ORIGINAL ARTICLE   **Open Access**

Catalpol protects mouse ATDC5 chondrocytes against interleukin-1β-induced catabolism

Chengkui Cai[1], Pengcheng Sun[1], Zhihui Chen[1], Chao Sun[1] and Liying Tian[2]
[1]Department of Orthopedics, The Second Affiliated Hospital of Shaanxi University of Chinese Medicine, Xianyang and
[2]Department of Anesthesiology, The Second Affiliated Hospital of Air Force Medical University, Xi' an, Shaanxi, PR China

Summary. Catalpol is a natural product with promising anti-inflammatory effects, however, its effects on chondrocytes and osteoarthritis (OA) have not been well investigated. OA is a painful and debilitating joint disease that affects people worldwide. Traditional Chinese Medicine has been sought to treat OA, including the Rehmannia extract, Catalpol. Here, we examined the effects of Catalpol, a plant derivative used in traditional Chinese medicine, on ATDC5 chondrocytes originating from mouse teratocarcinoma cells stimulated with interleukin-1β (IL-1β) to mimic the OA cellular environment. Catalpol significantly reduced matrix metalloproteinase-1, -3, -13 (MMP-1, -3, -13), a disintegrin and metalloproteinase with thrombospondin motifs -4, -5 (ADAMTS-4, -5) against IL-1β, demonstrating a likely anti-cartilage degradation activity. We also found that Catalpol exerted a significant anti-oxidative stress effect by downregulating the production of inducible nitric oxide synthase (iNOS), nitric oxide (NO), reactive oxygen species (ROS), and malondialdehyde (MDA). Catalpol treatment significantly reduced the levels of several key inflammatory factors, including Prostaglandin E_2 (PGE2), cyclooxygenase-2 (COX-2), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1). We further demonstrate that the effects of Catalpol were mediated by the nuclear factor -κB (NF-κB) pathway via downregulation of the phosphorylation of inhibitor of nuclear factor κB-α (IκBα). This was confirmed by measuring p38 and p65 protein levels as well as the luciferase activity of NF-κB. Altogether, we demonstrate the potential of Catalpol as a novel treatment agent against cartilage matrix degradation, oxidative stress, and inflammation in OA.

Corresponding Author: Liying Tian, Department of Anesthesiology, The Second Affiliated Hospital of Air Force Medical University, No. 1, Xinsi Road, Baqiao District, Xi'an, Shaanxi, 710038, PR China. e-mail: tianly0323@yeah.net

Introduction

Osteoarthritis (OA) is a debilitating disease characterized by excessive degradation of the articular extracellular matrix (ECM) and is most prominent in the knee and hip joints. Recent work has indicated that the pathology of OA also involves the cellular and biomechanical stresses causing bone remodeling and the synovial inflammation in joints. While numerous factors can contribute to OA progression, advanced age is the most significant factor (Huddleston et al., 2012; Loeser et al., 2016). Recent research has focused on preventing or halting the pathogenesis of OA at the molecular level through the use of pharmaceuticals, but a reliable treatment has not been discovered yet. This is, in large part, due to the complicated etiology of the disease. Chondrocytes are the only cell type in joint cartilage and regulate the release of catabolic substances such as degradative enzymes, oxidants, and inflammatory cytokines. In normal conditions, chondrocytes maintain the gradual turnover of the ECM, but in OA, they become activated to induce a shift toward a pathological phenotype, increased apoptosis, and the expression of catabolic genes (Goldring and Otero, 2011).

Interleukin-1β (IL-1β) is well-recognized as playing a key role in the pathogenesis of OA by promoting the expression of degradative enzymes, including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), which target type II collagen and aggrecan for degradation, respectively (Ji et al., 2016; Makki and Haqqi, 2016). In addition to promoting cartilage degradation, IL-1β induces an inflammatory response by upregulating the expression of other proinflammatory cytokines and chemokines such as cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), monocyte chemoattractant protein-1 (MCP-1), and interleukin-8 (IL-8) (Chen et al., 2006; Sieghart et al., 2015; Zheng et

al., 2017). COX-2 is an isoenzyme that plays a role in the synthesis of PGE2, amongst other inflammatory and pain-sensitizing factors. Specific inhibition of COX-2 has long been regarded as a promising therapy against pain and swelling associated with OA (Lane, 1997). PGE2, the end product of arachidonic acid metabolism, is associated with the pathogenesis of OA, joint pain, and cartilage destruction, and preventing its expression is also considered a treatment target for OA (Akhtar et al., 2017). The IL-1β-induced release of MCP-1 and IL-8 plays a critical role in perpetuating the inflammatory response in osteoarthritic chondrocytes. The chemokine MCP-1 recruits monocytes to the joints. It is involved in the initiation and progression of OA by promoting cartilage degradation and chondrocyte degeneration, while IL-8 has been shown to be highly correlated with the severity of OA (Yan et al., 2016; Scanzello, 2017; Garcia-Cirera et al., 2019). Therefore, inhibiting the expression of these pro-inflammatory factors is another strategy against OA.

Oxidative stress occurs when the ratio of oxidants to antioxidants is disturbed and is another major contributor to the pathogenesis of OA. iNOS has been shown to reduce cartilage matrix synthesis and promote the expression of proteases. Nitric oxide (NO) is a product of iNOS and plays a role in altering the phenotype of OA chondrocytes as well as in bone remodeling and metabolism (de Andrés et al., 2016). NO has also been shown to exert catabolic effects in OA by mediating inflammation and inducing oxidative damage, apoptosis, extracellular matrix (ECM) degradation, and inhibiting the synthesis of type II collagen and aggrecan (Yudoh et al., 2005; Abramson, 2008). Overproduction of reactive oxygen species (ROS) is detrimental in OA as it leads to oxidative damage to collagen, proteoglycans, and hyaluronan, resulting in the production of malondialdehyde (MDA) (Wei et al., 2018). Various intracellular signaling pathways have been shown to influence the pathogenesis of OA. Notably, the NF-κB pathway is recognized as the master regulator of inflammation and has been explored as a treatment target to slow the progression of OA. Under oxidative stress or upon stimulation by cytokines such as IL-1β, NF-κB signaling promotes the expression of inflammatory cytokines and catabolic factors by chondrocytes and affects chondrocyte differentiation (Roman-Blas et al., 2006; Lepetsos et al., 2019). In chondrocytes, oxidative stress and inflammation are closely related to each other through activating the production of matrix metalloproteinases and aggrecanases. The coordinated activation of MMPs and aggrecanases could lead to cartilage degradation and OA development (Lepetsos et al., 2019). It has been recently reported that ROS may indirectly affect ECM catabolism by up-regulating MMPs. When stimulated by IL-1β, MMP-1 was produced by chondrocytes via a NOX4-mediated ROS-dependent pathway (Roman-Blas et al., 2006). Additionally, ROS play a key role in mediating MMP-13 gene expression caused by cytokines (Park,

2016).

Rehmannia glutinosa (Dihuang) is a plant commonly used in combination with other herbs in Traditional Chinese Medicine. Rehmannia is prescribed for diabetes, cancer, osteoporosis, allergies, and many other diseases (Zhang et al., 2008). Catalpol (Fig. 1A), an iridoid glycoside derived from Rehmannia, has been shown to exert anti-inflammatory effects in various diseases and cell types, including intestinal cells, neurons, astrocytes, and kidney cells (Tian et al., 2006; Park, 2016). It is known that Rehmannia extracts enhance bone metabolism (Oh, 2003). Interestingly, Catalpol has also been shown to influence the osteogenic differentiation of bone marrow mesenchymal stem cells and stromal cells (Chen et al., 2017; Zhu et al., 2019). Most recent works demonstrate that Catalpol inhibits chondrocytes catabolism and inflammation, but promotes bone formation (Zeng et al., 2019; Zhao et al., 2021). Based on previous findings, we hypothesized that Catalpol might have a regulatory effect on chondrocytes, and the supplement of Catalpol could have certain beneficial effects on OA. However, the mechanism of Catalpol on chondrocytes remains to be investigated. The present study examined the effect of Catalpol on oxidative stress, inflammation, and cartilage degradation induced by IL-1β in ATDC5 chondrocytes.

