTACCAGCTCATACAGTTCCC-3' Reverse: 5'-CTACAACTTGGGTGAAGACGT-3'	NM_001134530.1
MMP-3	Forward: 5'- GTCTGAAAAGGATGTGAAGCAG-3' Reverse: 5'- CTCGAACACTATGGAGCTGATG-3'	NM_133523.3
GAPDH	Forward: 5'- AACCCATCACCATCTTCCAG-3' Reverse: 5'- CTGGTGCTGAGTATGTCGTG-3'	NM_017008.4

Gelatin Zymography

Gelatin zymography was performed to assess the activation of MMPs in primary rat chondrocytes. Briefly, primary rat chondrocytes were treated with 50 or 100 µM acteoside in the presence or absence of 10 ng/mL IL-1β for 24 h. Thereafter, an equal volume of conditioned medium was electrophoresed on a 10% polyacrylamide gel copolymerized with 0.2% (1 mg/mL) porcine skin gelatin. After electrophoresis, the gel was incubated in zymogram renaturing buffer [50 mM Tris-HCl (pH 7.6), 10 mM CaCl₂, 50 mM NaCl, and 0.05% Brij-35] at 37 °C for 72 h. After renaturation of MMPs, the gel was stained with 0.1% Coomassie Brilliant Blue R250. Gelatinolytic bands were revealed as clear bands on a background uniformly stained light blue and then imaged using a digital camera.

Measurement Of Nitric Oxide (NO)

Primary rat chondrocytes were treated with 50 or 100 µM acteoside in the presence or absence of 10 ng/mL IL-1β for 24 h. Thereafter, 50 µL of the conditioned medium was reacted with 50 µL each of sulfanilamide and N-1-naphthylethylenediamine dihydrochloride. Absorbance was then measured at 540 nm wavelength using a spectrophotometer (Epoch Spectrophotometer, BioTek, Winooski, VT, USA).

Prostaglandin E₂ (PGE₂) Assay

Primary rat chondrocytes were treated with 50 or 100 µM acteoside in the presence or absence of 10 ng/mL IL-1β for 24 h. Thereafter, PGE₂ concentration was measured using a PGE₂ parameter assay kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Cytokine Array

Primary rat chondrocytes were treated with 50 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, total proteins were extracted and quantified as previously described [13]. Next, cytokine array was performed to investigate alteration in cytokine production, according the manufacturer's instructions (RayBiotech, Inc., Norcross, GA, USA).

Nuclear Translocation Assay

Primary rat chondrocytes were treated with 50 and 100 μ g/mL acteoside in the presence of 10 ng/mL IL-1 β . After 30 min, primary rat chondrocytes were fixed with 1% paraformaldehyde, permeabilized in 0.2% Triton X-100, and extensively washed with phosphate buffered saline. Non-specific signals were blocked using normal goat serum. After multiple washes, the chondrocytes were incubated with rabbit anti-NF κ B antibodies followed by incubation with FITC-conjugated goat anti-rabbit IgG (ThermoFisher Scientific, Waltham, MA, USA) overnight at 4 °C. Thereafter, stained cells were imaged using a laser confocal scanning microscope system (Leica Microsystems, Wetzlar, Germany) at the Gwangju branch of Korea Basic Science Institute (Gwangju, Republic of Korea).

Generation Of Osteoarthritic Animals

To generate osteoarthritic animals, the medial meniscus (DMM) was surgically destabilized in the knee joints of BALB/c mice (average body weight 19.3 ± 0.5 g) in accordance with IACUC guidelines (CIACUC2019-A0029). The OA-induced animals were treated orally with 5 and 10 mg/kg acteoside resolved in 5% ethanol (experimental group; $n = 5$) or vehicle (5% ethanol) (DMM group; $n = 5$) every other day for 8 weeks. At the end of the culture period, knee joints were dissected and fixed using 5% paraformaldehyde for 7 days to perform histological assessments. After safranin-O and fast green staining, imaged tissues of articular cartilage were examined in accordance with the Mankin's grade [14, 15].

Statistical analysis

The experimental data are presented as the mean \pm standard deviation and were compared using analysis of variance, followed by post-hoc multiple comparison (Tukey's test) using SPSS software version 25 (IBM Corp.) All the data, except the animal study, were obtained from three independent experiments.

Results

Acteoside does not affect L929 cell and primary rat chondrocyte viability

The mouse fibroblast cell line L929 used as normal cells was treated with 2.5, 5, 10, 25, 50, and 100 μM acteoside for 24 h. Thereafter, the MTT assay was performed to assess the cytotoxicity of acteoside on L929 cells. As shown in Fig. 2A, relative viabilities of L929 cells were determined to be $94.8 \pm 8\%$, $93.6 \pm 7\%$, $100 \pm 5\%$, $103.9 \pm 5\%$, $126.1 \pm 8\%$, and $122.7 \pm 4\%$ at 2.5, 5, 10, 25, 50, and 100 μM acteoside, respectively, compared with control ($100.02 \pm 3\%$). Furthermore, to verify the cytotoxicity of acteoside on primary rat chondrocytes, the MTT assay was performed as shown in Fig. 2B. The viabilities of primary rat chondrocytes treated with 2.5, 5, 10, 25, 50, and 100 μM acteoside were determined as $114 \pm 4\%$, $117.8 \pm 6\%$, $123.9 \pm 5\%$, $132.6 \pm 4\%$, $153.1 \pm 7\%$, and $142.1 \pm 6\%$, respectively, compared with control ($100.4 \pm 5\%$). Furthermore, to confirm the effect of acteoside on the viability of both L929 cells and primary rat chondrocytes, Cell Live/Dead assay was performed as shown in Fig. 2C. The number of dead cells stained as red fluorescence did not increase for both L929 cells and primary rat chondrocytes treated with 50 and 100 μM acteoside for 24 h. These data consistently demonstrated that defined dosage of acteoside did not affect the viability of L929 cells and primary rat chondrocytes. Thus, 50 and 100 μM acteoside, which are non-toxic doses in both L929 cells and primary rat chondrocytes, were used to verify its anti-catabolic effects in *in vitro* studies using primary rat chondrocytes.

Acteoside Counteracts IL-1 β -induced Proteoglycan Loss In Primary Rat Chondrocytes

Primary rat chondrocytes embedded in alginate beads were treated with 50 and 100 μM acteoside in presence or absence of 1 ng/ml IL-1 β for 21 days. Thereafter, DMMB assay was performed to assess the alteration in proteoglycan content as shown in Fig. 3A. The relative proteoglycan contents were determined as $88.3 \pm 18.1\%$ and $86.8 \pm 16.3\%$ in the primary rat chondrocytes treated with 50 and 100 μM acteoside, respectively, compared with control ($103.8 \pm 32.3\%$). Although the relative proteoglycan contents were decreased by acteoside, these results were not significant. However, the relative proteoglycan content significantly decreased by $37.1 \pm 14.7\%$ in the primary rat chondrocytes treated with 1 ng/ml IL-1 β , but 50 and 100 μM acteoside significantly reduced the proteoglycan content by $57 \pm 12.4\%$ and $64 \pm 14.5\%$, respectively, in presence of 1 ng/ml IL-1 β . Subsequently, to verify whether acteoside suppresses the IL-1 β -induced proteoglycan loss, articular cartilage dissected from rat knee joints was treated with 100 μM acteoside in presence or absence of 10 ng/ml IL-1 β for 7 days. Thereafter, histological assessments using H&E staining and safranin-O and fast green staining were performed as shown in Fig. 3B. Morphological alteration was not observed using H&E staining; however, safranin-O and fast green staining revealed that the proteoglycan stained as red color did not alter in the articular cartilages treated with 100 μM acteoside compared with that in control. However, severe proteoglycan loss was induced by 10 ng/ml IL-1 β in the articular cartilage, and 100 μM acteoside significantly suppressed the proteoglycan loss in the articular cartilage treated with 10 ng/ml IL-1 β . Collectively, these

data consistently show that acteoside has an anti-catabolic effect that retards the degeneration of articular cartilage through counteracting IL-1 β -induced proteoglycan loss.

Acteoside has an anti-catabolic effect that suppresses MMP expression and activation in primary rat chondrocytes treated with IL-1 β

To investigate whether acteoside-induced anti-catabolic effect is associated with the suppression of MMP expression and activation, primary rat chondrocytes were treated with 50 and 100 μ M acteoside in presence or absence of 10 ng/ml IL-1 β for 24 h. Thereafter, the alterations in MMPs were investigated. As shown in Fig. 4A, although the expression of cartilage-degrading enzymes such as MMP-13, MMP-1, and MMP-3 was significantly increased in the conditioned media of primary rat chondrocytes treated with 10 ng/ml IL-1 β , it was decreased by acteoside in a dose-dependent manner. Furthermore, results of both qPCR (Fig. 4B) and qRT-PCR (Fig. 4C) revealed that IL-1 β significantly increased the mRNA levels of MMPs such as MMP-13, MMP-1, and MMP-3 in the primary rat chondrocytes. However, they decreased dose dependently in the primary rat chondrocytes treated with 50 and 100 μ M acteoside. Moreover, 50 and 100 μ M acteoside effectively suppressed the activation of MMPs in the rat primary chondrocytes treated with 10 ng/ml IL-1 β . Taken together, these data consistently indicate that acteoside has an anti-catabolic effect that suppresses the expression and activation of cartilage-degrading enzymes.

