Carpomitra costata Extract Suppresses Interleukin-1β-Induced Inflammatory Response in SW1353 Human Chondrocytes through Suppressing NF-κB Signaling Pathway

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Abstract Osteoarthritis (OA) is an inflammatory degenerative joint disease that is accompanied by irreversible joint cartilage destruction. Recently, the antioxidant effects of *Carpomitra costata*, which is a type of brown algae, have been reported, but their effects on OA have not been investigated. In this study, the anti-osteoarthritic effect of the ethanol extract of *C. costata* (EECC) on SW1353 human chondrocytes was studied. Results showed that EECC significantly attenuated the interleukin-1β (IL-1β)-induced release of pro-inflammatory mediators, including prostaglandin E_2 and nitric oxide (NO), as well as expressions of cyclo-oxygenase-2 and inducible NO synthase. EECC also inhibited the IL-1β-induced expressions of matrix metalloproteinase-1, -3, and -13 in SW1353 chondrocytes, which reduced their extracellular secretion. In addition, the oxidative stress induced by IL-1β was confirmed to be blocked by EECC due to the inhibition of reactive oxygen species generation. Moreover, EECC suppressed IL-1β-mediated translocation of nuclear factor-kappa B (NF-κB) from cytosol into the nucleus and the degradation of IκB-α, which indicates that EECC exhibits anti-inflammatory effects by inhibiting the NF-κB signaling pathway. These results are the first to demonstrate the anti-inflammatory activities of *C. costata* extracts in chondrocytes, thus suggesting that this algae extract may be used in the treatment of OA.

Keywords : Osteoarthritis; *Carpomitra costata*; IL-1β; inflammation; NF-κB

Introduction

Osteoarthritis (OA), the most common joint disease associated with joint trauma and aging, is characterized by the destruction of progressive articular cartilage [1,2]. Among the commonly known causes of OA, inflammation is an important contributor to the pathogenesis of OA that causes an imbalance of the metabolic pathway of chondrocytes, leading to the loss of metabolic homeostasis and the degradation of the extracellular matrix (ECM) [3-5].

Among inflammatory cytokines, interleukin-1 beta (IL-1β) plays a pivotal role in inducing the production and secretion of inflammatory and catabolic factors associated with OA pathogenesis [6-8]. The levels of IL-1β are significantly higher in the synovial fluid and cartilage of OA patients than in normal individuals [9,10]. When chondrocytes are exposed to IL-1β, the expression of inflammatory genes including inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), and matrix metalloproteinases (MMPs), which are major proteases directly involved in cartilage degradation,

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are increased *via* activation of nuclear factor-kappa B (NF-κB) [11-13]. Therefore, NF-κB is a critical target of therapeutic strategies aimed at the treatment of OA. In addition, excessive oxidative stress may be considered as another important factor triggering OA following chondrocyte senescence and apoptosis [14,15]. Although non-steroidal anti-inflammatory drugs are generally widely used to relieve symptoms of OA, research on reliable and effective drugs is required due to side effects caused by long-term use [16,17].

Recently, attention has been focused on the use of marine species as a resource for suppressing various inflammatory diseases. Among them, seaweeds have a great potential for the development of OA prevention agents because of the abundance of physiologically active substances with various pharmacological actions [18]. For example, in a small clinical study on humans, the oral administration of fucoidan-rich brown algae extract significantly inhibited OA symptoms, and it was associated with the reduction of IL-6, a chronic inflammatory marker [19]. In addition, the phlorotannin-rich extract of *Ecklonia cava* markedly suppressed the production of prostaglandin E_2 (PGE$_2$) in lipopolysaccharide-treated RAW 246.7 cells and attenuated the IL-1α-induced proteolytic degradation [20]. However, up to now, studies on the effect of seaweed extract on OA have been limited in comparison with those on its diversity. Therefore, it was studied the effect of the ethanol extract of *Carpomitra costata* (Stackhouse) Batters (EECC), a kind of brown algae, on the IL-1β-mediated inflammatory responses, MMPs, and reactive oxygen species (ROS) production in SW1353 human chondrocytes as part of the discovery of new marine biomaterials for OA treatment.

