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ORIGINAL ARTICLE

Formononetin Antagonizes the Interleukin-1β-Induced Catabolic Effects Through Suppressing Inflammation in Primary Rat Chondrocytes

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Abstract— In the present study, we demonstrated the anti-catabolic effects of formononetin, a phytoestrogen derived from herbal plants, against interleukin-1β (IL-1β)-induced severe catabolic effects in primary rat chondrocytes and articular cartilage. Formononetin did not affect the viability of primary rat chondrocytes in both short- (24 h) and long-term (21 days) treatment periods. Furthermore, formononetin effectively antagonized the IL-1β-induced catabolic effects including the decrease in proteoglycan content, suppression of pericellular matrix formation, and loss of proteoglycan through the decreased expression of cartilage-degrading enzymes like matrix metalloproteinase (MMP)-13, MMP-1, and MMP-3 in primary rat chondrocytes. Moreover, catabolic oxidative stress mediators like nitric oxide, inducible nitric oxide synthase, cyclooxygenase-2, and prostaglandin E_2 were significantly downregulated by formononetin in primary rat chondrocytes treated with IL-1β. Sequentially, the upregulation of pro-inflammatory cytokines (like IL-1α, IL-1β, IL-6, and tumor necrosis factor α), chemokines (like fractalkine, monocyte chemoattractant protein-1, and macrophage inflammatory protein-3α), and vascular endothelial growth factor were significantly downregulated by formononetin in primary rat chondrocytes treated with IL-1β. These data suggest that formononetin may suppress IL-1β-induced severe catabolic effects and osteoarthritic condition. Furthermore, formononetin may be a promising candidate for the treatment and prevention of osteoarthritis.

KEY WORDS: osteoarthritis; articular cartilage; chondrocyte; inflammation; formononetin.

INTRODUCTION

As a representative chronic degenerative disease in the elderly population (over the age of 65), osteoarthritis (OA) is one of the most common clinical diseases and is characterized by chronic joint pain caused by progressive articular cartilage degeneration in the synovial joints such as knees, hands, hips, and spine [1, 2]. Furthermore, chronic joint pain caused by OA restricts the mobility of the mechanical joint and decreases the quality of life and social

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activities of the elderly population. Therefore, OA is emerging as a socioeconomic problem that requires an urgent solution [3]. Although the pathophysiological etiologies of OA are closely associated with aging processes combined with multifactorial risk factors such as individual heredity, obesity, severe mechanical joint movement, synovial joint infection, and traumatic joint injuries, the underlying mechanism behind the disease is still largely unknown [2]. The major clinical symptoms of OA are chronic joint pain, stiffness, joint instability and deformities, and joint space narrowing [4]. Due to the lack of effective clinical medications for patients with OA, clinical OA treatments are focused on alleviating chronic joint pain and the maintenance of functional joint capacities through weight loss, joint physiotherapy, administration of pain killers, and joint surgery [5]. This indicates that the prevention of progressive articular cartilage degeneration in the synovial joints is critical in the fight against OA.

The articular cartilage of the synovial joint is a highly specialized connective tissue composed of a dense extracellular matrix (ECM) that transmits loads with a low frictional coefficient [6]. The ECM is mainly composed of collagen type II and proteoglycans, which are synthesized from specialized cells called as chondrocytes [6]. The homeostasis of articular cartilage is precisely regulated and balanced by anabolism (synthesis) and catabolism (degradation) [7]. However, the upregulation of catabolic factors such as inflammation mediators, pro-inflammatory cytokines, and catabolic growth factors shift this balance [8]. Subsequently, these catabolic factors initiate the breakdown of the ECM through the expression of cartilage-degrading enzymes such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) enzymes from chondrocytes [9]. Therefore, strategies that would suppress catabolic factors and cartilage-degrading enzymes may be promising in the prevention of OA.

Formononetin (7-hydroxy-3-(4-methoxyphenyl)chromen-4-one, $C_{16}H_{12}O_4$, CAS number 485-72-3) with a similar chemical structure to estrogen, is a phytoestrogen purified from natural herbal plants such as *Astraglus membranaceus* [10] and *Trifolium pretense* [11]. Recently, the antioxidant activity [11], anti-inflammation [12], anti-neovascularization [13], and estrogenic effects [14] of formononetin have been investigated, suggesting a possible preventive role in articular cartilage damage. However, the chondroprotective effect of formononetin and its physiological mechanism are yet to be investigated in articular cartilage. Therefore, the aim of this study is to investigate the formononetin-induced chondroprotective effects and its physiological mechanism in primary rat chondrocytes.

MATERIALS AND METHODS

Isolation and Culture of Primary Rat Chondrocytes

The studies outlined here were performed in accordance with the protocol (CIACUC2017-S0053) approved by the Institutional Animal Care and Use Committee of Chosun University, Gwangju, Republic of Korea. Chondrocytes were isolated from the knee joint articular cartilage of 5-day-old Sprague–Dawley rats by enzymatic digestion in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) culture medium (Thermo Scientific, Rockford, IL, USA) with sequential treatments with 0.2% pronase and 0.025% collagenase P. For monolayer short-term culture, isolated primary rat chondrocytes were cultured at a density of 8×10^5 cells/mL in DMEM/F12 culture medium containing 10% fetal bovine serum (FBS), antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin), and 50 μg/mL ascorbic acid. For the 21-day long-term alginate bead culture, 2×10^6 primary rat chondrocytes were resuspended in 1.2% alginate and then added dropwise to 105 mM calcium chloride ($CaCl_2$) solution to form the beads. The chondrocytes embedded in the alginate beads were cultured in DMEM/F12 culture medium containing 10% FBS, antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin), and 50 μg/mL ascorbic acid.

Cytotoxicity Assay

Dimethyl thiazolyl diphenyl tetrazolium salt (MTT) assay was performed to examine the cytotoxicity of formononetin in isolated primary rat chondrocytes. Briefly, isolated primary rat chondrocytes were plated at a density of 8×10^5 cells/mL in 96-well culture plates and allowed to adhere overnight. Thereafter, cultured primary rat chondrocytes were stimulated with 5, 10, 25, 50, and 100 μM formononetin for 24 h. After incubation under the defined conditions, chondrocytes were incubated for another 4 h in 20 μL of 5 mg/mL MTT (Life Technologies, Grand Island, NY, USA). The supernatant was subsequently removed, and MTT crystals were dissolved in 200 μL/well dimethyl sulfoxide. Thereafter, optical density was measured at 570 nm using a spectrometer. The mean optical density (OD) ± standard deviation (SD) for each group of replicates was calculated. The inhibitory rate of cell growth was calculated using the following equation:

growth inhibition (%) = ([1 − OD extract treated]/[OD negative control]) × 100.

Cell Live and Dead Assay

Cell survival was measured using green calcein AM and ethidium homodimer-1 (Life Technologies, Grand Island, NY, USA), which stain live and dead cells, respectively. To evaluate cell survival, isolated primary rat chondrocytes were plated on chamber slides, stimulated with 25 and 50 μM formononetin for 24 h, and then stained with green calcein AM and ethidium homodimer-1 according to the manufacturer's protocol. Cells were then examined and imaged using fluorescence microscopy (Eclipse TE200; Nikon Instruments, Melville, NY). Thereafter, at least 100 cells were counted in triplicate for each data point to access the survival rate. The survival rate was calculated using the following equation: relative survival rate (%) = (the number of live cells/[the number of live cells + the number of dead cells]) × 100.