Acteoside suppresses the expression and production of IL-1 β -induced catabolic inflammatory mediators and proinflammatory cytokines in primary rat chondrocytes

To determine whether acteoside has a preventive effect against OA, primary rat chondrocytes were treated with 50 and 100 μ M acteoside in the presence or absence of 10 ng/ml IL-1 β for 24 h. Thereafter, the alterations in inflammatory mediators, representative catabolic factors such as iNOS, COX-2, and PGE₂, were investigated. The mRNA levels of iNOS, COX-2, and PTGS-2 were significantly increased by IL-1 β in the primary rat chondrocytes. However, they decreased dose-dependently in the primary rat chondrocytes treated with acteoside (Fig. 5A). Furthermore, acteoside not only suppressed the expression of iNOS and COX-2 in the primary rat chondrocytes treated with IL-1 β (Fig. 5B) but also significantly decreased the relative production of NO and PGE₂ as shown in Fig. 5C and 5D, respectively. These data suggest that acteoside suppresses the expression of inflammatory mediator-induced pro-inflammatory cytokines that act as catabolic factors to induce the progressive degeneration of articular cartilage. Hence, to investigate the expressional alteration of pro-inflammatory cytokines by 50 μ M acteoside in the primary rat chondrocytes treated with 10 ng/ml IL-1 β , cytokine array was performed as shown in Fig. 6. Acteoside suppressed the expression of cytokine-induced neutrophil chemoattractant (CINC)-2, CINC-3, ciliary neurotrophic factor (CNTF), fractalkine (CX3CL1), IL-1 α , IL-1 β , leptin, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-3 α , and β -nerve growth factor (NGF) in the primary rat chondrocytes treated with IL-1 β compared with IL-1 β alone. Taken together, these data suggest consistently that acteoside prevents the progressive degeneration of articular cartilage through suppression of inflammatory mediators and pro-inflammatory cytokines against the IL-1 β -induced catabolic effects in primary rat chondrocytes.

Acteoside suppresses MAPK and NF κ B phosphorylation in primary rat chondrocytes treated with IL-1 β

To investigate the cellular signaling pathways associated with acteoside-induced anti-catabolic effects against pro-inflammatory cytokine IL-1 β alteration of MAPK and NF κ B, primary rat chondrocytes were treated with 50 and 100 μ M acteoside in the presence or absence of IL-1 β for 24 h. Thereafter, total protein was extracted and electrophoresed on the SDS-PAGE gel to perform the western blot. As shown in Fig. 7, MAPK such as ERK1/2, p38, and JNK were significantly phosphorylated in the primary rat chondrocytes treated with IL-1 β . Whereas, 50 and 100 μ M acteoside did not significantly induce the phosphorylation of MAPK compared to the control in primary rat chondrocyte. However, 50 and 100 μ M acteoside dose-dependently suppressed the IL-1 β -induced MAPK phosphorylation in primary rat chondrocytes. Furthermore, the phosphorylation of NF κ B in the primary rat chondrocytes treated with 10 ng/mL IL-1 β was gradually decreased by acteoside in a dose dependent manner. These data indicate that MAPK and NF κ B cellular signaling pathways are closely involved with the acteoside-induced anti-catabolic effects against to IL-1 β in primary rat chondrocytes.

Acteoside suppresses translocation of NF κ B from the cytosol to the nucleus through suppression of IL-1 β -induced NF κ B phosphorylation in primary rat chondrocytes

To investigate whether acteoside suppresses the translocation of NF κ B from the cytosol to the nucleus, primary rat chondrocytes were treated with 50 and 100 μ g/mL acteoside in the presence or absence of 10 ng/mL IL-1 β . As shown in Fig. 8A, NF κ B was significantly translocated to the nucleus from the cytosol of the primary rat chondrocytes treated with 10 ng/mL IL-1 β . However, it was significantly inhibited by acteoside in a dose-dependent manner. Furthermore, although the NF κ B level was increased in the nuclear fraction extracted from the primary rat chondrocytes treated with 10 ng/mL IL-1 β , it was dose-dependently decreased by acteoside as shown in Fig. 8B. NF κ B level was decreased in the cytosolic fraction extracted from the primary rat chondrocytes treated with 10 ng/mL IL-1 β , but it was dose-dependently increased by acteoside. Taken together, these data consistently indicate that acteoside-induced anti-catabolic effects against IL-1 β are involved in the suppression of translocation from the cytosol to the nucleus on the modulation of the NF κ B signaling pathway in primary rat chondrocytes.

Acteoside attenuates progressive degeneration of articular cartilage in the surgical DMM-induced knee joint OA animals

To elucidate the acteoside-induced anti-catabolic effects *in vivo*, OA-induced animals generated by the surgical DMM performed on the knee joint of BALB/c mice were orally administrated 5 and 10 mg/kg acteoside resolved in 5% ethanol every other day for 8 weeks. Thereafter, knee joints were histologically assessed using safranin-O and fast green staining as shown in Fig. 9. The proteoglycan loss and injury of articular cartilage surface were significantly increased in the knee joint dissected from DMM-induced OA animals. However, the oral administration of acteoside suppressed the proteoglycan loss and less injury of articular cartilage compared with vehicle only. Furthermore, Mankin's grading score was significantly increased in the OA animal group ($n = 5$, 3 ± 0.7) supplied vehicle only compared with Naïve ($n = 5$, 0.67 ± 0.5). However, the oral administration of 5 and 10 mg/Kg acteoside into OA animal group (n

= 5) decreased the Mankin's grading score by 2 ± 0.7 and 1.67 ± 0.5 , respectively, compared with vehicle only. Taken together, these data indicate that the oral administration of acteoside attenuates the progressive degeneration of articular cartilage in synovial joint with catabolic conditions.

Discussion

The synovial (diarthrosis) joint is a complex anatomical structure consisting of several different types of tissues located at the potential space between bones to permit mobility and stability at the body through counteracting the different intensities of mechanical loading and control fine movements [16]. As the elderly population is increasing worldwide, OA is emerging as a degenerative diseases associated with psychological and socio-economic problems that are to be solved urgently [17]. Unfortunately, there are still no effective medications for OA; therefore, the prevention of articular cartilage degeneration is the most important to maintain the mechanical joint functions associated with the permission of body mobility and stability.

Generally, the synovial joint is composed of two bones to provide stability and support the muscle by ligament and tendons and is surrounded by a synovial fibrous joint capsule filled with synovial fluid to reduce friction between the articular cartilages located on the articular surfaces of the joined bones [16]. Especially, the extracellular matrix (ECM) of articular cartilage is composed mainly of type II collagen and proteoglycans that are synthesized and regulated by specialized cells called as chondrocytes. The homeostasis of articular cartilage is precisely balanced between anabolism (synthesis of ECM) and catabolism (degeneration of ECM) in synovial joints [18]. Generally, catabolic factors such as pro-inflammatory cytokines and inflammatory mediators induce the progressive degeneration of articular cartilage through the expression of cartilage degrading enzymes such as matrix metalloproteinase (MMPs) and metalloproteinase with thrombospondin motifs (ADAMTs) from chondrocytes [18]. Hence, recent biochemical strategies to prevent or attenuate the progressive degeneration of articular cartilage have targeted the suppression of cartilage degrading enzymes, pro-inflammatory cytokines, and inflammatory mediators based on the long-term biological safeties in synovial joints [19, 20]. Recent studies demonstrate that natural products, originating from herbal or oriental medicine, possess long-term biological safeties, anti-inflammatory, and anti-oxidative properties, and may promote the joint health and managing OA through suppressing the release of pro-inflammatory cytokines [21].

Acteoside (called as verbascoside; $C_{29}H_{36}O_{15}$) is a glycoside that is isolated from the flowers or leaves of many herbal plants such as *Scrophularia ningpoensis*, *Cistanche deserticola*, *Digitalis purpurea*, and *Osmanthus fragrans* [22, 23]. Recently, Henn et al., reported that the high concentration (100 μ g/mL) of acteoside isolated from the leaves of *Plantago australis* did not only show a less cytotoxicity in V79 Chinese hamster cells used as a normal cells but also did not have mutagenic or genotoxic activities and phototoxic properties [6]. Furthermore, Perucatti et al., have reported that *in vivo* cytogenetic tests that is feeding 5 mg/Kg acteoside to rabbit (*Oryctolagus cuniculus*) for 80 days revealed no toxicity with any other mutagenic activity, resulting in no cytotoxic for the animals [24]. These studies suggest that acteoside is a bioactive material that can be used in both animal and human diets [6, 24]. As shown in

Fig. 2, similar with previous studies, 100 μ M (62.459 μ g/ml) acteoside did not affect the viabilities of mouse fibroblast cell line L929 used as a normal cells and primary rat chondrocytes in present study. Hence, these data indicate that acteoside may have secured a potential biological safety and can be used as a supplement.

ECM, a large amount up to 98% of cartilage volume, is a highly organized network of hyaluronan, proteoglycans, and type II collagen [25]. Especially, proteoglycans are proteins glycosylated with sulfated glycosaminoglycan to form aggregating network that generate a static charge density to counteract compressive forces during the mechanical function of synovial joints [25]. Hence, the loss of proteoglycan in the articular cartilage of synovial joints leads to disability of mechanical joint function [25]. Degeneration of articular cartilage due to the loss of proteoglycan results in the imbalance between anabolic and catabolic process. Hence, recent biological strategies related with the regeneration of articular cartilage and the prevention or attenuation of progressive articular cartilage degeneration are considering the increase of anabolic process through the synthesis of major articular cartilage component such as proteoglycan and type II collagen and the increase of anti-catabolic process against catabolic factors such as pro-inflammatory cytokines, inflammation mediators, and catabolic growth factors. As shown in Fig. 3, acteoside did not only recover the proteoglycan content through the counteraction against to pro-inflammatory cytokine IL-1 β -induced proteoglycan depletion in the primary rat chondrocytes but also suppressed the proteoglycan loss in the articular cartilage tissues treated with IL-1 β for 7 days. Taken together, these data indicate consistently that acteoside may protect or attenuate the progressive degeneration of articular cartilage through counteract to pro-inflammatory cytokine-induced catabolic process in the articular cartilage of synovial joint.

