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Master's Thesis

Effects of 4,5-Dicaffeoylquinic acid on osteoarthritis *in vitro* and *in vivo*

Graduate School of Chosun University

Department of Biodental Engineering

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퇴행성 관절염에 미치는 효능 연구

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ABSTRACT

*In vitro*와 *In vivo*에서 4,5-Dicaffeoylquinic acid가 퇴행성 관절염에 미치는 효능 연구

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퇴행성 관절염은(Osteoarthritis) 관절의 오랜 사용에 따른 연골 마모에 의해 염증이 유발 되어 생기는 질환으로, 55세 이상 사람의 80% 이상에서 나타난다. 이 퇴행성 관절염은 subchondrol bone remodeling, osteophyte 등의 특징을 보인다. 퇴행성 관절염의 치료법으로는 비약물성 치료법과 약물성 치료법이 있으며, 비약물성 치료법으로는 연골 대체 수술이 있으나 최후의 수단이며 사회/경제적 부담이 따른다. 약물성 치료법에는 acetaminophen과 NSAIDs이 처방되고 있으나, 이러한 약물의 장기간 복용은 위장 및 신장 장애와 같은 부작용으로 인해 사용에 제한을 두고 있다. 이 때문에 약리 효과가 뛰어난 전통약용작물에서 그 해답을 찾고 있다. 그래서, 여러 질병 완화에 효능이 있다고 알려져 있으며, 식물에 다량 함유 되어있는 polyphenols이나 flavonoids과 같은 phytochemical 성분인 dicaffeoylquinic acid(diCQA)이 퇴행성 관절염에 미치는 효능을 알아보았다. DiCQA는 2개의 caffeic acid 분자와 1개의 quinic acid 분자가 ester 결합으로 이루어져 있으며, 이는 커피콩, 과일, 식물 잎 등에 많이 포함되어 있다. 그리고, 항산화, 항염증, 손상된 각질 세포 보호, 항간독성, 신경 보호 등에 효과가 있다고 알려져 있다. 하지만, 많은 효과가 있음에도 diCQA의 퇴행성 관절염 효능에 대한 연구가 미비하다.

본 연구에서는, diCQA 중 항산화 효과가 가장 좋다고 알려진 4,5-diCQA의 퇴행성 관절염 효능을 연구하였다. IL-1β에 의해 염증이 유도된 랫트의 연골 세포에서 4,5-diCQA를 전처리하여 퇴행성 관절염 완화 효과가 있는지 확인하였다. 염증 cytokine인 nitric oxide와 PGE_2와 연골 주 구성 성분인 aggrecan의 발현 정도를

ELISA를 통해 보았으며, iNOS, COX-2, TNF-α, NF-κB, IκB-α, aggrecan, 그리고 연골을 분해하는 효소들(MMPs와 ADAMTS-4)의 발현 정도를 보기 위해 western blot을 수행하였다. 이 결과에서, 4,5-diCQA가 염증 cytokine 및 연골 분해 효소들의 발현을 억제하는 효과가 있음을 확인함으로써, 퇴행성 관절염 완화에 효과가 있는 것을 확인하였다. *In vivo*에서도 DMM 수술에 의해 퇴행성 관절염이 유도된 랫트에 4,5-diCQA를 격일로 총 2주 동안 경구 투여한 결과, 연골 보호 효과가 있음을 Safranin O/Fast green stain을 통해 확인하였다. 이로써, 4,5-diCQA가 염증 및 퇴행성 관절염에 효과가 있음을 증명하였으며, 퇴행성 관절염 치료제 개발을 위한 후보 물질이 될 수 있음을 시사한다.

I. Introduction

Osteoarthritis (OA) is a disease induced by cartilage wear associated with long-term use of joints, and is present in more than 80% of people over the age of 55 [1]. The OA is characterized by subchondral bone remodeling and osteophyte as cartilage is damaged by cartilage abrasion and inflammation, and ultimately interferes with well-being life by accompanying pain in movement [2]. In a molecular biological point, OA is known to be induced by promoting extracellular matrix (ECM) degradation of chondrocytes by cartilage-destroying factors such as oxidative stress and overexpression of inflammatory mediators [3]. Among them, interleukin 1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are main factors accelerating degenerative arthritis by inducing the expression of other cartilage ECM degrading factors (iNOS, PGE_2, MMPs, and ADAMTS-4) [4].

In particular, inflammation-induced MMPs and ADAMTS-4 degrade proteoglycan (such as, aggrecan), which are the main components of cartilage ECM, and interfere with the normal function of cartilage [5]. In addition, these degradation products influence the inflammatory environment in synovial membranes as inflammation inducer and accelerate the expression of cartilage ECM degrading factors [4]. Therefore, suppression of their expression is considered to be an important marker for the alleviation of OA, and many studies on the treatment of OA insist the improvement effect of OA through suppression of their expression.

Currently, as treatment for OA, there are non-pharmacological methods and pharmacological treatments. As non-pharmacological treatments, include cartilage replacement surgery, but is the last resort, and social/economic burden follows. Although acetaminophen and NSAIDs are prescribed for pharmacological treatment [9], these drugs are limited in long-term use as a result of side effects such as gastrointestinal and renal disorders [6,7,8]. In addition, there are functional foods such as glucosamine and methylsulfonylmethane for preventing OA [10], but the functional food's OA improvement effect has been raised as problems.

In order to solve this problem, traditional medicinal plants were studied with

significantly lower side effects and superior pharmacological effects compared to synthetic preparations [11,12]. This traditional medicinal crops have been used for a long time to treat various diseases due to their excellent pharmacological effects [12], as well as their safety and pharmacological effects. Previously in our study, we demonstrated the stability and alleviation effects of OA with the *Anthriscus sylvestri'*s leaves, one of the traditional medicinal plants, and presented it as a strong candidate for the development of functional foods [13,14]. However, the seven active ingredients ((Luteolin-7-O-glucoside (Cynaroside), Chlorogenic acid (3-CQA), Crypto-chlorogenic acid (4-CQA), 3,4-Di-caffeoylquinic acid (3,4-diCQA), 3,5-Di-caffeoylquinic acid (3,5-diCQA), 4,5-Di-caffeoylquinic acid (4,5-diCQA), Luteolin-7-O-(6"-malonylglucoside)) contained in the water extract from *Anthriscus sylvestri'*s leaves has not been sufficiently examined for alleviation of OA.

The phytochemicals, contained in the plant-derived drugs such as polyphenols and flavonoids, are known to be effective in alleviating various diseases. Among them, diCQA is connected by an ester bond between two caffeic acid molecules and one quinic acid molecule [15], and is abundant in coffee beans, fruits, and vegetables such as *Gynura divaricata* leaves [16]. The diCQA have numerous isomers depending on the position of the double bond, and such other double bond sites represent different cell absorption rates and efficacy. The effects of diCQAs has anti-inflammatory, oxidative-damaged human keratinocyte protection, anti-hepatotoxicity, and neuroprotection [17]. It was analyzed that 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA (diCQAs) were contained in the hydrothermal extract of *Anthriscus sylvestri'*s leaves, and among them, 4,5-diCQA was more effective than chlorogenic acid, an excellent antioxidants. However, despite this high antioxidant effect, studies have not been reported on chondroprotective effects of the diCQA for the alleviation of OA. Therefore, in this study, we investigate whether 4,5-diCQA has a chondroprotective effect on rat primary chondrocytes and DMM-induced rat OA model for the mitigation of OA.