Materials and methods

Cell culture and treatment

Mouse chondrogenic cell line ATDC5 cells were from ECACC, UK, and cultured in DMEM supplemented with 10% FBS, and 1% penicillin/ streptomycin (P/S) in a 5% CO_2 incubator until mature. The protocol of this study was supported by the Ethical Commitiee of The Second Affiliated Hospital of Air Force Medical University (SAHAFMU- EA018A03). IL-1β was purchased from R&D Systems (Minneapolis, USA). Catalpol was purchased from Sigma-Aldrich (Shanghai, China). Both IL-1β and Catalpol were prepared by dissolving in PBS and used for fresh aliquots. For the cell treatment experiment, ATDC5 cells were seeded onto a 6-well plate overnight with a density of 0.2×10^6 cells/well. The next day, the cells were incubated with IL-1β (10 ng/mL) in the presence or absence of 15 or 30 μM catalpol (Xu et al., 2018; Hu et al., 2019) for 48h. For the control, the same amount of PBS was added to the cell culture media.

Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from ATDC5 chondrocytes using Qiazol reagent (Qiagen, USA) (Wang et al., 2022). Then, cDNA was generated using 2 μg of purified RNA for reverse-transcription PCR with a cDNA synthesis kit (Bio-Rad, USA). Quantitative real-time PCR using SYBR Green Master Mix was performed to measure the expression of the target genes (Roche Diagnostics,

Switzerland). The mRNA expressions of MMP-1, MMP-3, MMP-13, ADAMTS-4, ADAMTS-5, COX2, iNOS and GAPDH were examined. The $2^{-\Delta\Delta Ct}$ method was used to calculate the levels of the target genes. The following primers were used in this study: MMP-1 (forward: 5'-AGGAAGGCGATATTGTGCTCTCC-3', reverse: 5'- TGGCTGGAAAGTGTGAGCAAGC -3'); MMP-3 (forward:5'- CTCTGGAACCTGAGAC ATCACC-3', reverse: 5'- AGGAGTCCTGAGAG ATTTGCGC-3') , MMP-13 (forward:5'- GATGACCTG TCTGAGGAAGACC-3', reverse: 5'- GCATTTCTCG GAGCCTGTCAAC-3'), ADAMTS-4 (forward:5'- GAACGGTGGCAAGTATTGTGAGG-3′, reverse: 5′-TTCGGTGGTTGTAGGCAGCACA-3′), ADAMTS-5 (forward: 5'- CTGCCTTCAAGGCAAATGTGTGG-3', reverse: 5'-CAATGGCGGTAGGCAAACTGCA -3'), COX2 (forward: 5' - GCGACATACTCAAGCAGG AGCA-3', reverse: 5'-AGTGGTAACCGCTCAGG TGTTG-3'), iNOS (forward: 5'-GCGACATACTCAA GCAGGAGCA-3', reverse: 5'-AGTGGTAACCGC TCAGGTGTTG-3'), GAPDH: forward: 5'-AAGACC TCTATGCCAACACAGT-3', reverse, 5'- AGCCAGA GCAGTAATCTCCTTC-3').

Western blot analysis

Cell lysis buffer was used to isolate the protein. Total protein concentration was measured using a commercial BCA protein assay kit. Then, 20 μg protein samples were separated using 10% SDS-PAGE and transferred onto PVDF membranes (Wu et al., 2021). To block non-specific sites on the membranes, the membranes were blocked with 5% non-fat milk at RT and probed overnight in a cold room with primary antibodies. The antibodies used in this study include COX2 (1:1000, Santa Cruz Biotechnology, #sc-19999), iNOS (1:1000, Santa Cruz Biotechnology, #sc-7271), p-38 (1:1000, Cell Signaling Technology, #9211), total-p38 (1:1000, Cell Signaling Technology, #9212), p-IκBα (1:1000, Cell Signaling Technology, #2859), total-IκBα (1:2000, Cell Signaling Technology, #4812), p65 (1:2000, Cell Signaling Technology, #6956), Lamin B (1:2000, Cell Signaling Technology, #12586), and β-actin (1:5000, Santa Cruz Biotechnology, #sc-8432). The membranes were then washed 3 times with PBST and incubated with HRP-conjugated anti-mouse secondary antibody (1:5000, Santa Cruz Biotechnology, #sc-2005) or anti-rabbit antibodies (1:5000, Santa Cruz Biotechnology, #sc-2004).

ELISA

The cells were lysed with RIPA buffer (Tris-HCl 50 mM, NaCl 150 mM, 1% Triton X-100, Sodium Deoxycholate 1%, SDS 0.1%, EDTA 2 mM, PH=7.5) with protease inhibitors (Complete EDTA−free protease inhibitor cocktail, Roche, Shanghai) and phosphatase inhibitor cocktail (PhosSTOP, Roche, Shanghai). Total lysates were clarified by centri-

fugation at 14,000 g for 5 minutes and the soluble proteins were collected and quantified using BCA (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The commercial ELISA kits were purchased to assess the expression levels of MMP-1 (LSBio, #F5523), MMP-3 (R&D Systems, #MMP300), MMP-13 (Qarigo, ARG82284), ADAMTS-4 (LSBio, #F18571), ADAMTS-5 (MBiosource, #MBS7606113), PGE2 (R&D Systems, #KGE004B), IL-8 (MBiosource, #MBS7606860), and MCP-1 (R&D Systems, #MJE00B). The levels of these proteins were measured according to their manufacturer's instructions.

Measurement of intracellular NO

Cell-permeable fluorescent probe staining was used to assess the levels of intracellular NO. The fluorescent dye DAF-FM DA was used to probe cells for NO (Sigma-Aldrich, St. Louis, MO). Briefly, 10 μL DAF-FM DA was added and incubated for 30 min. The cells were then washed twice with PBS and images were recorded with a Leica TCS SP2 microscope (Leica, USA). The fluorescence intensity was quantified using Image J software (NIH, USA). For each group, 10 regions of interest (ROI) were randomly assigned, the integrated density value (IDV) of the ROI and the number of cells in the ROI was obtained by specific function, and the fluorescence level of the reaction was normalized by the cell numbers, the data of replicates were aggregated and presented as fold change.

DCFH-DA staining

The level of production of ROS was measured by probing the cells with DCFH-DA. After necessary treatment, the cells were loaded with the dye DCFH-DA (5 μmol/L) for 30 min. After being washed 3 times, fluorescent images were captured with a Leica TCS SP2 fluorescence microscope (Leica, USA). The ROS levels were quantified with a similar method as described in 2.5.

Luciferase activity

The NF-κB luciferase promoter vector used in this study was purchased from Thermo Fisher Scientific, USA. Briefly, the cells were co-transfected with NF-κB promoter and firefly luciferase vector using Lipofectamine 3000. After treatment with IL-1β (10 ng/mL) or catalpol (15 and 30 μM) for 48h, the cells were lysed using a lysis buffer, and the luciferase activity of NF-κB was measured using a dual-luciferase reporter assay system (Promega, USA).

MTT

The cells were plated in a 96 well culture plate

(10000 cells/200 µl/well density) and incubated in a CO_2 incubator. The next day, the cells were treated according to the experimental design. At the end of the treatment, 50 µl MTT solutions from the Stock (5 mg/ml, Sigma-Aldrich) were added and the cells were incubated in the dark for 4h. Then the medium was removed and Formazan crystals formed by the cells were dissolved using 200 µl of DMSO. The absorbance value was read at 570 nm using 630 nm as the reference wavelength on a microplate plate reader (Bioteck, USA). The data were presented as fold change.

Statistical analysis

All experimental data are presented as mean ± standard error of measurement (S.E.M.). Statistical analysis was performed using SPSS. ANOVA followed by the post hoc Bonferroni test was used. A value of P<0.05 was considered statistically significant.

Results

In the current study, we investigated the role of Catalpol in pro-inflammatory IL-1β-elicited chondrocytes. Our results show that Catalpol possesses anti-oxidative and anti-inflammatory effects in activated chondrocytes. As a result, the presence of Catalpol in chondrocytes suppressed cartilage matrix degradation. We also elucidate the involvement of the NF-κB signaling pathway in mediating the effects of Catalpol.

*Catalpol reduces IL-1β-induced expression of MMPs and ADAMTS*

We first tested the dose-related toxicity of Catalpol in cultured ATCD5 chondrocytes. A series of dose-responsive MTT assays showed that 0.5, 5, 15, and 30 µM Catalpol treatment for 48 hours did not have obvious effects on the cell viability, but 150 and 300 µM Catalpol treatment for the same time resulted in 12.2% and 18.5% reductions in ATDC cell viability, respectively (Fig. 1B). Therefore, we adopted 15 and 30 µM Catalpol in the following experiments.