Elevated cartilage degrading enzymes including MMP-1, MMP-3, MMP-13, ADMATS-4, and ADAMTS-5 in the synovial fluid of patients with OA are the key enzymes responsible for the progressive degeneration of articular cartilage through degradation of collagen and ECM component [26, 27]. Hence, the inhibition of MMPs expression and activation seems to be an attractive therapeutic strategy to prevent and attenuate the progressive degeneration of articular cartilage for maintaining the mechanical function of synovial joints [26]. In present study, acteoside effectively suppressed the expression and activation of cartilage degrading enzyme in the primary rat chondrocytes treated with pro-inflammatory cytokine IL-1 β as shown in Fig. 4. These data indicate that acteoside may attenuate the progressive degeneration of articular cartilage through suppressing the expression and activation of articular cartilage in the synovial joint with catabolic conditions.

The inflammatory mediators such as iNOS, NO, COX-2, and PGE₂ are integral to OA pathogenesis [28]. Especially, pro-inflammatory cytokines such as IL-1 β and TNF α upregulate the production of NO and PGE₂ through the increase of iNOS and COX2, respectively, in the synovial joint with OA [29, 30]. Upregulated NO inhibits the synthesis of ECM component such as type II collagen and proteoglycan. Besides, increased PGE₂ inhibits the proliferation of chondrocytes and reduces the synthesis of ECM [28]. Hence, suppression of inflammatory mediators may attenuate the progressive degeneration of articular cartilage through the inhibition of ECM reduction in the synovial joint with OA. In present study, acteoside

effectively suppressed the upregulation of inflammatory mediators as shown in Fig. 5. These data indicate consistently that acteoside may attenuate the progressive degeneration of articular cartilage through the suppression of inflammatory mediators in the synovial joint with OA.

Moreover, the overexpression of pro-inflammatory cytokines by the inflamed synovium and chondrocytes is major risk pathogenic factor in OA pathogenesis. Especially, the expression of pro-inflammatory cytokine is thought to be generated by the synovial membrane at the stage of OA initiation. Sequentially, upregulated pro-inflammatory cytokines activate chondrocytes to express their own expression and to synthesize the cartilage degrading enzymes, chemokines, inflammatory mediators [31]. Therefore, the suppression of pro-inflammatory cytokines can prevent OA and may attenuate the progressive degeneration of articular cartilage through the inhibition of other pro-inflammatory cytokines, inflammatory mediators, and cartilage degrading enzymes. In present study, acteoside suppressed the production of pro-inflammatory cytokines such as CINC-2, CINC-3, CNTF, fractalkine, IL-1 α , IL-1 β , leptin, MCP-1, MIP-3 α , and β -NGF in primary rat chondrocytes treated with IL-1 β compared with IL-1 β alone, as shown in Fig. 6.

Gouze et al., reported that CINC-2 was significantly increased in chondrocytes treated with IL-1 β as similar with our study [32]. However, recent study showed that spinal processing of painful inputs is closely altered during OA pathogenesis [33]. With regard to joint pain, CINC-2 and CINC-3 were significantly upregulated in the spinal dorsal horn of OA animals generated by the intra-articular injection of monosodium iodoacetate into knee joint [34, 35]. Although the pathophysiological role of CINC-2 and CINC-3 in OA pathogenesis is still largely unknown, these studies indicate that the expression of CINC-2 and CINC-3 in spinal dorsal horn under OA conditions may be closely associated with the development of joint pain during OA pathogenesis.

CNTF, which is a pluripotent neurotropic factor and is related with the cytokine family that includes IL-6, IL-11, leukemia inhibitory family, and oncostatin, bind and signal to maintain the bone homeostasis through the gp130 co-receptor subunit [36]. Although the biological function of CNTF is still largely unknown in OA, recent studies have shown that CNTF-gp130 signaling may be associated with the pathologic bone remodeling evident in rheumatoid arthritis (RA), periodontal disease, spondyarthropathies, and OA through regulating the differentiation and activity of osteoblast, osteoclast, and chondrocytes [36]. In addition, recent study showed that β -NGF, a neurotrophic factor involved with the physiological regulation of neuronal cells, was upregulated in blood and synovial fluid in the patient with OA [37]. However, several studies have reported that the blockade of NGF reduce the OA pain [38–40]. Therefore, neurotropic factors including CNTF and NGF not only are considered as a pathogenic risk factor of OA progression but also provide the neurological linkage between the progressive degeneration of articular cartilage and the development of chronic OA pain. Furthermore, it has been considered as a therapeutic targeting molecule to reduce the chronic OA pain.

Fractalkine also known as chemokine CX3CL1 is exuberantly expressed in both adult human and rat articular chondrocytes treated with IL-1 β [41, 42]. Recent studies have reported that fractalkine promotes

the expression of MMP-3 through the CX3CR1, c-Raf, MEK, ERK, and NF κ B cellular signaling pathways in the synovial tissue obtained from the patients with OA [43]. Furthermore, the genomic-wide DNA methylation analysis in OA chondrocytes revealed that fractalkine gene was not only hypomethylated but also constantly correlated with its mRNA expression [44]. MCP-1, a member of chemokine family to induce the inflammation, trigger the chemotaxis and transendothelial migration of monocyte to inflammatory lesion. Recently, Xu et al., have reported that MCP-1 and chemokine (C-C motif) receptor 2 axis are involved with the degradation of articular cartilage through the expression of MMP-13 and the increase of OA chondrocyte apoptosis [45]. Furthermore, MIP-3 α also called as a chemokine CCL20 is abundantly expressed in the articular cartilage of patients with OA and increase the progressive degeneration of articular cartilage through the expression of cartilage degrading enzymes such as MMP-1 and MMP-3, inflammatory mediator such as PGE₂ and pro-inflammatory cytokine IL-6 [46]. Hence, chemokines such as fractalkine, MCP-1, and MIP-3 α have been also considered as a pathophysiological risk factor to initiate the progression of OA.

Leptin is a peptide hormone belonging to adipokines, which are cytokines secreted by adipose tissue [47]. Recent studies have reported that the level of leptin is not only elevated significantly in the human body with obesity but also increased in the serum and synovial fluid collected from the patients with OA that is correlated with the severity of OA [48]. Hence, resent studies have suggested that the expressions of leptin and its receptor have been considered positively as a risk factor associated with the development of OA [49–51].[52]

IL-1 family, including IL-1 α and IL-1 β , is considered the most key cytokine associated with the pathogenesis of OA that induce the inflammatory catabolic process combined with other catabolic factors such as aging, obesity, and traumatic joint injury [53]. Generally, the level of IL-1 family in the synovial fluid, synovial membrane, articular cartilage, and subchondral bone is elevated in the synovial joint of patients with OA [54]. After IL-1 family binds onto their receptors, it manifests the progressive degeneration of articular cartilage by the expression of other cytokines, chemokines, adhesion molecules, inflammatory mediators, and cartilage degrading enzymes through the phosphorylation of cellular signaling transcriptional factors such as the NF κ B and MAPKs [54]. As shown in Fig. 7, acteoside not only reduced the phosphorylation of ERK1/2, p38, and JNK but also inhibited the phosphorylation of NF κ B in the primary rat chondrocytes treated with IL-1 β . Moreover, Fig. 8 shows that acteoside inhibited the translocation of NF κ B from cytosol to nucleus in the primary rat chondrocytes treated with IL-1 β . Therefore, our results consistently indicate that acteoside counteract the IL-1 β -induced catabolic effects such as the expression of cartilage degrading enzymes and the production of pro-inflammatory cytokines and inflammatory mediators through the inactivation of cellular signaling pathways such as MAPK and NF κ B in the primary rat chondrocytes. Recently, similar with our study, Qiao et al., have reported that acteoside inhibits inflammatory response in OA-induced animals [55]. They showed the suppression of inflammatory cytokines through the inactivation of JAK/STAT signaling pathway in the synovial tissue of DMM-induced OA animals that were administered intraperitoneal injection of acteoside [55]. However, to estimate the effectiveness of acteoside as an OA preventive supplement, acteoside were orally

administered to DMM-induced OA animals in present study. Thereafter, the alteration of articular cartilage was histological assessed as shown in Fig. 9. Our histological assessment showed that the oral administration of acteoside consistently prevented the progressive degeneration of articular cartilage through the inhibition of proteoglycan loss in DMM-induced OA animals.

Conclusions

Our findings suggests that acteoside is capable for oral administration and may be used as an effective supplement to prevent or attenuate OA based on the biological safety and anti-catabolic effects against to pro-inflammatory cytokines.

Abbreviations

ADAMTs: a disintegrin and metalloproteinase with thrombospondin motifs; β -NGF: β -nerve growth factor; CINC: cytokine-induced neutrophil chemoattractant; CNTF: ciliary neurotrophic factor; COX-2: cyclooxygenase-2; CX3CL1: fractalkine; DMEM/F12: Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12; DMM: destabilization of the medial meniscus; DMMB: dimethylmethylen blue; ECM: extracellular matrix; FBS: fetal bovine serum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; IACUC: Institutional Animal Care & Use Committee; IL-1 β : interleukin-1 β ; iNOS: inducible nitric oxide synthase; MAPK: mitogen-activated protein kinase; MCP-1: monocyte chemoattractant protein-1; mini-ITS: mini-insulin-transferrin-selenium; MIP: macrophage inflammatory protein; MMP: matrix metalloproteinase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF κ B: nuclear factor-kappa B; NO: nitric oxide; OA: osteoarthritis; PGE₂: Prostaglandin E₂; PTGS2: prostaglandin-endoperoxide synthase 2; qPCR: quantitative polymerase chain reaction; qRT-PCR: quantitative real time polymerase chain reaction; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Declarations

Authors' contributions

HL, THK, KRK, JYS, HWK and GUJ carried out the cell assay, *ex vivo* assay, *in vivo* assay using animal model, data preparation, and manuscript preparation. DKK, SSC, YY, YYC, JTL, CJO, DSO, and HSC carried out data interpretation, writing-review & editing. JSK designed and carried out supervision, investigation, formal analysis, original draft, and writing-review & editing.