II. Materials and Methods

2.1. Reagents

Lipopolysaccharide (LPS), carrageenan, 4,5-Di-O-caffeoylquinic acid, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-1β was purchased from ProSpec protein specialists (Rehovot, Israel). Aggrecan ELISA kit were purchased from MyBioSource (San Diego, CA, USA). The prostaglandin E_2 (PGE_2) ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA). Collagenase Type 2 was purchased from Worthington Biochemical Corporation (Lakewood, NJ, U.S.A.). Dulbecco's modified Eagle's medium/Nutrient mixture F-12 (DMEM/F12) and penicillin-streptomycin solution were purchased from WelGene (Daegu, Republic of Korea). Fetal bovine serum (FBS) was purchased from ATLAS Biologicals (Fort Collins, CO, USA). Primary and secondary antibodies were purchased from, MMP-13, ADAMTS-4, and iNOS (Abcam (Cambridge, MA, USA)), anti-alpha-tubulin (Thermo Fisher Scientific, Waltham, MA, USA), MMP-1 (Lifespan Biosciences, Seattle, WA), MMP-3, COX-2, and TNF-α (Cell Signaling Technology, Danvers, MA, USA), p-IκB-α, IκB-α, and NF-κB (Invitrogen, Carlsbad, Germany), and PCNA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

2.2. Cell Culture

Murine macrophage RAW 264.7 cells were obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Cells were cultured 2×10^6 cells/mL into 6-well cell culture plates with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37 ℃. RAW 264.7 cells were cultured up to 90% of cell confluency. For rat primary chondrocytes isolation, Sprague-Dawley rats of 5-day-old were dissected articular cartilage by digested using 0.3% (w/v) collagenase type Ⅱ dissolved in DMEM/F12 at 37 ℃, overnight. All of these animal management and procedures have been approved by the Chosun University Institutional Animal Care

and Use Committee (CIACUC2021-S0013). Cells and debris were filtered through a cell strainer (0.45 μm). Chondrocytes were seeded 2 × 10^6 cells/mL into 6-well cell culture plates with DMEM/F12 with 10% FBS and 1% penicillin/streptomycin in the same environment. Chondrocytes were cultured up to 90% of cell confluency and were not passaged during the experiment.

2.3. Cell viability

The viability analysis of the 4,5-diCQA on RAW 264.7 cells and chondrocytes was determined through MTT assay following the manufacturer's protocol. RAW 264.7 cells were treated by various concentrations of those 4,5-diCQA (2.5, 5, 10, 20, and 40 μM) for 24 h. Chondrocytes were treated by 4,5-diCQA (10, 20, 40, 100 and 200 μM) for 24 h. Post incubation, each well (100 μL/well) was added MTT solution (5 mg/mL), and cells were incubated for another 2 h at 37 ℃. First of all, remove the cell culture medium including the MTT solution, then each well was added DMSO (1 mL/well) and was measured on the absorbance at 565nm.

Cell viability (%) = (Absorbance of Treat group / Average absorbance of control group) × 100

2.4. Measurement of Nitric oxide and PGE_2 prodution

Raw 264.7 cell were pretreated with varying concentration of the 4,5-diCQA (1, 2 and 4 μM) for 1h, and then stimulated LPS (50 ng/mL) for 24 h. Chondrocytes were pretreated with the 4,5-diCQA (10, 20 and 40 μM) for 1h, and then stimulated IL-1β (5 ng/mL) for 24 h. Nitric oxide production was determined by measuring the accumulation of nitrite in cultured medium. In brief, the production was evaluated that cultured medium (100 μL) was mixed with 100 μL the Griess reagent [1% sulfanilamide in 5% phosphoric acid, 0.1% α-naphtylamide in H_2O], and then measured at 540 nm using a microplate reader (Epoch BioTek Instruments Inc., Winooski, VT, USA). The production of PGE_2 was measured with a Parameter™ prostaglandin E_2 assay kit, according to manufacturer's protocol.

2.5. Western Blot Analysis

Raw 264.7 cell were pretreated with varying concentration of the 4,5-diCQA (1, 2 and 4 μM) for 1h, and then stimulated LPS (50 ng/mL) for 30 min or 24 h. Chondrocytes were pretreated with the 4,5-diCQA (10, 20 and 40 μM) for 1h, and then stimulated IL-1β (5 ng/mL) for 30 min or 24 h. Once cells were washed by using 1X phosphate buffered saline (PBS), and then lysed with a PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam-si, Republic of Korea) to isolate whole protein for 30 min in ice. To isolate cytoplasmic and nucleic proteins were used NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA) with modifying the manufacturer's instructions. Protein concentrations were quantified through a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Equivalent amounts of lysate Protein (10 μg or 20 μg) were separated on 6%, 8%, 10% or 12% sodium dodecyl sulphate polyacrylamide (SDS) gel and then transferred to a polyvinylidene difluoride membrane (Bio-Rad laboratories, Hercules, CA, USA). The transblotted membranes were blocked with 5% bovine serum albumin in tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 1 h, then incubate the membranes with primary antibodies (1:1000) at 4 ℃, overnight. The membranes were washed with TBST thrice and were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 10000) at room temperature for 1 h. The immunoreactive bands were detected using an enhanced chemiluminescence (ECL) kit (Millipore, Bedford, MA, USA) and visualized using a MicroChemi 4.2 imager (DNR Bioimaging Systems, Jerusalem, Israel).

2.6. Aggrecan ELISA analysis

Chondrocytes were pretreated with the 4,5-diCQA (10, 20 and 40 μM) for 1 h, and then stimulated with IL-1β (5 ng/mL) for 24 h. Then cultured medium was collected to access aggrecan content, according to aggrecan ELISA kits. All assays were performed in duplicate.

2.7. Gelatin Zymography

Chondrocytes were pretreated with the 4,5-diCQA (10, 20 and 40 μM) for 1 h and cultured with IL-1β (5 ng/ml) for 24 h. Supernatants from cultured media (30 μL)

were mixed with non-reducing sample buffer (250 mM Tris-HCl (pH 6.8), 40% (v/v) glycerol, 8% (w/v) SDS, 0.01% (w/v) bromophenol blue) and resolved using 8% SDS-PAGE containing gelatin. After electrophoresis, gels were exerted to rinse with 2.5% (v/v) Triton X-100 by gently shaking for 30 min, followed by washed with distilled water (DW) for 15 min, three times. The gels were incubated with zymogram renaturing buffer (10 mM $CaCl_2$, 50 mM Tris-HCl (pH7.6), 0.15 M NaCl) at 37 °C for 72 h. After renaturation of the cartilage degrading enzymes, gels were stained with 0.1% (w/v) coomassie brilliant blue R-250 for 30 min at room temperature, then destaining until clear bands were visible, and imaging the gel by photography with a digital camera (G16, Canon, Tokyo, Japan). Clearance white zone in band indicated gelatin degradation.

2.8. Animals

Male specific pathogen-free Sprague–Dawley rats, weighing 200–220 g, were purchased from Damool Science (Daejeon, Republic of Korea). The animals were housed in a controlled environment (temperature: 21 ± 1° C; humidity: 55 ± 5%; 12-h light/dark cycle) and were allowed free access to commercial pellets and water. All animal-handling procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [18]. Carrageenan-induced rat paw edema experiment was approved by the Chosun University Institutional Animal Care and Use Committee (CIACUC2021-S0016). In addition, Sprague-Dawley rats (8 weeks old) for destabilization of the medical meniscus (DMM) surgery have also been housed in the same environment. Prior to the experiment, the rats were adapted to the environment for 1 week for acclimatization. DMM surgery was approved by Chosun University Institutional Animal Care and Use Committee (CIACUC2021-S0015).

2.9. Carrageenan-induced inflammation model in rats

The inhibitory effect of 4,5-diCQA on carrageenan-induced rat paw edema was evaluated with minor modifications as described by Akinnawo *et al*. [19]. The rats (n = 24) were randomly divided into six groups: group 1 (non-inflamed, 0.9% normal saline, as the negative control), group 2 (paw edema, 0.9% normal saline), group 3–5 (paw

edema, 5, 10, and 20 mg/kg 4,5-diCQA, respectively), and group 6 (paw edema, 10 mg/kg diclofenac sodium, as the positive control). Edema was induced by the injection of 1% (w/v) carrageenan suspension (0.1 mL) in 0.9% normal saline into the sub-plantar surface of both hind limbs. The treated groups were orally administered 4,5-diCQA and diclofenac sodium 1 h prior to injecting carrageenan. The thickness of the middle of the paw was measured using a digital caliper at specific time intervals, from 0 (before carrageenan injection) to 24 h after injection. The inhibition rate of paw edema was calculated using the following

$$\text{Inhibition rate}(\%) = (Ct - C0)_{\text{control}} - (Ct - C0)_{\text{treated}} / (Ct - C0)_{\text{control}} \times 100$$

where Ct = paw thickness (mm) of the left hind limb at time t, C0 = paw thickness (mm) of the left hind limb before carrageenan injection, $(Ct - C0)_{control}$ = increase in rat paw size after injecting carrageenan at time t, and $(Ct - C0)_{treated}$ = increase in paw size after injecting carrageenan into control or treated rats at time t.