Materials and Methods

Preparation of EECC
EECC used in the current study was provided by Dr DS Lee (National Marine Biodiversity Institute of Korea, Seocheon, Republic of Korea). For the preparation of EECC, *C. costata* was collected from offshore Ulleung Island, Republic of Korea in March 2016. Collected *C. costata* was washed with tap water and stored at −20°C. Samples were homogenized using a grinder and dried prior to extraction. The dried powder was extracted 5 times with 70% EtOH (1:10 w/v) at 1 h intervals by sonication. Subsequently, the extract (EECC) was evaporated in vacuo, and then dissolved in dimethyl-sulfoxide (DMSO, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) before use in the experiment.

Cell culture
SW1353 chondrocytes, which were purchased from American Type Culture Collection (Manassas, VA, USA), were maintained in humidified air at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin and streptomycin, and 10% fetal bovine serum. All materials required for cell culture were purchased from WelGENE Inc. (Daegu, Republic of Korea).

Cell viability assay
The cytotoxicity of EECC to SW1353 chondrocytes was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. In brief, cells were incubated in 96-well plates for 24 h at a density of 1×10^4 cells, and then treated with different concentrations of EECC or IL-1β (40 ng/ml, R&D Systems, Minneapolis, MN, USA) alone or pretreated with different concentrations of EECC for 1 h before IL-1β treatment for 24 h. Then, the medium was removed, and MTT solution (0.5 mg/ml, Sigma-Aldrich Chemical Co.) was dispensed into each well and reacted at 37°C. After 3 h, the supernatant was removed and DMSO was added to dissolve blue formazan crystals for 10 min. The absorbance per well was quantified at a wavelength of 540 nm using an enzyme-linked immunosorbant assay (ELISA) reader (Dynatech Laboratories, Chantilly, VA, USA).

Measurement of NO, PGE$_2$ and MMPs

SW1353 chondrocytes were treated with various concentrations of EECC for 1 h and then stimulated with IL-1β for 24 h. Nitrite levels in the media were evaluated by a Griess reaction (Sigma-Aldrich Chemical Co.). Briefly, 100 μL of the cell conditioned medium was mixed with the same amount of Griess reagent for 10 min, and then the absorbance was measured with an ELISA reader at a wavelength of 540 nm. To investigate PGE_2 levels, the culture supernatants were collected and assayed using a commercially available ELISA kit (R&D Systems) with the instructions of the manufacturer. The levels of levels of MMPs released from cultured chondrocytes were calculated by specific ELISA kits (R&D Systems) in accordance with the instructions of the manufacturer.

Protein isolation and immunoblotting

Nuclear extraction reagents purchased from Pierce (Rockford, IL, USA) were used to isolate proteins from the nucleus and cytoplasm according to the manufacturer's protocol. The concentration of the isolated protein was measured using the Bio-Rad protein assay kit obtained from Bio-Rad Laboratories (Hercules, CA, USA). Equal amounts of protein were separated by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins in the gel were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Schleicher and Schuell GmbH, Keene, NH, USA). The protein-transferred membrane was blocked with non-fat dry milk solution (5%) at room temperature for 1 h, and then reacted with primary antibodies overnight at 4°C. Antibodies against NF-κB p65 (sc-109), IκB-α (sc-371), lamin B (sc-6216) and β-actin (sc-1615) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The membrane was washed 3 times for 5 min with Tris buffered saline (0.1% Tween-20) and then incubated with goat anti-rabbit IgG-horseradish-peroxidase (HRP) and goat anti-mouse IgG-HRP secondary antibodies (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. The membrane was reacted with an enhanced chemiluminescent solution purchased from Amersham Corp.