Availability of data and materials

Please contact author for data requests.

Ethics approval and consent to participate

Not applicable

Funding

This study was supported by the Korean Forestry Promotion Institute (2019141A00-1921-AB02), Republic of Korea.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author details

¹Institute of Dental Science, ²Departments of Biomedical Science, Chosun University, Gwangju 61452, Republic of Korea

³Department of Pharmacy, College of Pharmacy, Mokpo National University, Muan, Jeonnam 58554, Republic of Korea

⁴Chung-Yeon Medical Institute, Gwangju 61949, Republic of Korea

⁵Research and Development Institute, CY Pharma Co., Seoul 06224, Republic of Korea

⁶Jeollanamdo Forest Resources Institute, Naju, Jeollanamdo, 58213, Republic of Korea

[†]These authors contributed equally to this study

References

1. Chen D, Shen J, Zhao W, Wang T, Han L, Hamilton JL, Im HJ. Osteoarthritis: toward a comprehensive understanding of pathological mechanism. *Bone Res.* 2017;5:16044.
2. Musumeci G, Aiello FC, Szychlinska MA, Di Rosa M, Castrogiovanni P, Mobasher A. Osteoarthritis in the XXIst century: risk factors and behaviours that influence disease onset and progression. *Int J Mol Sci.* 2015;16(3):6093–112.
3. Ghouri A, Conaghan PG. **Prospects for Therapies in Osteoarthritis.** *Calcif Tissue Int* 2020.
4. Klimek B. 6'-O-apiosyl-verbascoside in the flowers of mullein (*Verbascum* species). *Acta Pol Pharm.* 1996;53(2):137–40.
5. Pardo F, Perich F, Villarroel L, Torres R. Isolation of verbascoside, an antimicrobial constituent of *Buddleja globosa* leaves. *J Ethnopharmacol.* 1993;39(3):221–2.

6. Henn JG, Steffens L, de Moura Sperotto ND, de Souza Ponce B, Verissimo RM, Boaretto FBM, Hassemer G, Peres VF, Schirmer H, Picada JN, et al. Toxicological evaluation of a standardized hydroethanolic extract from leaves of *Plantago australis* and its major compound, verbascoside. *J Ethnopharmacol.* 2019;229:145–56.
7. Khullar M, Sharma A, Wani A, Sharma N, Sharma N, Chandan BK, Kumar A, Ahmed Z. Acteoside ameliorates inflammatory responses through NFkB pathway in alcohol induced hepatic damage. *Int Immunopharmacol.* 2019;69:109–17.
8. Hwang TW, Kim DH, Kim DB, Jang TW, Kim GH, Moon M, Yoon KA, Choi DE, Park JH, Kim JJ. Synergistic anticancer effect of acteoside and temozolomide-based glioblastoma chemotherapy. *Int J Mol Med.* 2019;43(3):1478–86.
9. Li X, Xie Y, Li K, Wu A, Xie H, Guo Q, Xue P, Maleshibek Y, Zhao W, Guo J, et al: **Antioxidation and Cytoprotection of Acteoside and Its Derivatives: Comparison and Mechanistic Chemistry.** *Molecules* 2018, 23(2).
10. Li M, Zhou F, Xu T, Song H, Lu B. Acteoside protects against 6-OHDA-induced dopaminergic neuron damage via Nrf2-ARE signaling pathway. *Food Chem Toxicol.* 2018;119:6–13.
11. Santos-Cruz LF, Avila-Acevedo JG, Ortega-Capitaine D, Ojeda-Duplancher JC, Perdigon-Moya JL, Hernandez-Portilla LB, Lopez-Dionicio H, Duran-Diaz A, Duenas-Garcia IE, Castaneda-Partida L, et al. Verbascoside is not genotoxic in the ST and HB crosses of the *Drosophila* wing spot test, and its constituent, caffeic acid, decreases the spontaneous mutation rate in the ST cross. *Food Chem Toxicol.* 2012;50(3–4):1082–90.
12. Negoro K, Kobayashi S, Takeno K, Uchida K, Baba H. Effect of osmolarity on glycosaminoglycan production and cell metabolism of articular chondrocyte under three-dimensional culture system. *Clin Exp Rheumatol.* 2008;26(4):534–41.
13. You JS, Cho IA, Kang KR, Oh JS, Yu SJ, Lee GJ, Seo YS, Kim SG, Kim CS, Kim DK, et al. Coumestrol Counteracts Interleukin-1beta-Induced Catabolic Effects by Suppressing Inflammation in Primary Rat Chondrocytes. *Inflammation.* 2017;40(1):79–91.
14. Pauli C, Whiteside R, Heras FL, Nesic D, Koziol J, Grogan SP, Matyas J, Pritzker KP, D'Lima DD, Lotz MK. Comparison of cartilage histopathology assessment systems on human knee joints at all stages of osteoarthritis development. *Osteoarthritis Cartilage.* 2012;20(6):476–85.
15. Henson FM, Vincent TA. Alterations in the vimentin cytoskeleton in response to single impact load in an in vitro model of cartilage damage in the rat. *BMC Musculoskelet Disord.* 2008;9:94.
16. Corciulo C, Cronstein BN. Signaling of the Purinergic System in the Joint. *Front Pharmacol.* 2019;10:1591.
17. Neogi T. The epidemiology and impact of pain in osteoarthritis. *Osteoarthritis Cartilage.* 2013;21(9):1145–53.
18. Hall AC. The Role of Chondrocyte Morphology and Volume in Controlling Phenotype-Implications for Osteoarthritis, Cartilage Repair, and Cartilage Engineering. *Curr Rheumatol Rep.* 2019;21(8):38.

19. Leong DJ, Hardin JA, Cobelli NJ, Sun HB. Mechanotransduction and cartilage integrity. *Ann N Y Acad Sci.* 2011;1240:32–7.
20. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol.* 2011;7(1):33–42.
21. Henrotin Y, Mobasher A. Natural Products for Promoting Joint Health and Managing Osteoarthritis. *Curr Rheumatol Rep.* 2018;20(11):72.
22. He J, Hu XP, Zeng Y, Li Y, Wu HQ, Qiu RZ, Ma WJ, Li T, Li CY, He ZD. Advanced research on acteoside for chemistry and bioactivities. *J Asian Nat Prod Res.* 2011;13(5):449–64.
23. Xiong L, Mao S, Lu B, Yang J, Zhou F, Hu Y, Jiang Y, Shen C, Zhao Y. *Osmanthus fragrans* Flower Extract and Acteoside Protect Against d-Galactose-Induced Aging in an ICR Mouse Model. *J Med Food.* 2016;19(1):54–61.
24. Perucatti A, Genualdo V, Pauciullo A, Iorio C, Incarnato D, Rossetti C, Vizzarri F, Palazzo M, Casamassima D, Iannuzzi L, et al. Cytogenetic tests reveal no toxicity in lymphocytes of rabbit (*Oryctolagus cuniculus*, 2n = 44) feed in presence of verbascoside and/or lycopene. *Food Chem Toxicol.* 2018;114:311–5.
25. Thielen NGM, van der Kraan PM, van Caam APM. **TGFbeta/BMP Signaling Pathway in Cartilage Homeostasis.** *Cells* 2019, 8(9).
26. Mehana EE, Khafaga AF, El-Blehi SS. The role of matrix metalloproteinases in osteoarthritis pathogenesis: An updated review. *Life Sci.* 2019;234:116786.
27. Thorson C, Galicia K, Burleson A, Bouchard O, Hoppensteadt D, Fareed J, Hopkinson W. Matrix Metalloproteinases and Their Inhibitors and Proteoglycan 4 in Patients Undergoing Total Joint Arthroplasty. *Clin Appl Thromb Hemost.* 2019;25:1076029619828113.
28. Chow YY, Chin KY. The Role of Inflammation in the Pathogenesis of Osteoarthritis. *Mediators Inflamm.* 2020;2020:8293921.
29. Sasaki K, Hattori T, Fujisawa T, Takahashi K, Inoue H, Takigawa M. Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit articular chondrocytes. *J Biochem.* 1998;123(3):431–9.
30. Goggs R, Carter SD, Schulze-Tanzil G, Shakibaei M, Mobasher A. Apoptosis and the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. *Vet J.* 2003;166(2):140–58.
31. Rahmati M, Mobasher A, Mozafari M. Inflammatory mediators in osteoarthritis: A critical review of the state-of-the-art, current prospects, and future challenges. *Bone.* 2016;85:81–90.
32. Gouze JN, Gouze E, Popp MP, Bush ML, Dacanay EA, Kay JD, Levings PP, Patel KR, Saran JP, Watson RS, et al. Exogenous glucosamine globally protects chondrocytes from the arthritogenic effects of IL-1beta. *Arthritis Res Ther.* 2006;8(6):R173.
33. Zhang RX, Ren K, Dubner R. Osteoarthritis pain mechanisms: basic studies in animal models. *Osteoarthritis Cartilage.* 2013;21(9):1308–15.