Real-time PCR analysis demonstrated that the mRNA levels of MMP-1, -3, -13 were elevated to around 4.5-, 5.8-, and 7.3-fold, respectively by IL-1β stimulation. However, they were decreased to 2.7-, 3.2-, and 3.1-fold by 15 µM of Catalpol, respectively, while 30 µM Catalpol further reduced them to only 1.8-, 1.8-, and 1.5-fold, respectively (Fig. 2A). For their protein levels, IL-1β alone induced 5.2-, 5.5-, and 5.7-fold productions of MMP-1, MMP-3, and MMP-13, respectively. However, the two doses of Catalpol reduced their production. When 15 µM Catalpol was added, the productions of MMP-1, MMP-3, and MMP-13 were reduced to only 1.9-,1.5-, and 1.5-fold, respectively (Fig. 2B).

Next, we assessed the effects of Catalpol on ADAMTS-4, -5, the two main aggrecanases. Exposure to IL-1β alone upregulated the mRNA of ADAMTS-4 to 4.1-fold and that of ADAMTS-5 to 5.3-fold (Fig. 3A). However, the two doses of Catalpol reduced the expressions of ADAMTS-4 to only 2.6- and 1.7- fold, and those of ADAMTS-5 to 2.8- and 1.9-fold, respectively. Similarly, the results of ELISA in Fig. 3B show that the protein concentrations of ADAMTS-4 and ADAMTS-5 increased 5.7 and 5.0-fold, respectively, in the presence of IL-1β alone, while the two doses of Catalpol reduced their production. When 30 µM Catalpol was added, the productions of ADAMTS-4 and ADAMTS-5 were reduced to only 2.1- and 1.5-fold, respectively. As a result, IL-1β treatment resulted in about a 47% reduction of type 2 collagen, but the presence of Catalpol mitigated its reduction and resulted in about only 8% reduction (Fig. 4). Meanwhile, IL-1β treatment resulted in about a 41% reduction of aggrecan but the presence of Catalpol significantly ameliorated this to only about 8% aggrecan

(A)
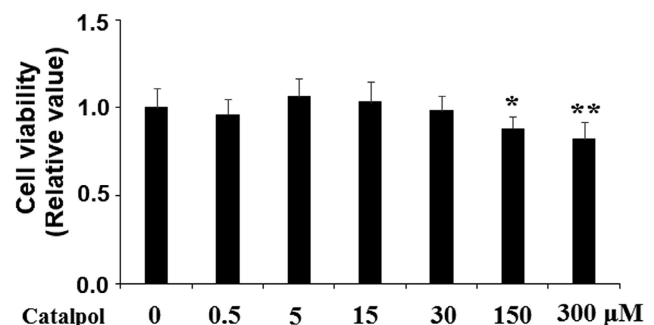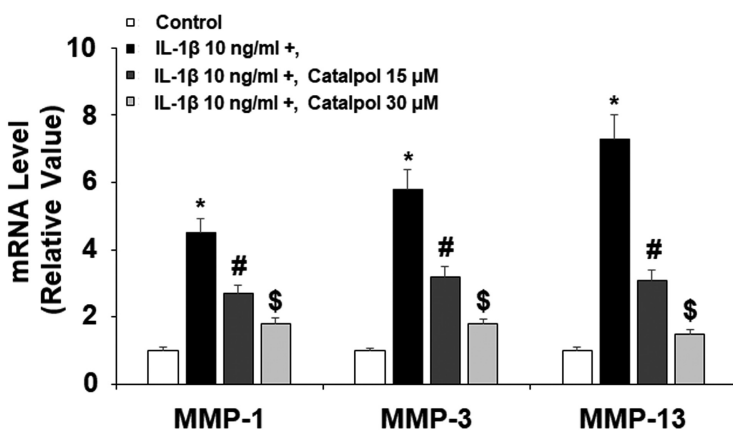

(B)
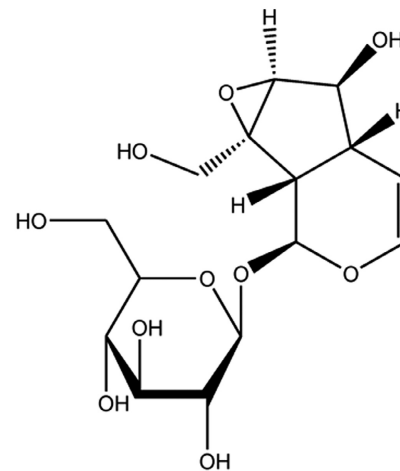
Fig. 1. A. Catapol structure. **B.** The effects of catalpol on cell viability of chondrocytes. Cells were treated with catalpol at concentrations of 0.5, 5, 15, 30, 150, 300 µM for 48h. Cell viability was measured by MTT assay (* and **, P<0.01, 0.001 vs. vehicle group by ANOVA with Bonferroni's post hoc correction, respectively, N=5,).
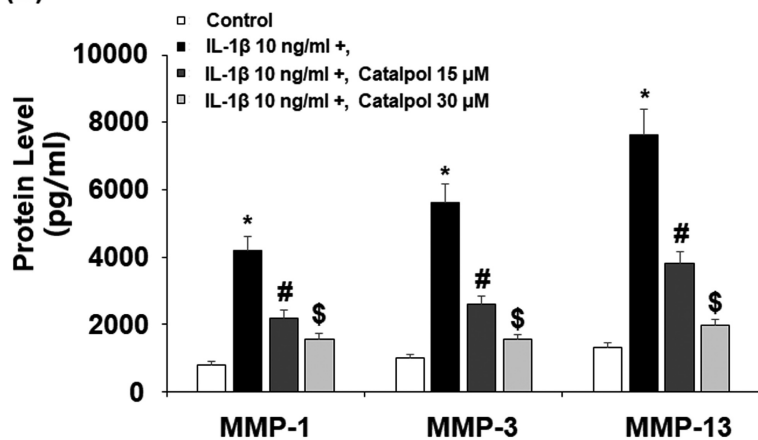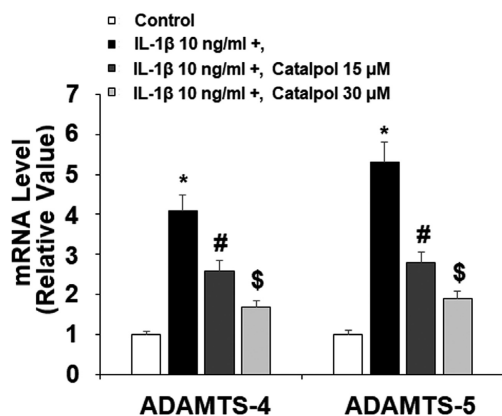
(A)

(B)

Fig. 2. Catalpol reduces IL-1β-induced expression of metalloproteinases (MMPs) in ATDC5 chondrocytes. **A.** mRNA of MMP-1, MMP-3, and MMP-13 by RT-PCR (N=5). **B.** Protein levels of MMP-1, MMP-3, and MMP-13 by ELISA assay (*, P<0.01 vs. vehicle group; #, P<0.01 vs. IL-1β group; &, P<0.01 vs. IL-1β+15 μM catalpol group; ANOVA with Bonferroni's post hoc correction, N=5).
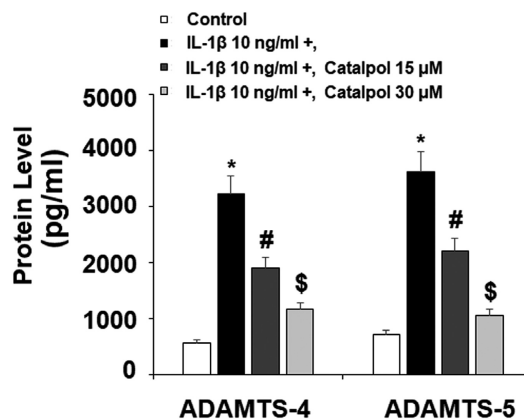
(A)

(B)

Fig. 3. Catalpol mitigates IL-1β- induced expression of Disintegrin-like and metalloproteinases with thrombospondin motifs (ADAMTS) in ATDC5 chondrocytes. **A.** mRNA of ADAMTS-4 and ADAMTS-5 by RT-PCR. **B.** Protein levels of ADAMTS-4 and ADAMTS-5 by ELISA assay (*, P<0.01 vs. vehicle group; #, P<0.01 vs. IL-1β group; &, P<0.01 vs. IL-1β+15 μM catalpol group; ANOVA with Bonferroni's post hoc correction, N=4).

production reduction (Fig. 4).