(Arlington Heights, IL, USA) and then exposed to an X-ray film to visualize the corresponding proteins. Bands were quantified using an ImageJ program (Ver. 1.46; NIH, Bethesda, MD, USA) and normalized to β-actin, and the ratio was determined.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total RNA was extracted from the cells using TRIzol reagents (Invitrogen Life Technologies, Carlsbad, CA, USA), following to the manufacturer's and quantified. The isolated total RNA (1 μg) was used to synthesize cDNA using AccuPower® RT PreMix (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. The cDNA generated at RT was amplified using selected primers. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The PCR primers were as follows: iNOS, 5′-GTG-AGG-ATC-AAA-AAC-TGG-GG-3′ (sense) and 5′-ACC-TGC-AGG-TTG-GAC-CAC-3′ (anti-sense); COX-2, 5′-TCA-GCC-ACG-CAG-CAA-ATC-CT-3′ (sense) and 5′-GTG-ATC-TGG-ATG-TCA-CG-3′ (anti-sense); GAPDH, 5′-CGA-TGC-TGG-GCG-TGA-GTA-C-3′ (sense) and 5′-CGT-TCA-GCT-CAG-GGA-TGA-CC-3′ (ant-isense); MMP-1, 5′-CTG-TTC-AGG-GAC-AGA-ATG-TGC-3′ (sense) and 5′-TTG-GAC-TCA-CAC-CAT-GTG-TT-3′ (anti-sense); MMP-3, 5′-TGC-GTG-GCA-GTT-TGC-TCA-GCC-3′ (sense) and 5′-GAA-TGT-GAG-TGG-AGT-CAC-CTC-3′ (anti-sense), and MMP-13, 5′-GGC-TCC-GAG-AAA-TGC-AGT-CTT-TCT-T-3′ (sense) and 5′-ATC-AAA-TGG-GTA-GAA-GTC-GCC-ATG-C-3′ (anti-sense). The images of RT-PCR were also analyzed with an ImageJ program and normalized to GAPDH, and the ratio was determined.

Detection of ROS levels

The generation of ROS was measured using 5,6-carboxy-2',7'-dichlorofluorescin diacetate (DCF-DA, Sigma-Aldrich Chemical Co.). Briefly, SW1353 chondrocytes were pretreated with 300 μg/ml EECC for 1 h and then incubated for 2 h in the absence or presence

of IL-1β with or without an ROS scavenger, N-acetyl cysteine (NAC, Sigma-Aldrich Chemical Co.). The cells were stained with 10 μM DCF-DA for 15 min in the dark at 37°C. The cells were then washed with PBS and immediately used for flow cytometry (BD Biosciences, San Jose, CA, USA) as previous described [21].

To compare the degree of ROS generation through fluorescence microscopic observation, the cells were stained with DCF-DA for 15 min at 37°C and then fixed with paraformaldehyde solution (4%, pH 7.4) for 20 min. The cells were washed with PBS and analyzed for ROS fluorescence intensity using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) as previously described [22].

Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). The analyzed results were expressed as mean ± standard deviation (SD), and the difference between groups was evaluated through analysis of variance followed by ANOVA-Tukey's *post hoc*. $p < 0.05$ was considered to represent a statistically significant difference.

Results

Effect of EECC on SW1353 chondrocyte cytotoxicity

The cytotoxic effects of EECC on the SW1353 chondrocytes were determined by the MTT assay. As shown in Fig. 1A, EECC at the concentration range of 50−300 μg/ml did not show a cytotoxic effect on the SW1353 cells, but significant cytotoxicity was observed in the 400 μg/ml EECC treated group. Subsequent experiments did not show any adverse effect on cell viability when EECC was administered to the 40 ng/ml IL-1β -treated SW1353 cells at a concentration of 300 μg/ml or less (Fig. 1B). Thus, EECC concentrations up to 300 μg/ml were used in the following experiments.

EECC inhibited the IL-1β-induced NO and PGE$_2$

production in the SW1353 chondrocytes

The effects of EECC on the levels of released inflammatory mediators (NO and PGE$_2$) were detected to assess the anti-inflammatory effects of EECC. The SW1353 cells were pretreated with various concentrations of EECC for 1 h before stimulation with 40 ng/ml IL-1β for 24 h. Then, the NO concentration in the cell suspension was assayed by the Griess reaction, and the level of PGE$_2$ was measured using an ELISA kit. As shown in the results in Fig. 2, IL-1β stimulation increased the release of NO and PGE$_2$ compared with the non-stimulated control, but NO and PGE$_2$ production was significantly decreased in the EECC-pretreated cells in a concentration-dependent manner.
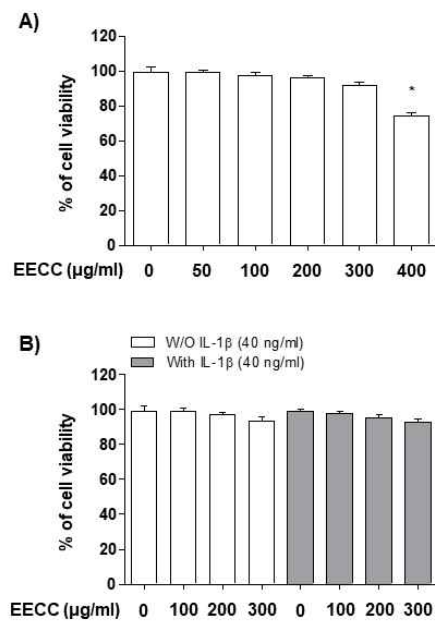