34. Im HJ, Kim JS, Li X, Kotwal N, Sumner DR, van Wijnen AJ, Davis FJ, Yan D, Levine B, Henry JL, et al. Alteration of sensory neurons and spinal response to an experimental osteoarthritis pain model. *Arthritis Rheum.* 2010;62(10):2995–3005.
35. Wu F, Zhang R, Shen X, Lao L. Preliminary study on pain reduction of monosodium iodoacetate-induced knee osteoarthritis in rats by carbon dioxide laser moxibustion. *Evid Based Complement Alternat Med.* 2014;2014:754304.
36. Sims NA, Walsh NC. GP130 cytokines and bone remodelling in health and disease. *BMB Rep.* 2010;43(8):513–23.
37. Montagnoli C, Tiribuzi R, Crispoltori L, Pistilli A, Stabile AM, Manfreda F, Placella G, Rende M, Cerulli GG. beta-NGF and beta-NGF receptor upregulation in blood and synovial fluid in osteoarthritis. *Biol Chem.* 2017;398(9):1045–54.
38. Miyagi M, Ishikawa T, Kamoda H, Suzuki M, Inoue G, Sakuma Y, Oikawa Y, Orita S, Uchida K, Takahashi K, et al. Efficacy of nerve growth factor antibody in a knee osteoarthritis pain model in mice. *BMC Musculoskelet Disord.* 2017;18(1):428.
39. Berenbaum F. Targeting nerve growth factor to relieve pain from osteoarthritis: What can we expect? *Joint Bone Spine.* 2019;86(2):127–8.
40. Miller RE, Block JA, Malfait AM. Nerve growth factor blockade for the management of osteoarthritis pain: what can we learn from clinical trials and preclinical models? *Curr Opin Rheumatol.* 2017;29(1):110–8.
41. Sandell LJ, Xing X, Franz C, Davies S, Chang LW, Patra D. Exuberant expression of chemokine genes by adult human articular chondrocytes in response to IL-1beta. *Osteoarthritis Cartilage.* 2008;16(12):1560–71.
42. Cho IA, Kim TH, Lim H, Park JH, Kang KR, Lee SY, Kim CS, Kim DK, Kim HJ, Yu SK, et al. Formononetin Antagonizes the Interleukin-1beta-Induced Catabolic Effects Through Suppressing Inflammation in Primary Rat Chondrocytes. *Inflammation.* 2019;42(4):1426–40.
43. Hou SM, Hou CH, Liu JF. CX3CL1 promotes MMP-3 production via the CX3CR1, c-Raf, MEK, ERK, and NF-kappaB signaling pathway in osteoarthritis synovial fibroblasts. *Arthritis Res Ther.* 2017;19(1):282.
44. Zhao L, Wang Q, Zhang C, Huang C. Genome-wide DNA methylation analysis of articular chondrocytes identifies TRAF1, CTGF, and CX3CL1 genes as hypomethylated in osteoarthritis. *Clin Rheumatol.* 2017;36(10):2335–42.
45. Xu YK, Ke Y, Wang B, Lin JH. The role of MCP-1-CCR2 ligand-receptor axis in chondrocyte degradation and disease progress in knee osteoarthritis. *Biol Res.* 2015;48:64.
46. Alaaeddine N, Antoniou J, Moussa M, Hilal G, Kreichaty G, Ghanem I, Abouchedid W, Saghbini E, Di Battista JA. The chemokine CCL20 induces proinflammatory and matrix degradative responses in cartilage. *Inflamm Res.* 2015;64(9):721–31.
47. Hamrick MW, Herberg S, Arounleut P, He HZ, Shiver A, Qi RQ, Zhou L, Isales CM, Mi QS. The adipokine leptin increases skeletal muscle mass and significantly alters skeletal muscle miRNA expression

- profile in aged mice. *Biochem Biophys Res Commun.* 2010;400(3):379–83.
48. Ku JH, Lee CK, Joo BS, An BM, Choi SH, Wang TH, Cho HL. Correlation of synovial fluid leptin concentrations with the severity of osteoarthritis. *Clin Rheumatol.* 2009;28(12):1431–5.
49. Yan M, Zhang J, Yang H, Sun Y. The role of leptin in osteoarthritis. *Medicine.* 2018;97(14):e0257.
50. Kroon FPB, Veenbrink AI, de Mutsert R, Visser AW, van Dijk KW, le Cessie S, Rosendaal FR, Kloppenburg M. The role of leptin and adiponectin as mediators in the relationship between adiposity and hand and knee osteoarthritis. *Osteoarthritis Cartilage.* 2019;27(12):1761–7.
51. Gao YH, Zhao CW, Liu B, Dong N, Ding L, Li YR, Liu JG, Feng W, Qi X, Jin XH. An update on the association between metabolic syndrome and osteoarthritis and on the potential role of leptin in osteoarthritis. *Cytokine.* 2020;129:155043.
52. Acuna AJ, Samuel LT, Jeong SH, Emara AK, Kamath AF. Viscosupplementation for hip osteoarthritis: Does systematic review of patient-reported outcome measures support use? *J Orthop.* 2020;21:137–49.
53. Sokolove J, Lepus CM. Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. *Ther Adv Musculoskelet Dis.* 2013;5(2):77–94.
54. Wojdasiewicz P, Poniatowski LA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm.* 2014;2014:561459.
55. Qiao Z, Tang J, Wu W, Tang J, Liu M. Acteoside inhibits inflammatory response via JAK/STAT signaling pathway in osteoarthritic rats. *BMC Complement Altern Med.* 2019;19(1):264.

Figures

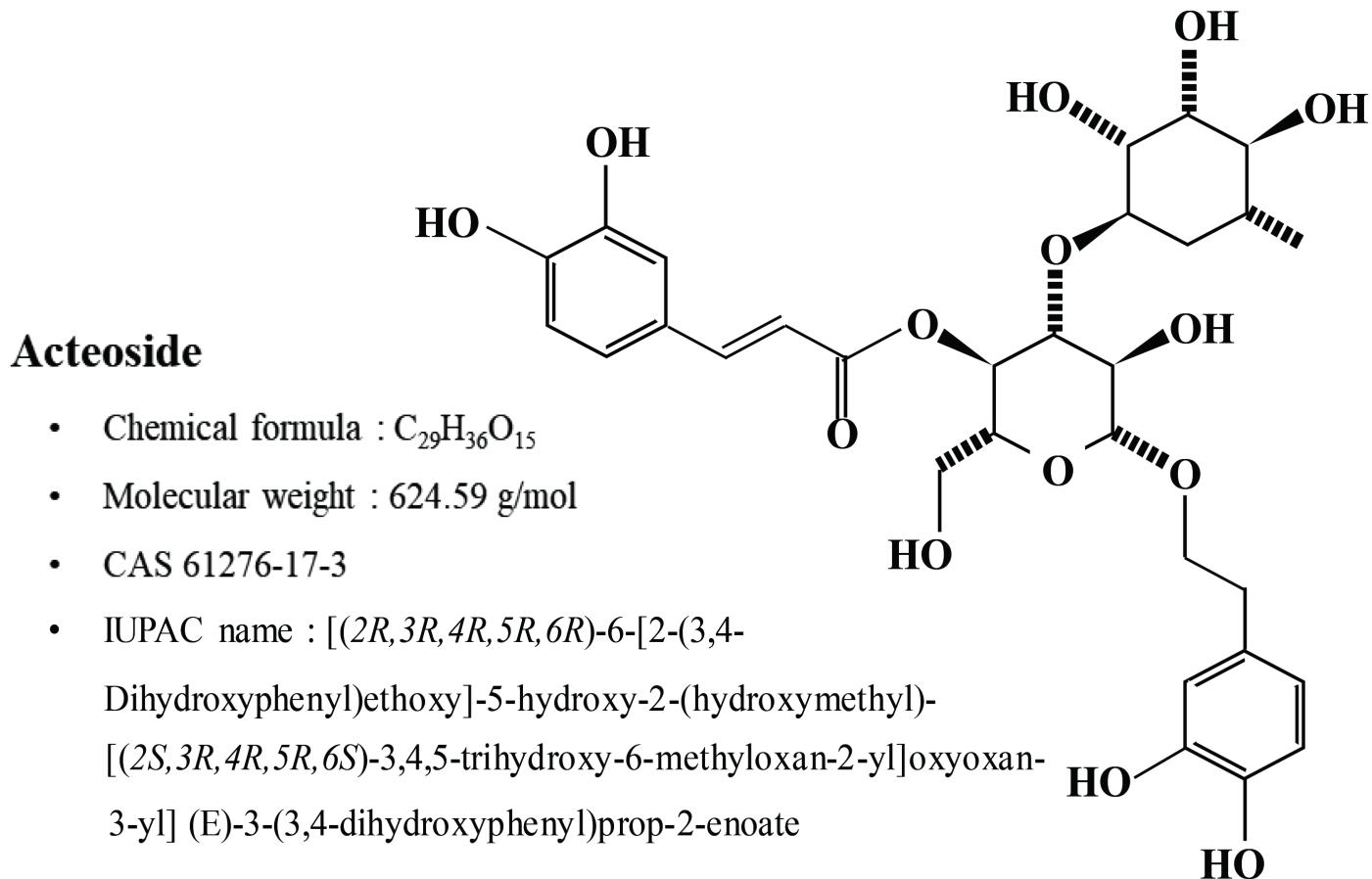


Figure 1

Chemical structure and information of acteoside

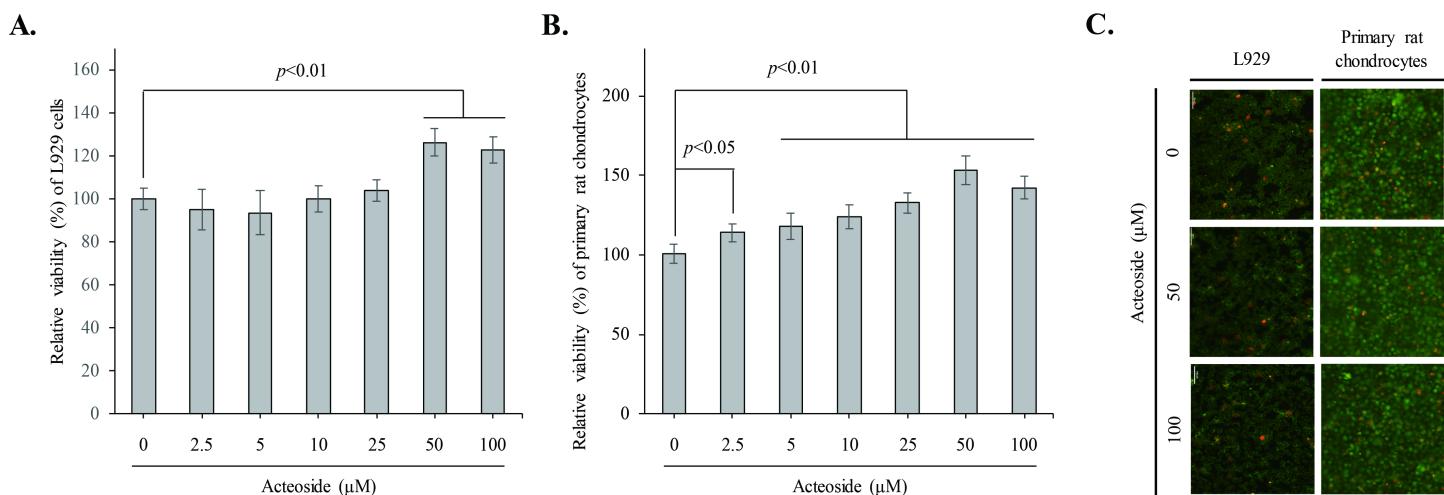


Figure 2

Acteoside does not affect L929 mouse fibroblast cell and primary rat chondrocyte viability. A, acteoside did not affect the viability of L929 mouse fibroblast cells. B, acteoside did not decrease the viability of primary rat chondrocytes. Mouse fibroblast cell line L929 used as a normal cells and primary rat