DMMB Assay

Isolated primary rat chondrocytes (2×10^6 cells) were resuspended in 1 mL of 1.2% alginate and then added dropwise to a 105 mM $CaCl_2$ solution to form the beads. The chondrocytes embedded in the alginate beads were cultured in DMEM/F12 culture medium containing 10% FBS, antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin), and 50 μg/mL ascorbic acid for 24 h. Thereafter, alginate beads containing primary rat chondrocytes were cultured in DMEM/F12 culture medium containing 1% mini-ITS (insulin-transferrin-selenium) and 50 μg/mL ascorbic acid for 24 h. Subsequently, alginate beads containing primary rat chondrocytes were treated with 2.5 and 5 μM formononetin in the presence or absence of 1 ng/mL interleukin-1β (IL-1β) for 21 days. In addition, the survival rate of alginate bead-embedded primary rat chondrocytes was assessed by the cell live/dead assay (Molecular Probes, Carlsbad, CA, USA) every week during the culture period. At least 100 cells were counted in triplicate for each data point. At the end of culture period, the proliferation rates of alginate bead-embedded primary rat chondrocytes for 21 days were determined by DNA assay using PicoGreen (Molecular Probes, Carlsbad, CA, USA) according to the manufacture's protocol. Thereafter, the alginate bead-embedded primary rat chondrocytes were collected to assess the proteoglycan content using the dimethylmethylene blue (DMMB) assay. The proteoglycan content measured in the cell-associated matrix were quantified using DNA measurements to determine the total amount of proteoglycans produced and retained in the alginate bead-embedded primary rat chondrocytes.

Particle Exclusion Assay

To visualize the altered pericellular matrix of alginate-embedded chondrocytes treated with formononetin in the presence or absence of IL-1β for 21 days, a particle exclusion assay was performed. Briefly, at the end of the culture period, chondrocytes were collected by centrifugation, after the resolution of alginate using 55 mM sodium citrate (pH 6.8). Collected chondrocytes were resuspended in DMEM/F12 culture media, and then plated on a 6-well culture plate. Thereafter, plated chondrocytes were allowed to settle and attach to the plates for 12 h. Formalin-fixed erythrocytes were then added and allowed to settle for 15 min. The chondrocytes were then observed, and the images were acquired with an inverted phase-contrast microscope (Eclipse 2000, Nikon, Melville, NY, USA).

qPCR and qRT-PCR

Total RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and its concentration was measured using a Nanodrop 2000 (Thermo Scientific, Rockford, IL, USA), Thereafter, 1 μg RNA was reverse transcribed using the ThermoScript reverse transcription-PCR system (Invitrogen, Carlsbad, CA, USA) for the first-strand cDNA synthesis according to the manufacturer's instructions. For quantitative polymerase chain reaction (qPCR), the cDNA was amplified using 2X TOPsimple™ DyeMIX-*n*Taq (Enzynomics, Seoul, Republic of Korea) using a TaKaRa PCR Thermal Cycler Dice (TaKaRa Bio Inc., Shiga, Japan). Gene induction was determined using agarose gel electrophoresis while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control for normalizing the reactions. For quantitative real-time PCR (qRT-PCR), cNDA was amplified using an Eco™ Real-Time PCR system (illumine Inc., San Diego, CA, USA). β-Actin was used as an internal control. The primer sequences used in the present study are summarized in Table 1.

Western Blotting

After treatment with 25 and 50 μM formononetin in the presence or absence of 10 ng/mL IL-1β for 24 h, the primary rat chondrocytes were harvested, lysed using lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease and phosphatase inhibitor cocktails,

Table 1. PCR primer sequences used in this study

Type of PCR	Gene	Primer sequences	NCBI gene no.
qRT-PCR	MMP-13	Forward: 5′-TGGGCCTTCTGGTCTTCTGG-3′ Reverse: 5′-TGGGAAACATCAGGGCTCCA-3′	NM_133530.1
	MMP-1	Forward: 5′-GATTCCCCACAGACATCCATAG-3′ Reverse: 5′-CCAGTCTCTTCCTCACAAACG-3′	NM_001134530.1
	MMP-3	Forward: 5′-AGGTGGATGCTGTCTTTGAAG-3′ Reverse: 5′-GGTCACTTTCCCTGCATTTG-3′	NM_133523.3
	β-Actin	Forward: 5′-CCCATCTATGAGGGTTACGC-3′ Reverse: 5′-TTTAATGTCACGCACGATTT-3′	NM_019212.2
qPCR	MMP-13	Forward: 5′-AAGATGTGGAGTGCCTGATG-3′ Reverse: 5′-CCAGTGTAGGTATAGATGGGAAC-3′	NM_133530.1
	MMP-1	Forward: 5′-GGTGAAGACGTCCAAGCTAAA-3′ Reverse: 5′-CTCTGTAGAAGGCGAACACAATA-3′	NM_001134530.1
	MMP-3	Forward: 5′-GTGGTACCCACCAAATCTAACT-3′ Reverse: 5′-ATCGATCTTCTGGACGGTTTC-3′	NM_133523.3
	GAPDH	Forward: 5′-TGACTCTACCCACGGCAAGTTCAA-3′ Reverse: 5′-TCTCGTGGTTCACACCCATCACAA-3′	NG_028301.1

and incubated for 1 h at 4 °C. Subsequently, the lysates were centrifuged at 14,000×*g* for 10 min at 4 °C, and the total protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL, USA).

In addition, the conditioned medium was collected to detect the cartilage-degrading enzymes secreted from chondrocytes. Thereafter, 5× loading buffer was added to equal amounts of protein and conditioned medium, the mixture was boiled at 90 °C for 10 min, and then separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking for 2 h with 5% bovine serum albumin in Tris-buffered saline containing Tween-20 at room temperature, the membranes were incubated with primary antibodies against MMP-13 (SC-30073, Santa Cruz Biotechnology Inc., Dallas, TX, USA), MMP-1 (SC-30069, Santa Cruz Biotechnology Inc., Dallas, TX, USA), or MMP-3 (SC-30070, Santa Cruz Biotechnology Inc., Dallas, TX, USA) overnight at 4 °C. Subsequently, horseradish peroxidase-conjugated secondary antibodies (SC-2004, Santa Cruz Biotechnology Inc., Dallas, TX, USA) were then added. The immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) system (Thermo Scientific, Rockford, IL, USA) and then were imaged by Microchemi device (DNR Bioimaging Systems, Jerusalem, Israel). Thereafter, the intensity of bands in western blot gel were analyzed using "ImageJ," which is an image analysis software (www.imagej.nih.gov). *p* Values were presented as mean ± standard deviation based on the cumulative data of two experiments.