Figure 1. Effect of EECC and IL-1β on the cell viability of the SW1353 chondrocytes. The cells were treated with various concentrations of EECC alone for 24 h (A) or pretreated with or without EECC for 1 h before 40 ng/ml IL-1β stimulation for 24 h (B). Cell viability was analyzed using an MTT assay (*$p < 0.05$ in comparison with the control group).

EECC attenuated the IL-1β-mediated increase in MMP-1, -3, and -13 production in the SW1353 chondrocytes

As catabolic factors play the important role of OA

pathogenesis, it was investigated whether EECC could avoid the generation of MMPs having an essential part in cartilage degradation. The results showed that the amount of MMPs (MMP-1, -3, and -13) released into the culture supernatant after stimulation with IL-1β was significantly increased. However, the enhanced production of these MMPs by IL-1β was suppressed by EECC pretreatment, and this effect was dependent on the EECC treatment concentration (Fig. 3).
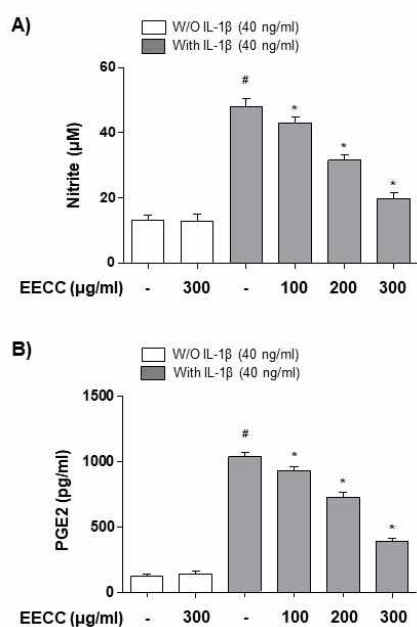
of EECC pretreatment (Fig. 4A). Subsequently, it was also investigated whether the inhibition of MMP production by EECC in the IL-1β-treated SW1353 chondrocytes was associated with the decreased expression of these genes. As shown in Fig. 4B, the expression of the three MMPs mRNAs was greatly enhanced by the IL-1β treatment, but their expression was dramatically reduced in the cells cultured under the condition of EECC pretreatment.
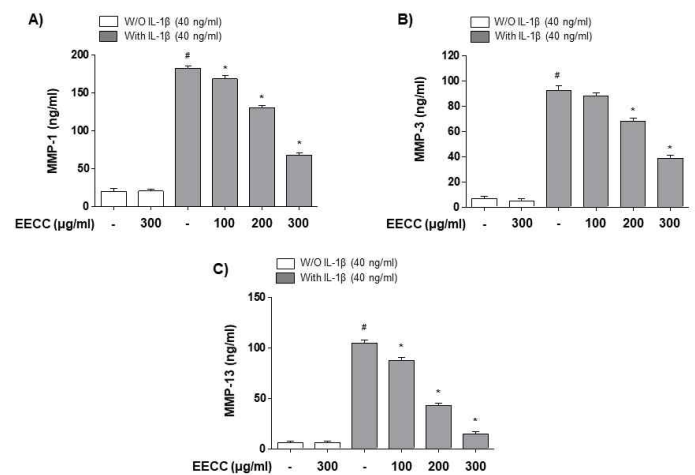

Figure 2. Inhibition of the IL-1β-induced NO and PGE$_2$ production by EECC in the SW1353 chondrocytes. The cells were pretreated with various concentrations of EECC for 1 h before 40 ng/ml IL-1β stimulation for 24 h. (A) The NO concentration in the culture medium was determined by the Griess reaction. (B) The PGE$_2$ concentration was determined by a commercial ELISA kit ([#]$p<0.05$ in comparison with the control group; *$p<0.05$ in comparison with the IL-1β group).