chondrocytes were treated with 2.5, 5, 10, 25, 50, and 100 μ M acteoside for 24 h. Thereafter, MTT assay was performed to assess the cytotoxicity of acteoside in L929 cells and primary rat chondrocytes. C, acteoside did not increase the cytotoxicity in both L929 and primary rat chondrocytes. To perform the Cell Live/Dead assay, L929 and primary rat chondrocyte were treated with 50 and 100 μ M acteoside for 24 h. Thereafter, Cell Live/Dead assay was performed. Stained cells were imaged using a fluorescence microscope (Eclipse TE200; Nikon Instruments, Melville, NY).

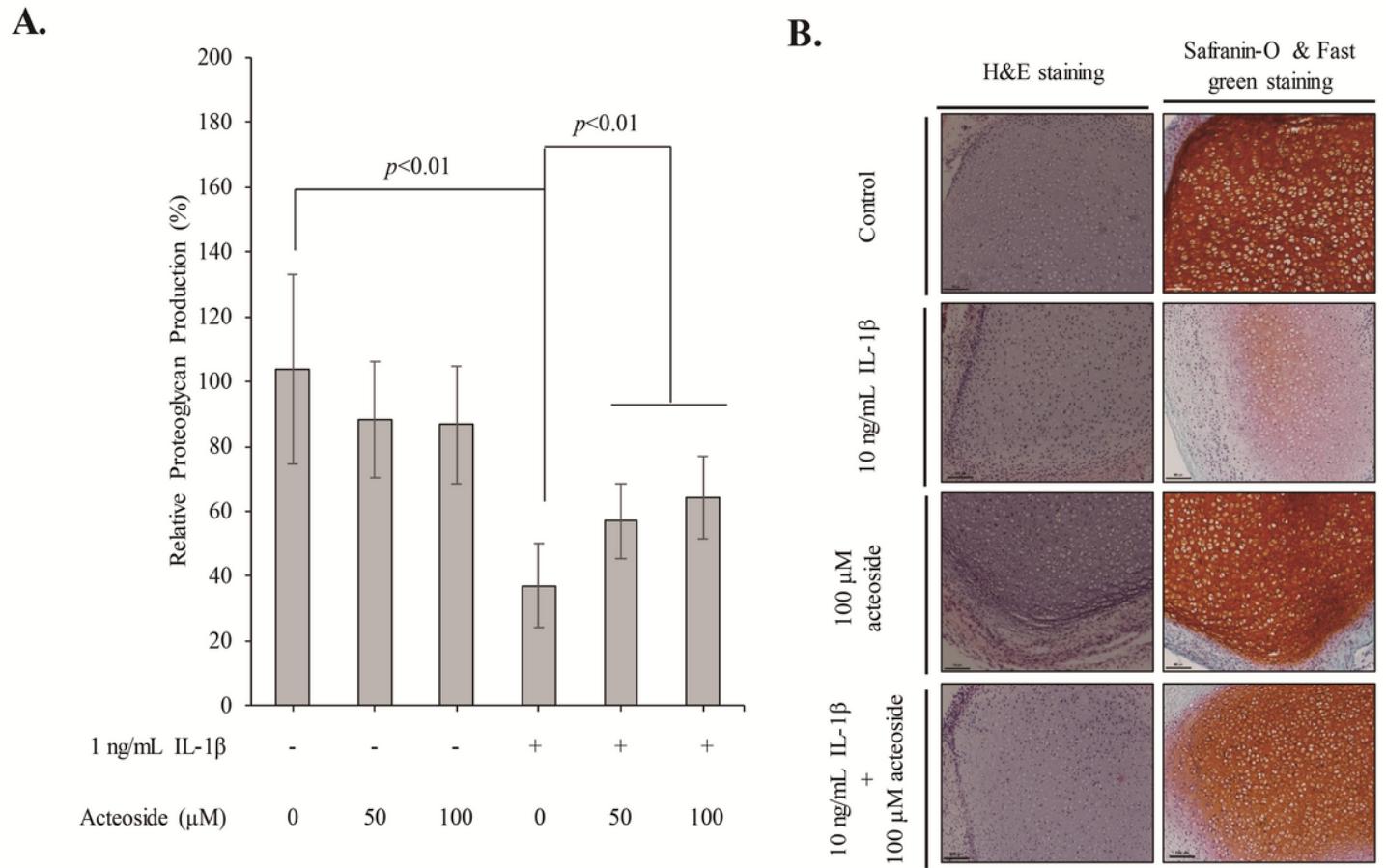


Figure 3

Acteoside counteracts IL-1 β -induced proteoglycan loss in primary rat chondrocytes. A, acteoside rescued the proteoglycan production in the primary rat chondrocytes treated with IL-1 β . Primary rat chondrocytes embedded in alginate were treated with 50 and 100 μ M acteoside in the presence or absence of 1 ng/mL IL-1 β for 21 days. Thereafter, DMMB assay was performed to verify the alteration of proteoglycan contents. B, acteoside inhibits the IL-1 β -induced proteoglycan loss in the articular cartilage dissected from rat knee joints. The tissues of articular cartilage were dissected from rat knee joint and were treated with 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 7 days. Thereafter, histological assessment using safranin-O and fast green staining was performed to verify the alteration of proteoglycan loss. Stained tissues were imaged using a microscope (Eclipse TE200; Nikon Instruments, Melville, NY).

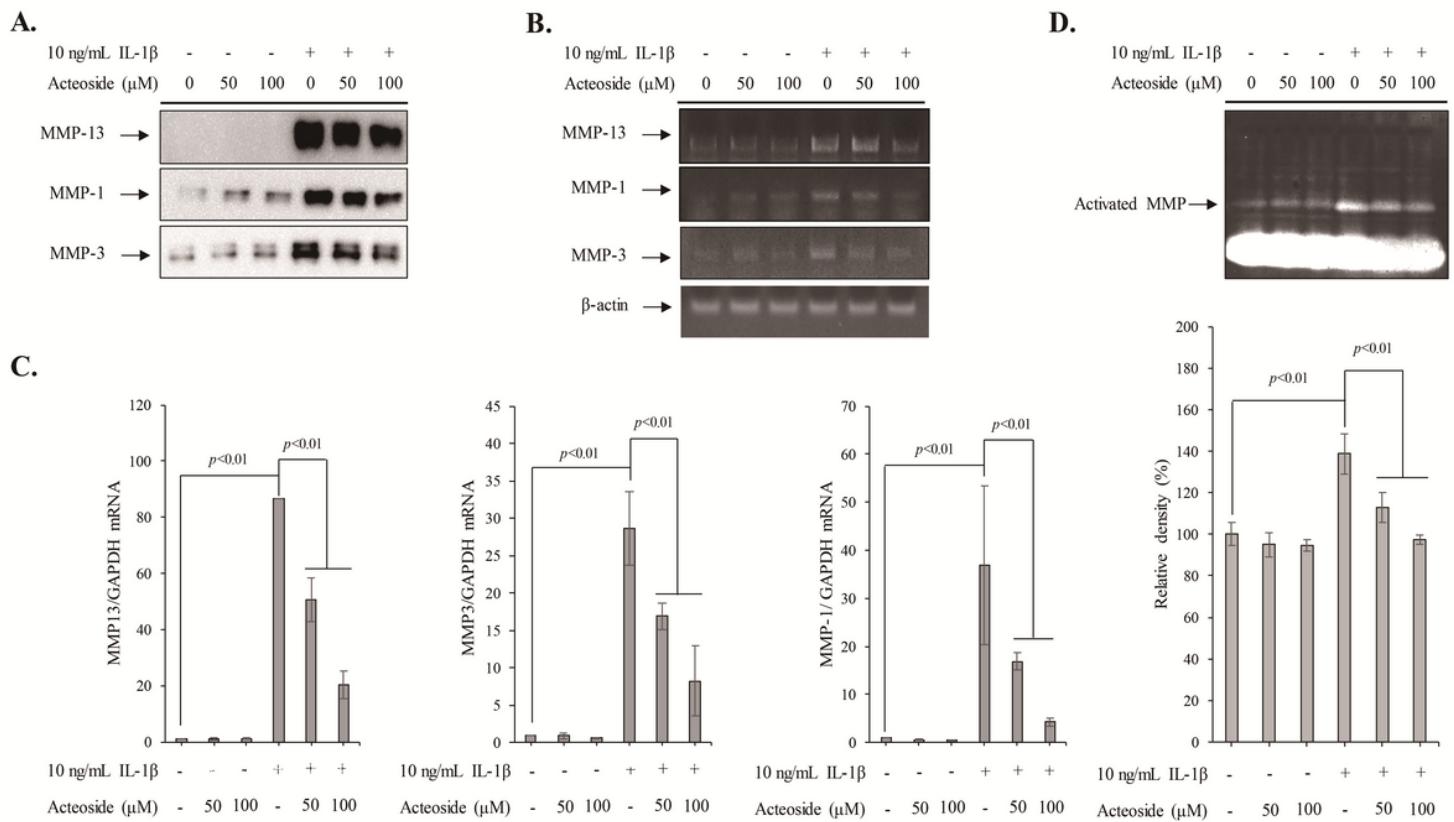


Figure 4

Acteoside has an anti-catabolic effect that suppresses MMP expression and activation in primary rat chondrocytes treated with IL-1 β . A–C, acteoside suppressed the expression of cartilage degrading enzymes such as MMP-13, MMP-1, and MMP-3 in the primary rat chondrocytes treated with IL-1 β . Primary rat chondrocytes were treated with 50 and 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, total proteins and total RNA were extracted to perform western blot (A), quantitative PCR (B), and quantitative real time-PCR (C). D, acteoside suppressed the activation of cartilage degrading enzymes. Primary rat chondrocytes were treated with 50 and 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, zymography was performed to verify the alteration of activated MMPs.