Gelatin Zymography

Gelatin zymography was performed to verify the altered activation of matrix-degrading enzymes secreted from primary rat chondrocytes treated with formononetin in the presence or absence of 10 ng/mL IL-1β for 24 h. Briefly, an equal volume of conditioned medium was mixed with a non-reducing sample buffer composed of 4% SDS, 0.15 M Tris (pH 6.8), and 20% (*v*/*v*) glycerol containing 0.05% (*w*/*v*) bromophenol blue. Thereafter, samples were electrophoresed on a 10% polyacrylamide gel containing copolymerized 0.2% (1 mg/mL) porcine skin gelatin. After electrophoresis of the conditioned medium samples, the gels obtained were washed with cold phosphate-buffered saline (PBS) containing 2.5% (*v*/*v*) Triton X-100 for 30 min and then washed twice with cold PBS for 15 min. Subsequently, the gels were incubated in zymogram renaturing buffer composed of 50 mM Tris-HCl (pH 7.6), 10 mM $CaCl_2$, 50 mM NaCl, and 0.05% Brij-35 at 37 °C for 72 h. After renaturation of the cartilage-degrading enzymes, the gels were stained with 0.1% Coomassie Brilliant Blue R250 and the gelatinolytic activity, which was revealed as a clear band on a background of uniform light blue staining, was assessed.

Ex Vivo Organ Culture of Rat Articular Cartilage

Articular cartilage was obtained from the knee joint of 5-day-old Sprague–Dawley rats and maintained for 7 days in DMEM/Ham's F-12 (1:1) culture medium containing 10% FBS with 50 μM formononetin in the presence or absence of 10 ng/mL IL-1β. At the end of the culture

period, the articular cartilage was harvested and fixed in 4% paraformaldehyde for histological evaluation.

Histological Analysis

The fixed articular cartilage samples were decalcified in ethylenediaminetetraacetic acid (EDTA), which was changed every 5 days. The decalcified articular cartilages were embedded in paraffin, serial 5-μm-thick sections were cut, and mounted on slides. The safranin-O and fast green staining was subsequently performed to assess the 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.

Measurement of Total NO Production

Nitric oxide (NO) production was assessed spectrophotometrically as formed nitrites (NO_2). Primary rat chondrocytes were plated in a 6-well plate and treated with 25 and 50 μM formononetin for 24 h in the presence or absence of 10 ng/mL IL-1β. Then, 50 μL of the conditioned medium was allowed to react with 50 μL each of sulfanilamide and N-1-napthylethylenediamine dihydrochloride. The absorbance was then measured at 540 nm using a spectrophotometer (Epoch Spectrophotometer, BioTek, Winooski, VT, USA).

PGE_2 Assay

To measure the prostaglandin E_2 (PGE_2) levels, primary rat chondrocytes were plated in a 6-well plate and treated with 25 and 50 μM formononetin for 24 h in the presence or absence of 10 ng/mL IL-1β. The PGE_2 concentration was then measured using the PGE_2 Parameter Assay kit (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

Cytokine Array

The isolated primary rat chondrocytes were counted, plated onto a 6-well culture plate at 8×10^5 cells/mL, and then treated with DMEM/F12 culture medium containing 50 μM formononetin in the presence or absence of 10 ng/mL IL-1β for 24 h. The primary rat chondrocytes were then lysed in lysis buffer containing protease and phosphatase inhibitor cocktails and incubated for 1 h at 4 °C. The lysates were centrifuged at 14,000×*g* for 10 min at 4 °C, and then the total protein concentrations were determined using the BCA protein assay. An array of cytokines (rat cytokine antibody array C1 [Cat#AAR-CYT-1-4], RayBiotech, Inc., Norcross, GA, USA) was used to determine the relative alterations in cytokine levels following the manufacturer's instructions.

Statistical Analysis

An analysis of variance (ANOVA) was performed using the StatView 5.0 software (Statistical analysis software, SAS Institute, Cary, NC), and p values < 0.05 were considered statistically significant in each test.

RESULTS

Formononetin Does Not Affect the Viability of Primary Rat Chondrocyte

Primary rat chondrocytes were treated with 5–100 μM formononetin for 24 h, and an MTT assay was performed to determine if cell viability was altered. As shown in Fig. 1a, the viability of primary rat chondrocytes treated with 5, 10, 25, 50, and 100 μM formononetin were 100 ± 5, 105 ± 3, 103 ± 3, 98 ± 4, 97 ± 8, and 96 ± 6, respectively. These data indicate that formononetin did not affect the cell viability of primary rat chondrocytes. To verify the survival of chondrocytes treated with formononetin, cell live/dead assay using green calcein AM and ethidium homodimer-1 was performed (Fig. 1b). The results of cell live/dead assays showed an increase in the living population of primary rat chondrocytes incubated with formononetin as compared with the untreated cells. Furthermore, relative cell survival rates were assessed as $112 \pm 10\%$ and $118 \pm 12\%$ for primary rat chondrocytes treated with 25 and 50 μM formononetin, respectively, compared with control cells ($100 \pm 9\%$); no statistical significance was observed between these groups. Taken together, these results indicate that the defined treatment concentrations of formononetin increased the viability in the primary rat chondrocytes.

Formononetin Counteracts the IL-1β-Induced Catabolic Effects on Proteoglycan Without Affecting the Proliferation of Primary Rat Chondrocytes

To determine if formononetin interferes with the IL-1β-induced catabolic effects on proteoglycan, primary rat chondrocytes embedded in 1% alginate beads were stimulated with 2.5 and 5 μM formononetin in the presence or absence of 1 ng/mL IL-1β for 21 days.

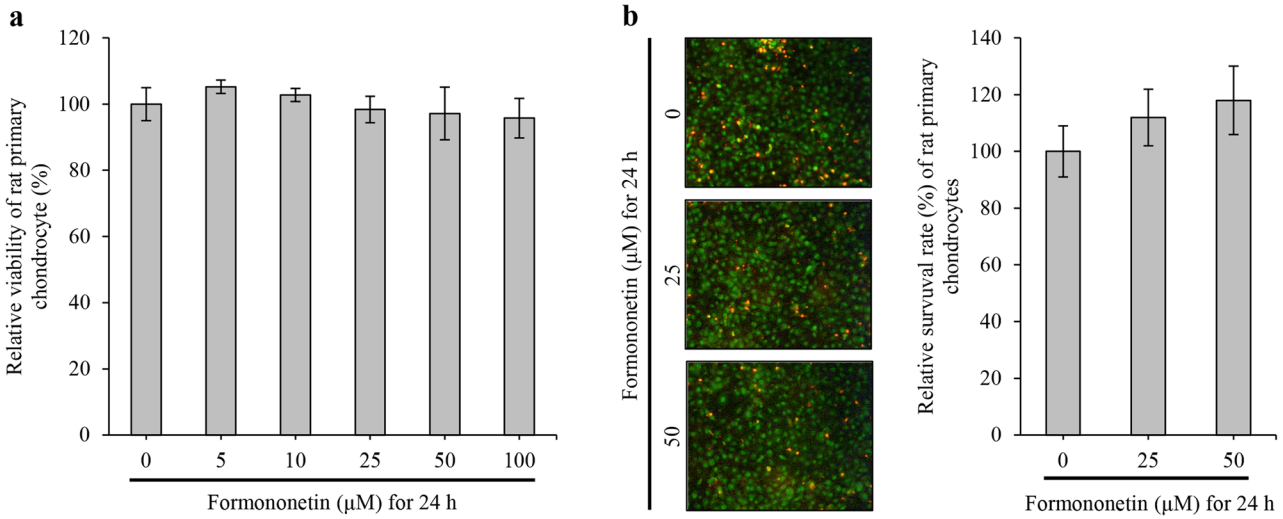

Fig. 1. Formononetin does not affect the viability of primary rat chondrocytes. **a** Formononetin did not affect the cell viability of primary rat chondrocytes. Primary rat chondrocytes were treated with 5–100 μM formononetin for 24 h. Under indicated treatment conditions, viability of primary rat chondrocyte was measured using an MTT assay. The deviations in samples represent three different donors in three separate experiments. **b** Formononetin increased in the living population of primary rat chondrocytes. Primary rat chondrocytes were treated with 25 and 50 μM formononetin for 24 h. Thereafter, cell survival was measured by cell live and dead assay using green calcein AM to staining live cells (green) and ethidium homodimer-1 to stain dead cells (red).