2.10. DMM-induced OA model in rats

The rats were divided into 7 groups of 4 rats each: Group 1 (normal), Group 2 (Sham, 0.9% saline), Group 3 (DMM, 0.9% saline), and Group 4–7 (5, 10, 20 mg/kg), 4,5-diCQA, and 10 mg/kg, diclofenac) each drug was administered as it was. DMM surgery was performed by incising the medial meniscotibial ligament (MMTL) to induce OA above the right and left knees. For this operation, rats were anesthetized with 2.5% isoflurane and then the MMTL was incised. In the Sham group, only the skin was incised, and the MMTL was not actually an incision. In order to induce sufficient OA, two weeks after DMM surgery, 4,5-diCQA groups were orally administered with concentration to the 4,5-diCQA (5, 10, 20 mg/kg) and diclofenac group were orally administeren with the diclofenac (10 mg/kg), respectively. Oral administration of these drugs was administered once every 2 days for 2 weeks. The sham group and the DMM-only group were orally administered with saline for the same period. After that, all rats were sacrificed the day.

2.11. Histology Analysis and Stain

Extracted articular cartilages were fixed in 10% neutral buffered formalin for 1 day at 4 ℃, and then decalcified with 0.5M EDTA (pH 7.4) for 7 days at 4 ℃. Following the steps, the articular cartilages were dehydrated through a series of ethanol solutions, and embedded in paraffin blocks. After that, lateral serial sections were sectioned at 4 μm thickness intervals, and stained with Safranin O/Fast Green. A EVOS Core microscope (Thermo Fisher Scientific, Waltham, MA) was used to digitally photograph the stained sections. The stained sections were scored established on the Osteoarthritis Research Society International (OARSI) advanced Osteoarthritis Cartilage Histopathology Assessment System (0-6.5) and used a summed OARSI score to analysis the degree of articular cartilage destruction.

2.12. Statistical analysis

All data were obtained from at least independent experiments. The results were expressed as the means ± standard deviation (SD). A one-way analysis of variance (ANOVA) from Dunnett's test, was employed for multiple comparisons using the GraphPad Prism (GraphPad Software Inc., CA, USA). Statistical significance of first subject about anti-inflammatory results was represented at ### $p < 0.01$ compared with control group; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with LPS-treated group. Plus, Statistical significance of second subject about anti-osteoarthritis results was set at ###$p < 0.005$ compared with control group; *$p < 0.5$, **$p < 0.05$, ***$p < 0.005$ compared with IL-1β-treated group.

Ⅲ. Results

1. Anti-inflammatory effects

3.1.1. Effect of 4,5-diCQA on RAW 264.7 cell viability.

Prior to assessing the anti-inflammatory effects of 4,5-diCQA, cytotoxicity was evaluated using the MTT assay. RAW 264.7 cells were treated with various concentrations of 4,5-diCQA (0, 2.5, 5, 10, 20, and 40 μM) for 24 h. Nitric oxide cytotoxicity was observed at concentrations up to 40 μM (Figure 1B).

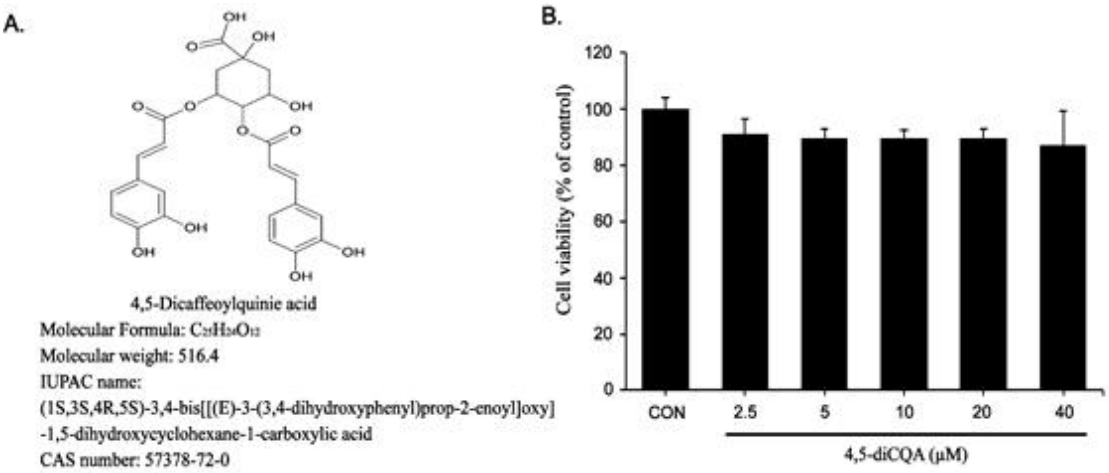

Figure 1. Effects of 4,5-diCQA on RAW 264.7 cells viability. (A) Chemical formula of 4,5-diCQA. (B) Cells were treated with 4,5-diCQA (2.5, 5, 10, 20, and 40 μM) for 24 h, and viability was determined by MTT assay. Cells incubated without 4,5-diCQA were used as controls and considered as 100% viable. Data are represented as the mean ± SD of three independent experiments. CON; control.

3.1.2. Inhibitory effects of 4,5-diCQA on nitrite production and iNOS protein expression in RAW 264.7 cells stimulated with LPS.

To verify the anti-inflammatory effects of 4,5-diCQA, nitrite production and iNOS expression were evaluated using Griess reagent and western blotting, respectively. As shown in Fig. 2A and C, LPS significantly induced nitrite production (56-fold, 56.6 ± 1.5 μM) compared to in the control (1.3 ± 0.3 μM). However, pretreatment with 4,5-diCQA reduced nitrite production in a dose-dependent manner (Fig. 2A). Consistent with the nitrite production results, LPS significantly increased iNOS protein expression, but the effect decreased gradually in response to 4,5-diCQA pretreatment in a concentration-dependent manner (Fig. 2C).

3.1.3. Inhibitory effects of 4,5-diCQA on PGE_2 production and COX-2 protein expression in RAW 264.7 cells stimulated with LPS.

As shown in Fig. 2B, LPS markedly increased PGE_2 production (22-fold, 2,237.9 ± 90.71 μM) compared to in the control (99.9 ± 393 μM); however, pretreatment with 4,5-diCQA significantly suppressed LPS-induced PGE_2 production in a dose-dependent manner, with an inhibition rate of 55% at 4 μM. Additionally, LPS significantly increased COX-2 protein expression, but this effect decreased gradually in response to 4,5-diCQA pretreatment in a concentration-dependent manner (Fig. 2C). These results suggest that 4,5-diCQA potently inhibits PGE_2 production and COX-2 protein expression.