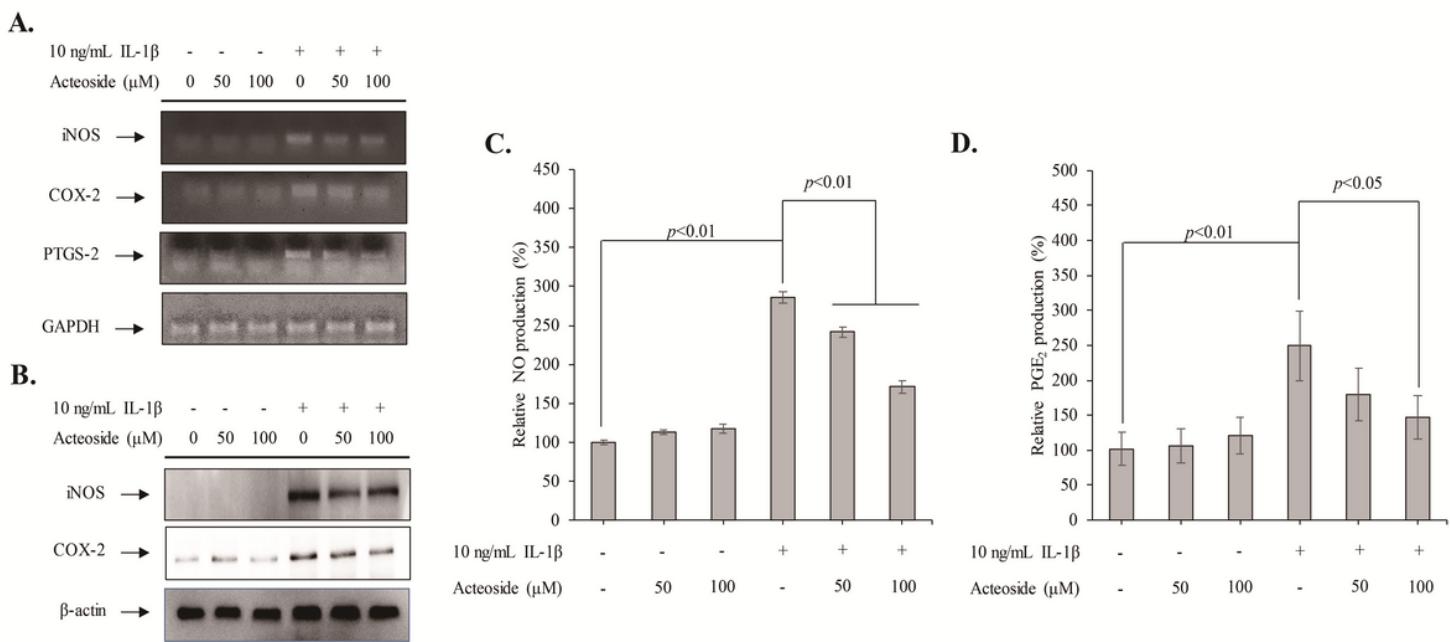


Figure 5

Acteoside suppresses the expression and production of IL-1 β -induced catabolic inflammatory mediators in primary rat chondrocytes. A & B, acteoside suppressed the expression of inflammatory mediators in the primary rat chondrocytes treated with IL-1 β . Primary rat chondrocytes were treated with 50 and 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, total proteins and total RNA were extracted to perform quantitative PCR (A) and western blot (B). C & D, acteoside suppressed the production of NO (C) and PGE2 (D) in the primary rat chondrocytes treated with IL-1 β . Primary rat chondrocytes were treated with 50 and 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, conditioned media performed the NO assay (C) and PGE2 assay (D).

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NGE	CINC-2	CINC-3	CNTF	Fractalkine (CX3CL1)
2								
3	GM-CSF	INF- γ	IL-1 α	IL-1 β	IL-4	IL-6	IL-10	LIX
4								
5	Leptin	MCP-1 (CCL2)	MIP-3 α (CCL20)	β -NGF	TIMP-1	TNF α	VEGF-A	BLANK
6								
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	POS
8								

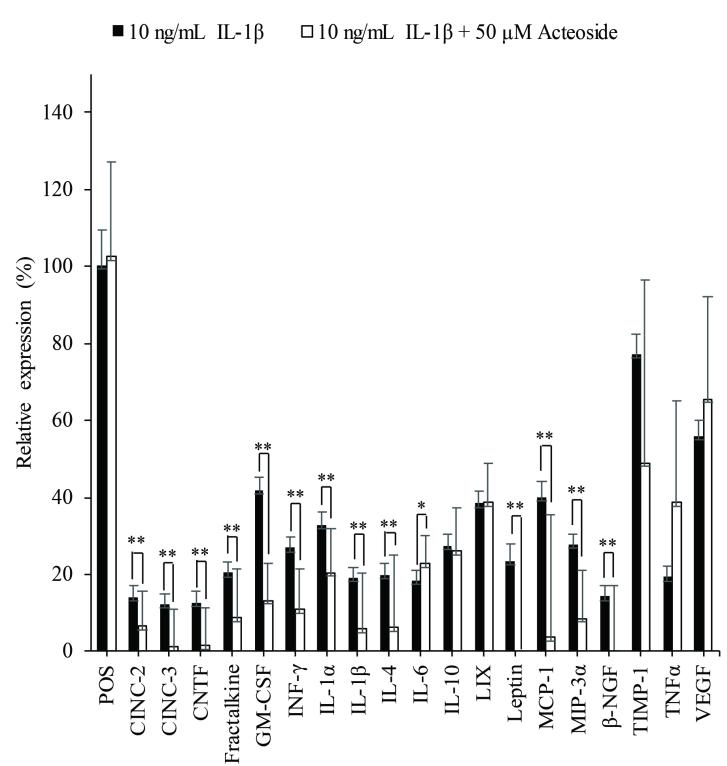
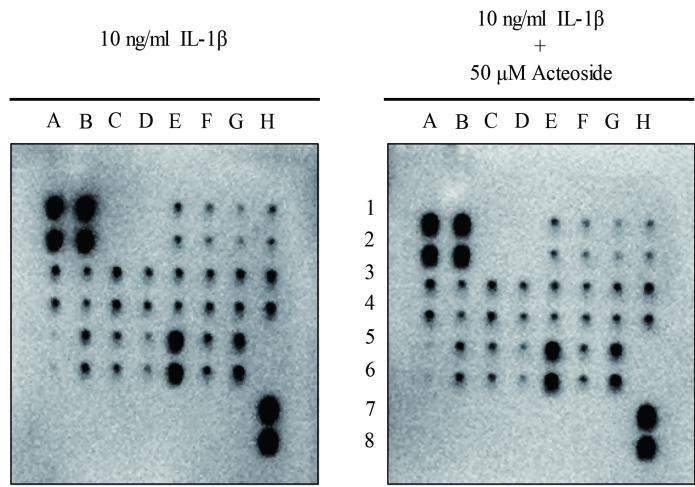


Figure 6

Acteoside suppressed the expression of IL-1 β -induced catabolic pro-inflammatory cytokines, chemokines, and growth factors in primary rat chondrocytes. Primary rat chondrocytes were treated with 50 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Total proteins were extracted and performed the cytokine array according the manufacturer's instructions.