At the end of culture periods, the proliferation of primary rat chondrocytes was also assessed by DNA assay using picogreen (Molecular Probes, Carlsbad, CA, USA). As shown in the Fig. 2a, values of relative cell proliferation were 92 ± 8%, 96 ± 6%, and 109 ± 12% for the primary rat chondrocytes treated with 1 ng/mL IL-1β, 2.5 μM, and 5 μM formononetin, respectively. Furthermore, in the presence of 1 ng/mL IL-1β, values of relative cell proliferation were 94 ± 8% and 98 ± 7% for the primary rat chondrocytes treated with 2.5 and 5 μM formononetin, respectively. These relative proliferation rates were not statistically

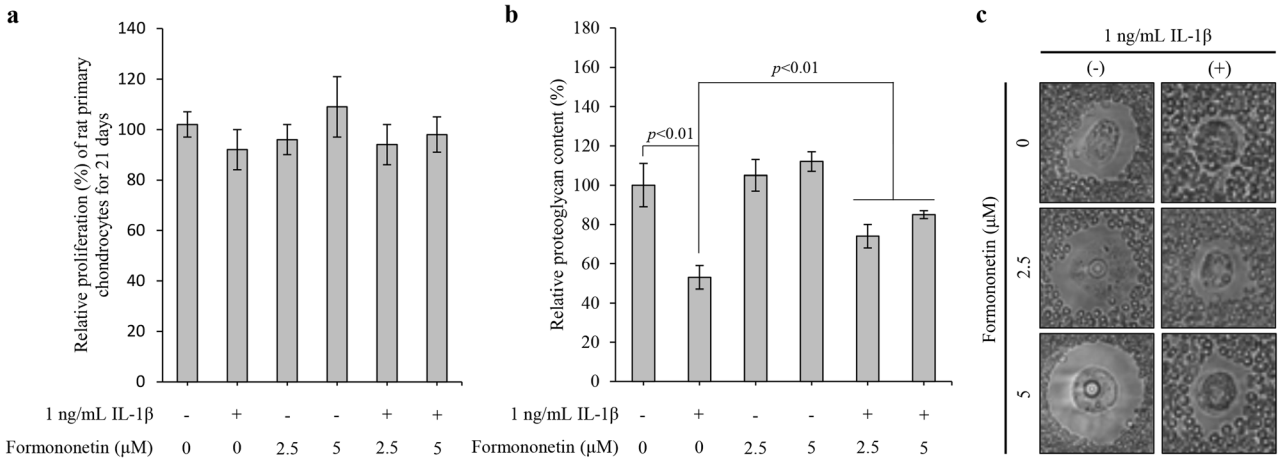

Fig. 2. Formononetin counteracts the IL-1β-induced catabolic effects on proteoglycan in primary rat chondrocytes. Primary rat chondrocytes were embedded in 1% alginate beads and were stimulated with 2.5 and 5 μM formononetin in the presence or absence of 1 ng/mL IL-1β for 21 days. At the end of treatment periods, cell proliferation (**a**), proteoglycan contents (**b**), and the formation of pericellular matrix (**c**) were measured by DNA assay using picogreen, dimethylmethylene blue (DMMB) assay, and particle exclusion assay, respectively.

different from the rate when cells were just treated with IL-1β. In addition, the results of the DNA assay were used to normalize the proteoglycan content in the DMMB assay. As shown in the Fig. 2b, the proteoglycan content decreased significantly (by 53 ± 2%) in primary rat chondrocytes embedded in alginate beads for a 21-day treatment with 1 ng/mL IL-1β as compared with the control. In contrast, the proteoglycan content in primary rat chondrocytes did not change significantly when cells were treated with both 2.5 and 5 μM formononetin for 21 days as compared with that in the control. Importantly, in the presence of 1 ng/mL IL-1β, 2.5 and 5 μM formononetin reversed the IL-1β-mediated suppression of proteoglycan accumulation by approximately 74 ± 6% and 85 ± 2%, respectively.

Next, to verify the alteration to the pericellular matrix in the primary rat chondrocytes treated with formononetin in the presence or absence of IL-1β for 21 days, a particle exclusion assay was performed (Fig. 2c). The chondrocyte pericellular matrix stimulated with 2.5 and 5 μM formononetin were similar to the control, whereas the pericellular matrix decreased significantly in chondrocytes treated with 1 ng/mL IL-1β for 21 days. However, in the presence of 1 ng/mL IL-1β, 2.5 and 5 μM formononetin rescued pericellular matrix formation in a dose-dependent manner.

Therefore, taken together, these data consistently suggest that formononetin is safe, does not affect chondrocyte proliferation, and counteracts the IL-1β-induced catabolic effects on proteoglycan in primary rat chondrocytes.

Formononetin Antagonizes IL-1β-Induced Proteoglycan Depletion in the *Ex Vivo* Organ Culture of Rat Articular Cartilage

Next, to verify the formononetin-induced anti-catabolic effects, articular cartilage dissected from rat knee joints were stimulated with 50 μM formononetin in the presence or absence of 100 ng/mL IL-1β for 7 days. Safranin-O and fast green staining was then performed to verify the proteoglycan loss from the ECM of the articular cartilage. As shown in Fig. 3, 100 ng/mL IL-1β induced severe proteoglycan loss from rat articular cartilage. By contrast, 50 μM formononetin did not induce proteoglycan loss as compared with the intact control. However, 50 μM formononetin attenuated the IL-1β-induced severe proteoglycan loss in the rat articular cartilages. These data indicate that formononetin has anti-catabolic effects that prevent the degeneration of articular cartilage under pro-inflammatory conditions.

Formononetin-Induced Anti-Catabolic Effect Is Mediated by the Suppression of Cartilage-Degrading Enzyme Expression and Activation in the Primary Rat Chondrocytes Treated with IL-1β

Proteoglycan loss is closely mediated by the expression of cartilage-degrading enzymes such as MMP-13, MMP-1, and MMP-3. Hence, to determine whether formononetin suppresses the expression of cartilage-degrading enzymes in primary rat chondrocytes treated with IL-1β, the alteration of expression and activation of MMPs associated with cartilage degeneration were