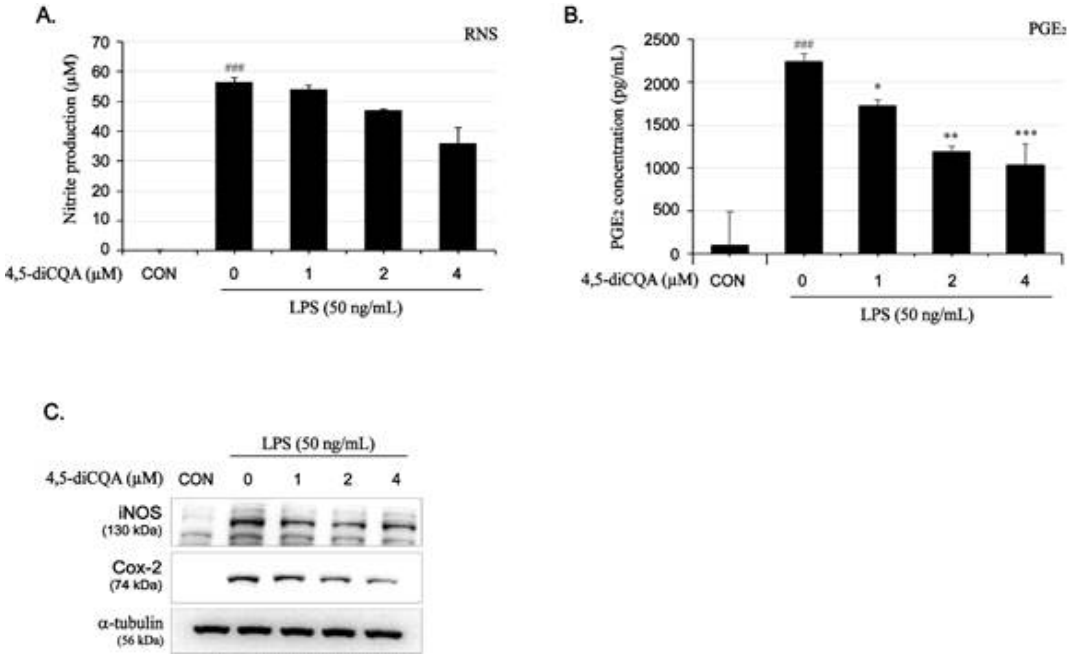

Figure 2. Inhibitory effects of 4,5-diCQA on LPS-induced nitrite, PGE_2, iNOS, and COX-2 in RAW 264.7 cells. Cells were pretreated with 4,5-diCQA (1, 2, and 4 μM) for 1 h, followed by LPS (50 ng/mL) stimulation for 24 h. (A) Nitrite production was determined in the culture medium using Griess reagent. (B) PGE_2 production was determined in the culture medium using ELISA. (C) Expression of iNOS and COX-2 was determined using western blot analysis. α-Tubulin served as an internal control. Expression results are represented as the mean ± SD of three independent experiments. ### $p < 0.01$ compared with control group; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with LPS-treated group. CON; control, RNS; reactive nitrogen species, PGE_2; prostaglandin E_2, iNOS; inducible nitrite oxide, COX-2; cyclooxygenase-2.

3.1.4. Inhibitory effects of 4,5-diCQA on pro-inflammatory cytokines in RAW 264.7 cells stimulated with LPS.

The effect of 4,5-diCQA investigated on LPS-induced expression of pro-inflammatory cytokines using a mouse cytokine array and western blotting. As shown in Fig. 3A, LPS significantly induced the expression of various immune- and inflammation-related factors. Among them, the expression of IL-6 and TNF-α, which play important roles in the inflammatory response, were significantly decreased by 4,5-diCQA; the inhibition rates were 20% and 40% at 4 μM, respectively (Fig. 3A). Furthermore, western blotting showed that LPS-induced protein expression of IL-6 and TNF-α was decreased by 4,5-diCQA in a dose-dependent manner (Fig. 3B). These results suggest that 4,5-diCQA suppressed LPS-induced IL-6 and TNF-α protein expression, supporting the hypothesis that 4,5-diCQA has anti-inflammatory activity.

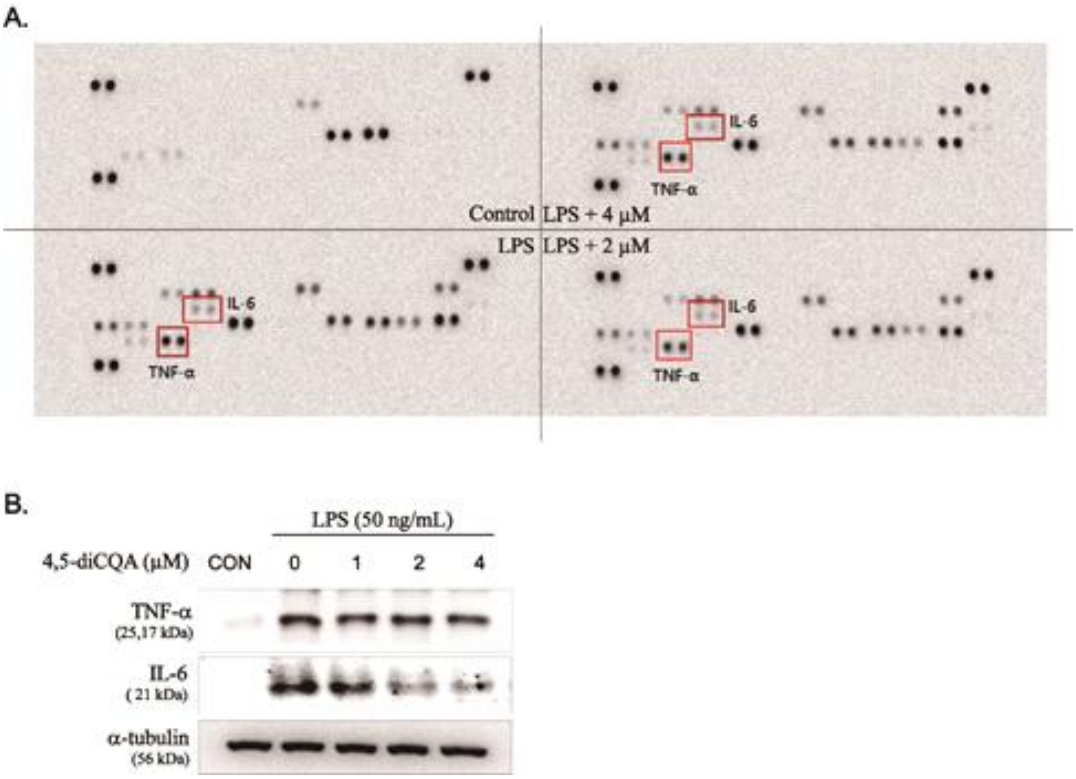

Figure 3. Inhibitory effect of 4,5-diCQA on LPS-induced TNF-α and IL-6 in RAW 264.7 cells-stimulated with LPS. Cells were pretreated with 4,5-diCQA (1, 2, and 4 μM) for 1 h, followed by LPS (50 ng/mL) stimulation for 24 h. (A) TNF-α and IL-6 were determined in the cultured medium using mouse cytokines array. (B) Expression of TNF-α and IL-6 was determined using western blot analysis. α-Tubulin served as an internal control. CON; control, TNF-α; tumor necrosis factor-alpha, IL-6; interleukin-6.

3.1.5. Suppression of NF-κB and MAPK signaling pathways by 4,5-diCQA in RAW 264.7 cells stimulated with LPS.

The critical role of the NF-κB and MAPK signaling pathways in regulating the expression of inflammatory mediators is well-known; thus, inhibition of these pathways is generally regarded as an important mechanism in the inflammatory response. The anti-inflammatory effect of 4,5-diCQA on inflammatory mediators and cytokines was affected by kinases in these pathways. As shown in Fig. 4A, LPS stimulation induced the phosphorylation and degradation of IκB-α in the cytoplasm, thereby enhancing translocation of NF-κB p65 from the cytoplasm to the nucleus. However, pretreatment with 4,5-diCQA inhibited LPS-induced nuclear translocation of NF-κB p65 by suppressing the phosphorylation and degradation of IκB-α. In addition, LPS alone induced phosphorylation of ERK, JNK, and p38 MAPK without affecting their total protein levels, whereas pretreatment with 4,5-diCQA significantly suppressed this effect in a dose-dependent manner (Fig. 4B). These results suggest that the NF-κB and MAPK pathways mediate the anti-inflammatory effect of 4,5-diCQA in RAW 264.7 cells stimulated by LPS.

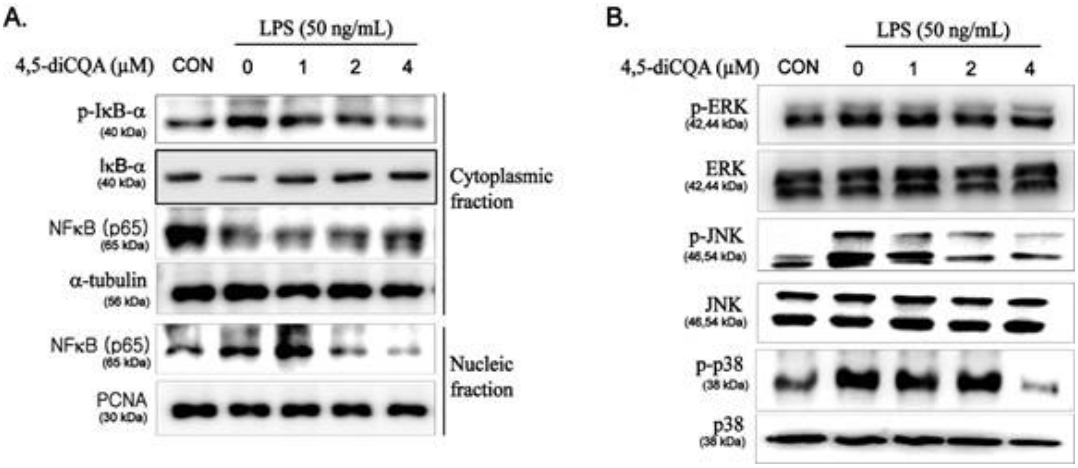

Figure 4. Effects of 4,5-diCQA on LPS-induced activation of NF-κB and phosphorylation of MAPKs in RAW 264.7 cells-stimulated with LPS. Cells were pretreated with 4,5-diCQA (1, 2, and 4 μM) for 1 h, followed by LPS (50 ng/mL) stimulation for 1 h. (A) Phosphorylation levels of IκB-α and NF-κB p65 translocation to nucleus were determined using western blot analysis. (B) Protein levels of phosphorylation of MAPKs (ERK, JNK, and p38) were determined using western blot analysis. α-Tubulin and PCNA were used as cytosolic and nuclear internal controls, respectively. CON; control, IκB-α; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, p65; NF-kappa-B p65 subunit, ERK; extracellular signal-regulated kinase, JNK; c-Jun N-terminal kinase.