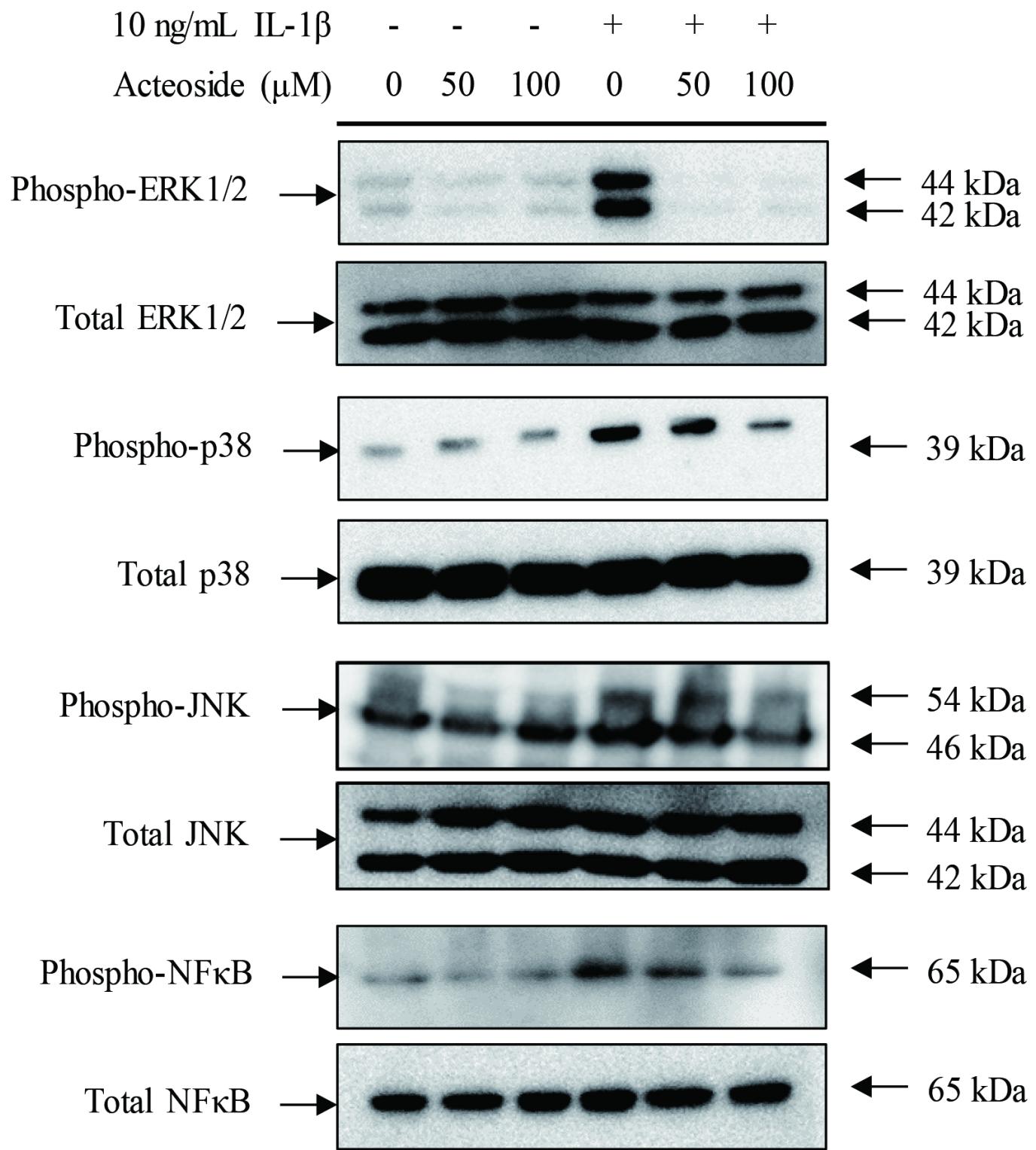


Figure 7

Acteoside suppresses MAPK and NF κ B phosphorylation in primary rat chondrocytes treated with IL-1 β . Primary rat chondrocytes were treated with 50 and 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, total proteins were extracted to perform western blot using MAPK and NF κ B antibody

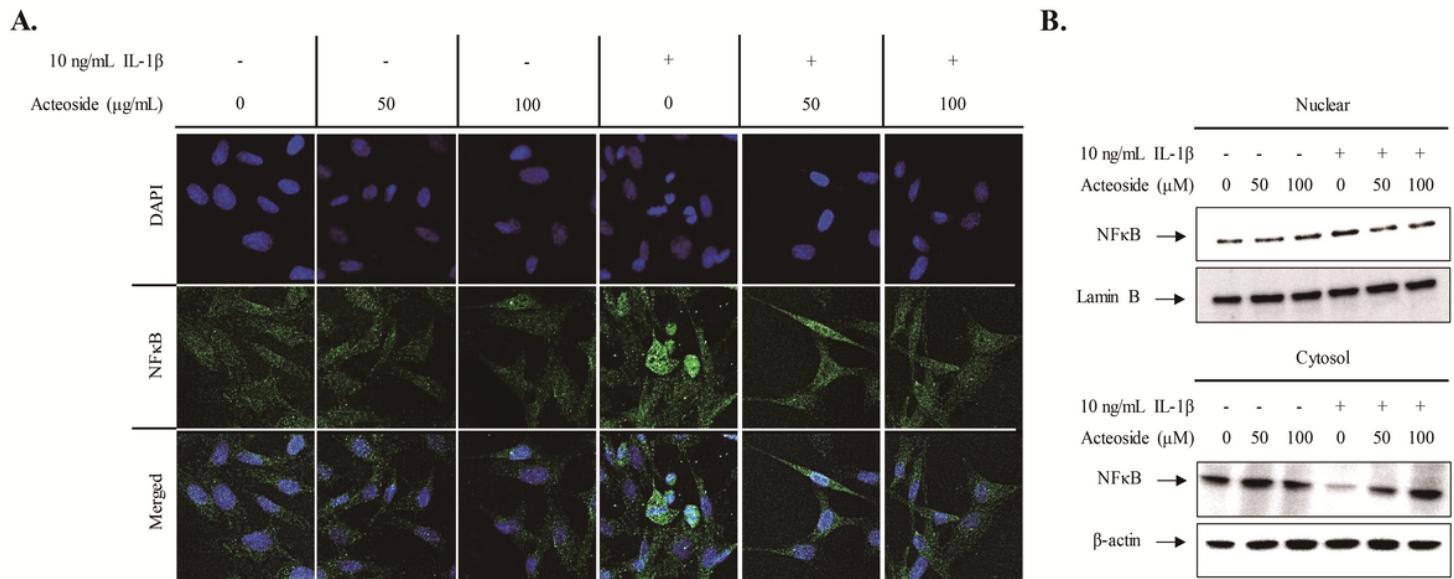


Figure 8

Acteoside suppresses translocation of NF κ B from the cytosol to the nucleus through suppression of IL-1 β -induced NF κ B phosphorylation in primary rat chondrocytes. A, acteoside suppressed the nucleus translocation of NF κ B in the primary rat chondrocytes treated with IL-1 β . Primary rat chondrocytes cultured on the chamber slide were treated with 50 and 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, the nucleus translocation was imaged using a laser confocal scanning microscope system (Leica Microsystems, Wetzlar, Germany). B, the translocation of NF κ B from cytosol to nucleus was suppressed by acteoside in primary rat chondrocytes treated with IL-1 β . Primary rat chondrocytes were treated with 50 and 100 μ M acteoside in the presence or absence of 10 ng/mL IL-1 β for 24 h. Thereafter, cytosolic and nucleus proteins were extracted and western blot was performed.

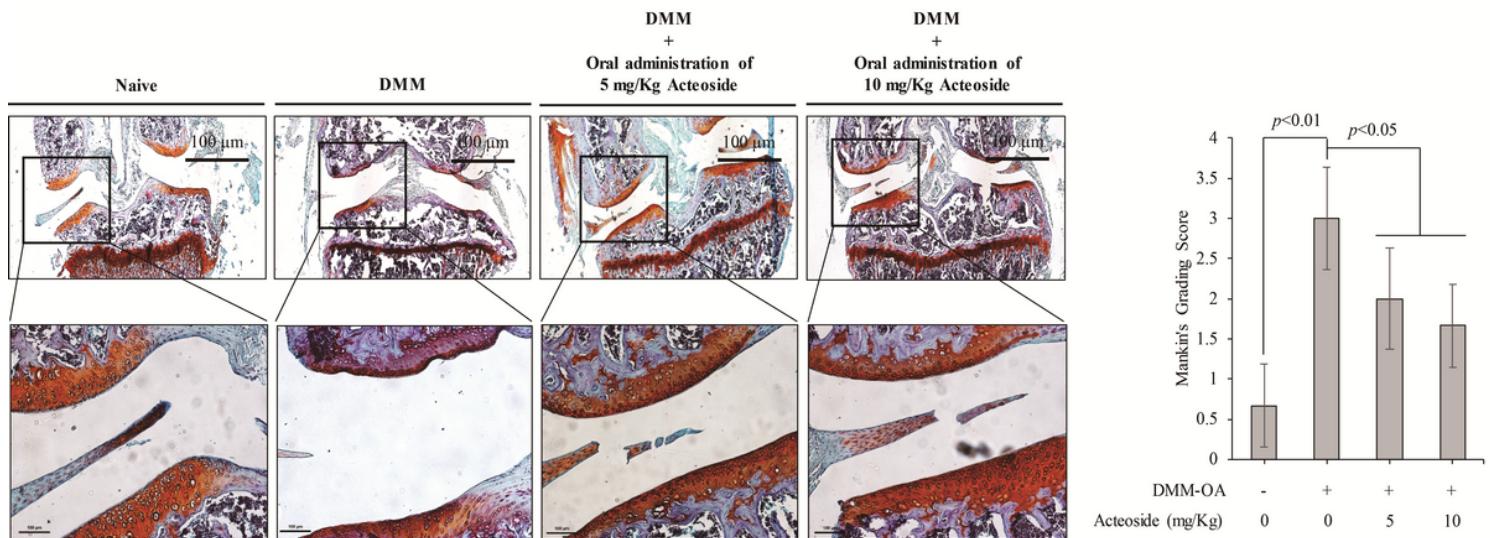


Figure 9

Acteoside attenuates progressive degeneration of articular cartilage in the surgical DMM-induced knee joint OA animals. A, progressive degeneration of articular cartilage were attenuated by the oral

administration of acteoside in the surgical DMM-induced knee joint OA animals. OA animals were generated by the DMM surgery on the knee joint and were orally administrated 5 and 10 mg/kg acteoside resolved in 5% ethanol every other day for 8 weeks. At the end of day, knee joint were dissected, fixed, decalcified, embedded, and sliced to performed safranin-O and fast green staining. Tissues were imaged using a microscope (Eclipse TE200; Nikon Instruments, Melville, NY). B, acteoside counteracted the Mankin's grading score in the surgical DMM-induced knee joint OA animals. After safranin-O and fast green staining, imaged tissues of articular cartilage were examined in accordance with the Mankin's grade.