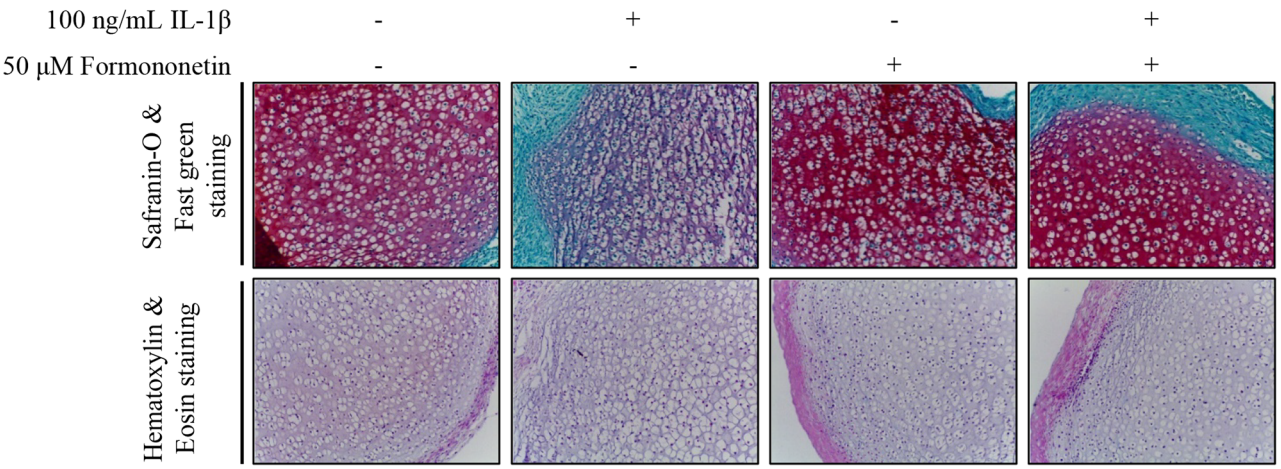

Fig. 3. Formononetin antagonizes IL-1β-induced proteoglycan depletion in the *ex vivo* organ culture of rat articular cartilage. Articular cartilage dissected from rat knee joints were stimulated with 50 μM formononetin in the presence or absence of 100 ng/mL IL-1β for 7 days. Safranin-O and fast green staining was then performed to verify the proteoglycan loss from the ECM of the articular cartilage.

investigated by qRT-PCR, qPCR, western blot using specific antibodies, and gelatin zymography (Fig. 4).

As shown in Fig. 4a–c, the mRNA and protein expression of MMP-13, MMP-1, and MMP-3 were significantly increased in primary rat chondrocytes treated with 10 ng/mL IL-1β for 24 h. However, 25 and 50 μM formononetin suppressed both the mRNA and protein expression of MMP-13, MMP-1, and MMP-3 in primary rat chondrocytes. Interestingly, the IL-1β-induced upregulation of these cartilage-degrading enzymes decreased significantly following formononetin treatment in a dose-dependent manner. Furthermore, the activities of the cartilage degrading enzymes were significantly inhibited by formononetin in primary rat chondrocytes treated with 10 ng/mL IL-1β (Fig. 4d).

Taken together, these data indicate that formononetin protects articular cartilage through the suppression of pro-inflammatory cytokine-induced cartilage-degrading enzyme expression and activation under pro-inflammatory cytokine-induced catabolic conditions. Hence, these data

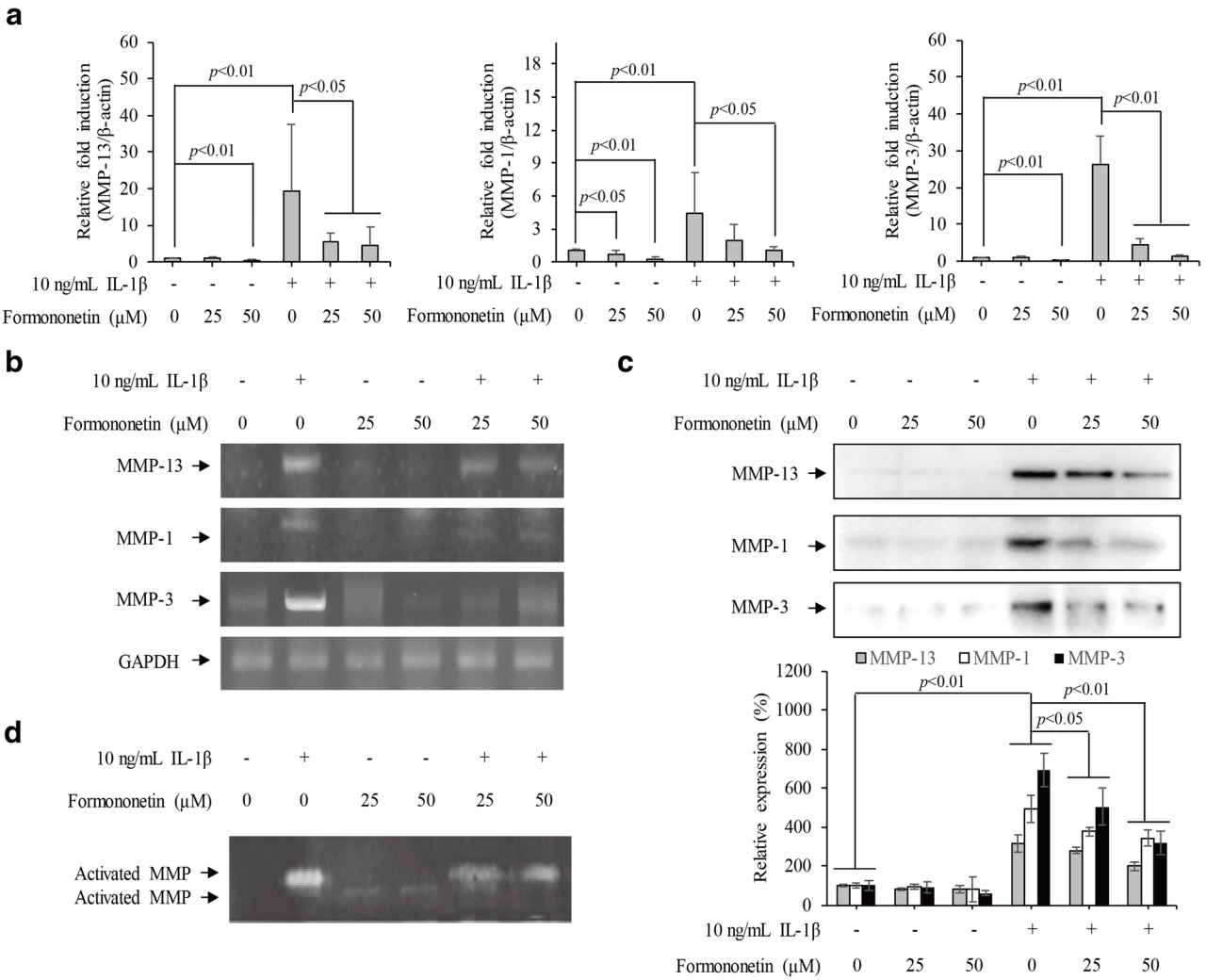

Fig. 4. Formononetin-induced anti-catabolic effect is mediated by the suppression of cartilage degrading enzyme expression and activation in the primary rat chondrocytes treated with IL-1β. Primary rat chondrocytes were treated with 25 and 50 μM formononetin in the presence or absence of 10 ng/mL IL-1β for 24 h. Thereafter, expression and activation of matrix metalloproteinase were assessed by qRT-PCR (**a**), qPCR (**b**), western blot (**c**), and gelatin zymography (**d**). Gene expression is expressed as mean ± SD compared with control level after normalizing with β-actin expression in qRT-PCR. A value of $p < 0.01$ indicates highly significant differences in ANOVA. In addition, the intensity of bands in western blot gel were analyzed using "ImageJ," which is an image analysis software (www.imagej.nih.gov). *p* Values were presented as mean ± standard deviation based on the cumulative data of two experiments.

suggest that formononetin is an anti-catabolic natural phytoestrogen that can be used to prevent the degeneration of articular cartilage.