3.1.6. Anti-inflammatory effects of 4,5-diCQA *in vivo*.

To verify the anti-inflammatory effect of 4,5-diCQA *in vivo*, suppression of carrageenan-induced paw edema was assessed. As shown in Fig. 5A, the inhibition rate of paw edema thickness by 4,5-diCQA (5, 10, and 20 mg/kg) and diclofenac sodium (positive control, 10 mg/kg) gradually increased over 5 h compared with that in the untreated control group. Furthermore, at 5 h, the effect of 4,5-diCQA (20 mg/kg) was comparable to that of diclofenac sodium (10 mg/kg) (Fig. 5A). As carrageenan causes acute inflammation, the spinal cord was isolated and performed by western blotting to analyze the expression levels of inflammation-related factors. These findings are consistent with the above paw edema results. Carrageenan injection significantly increased the protein expression of iNOS, COX-2, and TNF-α, and 4,5-diCQA markedly decreased carrageenan-induced protein expression. In addition, the inhibitory effect of 4,5-diCQA (20 mg/kg) on the protein expression of iNOS, COX-2, and TNF-α was comparable to that of diclofenac sodium (10 mg/kg). These results suggest that 4,5-diCQA has a potent anti-inflammatory effect.

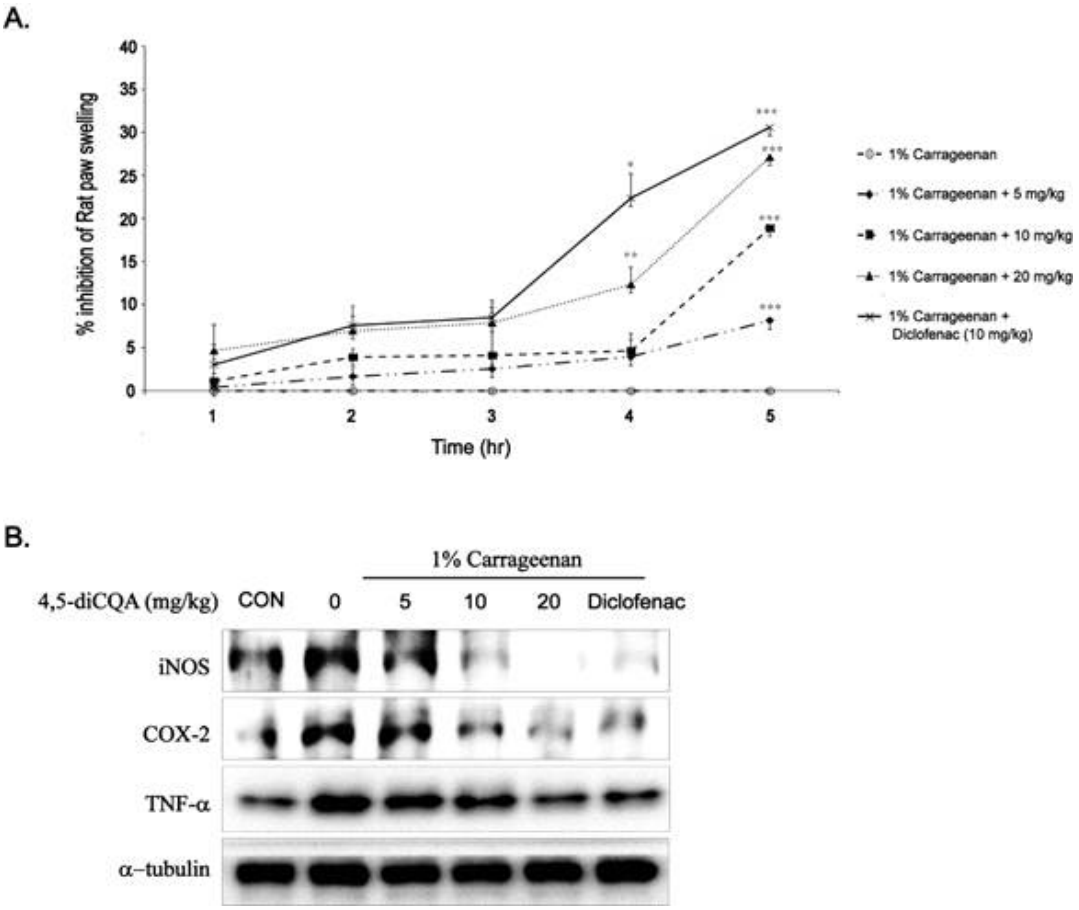

Figure 5. Effects of 4,5-diCQA on carrageenan-induced paw edema. Paw edema was induced by subcutaneous injection of a 1% carrageenan solution (100 μL/animal) into the hind paw, 1 h after oral administration of 4,5-diCQA (5, 10, and 20 mg/kg) or diclofenac sodium (10 mg/kg). (A) Paw thickness was measured at 0–5 h after carrageenan injection. Results are the means ± SD of four animals. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to the carrageenan group at the corresponding time points. (B) At the end point, expression of iNOS, COX-2, and TNF-α in the spinal cord of experimental animals was determined using western blot analysis. α-Tubulin served as an internal control. CON; control, iNOS; inducible nitrite oxide, COX-2; cyclooxygenase-2, TNF-α; tumor necrosis factor-alpha.

2. Anti-osteroarthritis effects

3.2.1. Effect of those 4,5-diCQA on viability of rat primary chondrocytes.

To investigate the toxicity of 4,5-diCQAs, this drug is treated in rat primary chondrocytes at concentrations of 10, 20, 40, 100, and 200 μM for 24 h. 4,5-diCQAs are non-toxic up to a concentration of 200 μM (Fig. 6B).

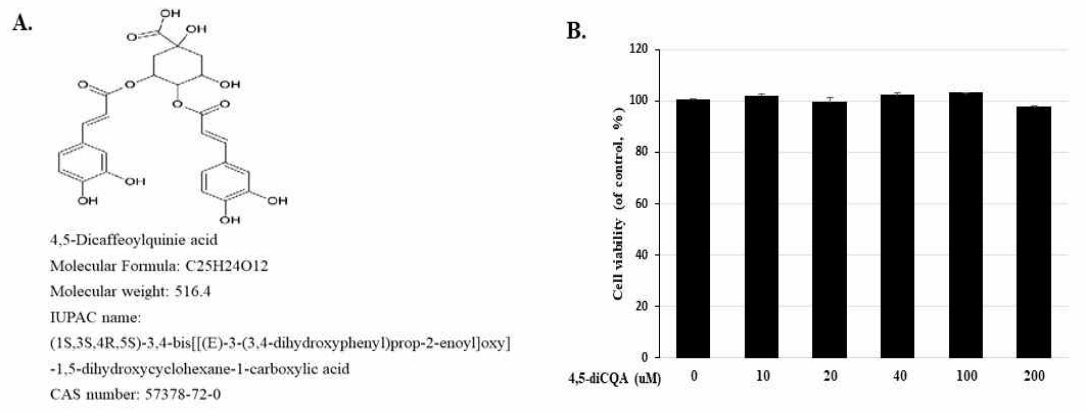

Figure 6. Effects of 4,5-diCQA on rat primary chondrocytes viability. (A) Chemical formula of 4,5-diCQA. (B) Cells were treated with 4,5-diCQA (10, 20, 40, 100, and 200 μM) for 24 h, and viability was determined by MTT assay. Cells incubated without 4,5-diCQA were used as controls and were considered 100% viable. Data are represented as mean ± SD of three independent experiments.