Catalpol reduces the expressions of COX2 and PGE2

The mRNA expression of COX-2 was upregulated to 5.1- fold by treatment with IL-1β alone. However, the addition of 15 and 30 μM Catalpol reduced the mRNA of COX-2 to only 3.2- and 1.7-fold, respectively (Fig. 5A). Concordantly, exposure to IL-1β alone increased the production of PGE$_2$ 6.3-fold, however, the addition of 15 and 30 μM Catalpol attenuated its production to only 3.3- and 1.9-fold, respectively (Fig. 5B).

Catalpol reduces IL-1β-induced production of iNOS and NO

IL-1β stimulation elevated the mRNA of iNOS 5.5-fold, which was then decreased to only 3.3- and 2.1-fold by 15 and 30 μM Catalpol, respectively (Fig. 6A). NO

generation was increased 4.3-fold by IL-1β, then decreased to only 2.6- and 1.7-fold by 15 and 30 μM Catalpol, respectively (Fig. 6B).

Catalpol inhibits the expression of proinflammatory chemokines

IL-1β treatment upregulated the mRNA levels of IL-8 and MCP-1 to 9.8- and 8.8-fold, respectively. However, the mRNA expressions of these cytokines were obviously inhibited by 15 and 30 μM Catalpol. IL-8 was reduced 4.5- and 2.5-fold, while MCP-1 expression was decreased 4.1- and 2.6-fold, respectively (Fig. 7A). Meanwhile, at the protein level, IL-1β treatment alone induced 6.2- and 6.1 increases in IL-8 and MCP-1 expressions, respectively. However, 15 and 30 μM Catalpol ameliorated this effect, with high dose Catalpol reducing IL-8 and MCP-1 to only 2.5- and 1.9-fold, respectively. Thus, Catalpol may exert a potent
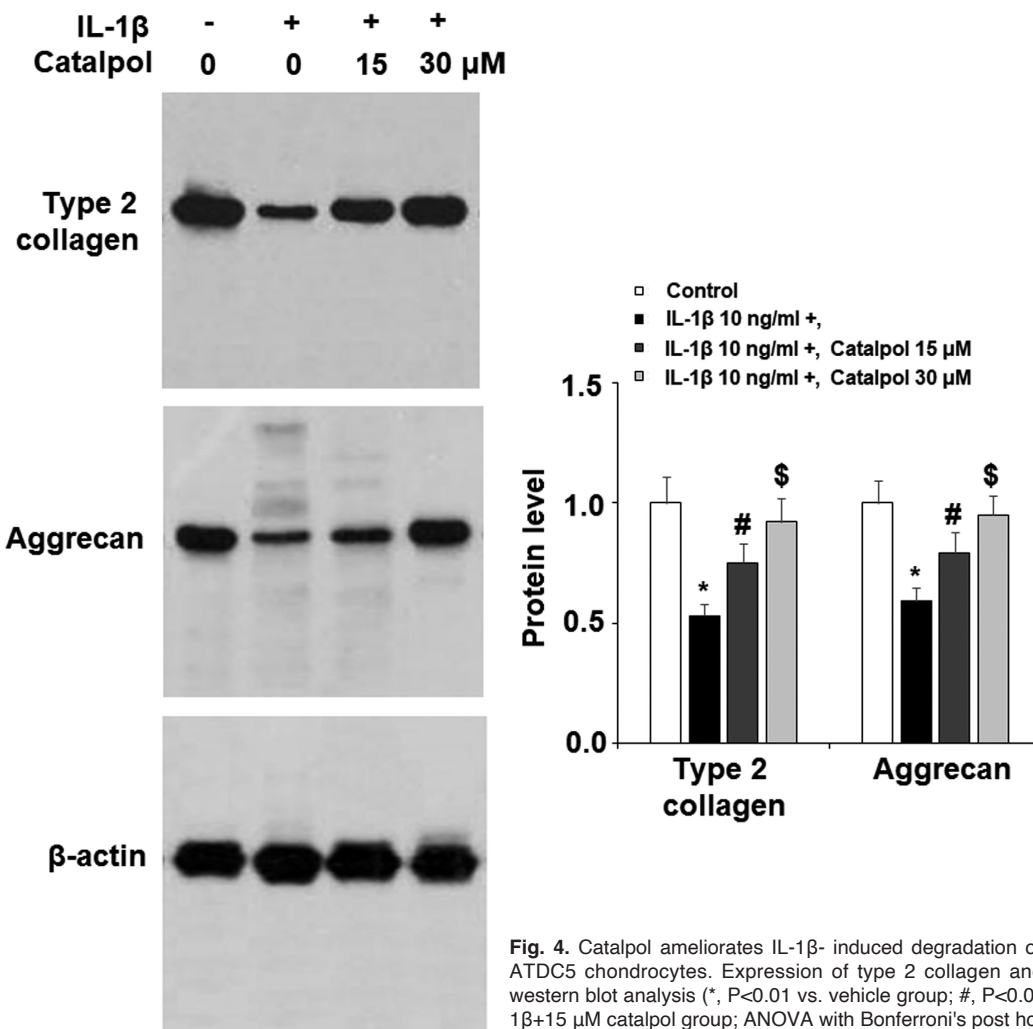

Fig. 4. Catalpol ameliorates IL-1β- induced degradation of type 2 collagen and aggrecan in ATDC5 chondrocytes. Expression of type 2 collagen and aggrecan were measured using western blot analysis (*, P<0.01 vs. vehicle group; #, P<0.01 vs. IL-1β group; &, P<0.01 vs. IL-1β+15 μM catalpol group; ANOVA with Bonferroni's post hoc correction, N=5).

anti-inflammatory effect in OA chondrocytes.

Catalpol reduces the IL-1β-induced expression of ROS and MDA

ROS and MDA are significant indicators of oxidative stress and lipid peroxidation. Production of ROS increased 4.1-fold, which was then ameliorated by 15 and 30 μM Catalpol, to only 2.4- and 1.5-fold. The concentration of MDA was determined using a commercial kit. As shown in Figure 8B, the concentration of MDA was increased from 2.3 to 9.2 nmol/mg by IL-1β alone, while the two doses of Catalpol significantly reduced the level of MDA to only 5.1 and 3.5 nmol/mg, respectively.

The effects of Catalpol are mediated through the NF-κB pathway

Finally, we investigated the involvement of the NF-κB signaling pathway in mediating the effects of Catalpol against IL-1β-induced insult. The phosphorylation and activation of p38 protein and IκBα were measured. The level of phosphorylated p38 was increased 3.8-fold by IL-1β alone, while 15 and 30 μM Catalpol reduced this level to only 2.4- and 1.6-fold, respectively (Fig. 9A). Similarly, the results in Fig. 9B indicate that the level of phosphorylated IκBα was increased 3.3-fold by IL-1β alone, while 15 and 30 μM Catalpol decreased this to only 2.2- and 1.5-fold, respectively, thereby indicating a potent capacity of Catalpol to inhibit the degradation of IκBα.

Next, we measured the luciferase activity of NF-κB to confirm whether the effects of Catalpol on IκBα phosphorylation indeed prevent activation of NF-κB. Phosphorylation of p65 protein increased 4.1-fold, which

was reduced by the addition of the two doses of Catalpol to only 2.5- and 1.5-fold, respectively (Fig. 10A). As shown in Figure 10B, the luciferase activity of NF-κB was upregulated 89.5-fold by IL-1β, while 15 and 30 μM Catalpol inhibited NF-κB activation to only 35.5- and 21.5-fold, respectively.