Formononetin Suppresses IL-1β-Induced Catabolic Inflammations in Primary Rat Chondrocytes

The expression of pro-inflammatory cytokines is closely regulated by pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and PGE_2. Hence, to determine whether formononetin suppresses the expression of IL-1β-induced pro-inflammatory mediators, NO assay, a western blot for iNOS and COX-2, and an ELISA for PGE_2 were performed (Fig. 5).

NO is known to be a catabolic pro-inflammatory mediator, and the relative NO production dose-dependently decreased by 93 ± 3% and 84 ± 6% in primary rat chondrocytes treated with 25 and 50 μM formononetin, respectively (Fig. 5a). Moreover, 10 ng/mL IL-1β significantly increased NO production by approximately 477 ± 24% as compared with that in the control alone, but it was dose-dependently decreased by 313 ± 18% and 274 ± 9% in the primary rat chondrocytes treated with 25 and 50 μM formononetin, respectively. Furthermore, as shown in Fig. 5b, 10 ng/mL IL-1β upregulated the expression of iNOS and COX-2 in primary rat chondrocytes, while 25 and 50 μM formononetin significantly downregulated the expression of iNOS and COX-2 in those cells treated with 10 ng/mL IL-1β. Moreover, the relative production of PGE_2 decreased by 98 ± 3% and 77 ± 2.2% in primary rat chondrocytes treated with 25 and 50 μM formononetin, respectively, as compared with the control alone. However, 10 ng/mL IL-1β significantly increased the production of PGE_2 by 247 ± 5% compared with the control alone. However, the relative production of PGE_2 was significantly suppressed by 163 ± 11% and 141 ± 14% in the presence of 10 ng/mL IL-1β and cotreated with 25 or 50 μM formononetin, respectively (Fig. 5c).

Taken together, these results consistently indicate that formononetin counteracts the IL-1β-induced catabolic pro-inflammatory effects in primary rat chondrocytes and may work against the IL-1β-induced articular cartilage degeneration.

Formononetin Alters the Expression of Catabolic Pro-inflammatory Cytokines in Primary Rat Chondrocytes Treated with IL-1β

Previously, we determined that formononetin suppressed the expression of catabolic mediators associated with the induction of pro-inflammatory cytokines in primary rat chondrocytes treated with IL-1β. Hence, to determine the alterations in the expression of pro-inflammatory cytokines such as cytokine-induced neutrophil chemoattractant (CINC)-2, CINC-3, ciliary neurotrophic factor (CNTF), fractalkine (chemokine (C-X3-C motif) ligand 1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (INF)-γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, LIX (C-X-C motif chemokine 5), leptin, monocyte chemoattractant protein-1 (MCP-1 as known as chemokine (C-C motif) ligand 2), macrophage inflammatory protein-3α (MIP-3α as known as chemokine (C-C motif) ligand 20), β-nerve growth factor (β-NGF), tissue inhibitors of metalloproteinase-1 (TIMP)-1, tumor necrosis factor α (TNFα), and vascular endothelial growth factor (VEGF), a cytokine array was performed using the rat cytokine antibody array C1 kit (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's instructions. As shown in Fig. 6, 10 ng/mL IL-1β increased the expression of CINC-2, CINC-3, fractalkine, GM-CSF, IL-1α, IL-1β, IL-4, IL-6, IL-10, LIX, MCP-1, MIP-3α, β-NGF, TIMP-1, TNFα, and VEGF in primary rat chondrocytes as compared with the control alone. In the primary rat chondrocytes treated with 50 μM formononetin, expressions of CINC-2, CINC-3, IFN-γ, IL-1β, IL-4, IL-6, LIX, TNFα, and VEGF were significantly downregulated as compared with the control alone. However, the expression of fractalkine, IL-1α, IL-1β, IL-6, IL-10, LIX, MCP-1, MIP-3α, TIMP-1, TNFα, and VEGF were downregulated in primary rat chondrocytes cotreated with 10 ng/mL IL-1β and 50 μM formononetin as compared with 10 ng/mL IL-1β alone. These results indicate that formononetin antagonizes IL-1β-induced cartilage degeneration through the suppression of pro-inflammatory cytokines that act as catabolic factors.

DISCUSSION

It is well accepted that aging is accompanied with the increase of pro-inflammatory state that has been termed "inflamm-aging" [15]. In this regard, recent studies have reported that age-related inflammation acts as prominent catabolic factors that induce the progressive breakdown of articular cartilage through the overexpression of cartilage-degrading enzymes in the synovial joints [16, 17]. Furthermore, recent findings provide convincing evidence that