3.2.2. Effects of those 4,5-diCQA on the expression of IL-1β-induced nitrite and PGE_2, in rat primary chondrocytes.

Inflammation is known to be the main cause of exacerbation of OA. Therefore, at first, the expression levels of nitrite and PGE_2 were examined from the supernatant of IL-1β -induced rat primary chondrocytes, respectively. First, 4,5-diCQA is pretreated to rat primary chondrocytes on each concentration (10, 20, and 40 μM), after 1 h, and then treated with IL-1β (5 ng/mL) for 24 h. In the group in which only IL-1β was induced, the expression levels of nitrite and PGE_2 were significantly increased (Fig. 7A and B). However, in the group pretreated with 4,5-diCQA, the expression levels of nitrite and PGE_2, were decreased in a concentration-dependent manner even when treated with IL-1β. In addition, inflammatory mediators such as iNOS, COX-2, and TNF-α, inflammatory cytokine, were increased in only IL-1β-induced rat primary chondrocytes, but not in the group pretreated with 4,5-diCQA (Fig. 7C). This result indicates that 4,5-diCQA has a potential anti-inflammatory effect by suppressing the IL-1β-induced inflammatory response.

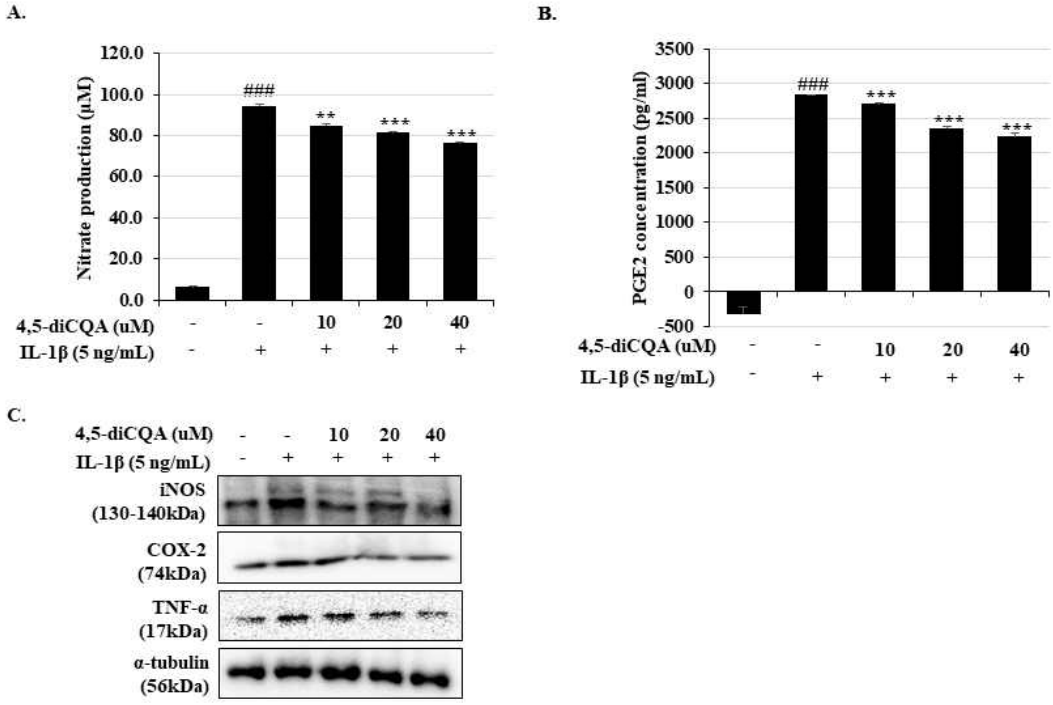

Figure 7. Inhibitory effects of 4,5-diCQA on IL-1β-induced nitrite, PGE_2, iNOS, COX-2, and TNF-α in rat primary chondrocytes. Cells were pretreated with 4,5-diCQA (10, 20, and 40 μM) for 1 h, followed by IL-1β (5 ng/mL) stimulation for 24 h. (A) Nitrite production was determined in the cultured medium using a Griess reagent. (B) PGE_2 production was determined in the cultured medium using an ELISA kit after 24 h. (C) Expression of the iNOS, COX2, and TNF-α was determined using western blot analysis. α-Tubulin served as an internal control. ### $p < 0.005$ vs. control group; **$p < 0.05$, and ***$p < 0.005$ compared with the IL-1β-treated group.

3.2.3. Effect of those 4,5-diCQA on expression of IL-1β-induced matrix-degrading enzymes in rat primary chondrocytes.

Inflammation mediators nitric oxide and PGE_2 promote the secretion of matrix-degrading enzymes such as MMPs and ADAMTS-4. MMPs and ADAMTS-4 are also degrading enzymes of aggrecan (ACAN), and this phenomenon of ECM degradation is a prominent feature of OA. Therefore, the efficacy of 4,5-diCQA was examined on IL-1β -induced rat primary chondrocytes through the expression levels of MMP-1, -3, 13, and ADAMTS-4. The expression level of MMP-1, MMP-3, MMP-13, and ADAMTS-4 increased in the group treated with only IL-1β. On the contrary, the expression level of these enzymes was significantly reduced in the group pretreated with 4,5-diCQA and induced with IL-1b (Fig. 8A and B). Moreover, the MMPs expression level increased in the IL-1β-only group in this gelatin zymography using the supernatant of the cultured medium (Fig. 8C). From these results, 4,5-diCQA inhibited cartilage degrading enzymes under IL-1β-induced conditions.

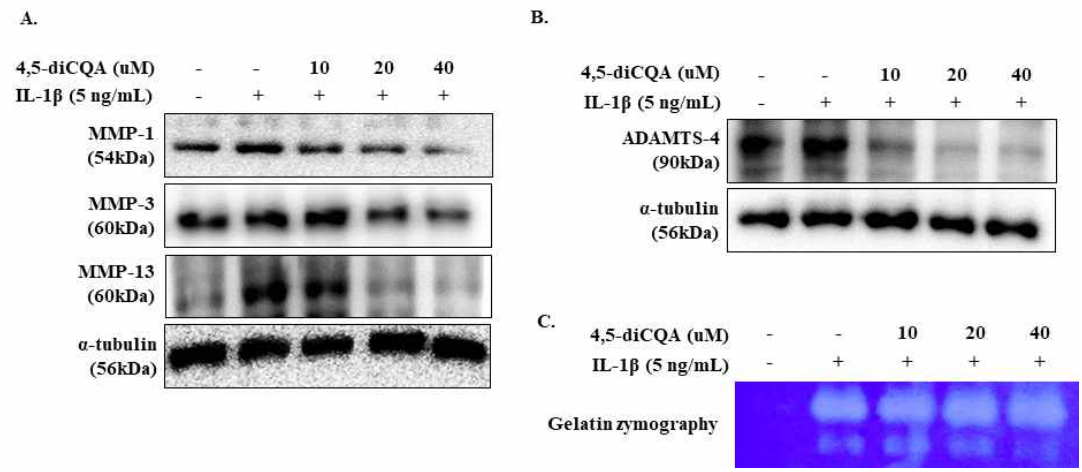

Figure 8. Inhibitory effect of 4,5-diCQA on IL-1β-induced MMP-1, MMP-13, and ADAMTS-4 in rat primary chondrocytes. Cells were pretreated with 4,5-diCQA (10, 20, and 40 μM) for 1 h, followed by IL-1β (5 ng/mL) stimulation for 24 h. (A and B) Protein levels of MMP-1, MMP-13, and ADAMTS-4 were determined using western blot analysis. α-Tubulin served as an internal control. (C) Activity of MMPs was measured in conditioned medium using gelatin zymography.

3.2.4. Effect of those 4,5-diCQA on IL-1β-induced ACAN degradation on rat primary chondrocytes.

ACAN is a component of cartilage ECM, but when IL-1b is induced, the degradation of ACAN is promoted by inflammation. This experiment was performed to confirm whether 4,5-diCQA has the effect of preventing the degradation of ACAN in rat primary chondrocytes. The expression level of ACAN was measured by ELISA in the supernatant of the cultured media and western blot in the cell lysates. The expression level of ACAN was decreased in the group induced only with IL-1β in the rat primary chondrocytes, but the group pretreated with 4,5-diCQA was significantly increased (Fig. 9). This result suggests that 4,5-diCQA has a potential chondroprotective effect by suppressing the degradation of ACAN in the IL-1β-induced group.