Figure 3. Inhibition of the IL-1β-induced MMPs production by EECC in the SW1353 chondrocytes. The cells were pretreated with various concentrations of EECC for 1 h before 40 ng/ml IL-1β stimulation for 24 h. Culture supernatants were then isolated, and the amounts of MMP-1 (A), -3 (B), and -13 (C) production were determined using commercial ELISA kits ([#]$p<0.05$ in comparison with the control group; *$p<0.05$ in comparison with the IL-1β group).

EECC alleviated the IL-1β-mediated generation of ROS in the SW1353 chondrocytes

Because oxidative stress also plays an important role in the degradation of ECM and in the induction of chondrocyte apoptosis, I investigated whether EECC could suppress IL-1β-induced oxidative stress using the DCF-DA probe. The flow cytometry results showed that the level of the intracellular ROS contents gradually increased with the stimulation of IL-1β, peaked at 2 h, and decreased thereafter (data not shown). However, the increase in ROS content in the SW1353 cells treated with IL-1β was dramatically reduced by

EECC decreased the expression of iNOS, COX-2 and MMP proteins increased by IL-1β in the SW1353 chondrocytes

I next examined whether EECC could inhibit the expression of iNOS and COX-2 by IL-1β. According to the RT-PCR results, the increased expression of iNOS and COX- mRNA by IL-1β was concentration-dependently decreased in the cells cultured under the condition

the addition of EECC. Moreover, as expected, the pretreatment of a ROS scavenger, NAC, completely protected the ROS production by IL-1β (Fig. 5A). Consistent with the results from the flow cytometry, the increase in the fluorescence intensity of DCF-DA observed in the cells treated with IL-1β was weakened by the pretreatment of EECC, as shown in Fig. 5B.
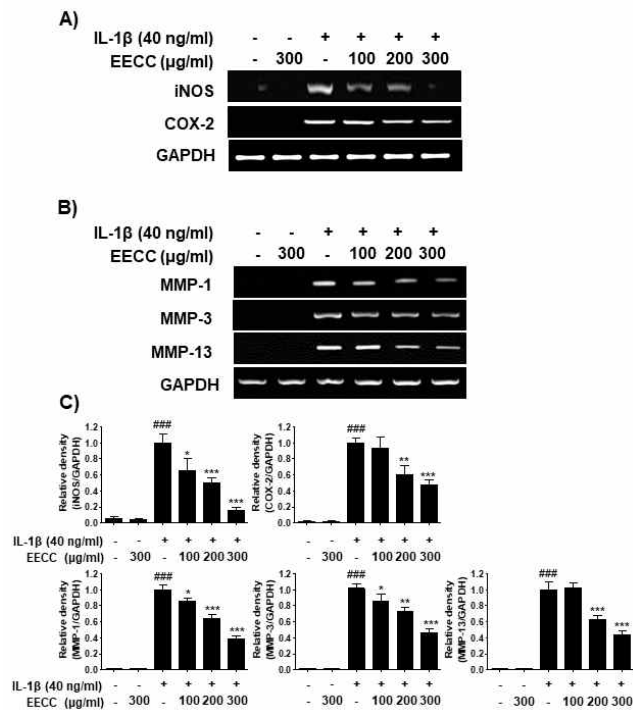

Figure 4. Inhibition of the iNOS, COX-2 (A) and MMPs (B) mRNA expression by EECC in the IL-1β-stimulated SW1353 chondrocytes. The cells were pretreated with various concentrations of EECC for 1 h and incubated with 40 ng/ml IL-1β for 24 h. RT-PCR was performed using the indicated primers and GAPDH was used as the internal control for the RT-PCR. (C) The relative density was calculated using ImageJ software, and normalized to GAPDH. The results are the average of three independent experiments and expressed as ± SD of the median. ###$p<0.001$ *vs*. the untreated group; ***$p<0.001$, **$p<0.01$, and *$p<0.05$ *vs*. the IL-1β-treated group.