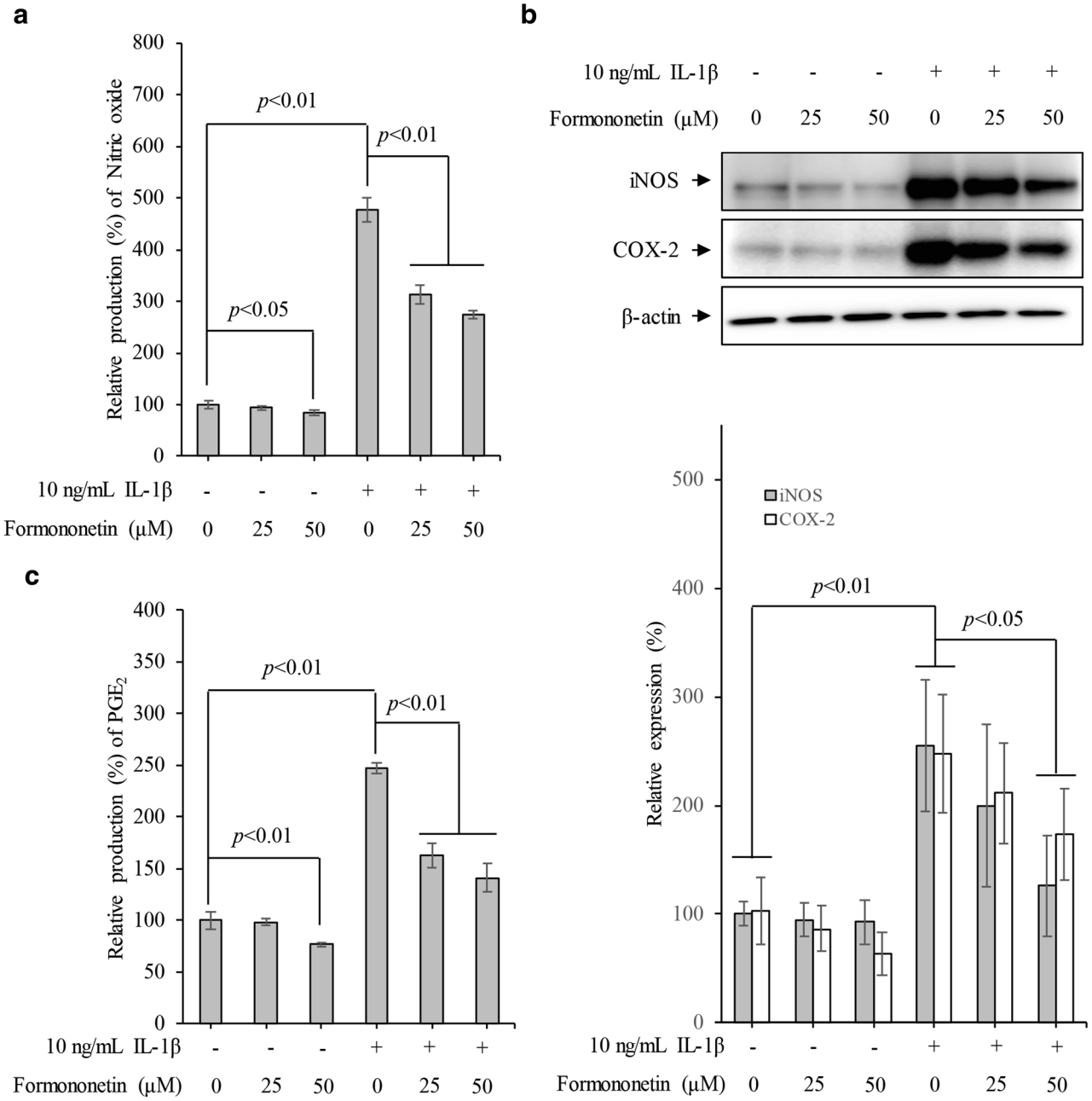

Fig. 5. Formononetin suppresses IL-1β-induced catabolic oxidative stresses in primary rat chondrocytes. Primary rat chondrocytes were treated with 25 and 50 μM formononetin in the presence or absence of 10 ng/mL IL-1β for 24 h. Thereafter, a nitric oxide (NO) assay (**a**), a western blot for iNOS and COX-2 (**b**), and an ELISA for PGE_2 (**c**) were performed. The relative production of NO and PGE_2 is expressed as mean ± SD compared with control level. A value of $p < 0.05$ and $p < 0.01$ indicate a significant and a highly significant difference, respectively, between groups in ANOVA. In addition, the intensity of bands in western blot gel were analyzed using "ImageJ," which is an image analysis software (www.imagej.nih.gov). *p* Values were presented as mean ± standard deviation based on the cumulative data of two experiments.

inflammation plays a pivotal role in the pathophysiological development of OA-related pain [18]. Hence, as there is no therapeutic medication for OA, counteracting to age-related inflammation is considering as a promising

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

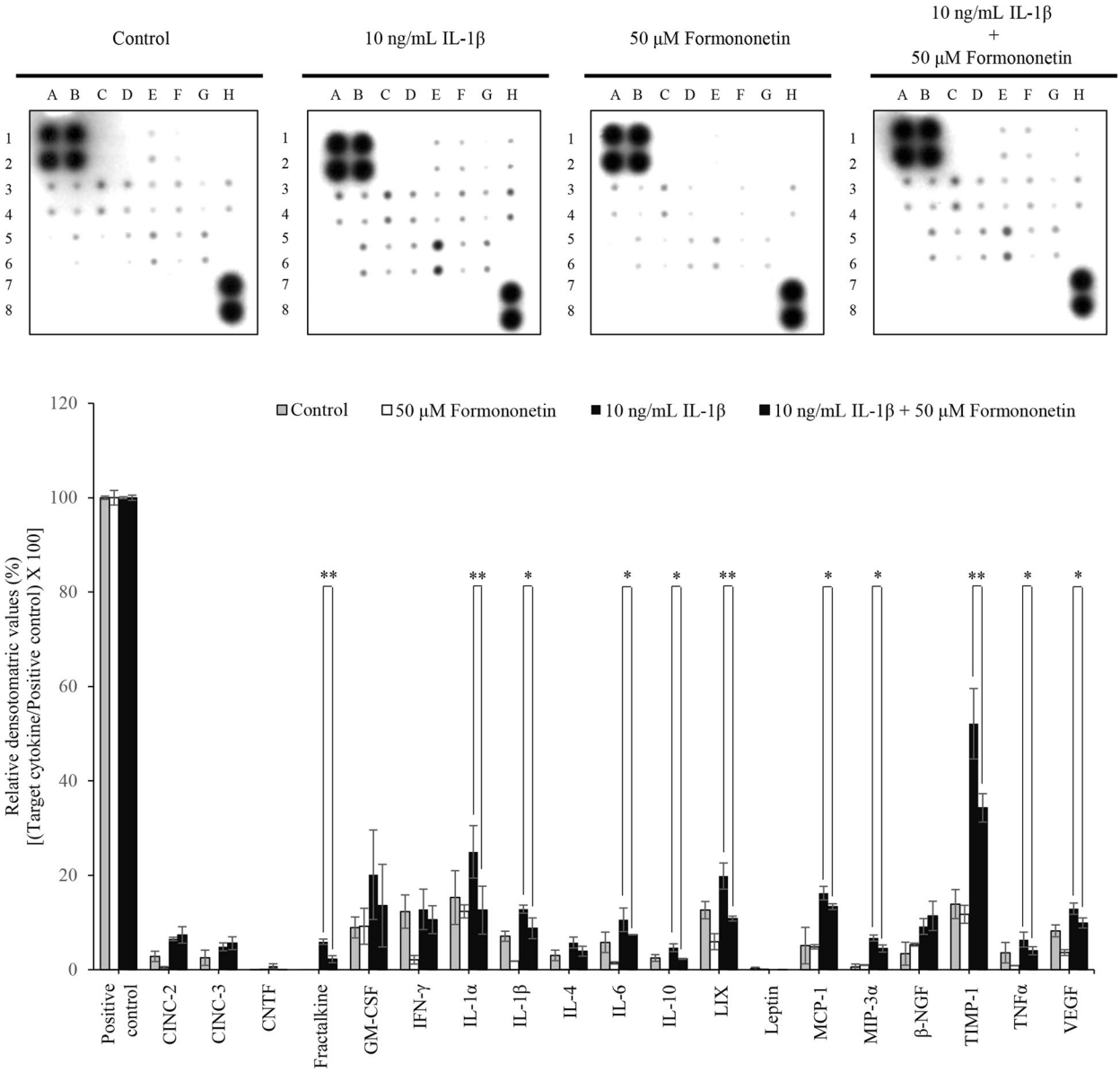

Fig. 6. Formononetin alters the expression of catabolic pro-inflammatory cytokines in primary rat chondrocytes treated with IL-1β. Primary rat chondrocytes were treated with 50 μM formononetin in the presence or absence of 10 ng/mL IL-1β for 24 h. Thereafter, an array of cytokines was used to determine the relative alterations in cytokine levels following the manufacturer's instructions.

biochemical strategy that is capable for the maintenance of biomechanical joint function through the prevention of progressive articular cartilage degeneration and OA-related pain development [19].

Although the cellular mechanisms associated with alleviating OA of herbal plants is not yet fully elucidated, but it is used for treating OA in traditional medicine. In present study, our results of MTT assay and cell live and dead assay showed that formononetin, a phytoestrogenic compound purified from a number of herbal plants [11, 13], did not affect the viability and survival of primary rat chondrocytes (Fig. 1). These data indicate that formononetin is nontoxic to primary rat chondrocytes. Furthermore, we demonstrated that formononetin counteracted the pro-inflammatory cytokine IL-1β-induced catabolic effects on proteoglycan accumulation and pericellular matrix formation through inhibiting the expression and activation of cartilage-degrading enzymes such as MMP-13, MMP-1, and MMP-3 in primary rat chondrocytes (Figs. 2, 3, and 4). Taken together, our data consistently demonstrate that formononetin has an anti-catabolic effects against the representative catabolic pro-inflammatory cytokine IL-1β in primary rat chondrocytes. Although cellular mechanisms of formononetin-induced anti-catabolic effects have not been yet fully elucidated, but these data suggest consistently that formononetin may prevent, retard, and decelerate the progressive degeneration of articular cartilage. In this regard, recent studies are providing a rational basis that the anti-inflammatory effectiveness of herbal plants and their biological active materials may counteract the inflammation-induced catabolic effects [15].