Discussion

The development of OA is a complex process involving the interaction of chondrocytes and joint tissue. Amongst all the risk factors, aging and inflammation are considered the most relevant events for the development of OA (Greene and Loeser, 2015). The age-related pro-inflammatory mediators and stress-induced chondrocyte senescence are important molecular mechanisms in the pathogenesis of OA. To develop safe and effective agents that can modulate the mechanisms involved in OA, many different traditional compounds have been sought. Catalpol has been studied as a pharmacologic agent since the early 1960s (Duff et al., 1965). In recent years, Catalpol has been extensively investigated due to its impressive pharmacological activities, such as anti-inflammatory activity. Here, we examined its effects in IL-1β-activated ATDC5 cells. Multiple studies have focused on the role of Catalpol in ameliorating neuronal damage resulting from ischemia-induced injury (Li et al., 2004, 2008). However, there is also evidence of its role in regulating bone metabolism and osteoblast differentiation (Wu et al., 2010; Lim and Kim, 2013), which prompted us to investigate its potential in modulating the activity of chondrocytes. Our results indicate that Catalpol may indeed hold promise as a safe and effective treatment against cartilage degradation and the progression of OA through multiple mechanisms.
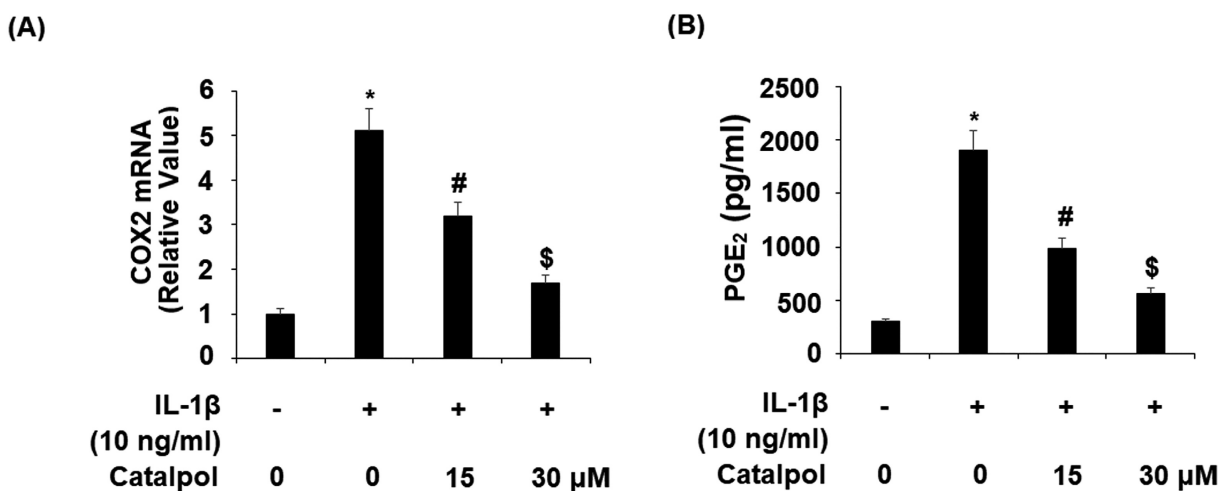
(A)

(B)

Fig. 5. Catalpol inhibits IL-1β-induced expressions of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) in ATDC5 chondrocytes. **A.** mRNA of COX2 by RT-PCR. **B.** Production of PGE2 by ELISA assay (*, P<0.01 vs. vehicle group; #, P<0.01 vs. IL-1β group; &, P<0.01 vs. IL-1β+15 μM catalpol group; ANOVA with Bonferroni's post hoc correction, N=5).

Firstly, our findings indicate that Catalpol can reduce IL-1β-induced increased expressions of collagenases (MMP-1, MMP-3, MMP-13) and aggrecanases (ADAMTS-4, ADAMTS-5), resulting in the amelioration of type 2 collagen and aggrecan degradation, suggesting a protective mechanism by Catalpol in chondrocytes. To our knowledge, this is the first study to demonstrate this kind of impact of Catalpol in chondrocytes. MMP-1, MMP-3, and MMP-13 are well-recognized as playing a pivotal role in the progression of OA through the catabolic breakdown of type II collagen (Koskinen et al., 2011). Previous studies have demonstrated a possible chemotherapeutic effect of Catalpol, as it was shown to reduce the expressions of MMP-2, MMP-9, and MMP-16, thereby inhibiting tumor angiogenesis in both colon and breast cancer models (Liu et al., 2015; Zhu et al., 2017). Our study provides evidence of its inhibitory effect against overexpression of the specific MMPs involved in OA. Furthermore, there is no previous research on the effects of Catalpol on the expressions of ADAMTS. ADAMTS-4 and ADAMTS-5 are the main degradative enzymes targeting aggrecan, which provides cartilage with its shock-absorptive property, for catabolic destruction (Song et al., 2007). Our findings show that Catalpol significantly ameliorates the IL-1β-induced expression of these key degradative enzymes and protects against degradation of key matrix proteins, indicating its
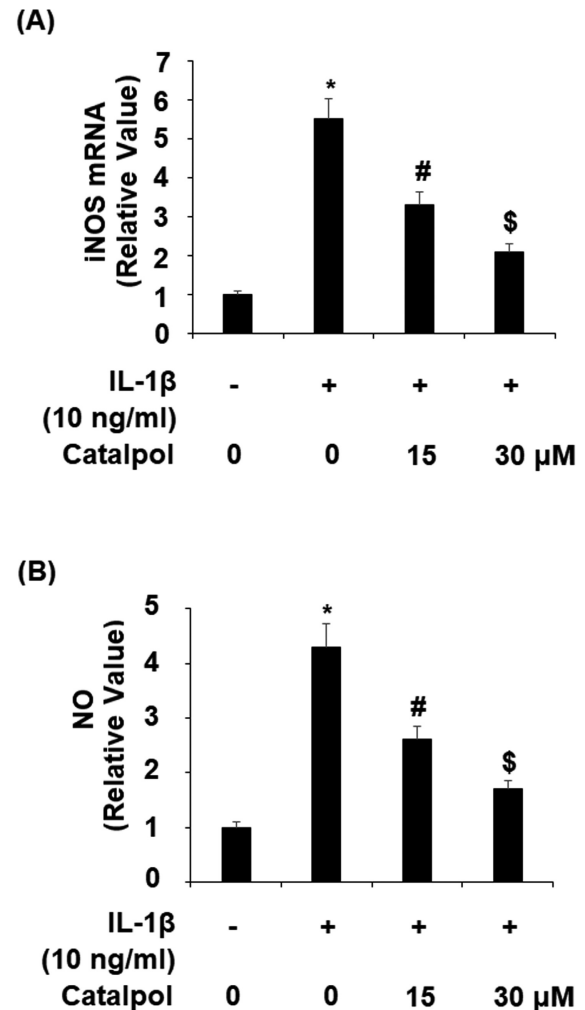

Fig. 6. Catalpol suppresses IL-1β-induced expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO) in ATDC5 chondrocytes. **A.** mRNA of iNOS by RT-PCR. **B.** Production of intracellular NO (*, $P < 0.01$ vs. vehicle group; #, $P < 0.01$ vs. IL-1β group; &, $P < 0.01$ vs. IL-1β+15 μM catalpol group; ANOVA with Bonferroni's post hoc correction, N=4).
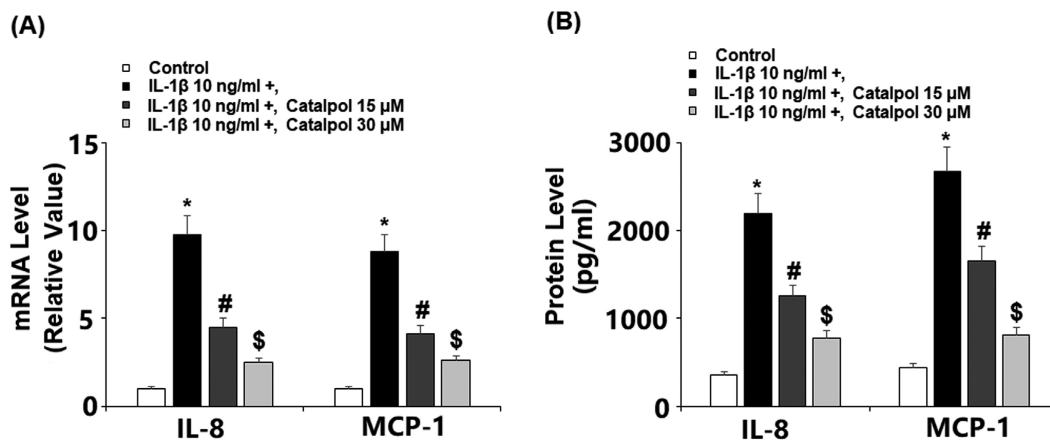

Fig. 7. Catalpol reduces IL-1β-induced expression of pro-inflammatory cytokines in ATDC5 chondrocytes. **A.** mRNA of IL-8 and MCP-1 by RT-PCR. **B.** Secretions of IL-8 and MCP-1 protein by ELISA assay (*, $P < 0.01$ vs. vehicle group; #, $P < 0.01$ vs. IL-1β group; &, $P < 0.01$ vs. IL-1β+15 μM catalpol group; ANOVA with Bonferroni's post hoc correction, N=5).

potential role in preventing excessive degradation of the articular ECM, which is the hallmark of OA.