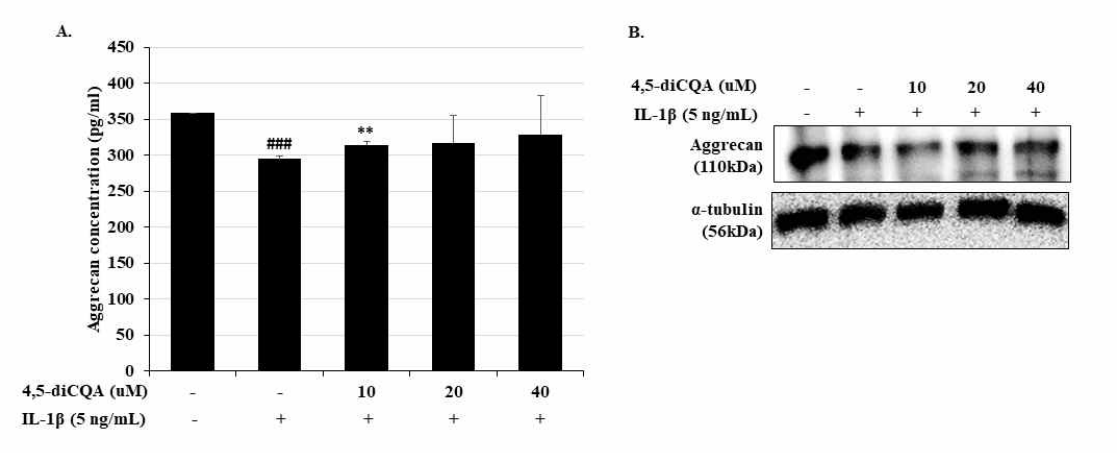

Figure 9. Inhibitory effect of 4,5-diCQA on IL-1β-induced ACAN degradation in rat primary chondrocytes. Cells were pretreated with 4,5-diCQA (10, 20, and 40 μM) for 1 h, followed by IL-1β (5 ng/mL) stimulation for 24 h. (A) Conditioned medium were prepared for ACAN ELISA assay. (B) Protein levels of ACAN were determined using western blot analysis. α-Tubulin served as an internal control. ANOVA and Dunnett test were used to evaluate the significance of the results. ###$p < 0.005$ compared with control group; **$p < 0.05$ compared with IL-1β-treated group.

3.2.5. Effect of those 4,5-diCQA on NF-κB signaling pathway in IL-1β-induced rat primary chondrocytes.

NF-κB is an important transcription factor that regulates the transcription of cartilage-degrading enzymes such as MMPs, ADAMTS familiy, and inflammatory mediators. Therefore, the efficacy of 4,5-diCQA was examined through the degree of NF-κB activity in the IL-1β-induced rat primary chondrocytes. When IL-1β is induced for 30 min, the transcription of the NF-κB p65 subunit moves from the cytoplasm to the nucleus, and the expression level increases (Fig. 10A). At the same time, phosphorylation and degradation of IκB occur together in the cytoplasm. However, in the group pretreated with 4,5-diCQA for 1 h in the IL-1β-induced condition, not only the phosphorylation and degradation of IκB were suppressed, but also the transfer of the NF-κB p65 subunit from the cytoplasm to the nucleus was suppressed (Fig. 10). This result suggests that the transcription of NF-κB was regulated by the chondroprotective effect of 4,5-diCQA.

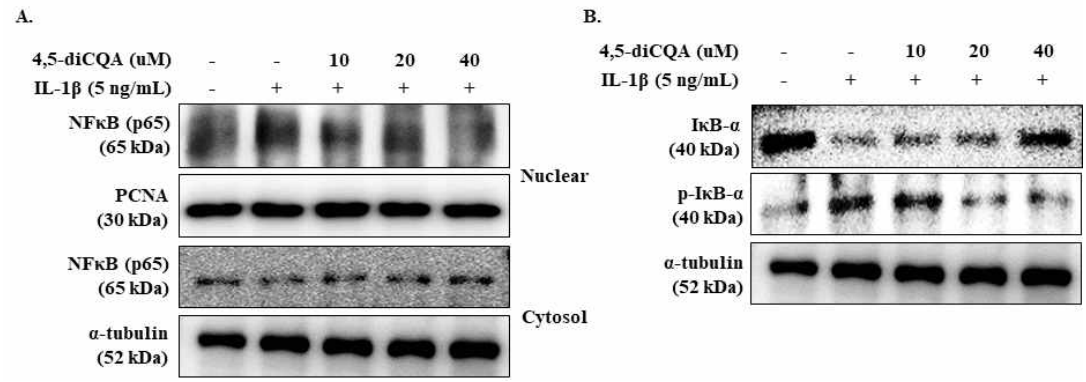

Figure 10. Effects of 4,5-diCQA on IL-1β-induced activation of NF-κB in rat primary chondrocytes. Cells were pretreated with 4,5-diCQA (10, 20, and 40 μM) for 1 h, followed by IL-1β (5 ng/mL) stimulation for 1 h. Phosphorylation levels of IκB-α and NF-κB p65 translocation to the nucleus were determined using western blot analysis. α-Tubulin and PCNA were used as cytosolic and nuclear internal controls, respectively.

3.2.6. Effect of 4,5-diCQA administration on macroscopic and histologic parameters in articular cartilage of rat OA model.

DMM is a widely used surgical technique by incising the medial meniscus and has a similar pathology to human OA. The effect of 4,5-diCQA was examined through DMM-induced OA cartilage structure using Safranin O/Fast green staining and OARSI score. Only DMM-induced OA group represented cartilage damage, whereas DMM-induced rats, which orally administered 4,5-diCQA, did not (Fig. 11A). The DMM-induced group of OA had an OARSI score of 16 ± 0.57, which indicates cartilage destruction and erosion. However, the 5, 10, and 20 of 4,5-diCQA-treated OA groups, the OARSI score was 10 ± 0.76, 5 ± 0.57, and 4 ± 0.57, respectively, indicating that cartilage destruction was significantly reduced (Fig. 11B). Consequently, these results suggest that 4,5-diCQA alleviates OA *in vivo*.