It is well accepted that reactive oxygen species (ROS) disrupts cartilage homeostasis and increases catabolism through the induction of chondrocyte death, breakdown of the ECM, and the inhibition of matrix synthesis [8]. NO, among of ROS, is biosynthesized endogenously from L-arginine by nitric oxide synthase (NOS) [20]. Moreover, the upregulated NO is closely related with the production of PGE_2 through the induction of COX-2 [21]. PGE_2 is known as a principal mediator of inflammation in chronic joint diseases such as rheumatoid arthritis and OA and is regulated by COX-2 [22]. Therefore, these studies suggest that the targeting of the iNOS-NO axis and its downstream target COX-2-PGE_2 axis is a promising biochemical strategy to decelerate and retard the progressive degeneration of articular cartilage. In the present study, formononetin significantly antagonized the IL-1β-induced upregulation of iNOS, NO, COX-2, and PGE_2 in primary rat chondrocytes (Fig. 5). These data consistently indicate that formononetin-induced anti-catabolic effects are mediated by the suppression and inactivation of cartilage degrading enzymes through the down-regulation of iNOS, NO, COX-2, and PGE_2 in primary rat chondrocytes treated with IL-1β.

Subsequently, our cytokine array data showed that the expression of representative catabolic pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNFα, and chemokines such as fractalkine (CX3CL1), LIX (CXCL5), MCP-1 (CCL2), and MIP-3α (CCL20), and catabolic growth factors such as VEGF were significantly downregulated by formononetin in the primary rat chondrocytes treated with IL-1β (Fig. 6).

Schuerwegh et al. reported that representative pro-inflammatory cytokines such as IL-1α and TNFα decreased the viability and proliferation of bovine chondrocytes [23]. Furthermore, IL-1α was able to enhance the production of NO in bovine chondrocytes in a dose-dependent manner [23]. More recently, human chondrocytes treated with pro-inflammatory cytokine IL-1α and TNFα secreted cathepsin S, which contributed to cartilage degradation through its potent proteolytic activity on the ECM [24]. IL-1β is a well-known catabolic pro-inflammatory cytokine that increases the levels of the cartilage degrading enzyme in cultured chondrocytes [25]. Furthermore, IL-1β increases the apoptotic death of normal and OA chondrocytes [26]. However, Mohtai et al. reported that IL-6 is expressed in OA chondrocytes, but not healthy chondrocytes [27]. Recently, Zhou et al. reported that IL-6 is closely associated with apoptosis in rat articular chondrocytes, and represents another pathophysiological cause of OA [28]. Although we did not investigate the formononetin-induced anti-apoptotic effects in primary rat chondrocytes, formononetin may rescue the viability of chondrocytes against chronic inflammation-mediated apoptosis.

Sandell et al. reported that chemokine genes, such as fractalkine (CX3CL1), LIX (CXCL5), MCP-1 (CCL2), and MIP-3α (CCL20), were exuberantly expressed in adult human articular chondrocytes in response to IL-1β [29]. Recent studies associated with the pathophysiological role of fractalkine in OA reported that the levels of fractalkine in synovial fluid and serum were significantly elevated in patients with knee joint OA as compared with patients with healthy knee joints, and that fractalkine might serve as an effective biomarker for the severity of OA [30, 31]. Furthermore, although recent studies have been reported that fractalkine acts as a chemoattractant for OA synovial fibroblasts [32] and is closely associated with inflammation in the synovial joints [33], the pathophysiological role of fractalkine in OA is still under

investigation. Recently, Xu et al. reported that MCP-1 is a potent attractor of monocytes and increases the expression of cartilage degrading enzymes. It also induces (or enhances) the apoptosis of chondrocytes derived from OA patients [34]. Alaaeddine et al. reported that MIP-3α, also known as chemokine CCL20, induced pro-inflammatory and matrix degradative responses in cartilage [35]. VEGF is a representative catabolic growth factor of progressive cartilage degeneration through the expression of cartilage degrading enzymes in OA [36]. Indeed, the inhibition of VEGF showed a deceleration and retardation of progressive articular cartilage degeneration [37, 38]. Taken together, our cytokine array data consistently suggest that formononetin-induced anti-catabolic effects are mediated by the suppression of catabolic pro-inflammatory cytokines, chemokines, and growth factors in primary rat chondrocytes treated with IL-1β.

In addition, one of the clinical symptoms of OA is chronic joint pain [39]. Recent studies have reported that inflammatory mediators, pro-inflammatory cytokines, and chemokines present in the osteoarthritic joints may not only promote synovitis and progressive articular cartilage destruction but may also activate innervating nociceptors that are sensory neurons related to pain perception [39]. However, the investigations into chronic joint pain caused by progressive articular cartilage degeneration and the upregulation of proalgesic pro-inflammatory cytokines and chemokines is ongoing in the hopes of developing novel OA-specific analgesic targets [39]. Moreover, Hamilton et al. reported that VEGF is not only involved in the pathological progression of OA but may also act directly on the sensory neurons to develop pain sensitization [40]. Furthermore, they suggest that the inhibition of VEGF signaling associated with hyperexcitability of sensory neurons may decrease pain sensitivity [40]. Hence, our cytokine array data suggest that formononetin may contribute the maintenance of mechanical joint functions through the analgesic effects resulted in the suppression of catabolic pro-inflammatory cytokines, chemokines, and growth factors that are closely associated with the development of joint pain.

However, although we demonstrated that formononetin was non-toxic to primary rat chondrocytes, and decelerated and retarded the progression articular cartilage degeneration through the suppression of cartilage-degrading enzymes, inflammatory mediators, pro-inflammatory cytokines, chemokines, and growth factors *in vitro*, and *ex vivo* studies from the present study, there are several recognized limitations of this study. Firstly, we have warranted addressing the anti-catabolic and analgesic effects of formononetin to maintain the mechanical joint function using animal models with pre-clinical OA symptoms that are chronic joint pain accompanied with the progressive degeneration of articular cartilage. Secondly, our findings were merely limited to the anti-catabolic effectiveness of formononetin against to IL-1β-induced catabolic inflammation. There are still underlying the specific cellular signaling pathway and molecular mechanism of formononetin on the maintenance of cartilage homeostasis. Therefore, further studies are required to investigate the precise cellular signaling pathways associated with formononetin-induced anti-catabolic and analgesic effectiveness through the *in vitro* using chondrocytes and *in vivo* using animal models with preclinical OA symptoms. Subsequent studies will allow us to elucidate that formononetin may be a promising candidate for the treatment and prevention of OA.

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AUTHOR'S CONTRIBUTIONS

I.A.C, T.H.K., K.R.K, H.L., J.H.P., S.Y.L., and J.S.K. contributed to the experimental design and collected the data. C.S.K., D.K.K., H.K.K., S.K.Y., S.G.K., and J.S.K. contributed to the data analysis and interpretation. I.A.C., K.R.K., and J.S.K. did the writing article.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

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