Oxidative stress and inflammation are key factors in a multitude of chronic diseases, including OA. The roles of iNOS, NO, and ROS in OA have been thoroughly studied, and antioxidant agents that act to reinstate the oxidant/antioxidant balance are widely sought after. Recently, there has been a considerable amount of research demonstrating a beneficial effect of Catalpol against oxidative stress induced by various substances and in several cell types (Mao et al., 2007; Tian et al., 2007; Bi et al., 2008). However, the vast majority of these studies have focused on the brain. In chondrocytes, recent work suggests that both catalpol and Rehmannia extract, a herb medicine that is rich in catalpol, possess anti-inflammatory and anti-oxidative effects. An *in vitro* experiment indicates that the presence of catalpol promotes mitochondrial biogenesis, suggesting that the agent may provide metabolic flexibility and promote chondrocyte survival in a joint stress environment (Chen et al., 2022). Another study shows that catalpol inhibits the NF-kB pathway, and reduces the production of inflammatory cytokines (IL-6, TNF-α), suggesting that it could have direct effects on the inflammatory pathway (Zeng et al., 2019). In accordance with these reports, we demonstrate for the first time that Catalpol can significantly ameliorate oxidative stress in mouse chondrocytes by decreasing IL-1β-induced productions of iNOS, NO, ROS, and MDA, thereby attenuating the progression of OA.

Ameliorating the expressions of cytokines and chemokines has been suggested as a potential treatment method to slow OA progression. These approaches include the inhibition of COX-2 and PGE_2 (Ma et al., 2015). Our findings demonstrate that Catalpol significantly downregulates the release of COX-2 and PGE_2 in chondrocytes exposed to IL-1β. IL-8 has been shown to promote the production of MMP-13, recruit leukocytes to the synovium, and trigger neutrophils to accumulate in the joint (Takahashi et al., 2015), while MCP-1 has been shown to increase the expression of MMP-3 and recruit memory T-lymphocytes, natural killer cells, and monocytes to the joint. Targeting chemokine-mediated pathways involved in OA has been suggested as a potential treatment method too (Scanzello, 2017). Here, our findings indicate that Catalpol may be a good candidate for cytokine- and chemokine-targeted treatment as it potently inhibited IL-1β-induced expressions of COX-2, PGE_2, IL-8, and MCP-1 in mouse chondrocytes.

Finally, we investigated the involvement of the NF-κB intracellular signaling pathway in mediating the effects of Catalpol. Numerous studies have suggested inhibition of NF-κB as an anti-inflammatory treatment strategy. A recent study demonstrated that Catalpol reduced lipopolysaccharide-induced expressions of IL-6, IL-8, MMP-1, PGE_2, and COX-2 in a rheumatoid arthritis model. Importantly, our study also shows that Catalpol suppressed the activation of the NF-κB

pathway by preventing the phosphorylation of IκBα (Bi et al., 2013). A previous study showed that Catalpol had a similar effect in astrocytes by ameliorating LPS-induced expressions of iNOS, COX-2, and Toll-like receptor 4 via inhibition of NF-κB activation (Bi et al., 2013). Concordantly, our findings show that this effect of Catalpol extends to chondrocytes.

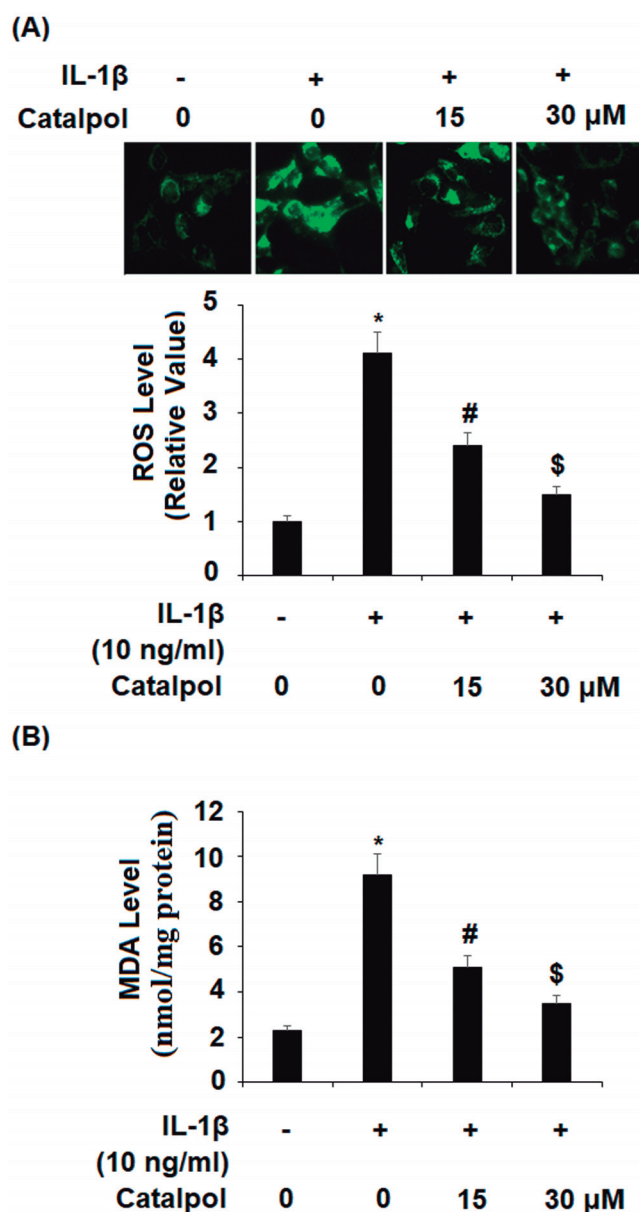The limitations of the study have to be discussed.

Fig. 8. Catalpol attenuates IL-1β-induced oxidative stress in ATDC5 chondrocytes. **A.** ROS was measured by DCFH-DA; Scale bar, 50 μm. **B.** levels of malonaldehyde (MDA) (*, P<0.01 vs. vehicle group; #, P<0.01 vs. IL-1β group; &, P<0.01 vs. IL-1β+15 μM catalpol group; ANOVA with Bonferroni's post hoc correction, N=5).

Firstly, the current study was only limited to tests *in vitro*, the effective dose of Catalpol *in vivo* remains to be defined precisely. We used relatively high doses of

Catalpol (15-30 µM), although similar concentrations were used in the function studies in endothelial and differentiated muscle (Xu et al., 2018; Hu et al., 2019).
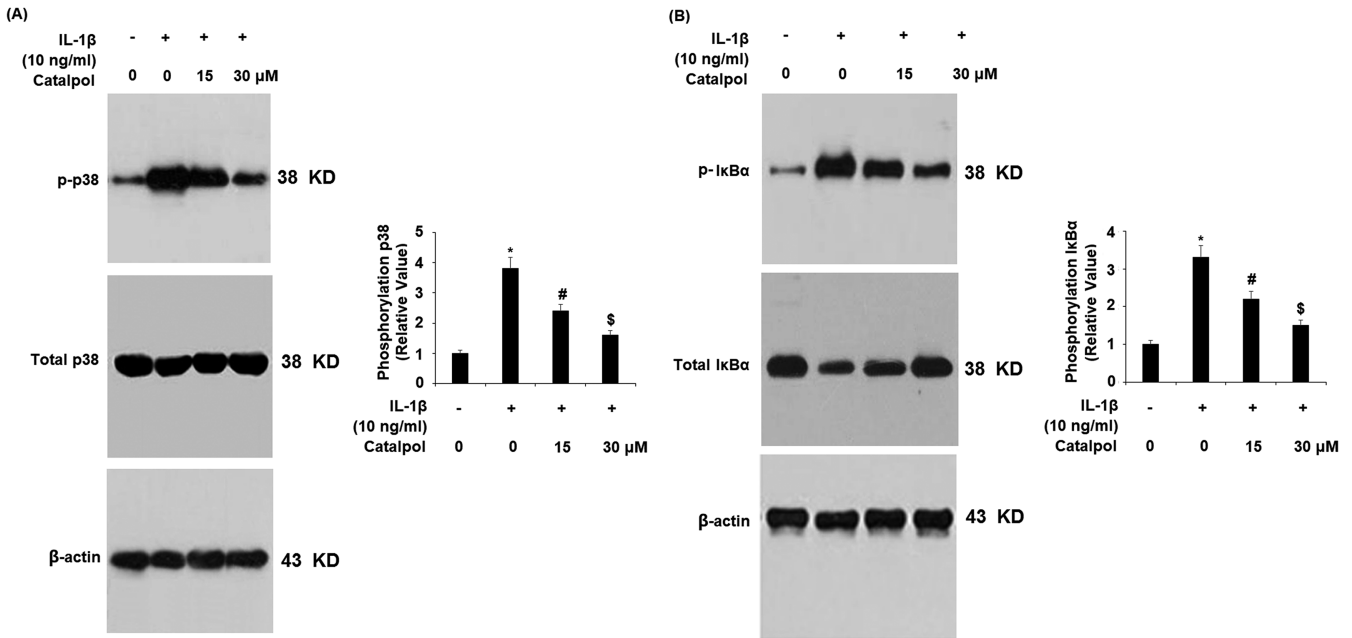

Fig. 9. Catalpol inhibits IL-1β-induced activation of p38 and IκBα. **A.** Phosphorylation and total levels of p38. **B.** Phosphorylation and total levels of IκBα (*, P<0.01 vs. vehicle group; #, P<0.01 vs. IL-1β group; &, P<0.01 vs. IL-1β+15 µM catalpol group; ANOVA with Bonferroni's post hoc correction, N=4).