EECC suppressed nuclear translocation of NF-κB in IL-1β-stimulated the SW1353 chondrocytes

It was further investigated whether EECC could inhibit the IL-1β-mediated activation of NF-κB because it is a critical mediator of IL-1β signaling in chon-

drocytes and the key factor controlling the transcription of inflammatory mediators and MMPs. As shown in Fig. 6, when the SW1353 cells were stimulated with IL-1β, the translocation of NF-κB p65 to the nucleus was significantly increased compared with the control group and peaked at 1 h. By contrast, the level of IκB-α in the cytoplasm was gradually decreased by the treatment of IL-1β. However, EECC significantly reduced the nuclear translocation of NF-κB p65 and the degradation of IκB induced by IL-1β (Fig. 6).
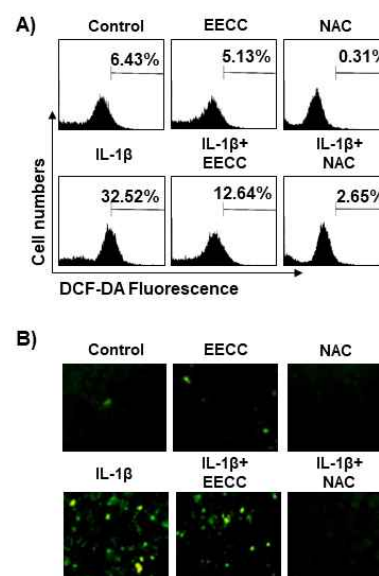

Figure 5. Effects of EECC on the IL-1β-induced ROS generation in the SW1353 chondrocytes. The cells were pretreated with 300 μg/ml EECC or 10 mM NAC for 1 h and then treatment with 40 ng/ml IL-1β for 2 h. (A) The DCF-DA-stained cells were collected, and then DCF fluorescence was calculated by a flow cytometry. The results shown are the average of two independent experiments. (B) The images were obtained using a fluorescence microscope and presented from one experiment.

Discussion

In this study, I investigated the effect of the ethanol extract of *C. costata* (EECC) on the IL-1β-induced inflammatory response in the SW1353 chondrocytes. According to the results, EECC remarkably reversed the production of IL-1β-mediated inflammatory mediators, such as NO and PGE$_2$, and MMP-1, -3, and -13

in the SW1353 chondrocytes. Moreover, EECC significantly attenuated the IL-1β-induced ROS generation and of NF-κB activation.
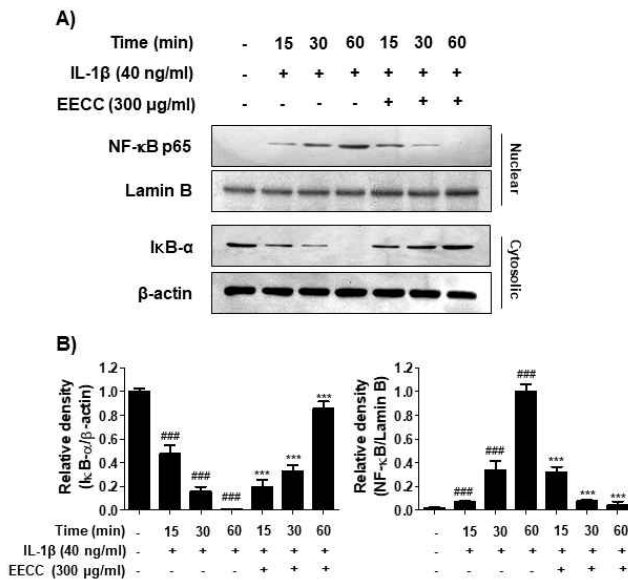

Figure 6. Inhibition of the NF-κB nuclear translocation by EECC in the IL-1β-stimulated SW1353 chondrocytes. The cells were pretreated with 300 μg/ml EECC for 1 h before the 40 ng/ml IL-1β treatment for the indicated times. (A) Nuclear and cytoplasmic proteins isolated from IL-1β-treated cells were isolated and the expression levels of NF-κB and IκB-α were determined by Western blotting. Lamin B and β-actin were presented as internal controls for each protein fraction. (B) Bands were quantified using ImageJ and normalized to lamin B and β-actin, respectively, and the ratio was determined. Data are expressed as mean ± SD. All experiments were repeated three times. $^{###}p<0.001$ vs. the untreated group; $^{***}p<0.001$ vs. the IL-1β-treated group.