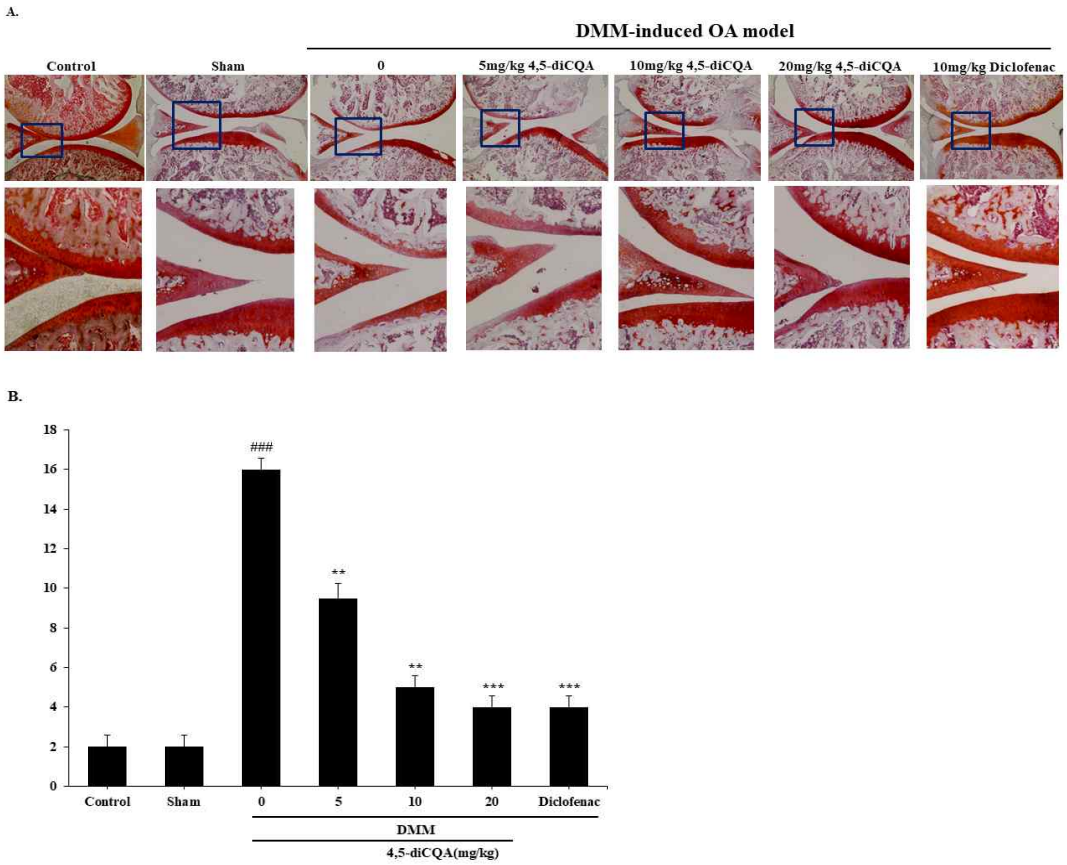

Figure 11. Histological evaluation of cartilage-protective effect of 4,5-diCQA against cartilage degradation in a DMM model. After sham or DMM surgery, rats received a gavage of distilled water, 4,5-diCQA (5, 10, and 20 mg/kg bodyweight) or diclofenac sodium (10 mg/kg bodyweight) every other day for 2 weeks. (A) Histological analysis of cartilage destruction was evaluated by Safranin O/Fast green staining. (B) Osteoarthritis Research Society International (OARSI) advanced Osteoarthritis Cartilage Histopathology Assessment System. ANOVA and Dunnett test were used to evaluate the significance of the results. ###p < 0.005 compared with sham group; **p<0.05, ***p<0.005 compared with DMM group.

DISCUSSION

Osteoarthritis (OA), a debilitating degenerative joint disease found primarily in people over the age of 65, is a major cause of disability that increases medical costs and reduces quality of life [20]. The pathogenic mechanism of osteoarthritis has not been elucidate, but accumulated research indicate that inflammation plays a very important role in the initiation and development of OA [20-22]. the imbalance in chondrocytes metabolism due to inflammation causes the overall shift toward catabolism over anabolism by excessively increasing the expression of inflammatory cytokines and substrate degrading enzymes, eventually leading to apoptosis and cartilage destruction [23,24]. Therefore, research on the mechanism of protecting chondrocytes may be one strategy for delaying or improving the development of OA, and plant-derived components with fewer side effects and excellent pharmacological effects are attracting attention as ideal drugs for OA [25,26]. In our previous study, water extract of *Anthriscus sylvestris's* leaves (AELAS) significantly suppressed the expression of IL-1β -induced OA-catabolic factors (nitric oxide, iNOS, COX-2, PGE_2, MMP-3, -13, and ADAMTS-4) and degradation of ACAN, collagen type II and proteoglycan in rat primary chondrocytes [29]. In addition, AELAS inhibited DMM surgery-induced cartilage destruction and proteoglycan loss [30]. Recently, As a result of components analysis to find the active ingredients of the AELAS, it was confirmed that a large amount of CQA-derived ingredients were contained. Therefore, this study suggest that 4,5-diCQA shows beneficial chondroprotective effects by inhibiting the expression of various pathological factors affecting OA, including nitrosative, degradation of articular ECM, and expression of proinflammatory cytokines and mediators via NF-κB and MAPK signaling pathways *in vitro* and *in vivo*.

IL-1β is a potent catabolic factor in OA pathogenesis that induces the expression of other OA-catabolic factors, such as iNOS, nitric oxide, COX-2, PGE_2, TNF-α, MMPs, and ADAMTSs, which contribute to chondrocytes dysfunction, ultimately accelerates the initiation and progression of ECM degradation from chondrocytes [22]. In particular, nitric oxide and PGE_2, which are highly expressed in OA patients, are the early

mediators by inflammation, and inhibit synthesis of collagen type II by inducing the expression of other catabolic factors [29,30]. Therefore, inhibition of IL-1β-induced inflammation mediators (iNOS, nitric oxide, COX-2, and PGE_2) had the ability to alleviate OA pathogenesis, reduce pain, inflammation, and proteoglycan loss [22,31]. In this study, the expression of nitric oxide, PGE_2, iNOS, and COX-2 increased upon IL-1 β treatment, but pretreatment with 4,5-diCQA rescued this effect. These results are consisntent with previous studies that analyzed the ameliorating effect of OA, Liu *et al*., reported that CQA-rich fraction of *Periploca forrestii* (CQAF) treatment significantely blocked IL-1β-induced expression of nitric oxide, PGE_2, COX-2, and iNOS in MH7A cells (human rheumatoid arthritis synovial cell line) [34]. MMPs are a family of proteinase that contribute to degradation of the collagen type II and proteoglycan, and elevated expression of MMP-13 is characteristic of OA chondrocytes [32,33]. ADAMTS is also enzyme that deeply involved in OA pathogenesis, and especially ADAMTS-4 considered as the primary aggrecanases [34]. In this study, we noted that IL-1β treatment increased the expression and activity of MMP-1, MMP-3, MMP-13, and ADAMTS-4. In addition, IL-1β treatment induced the degradation of ACAN. However, pretreatment with 4,5-diCQA inhibited degradation of ACAN by suppressing the IL-1β-induced activity of MMPs (1, 3, and 13) and ADAMTS-4. These effects of 4,5-diCQA were consistent with the results of other studies on anti-arthritis using natural products. Tran *et al*. reported that avenanthramide-C (Avn-C) isolated from Oats suppressed IL-1β-induced the expression of MMP-3, -12 and -13 in mouse articular chondrocytes, and Feng *et al*. reported oleuropein inhibits the IL-1β-induced expression of inflammatory mediators (nitrite oxide, PGE_2, COX-2, and iNOS) and ECM proteinase (MMP-1, MMP-3, MMP-13, and ADAMTS-5) [16,31]. These results clearly suggest that 4,5-diCQA has a chondroprotective effect against IL-1β-related induction and development of OA.

MAPK and NF-κB pathways are critical to some chronic inflammatory diseases, like OA [37,38]. The MAPK family includes extracellular signal-regulated kinases, of which ERK mediates chondrocytes proliferation and gene expression; p38 and JNK play an important role in inflammation and destruction of articular cartilage [40]. The phospholyated of MAPKs (p-ERK1/2, p-JNK, and p-P38) is regulated the cartilage

degradation and MMPs expression [40]. NF-κB pathway is activated by phosphorylation of MAPKs and is involved in the regulation of inflammatory mediators as well as OA progression [41,42]. Normally, NF-κB is localized to the cytoplasm with its inhibitor subunit IκB-α, but in presence of IL-1β, phosphorylation and degradation of IκB results in the translocation of NF-κB p65 into the nucleus, resulting in induction of expression of inflammatory mediators [41,43,44]. Therefore, inhibition of these pathways is considered to be crucial in suppressing inflammation, several studies reported that some plants and natural-derived compounds showed anti-arthritis activity by regulating the MAPK and NF-κB pathways, such as *Caragana sinica* root extract, *Punica granatum* extract, oleuropein, and CQAF [21,35,41,42]. In this study, 4,5-diCQA treatment inhibited the IL-1β-induced phosphorylation of MAPKs (ERK1/2, JNK, and p38), degradation of IκB-α, and translocation of the NF-κB p65 subunit. These results suggests that chondroprotective effect of 4,5-diCQA may mediated by MAPKs and NF-κB signaling pathway.

In *in vivo* experiments, we established a rat OA model through destabilization of the medial meniscus (DMM) to evaluate the protective effect of 4,5-diCQA on cartilage degradation. The 4,5-diCQA orally administration alleviated the severity of cartilage degradation in a DMM-induced OA model by suppressing catabolic activity and damage in chondrocytes, and this result was consistent with the OARSI score.

In conclusion, pretreatment with 4,5-diCQA effectively inhibited IL-1β-induced inflammatory factors (nitrite oxide, PGE_2, iNOS, and COX-2) and cartilage-degrading enzymes (MMP-1, -3 -13, and ADAMTS-4). Furthermore, 4,5-diCQA also protects ACAN, which are the constitutive components of chondrocytes ECM, from degradation by IL-1β treatment and DMM-surgery. These results suggest that 4,5-diCQA is an active ingredient showing the OA improvement effect of AELAS, and exerts chondroprotective effects in IL-1β-induced OA development.

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