Fig. 10. Catalpol inhibits IL-1β-induced activation of NF-κB. **A.** Nuclear translocation of NF-κB p65. **B.** Luciferase activity of NF-κB p65 promoter (*, P<0.01 vs. vehicle group; #, P<0.01 vs. IL-1β group; &, P<0.01 vs. IL-1β+15 µM catalpol group; ANOVA with Bonferroni's post hoc correction, N=4).
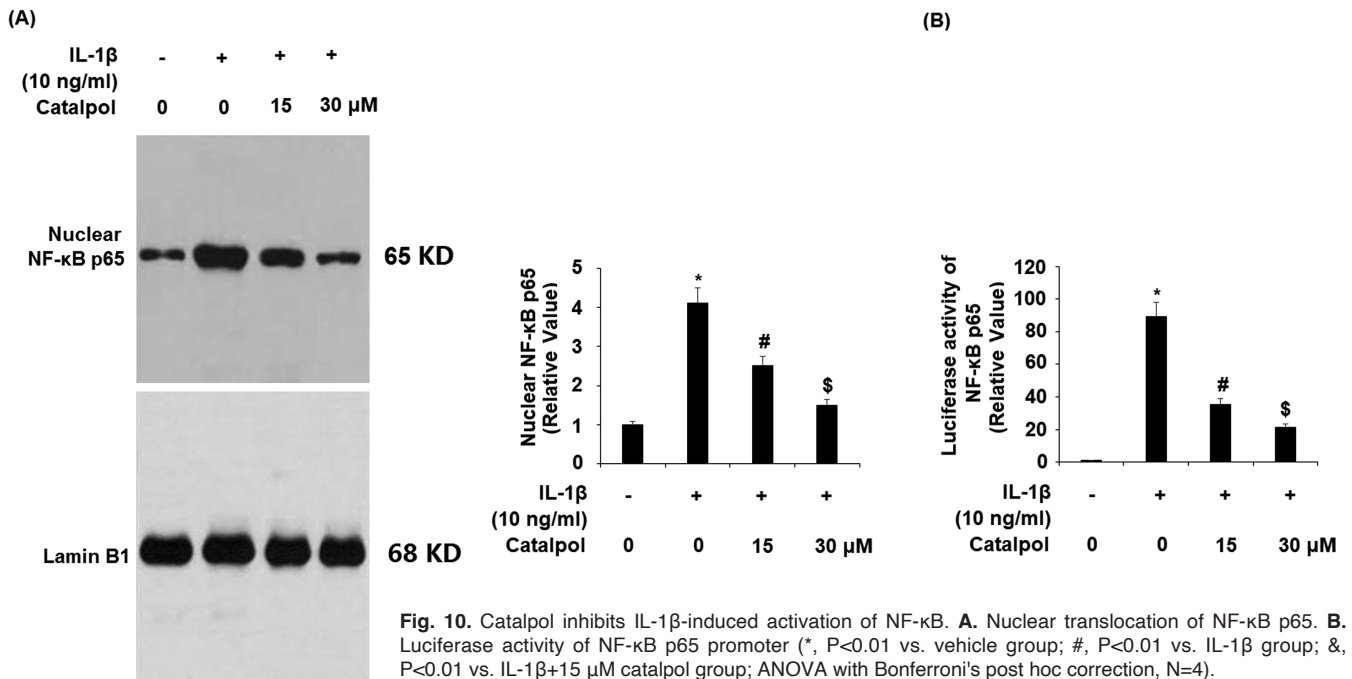
Oral administration from 10 to 200 mg/kg Catalpol for different periods was reported in animal models (Mao et al., 2007; Hu et al., 2019; Zhu et al., 2019). As the physiological level of Catalpol can be different from the concentration used in *in vitro* experiments, its concentration *in vivo* remains to be determined in future studies. Secondly, we have to be cautious about the anti-inflammatory effect of Catalpol. Its inhibitory effects on IL-1β-induced inflammation may be effective for suppression of inflammation but may increase susceptibility to infection due to immune-suppressing. The systematic investigation of Catalpol on the cytokine network in OA would be necessary to evaluate its effectiveness.

Conclusion

In conclusion, the present study demonstrates that Catalpol can suppress IL-1β-induced oxidative stress, cartilage degradation, and inflammation in mouse chondrocytes. Mechanistically, its effect on chondrocyte catabolism is mediated by the NF-κB pathway. Our findings indicate a promising role of the natural agent Catalpol on chondrocytes in OA development, further investigation is warranted to gain a more comprehensive understanding of its pharmacologic mechanisms in OA.

References

Abramson S.B. (2008). Nitric oxide in inflammation and pain associated with osteoarthritis. Arthritis Res. Ther. 10 (Suppl 2), S2.

Akhtar N., Khan N.M., Ashruf O.S. and Haqqi T.M. (2017). Inhibition of cartilage degradation and suppression of PGE2 and MMPs expression by pomegranate fruit extract in a model of posttraumatic osteoarthritis. Nutrition 33, 1-3.

Bi J., Jiang B., Liu J.H., Lei C., Zhang X.L. and An L.J. (2008). Protective effects of catalpol against H$_2$O$_2$-induced oxidative stress in astrocytes primary cultures. Neurosci. Lett. 442, 224-227.

Bi J., Jiang B., Zorn A., Zhao R.G., Liu P. and An L.J. (2013). Catalpol inhibits LPS plus IFN-γ-induced inflammatory response in astrocytes primary cultures. Toxicol. *In Vitro* 27, 543-550.

Chen T.H., Chen L., Hsieh M.S., Chang C.P., Chou D.T. and Tsai S.H. (2006). Evidence for a protective role for adiponectin in osteoarthritis. Biochim. Biophys. Acta 1762, 711-718.

Chen Z.M., Lu M., Zhao F.J., Sha X. and Ma H.S. (2017). Catalpol promotes proliferation and osteogenic differentiation of rat bone marrow stromal cells. Int. J. Clin. Exp. Med. 10, 11929-11936.

Chen D., Guo J. and Li L. (2022). Catalpol promotes mitochondrial biogenesis in chondrocytes. Arch. Physiol. Biochem. 2022 128, 802-808.

Garcia-Cirera S., Calvet J., Cristobal O., Navarro N., de Lara M.G. and Gratacos-Masmitja J. (2019). Synovial interleukin-8 levels are associalted to radiographic severity of knee osteoarthritis. A cross-sectional study. Ann. Rheum. Dis. 78 (Suppl 2), AB0803.

de Andrés M.C., Takahashi A. and Oreffo R.O. (2016). Demethylation of an NF-κB enhancer element orchestrates iNOS induction in osteoarthritis and is associated with altered chondrocyte cell cycle. Osteoarthritis Cartilage 24, 1951-1960.

Duff R.B., Bacon J.S., Mundie C.M., Farmer V.C., Russell J.D. and Forrester A.R. (1965). Catalpol and methylcatalpol: naturally occurring glycosides in Plantago and Buddleia species. Biochem. J. 96, 1-5.

Goldring M.B. and Otero M. (2011). Inflammation in osteoarthritis. Curr. Opin. Rheumatol. 23, 471-478.

Greene M.A. and Loeser R.F. (2015) Aging-related inflammation in osteoarthritis. Osteoarthritis Cartilage 23, 1966-1971.

Hu H., Wang C., Jin Y., Meng Q., Liu Q., Liu Z.H., Liu K., Liu X.Y. and Sun H.J. (2019). Catalpol inhibits homocysteine-induced oxidation and inflammation via inhibiting Nox4/NF-κB and GRP78/PERK pathways in human aorta endothelial cells. Inflammation 42, 64-80.

Huddleston J.I., Wang Y., Uquillas C., Herndon J.H. and Maloney W.J. (2012). Age and obesity are risk factors for adverse events after total hip arthroplasty. Clin. Orthop. Relat. Res. 470, 490-496.