Inflammation has emerged as a major cause of OA pathogenesis. In particular, IL-1β acts as a key mediator of articular cartilage degradation through the stimulation of inflammatory mediators, such as iNOS and COX-2 [6,8]. These mediators can accelerate the development of OA, and inhibiting their expression may impair OA development. In this study, the effects of EECC on the secretion of NO and PGE$_2$ were investigated to examine the inhibitory effect of EECC on the IL-1β-mediated inflammatory response. It was found that EECC significantly reduced the expression

of iNOS and COX-2 mRNA induced by IL-1β, which was associated with decreased NO and PGE$_2$ production. Therefore, the data demonstrate that the anti-inflammatory effect of EECC is due at least to the reduced expression of proteins involved in the generation of NO and PGE$_2$. Many previous studies showed that MMPs, the major proteases increased by chondrocytes according to IL-1β, contribute to the formation of the most important proteolytic system in the degradation of ECM [5,6]. In addition, their expression is increased in the cartilage and synovial tissue of most OA patients [3,4]. Therefore, the application of candidate substances that block the activity of MMPs could be applied as a new treatment strategy for OA. In the current study, MMPs such as MMP-1, -3, and -13, which are important enzymes for cartilage degradation, were significantly increased in the IL-1β-exposed chondrocytes. However, the elevated levels of the MMPs mRNA by IL-1β were obviously inhibited by the EECC treatment. These results indicate that EECC can protect the ECM degradation and chondrocytes by inhibiting the expression of MMPs.

The ROS levels are significantly greater in OA cartilage than in normal cartilage, and this has been reported in many clinical studies [14,23]. High levels of ROS in chondrocytes promote ECM degradation, cell dysfunction, and death. In addition, ROS stimulates the production of inflammatory cytokines and alters the function of critical cell signaling pathways [14,15], suggesting that oxidative stress acts as a risk factor for the pathogenesis of OA. As a result of examining the influence of EECC on oxidative stress by IL-1β, it was showed that EECC effectively repressed the production of ROS by IL-1β. Although the antioxidant potential of *C. costata* extract was reported in previous studies [24], the results of this study support the use of EECC as an antioxidant for OA management.

As noted in many studies, NF-κB plays a critical role in the control of inducible ant-inflammatory mediators, cytokines, and MMPs expression in OA pathogenesis [13,25]. Typically, NF-κB forms a complex with the inhibitory subunit IκB-α and remains inactive in the

cytoplasm. When IκB-α is phosphorylated and degraded through the upstream signaling systems by inflammatory stimulation, NF-κB migrates to the nucleus, triggering transcriptional activation of inflammation-inducing genes as well as catabolic enzymes [26,27]. Therefore, the efficacy of EECC on IL-1β-induced NF-κB activation was further evaluated because blocking the activity of NF-κB could be effective in treating OA. The current results showed that the translocation of NF-κB from the cytoplasm to the nucleus and the degradation of IκB-α were increased in IL-1β-treated SW1353 chondrocytes, but EECC effectively blocked the nuclear translocation of NF-κB, an essential step for NF-κB activation. Therefore, the inhibitory effect of EECC on the increased expression of iNOS, COX-2 and MMP in IL-1β-treated chondrocytes is due to blocking of the nuclear translocation of NF-κB.

However, further studies are needed to determine the role of other cellular signaling pathways that may be involved in the anti-inflammatory activity of EECC other than the NF-κB signaling pathway, and to determine the direct relationship with NF-κB signaling. In addition, the identification and role of other intracellular organelles in cells that are involved in ROS production by EECC should be continuously identified. It is also necessary to identify the anti-inflammatory active ingredients contained in EECC and re-confirm the anti-cancer efficacy of EECC through animal experiments.

Conclusion

In conclusion, the current study clearly indicates that EECC has the potential to inhibit the IL-1β-induced inflammatory response in chondrocytes, and that its anti-inflammatory effect is associated with the suppression of the NF-κB signaling pathway. Therefore, I suggest that EECC may have cartilage protection potential and can be used as a candidate for OA treatment.

Acknowledgements

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