Ji Q., Xu X., Zhang Q., Kang L., Xu Y., Zhang K., Li L., Liang Y., Hong T., Ye Q. and Wang Y. (2016). The IL-1β/AP-1/miR-30a/ADAMTS-5 axis regulates cartilage matrix degradation in human osteoarthritis. J. Mol. Med. 94, 771-785.

Koskinen A., Vuolteenaho K., Nieminen R., Moilanen T. and Moilanen E. (2011). Leptin enhances MMP-1, MMP-3 and MMP-13 production in human osteoarthritic cartilage and correlates with MMP-1 and MMP-3 in synovial fluid from OA patients. Clin. Exp. Rheumatol. 29, 57-64.

Lane N.E. (1997). Pain management in osteoarthritis: the role of COX-2 inhibitors. J. Rheumatol. Suppl. 49, 20-24.

Lepetsos P., Papavassiliou K.A. and Papavassiliou A.G. (2019). Redox and NF-κB signaling in osteoarthritis. Free Radic. Biol. Med. 132, 90-100.

Li D.Q., Duan Y.L., Bao Y.M., Liu C.P., Liu Y. and An L.J. (2004). Neuroprotection of catalpol in transient global ischemia in gerbils. Neurosci. Res. 50, 169-177.

Li Y., Bao Y., Jiang B., Wang Z., Liu Y., Zhang C. and An L. (2008). Catalpol protects primary cultured astrocytes from *in vitro* ischemia-induced damage. Int. J. Dev. Neurosci. 26, 309-317.

Lim D. and Kim Y. (2013). Dried root of *Rehmannia glutinosa* prevents bone loss in ovariectomized rats. Molecules 18, 5804-5813.

Liu C., Wu F., Liu Y. and Meng C. (2015). Catalpol suppresses proliferation and facilitates apoptosis of MCF-7 breast cancer cells through upregulating microRNA-146a and downregulating matrix metalloproteinase-16 expression. Mol. Med. Rep. 12, 7609-7614.

Loeser R.F., Collins J.A. and Diekman B.O. (2016). Ageing and the pathogenesis of osteoarthritis. Nat. Rev. Rheumatol. 12, 412-420.

Ma Z., Piao T., Wang Y. and Liu J. (2015). Astragalin inhibits IL-1β-induced inflammatory mediators production in human osteoarthritis chondrocyte by inhibiting NF-κB and MAPK activation. Int. Immunopharmacol. 25, 83-87.

Mao Y.R., Jiang L., Duan Y.L., An L.J. and Jiang B. (2007). Efficacy of catalpol as protectant against oxidative stress and mitochondrial dysfunction on rotenone-induced toxicity in mice brain. Environ. Toxicol. Pharmacol. 23, 314-318.

Makki M.S. and Haqqi T.M. (2016). Histone deacetylase inhibitor

vorinostat (SAHA) suppresses IL-1β-induced matrix metallo-peptidase-13 expression by inhibiting il-6 in osteoarthritis chondrocyte. Am. J. Pathol. 186, 2701-2708.

Oh K.O., Kim S.W., Kim J.Y., Ko S.Y., Kim H.M., Baek J.H., Ryoo H.M. and Kim J.K. (2003). Effect of *Rehmannia glutinosa* libosch extracts on bone metabolism. Clin. Chim. Acta 334, 185-195.

Park K.S. (2016). Catalpol reduces the production of inflammatory mediators via PPAR-γ activation in human intestinal Caco-2 cells. J. Nat. Med. 70, 620-626.

Roman-Blas J.A. and Jimenez S.A. (2006). NF-κB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. Osteoarthritis Cartilage 14, 839-848.

Scanzello C.R. (2017). Chemokines and inflammation in osteoarthritis: insights from patients and animal models. J. Orthop. Res. 35, 735-739.

Song R.H., Tortorella D.M., Malfait A.M., Alston J.T., Yang Z., Arner E.C. and Griggs D.W. (2007). Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. Arthritis Rheum. 56, 575-585.

Sieghart D., Liszt M., Wanivenhaus A., Bröll H., Kiener H., Klösch B. and Steiner G. (2015). Hydrogen sulphide decreases IL-1β induced activation of fibroblast-like synoviocytes from patients with osteoarthritis. J. Cell. Mol. Med. 19, 187-197.

Takahashi A., de Andrés M.C., Hashimoto K., Itoi E. and Oreffo R.O. (2015). Epigenetic regulation of interleukin-8, an inflammatory chemokine, in osteoarthritis. Osteoarthritis Cartilage 23, 1946-1954.

Tian Y.Y., Jiang B., An L.J. and Bao Y.M. (2007). Neuroprotective effect of catalpol against MPP+-induced oxidative stress in mesencephalic neurons. Eur. J. Pharmacol. 568, 142-148.

Tian Y.Y., An L.J., Jiang L., Duan Y.L., Chen J. and Jiang B. (2006). Catalpol protects dopaminergic neurons from LPS-induced neurotoxicity in mesencephalic neuron-glia cultures. Life Sci. 80, 193-199.

Wang S., Zha X.W., Ruan S.T., Yao S.L. and Zhang X.Y. (2022). Kruppel like factor 10 up-regulates PDZ and LIM domain containing protein 2 via nuclear factor kappa-B pathway to inhibit proliferation and inflammatory of fibroblastoid synovial cell in rheumatoid arthritis. Bioengineered 13, 1779-1790.

Wei Y., Jia J., Jin X., Tong W. and Tian H. (2018). Resveratrol ameliorates inflammatory damage and protects against osteoarthritis in a rat model of osteoarthritis. Mol. Med. Rep. 17, 1493-1498.

Wu M.S., Zhao S.Z., Li E. and Bai X. (2010). Effects of catalpol from Radix rehmanniae on proliferation, differentiation and matrix mineralization of MC3T3-E1 cells. Chin. Pharmacol. Bull. 26, 509-513.

Wu Y.H., Zhao M.L., Lin Z.H. (2021). Pyrroloquinoline quinone (PQQ) alleviated sepsis-induced acute liver injury, inflammation, oxidative stress and cell apoptosis by downregulating CUL3 expression. Bioengineered 12, 2459-2468.

Xu D., Wang L., Jiang Z., Zhao G., Hassan H.M., Sun L., Fan S., Zhou Z., Zhang L. and Wang T. (2018). A new hypoglycemic mechanism of catalpol revealed by enhancing MyoD/MyoG-mediated myogenesis. Life Sci. 209, 313-323.

Yan K., Bin W. and Jian-Hao L. (2016). Monocyte chemoattractant protein 1 induced chondrocytes degeneration and cartilage degradation in osteoarthritis. Osteoarthritis Cartilage 24, S140-S141.

Yudoh K., van Trieu N., Nakamura H., Hongo-Masuko K., Kato T. and Nishioka K. (2005). Potential involvement of oxidative stress in cartilage senescence and development of osteoarthritis: oxidative stress induces chondrocyte telomere instability and downregulation of chondrocyte function. Arthritis Res. Ther. 7, R380.

Zeng Y.F., Wang R., Bian Y., Chen W.S. and Peng L. (2019). Catalpol attenuates IL-1β induced matrix catabolism, apoptosis and inflammation in rat chondrocytes and inhibits cartilage degeneration. Med. Sci. Monit. 25, 6649-6659.

Zhang R.X., Li M.X. and Jia Z.P. (2008). *Rehmannia glutinosa*: review of botany, chemistry and pharmacology. J. Ethnopharmacol. 117, 199-214.

Zhao L., Du W., Zhao D., Ji X., Huang Y., Pang Y., Guo K. and Yin X. (2021). Catalpol protects against high glucose-induced bone loss by regulating osteoblast function. Front. Pharmacol. 12, 626621.

Zheng W., Feng Z., You S., Zhang H., Tao Z., Wang Q., Chen H. and Wu Y. (2017). Fisetin inhibits IL-1β-induced inflammatory response in human osteoarthritis chondrocytes through activating SIRT1 and attenuates the progression of osteoarthritis in mice. Int. Immunopharmacol. 45, 135-147.

Zhu P., Wu Y., Yang A., Fu X., Mao M. and Liu Z. (2017). Catalpol suppressed proliferation, growth and invasion of CT26 colon cancer by inhibiting inflammation and tumor angiogenesis. Biomed. Pharmacother. 95, 68-76.

Zhu Y., Wang Y., Jia Y., Xu J. and Chai Y. (2019). Catalpol promotes the osteogenic differentiation of bone marrow mesenchymal stem cells via the Wnt/β-catenin pathway. Stem Cell Res. Ther. 10, 37.