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Original article

Aqueous extract of *Codium fragile* alleviates osteoarthritis through the MAPK/NF-κB pathways in IL-1β-induced rat primary chondrocytes and a rat osteoarthritis model

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ABSTRACT

Background: *Codium fragile* (Suringar) Hariot has been used as a potential remedy in traditional medicine because of its anti-inflammatory and anti-oxidant effects. Osteoarthritis is a chronic progressive joint disease, characterized by complex mechanisms related to inflammation and degeneration of articular cartilage. In this study, we aimed to evaluate the cartilage protective effect of an aqueous extract of *Codium fragile* (AECF) using rat primary chondrocytes and the osteoarthritis animal model induced by destabilization of the medial meniscus (DMM).

Methods: *In vitro*, rat primary cultured chondrocytes were pre-treated with AECF (0.5, 1, and 2 mg/mL) for 1 h and then incubated with interleukin-1β (10 ng/mL) for 24 h. Nitrite production was detected by the Griess reagent. Alteration of the protein levels of iNOS, MMP-13, ADAMTS-4, ADAMTS-5, mitogen-activated protein kinases (MAPKs), and nuclear factor-κB (NF-κB) was detected by western blotting. *In vivo*, osteoarthritis was induced by DMM of Sprague Dawley (SD) rats. The rats subjected to destabilization of the medial meniscus (DMM) surgery were orally administered with AECF (50, 100, and 200 mg/kg bodyweight) or distilled water for 8 w. The severity of cartilage lesions was evaluated by safranin O staining and the Osteoarthritis Research Society International (OARSI) score.

Results: These results demonstrated that AECF significantly inhibited nitrite production and inhibited the levels of iNOS, MMP-13, ADAMTS-4, and ADAMTS-5 in interleukin-1β-induced rat primary cultured chondrocytes. Moreover, AECF suppressed interleukin-1β-induced NF-κB activation in the nucleus and phosphorylation of ERK1/2 and JNK in the cytosol. *In vivo*, the cartilage lesions in AECF-treated osteoarthritis rats exhibited less proteoglycan loss and lower OARSI scores.

Conclusions: These results suggested that AECF is a potential therapeutic agent for the alleviation of osteoarthritis progression.

1. Introduction

Osteoarthritis (OA) is one of the most common joint diseases and is characterized by articular cartilage degeneration, joint pain, and dysfunction [1]. The progression of OA is characterized by pathological changes, such as destruction of the articular cartilage, synovium inflammation, subchondral bone sclerosis, and osteophyte formation, leading to severe joint pain and loss of movement [2,3]. However, the etiology of OA is not clear; extracellular matrix (ECM) degradation and synovial fluid inflammation in articular cartilage were thought to be closely related to the pathogenesis of OA [4]. Currently, there are few effective treatments for joint diseases such as OA, e.g., joint replacement surgery [5]. Therefore, there is an urgent need to find an effective treatment to alleviate the progression of OA. Furthermore, molecular therapy for the progression of OA will become an important research direction in the future. The exact mechanism of the progression of OA is still unclear; however, an inflammatory response in the joint is thought to be involved [6]. Inflammation directly stimulates the catabolic

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effects of chondrocytes, which eventually results in the degradation of ECM [7]. Overproduction of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α play important roles in the progression of OA [8]. Among these inflammatory cytokines, IL-1β plays a crucial catabolic role in ECM degradation, in that it can trigger chondrocytes to induce the expression of matrix metalloproteinases (MMPs), a disintegrin, metalloproteinase with thrombospondin motifs (ADAMTS), and other catabolic enzymes [9]. There are two predominant matrix-degrading enzymes that can hydrolyze aggrecan core proteins; the MMPs and ADAMTS families [10]. The involvement of the ADAMTS family is inferred from their participation in the degradation of aggrecan in articular cartilage [11]. MMPs comprise at least 20 structurally related zinc metalloproteinases capable of degrading ECM components [12]. Among these MMPs, MMP-1, −2, −3, −7, −8, −9, and −13 are expressed in the articular cartilage of OA patients [13]. Moreover, several members of the ADAMTS family influence the progression of OA. Among 19 members of the ADAMTS family, ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-8, and ADAMTS-9 have been shown to degrade proteoglycan in joint tissues of patients with OA [14].

IL-1β induces the activation of pro-inflammatory transcriptional factor nuclear factor-κB (NF-κB) through phosphorylation of IκB kinase (IKK) and IκB [15]. Activation of NF-κB induces inflammation-related gene expression, such as those encoding the MMPs and ADAMTS families [16]. Transcriptional activation of MMPs and ADAMTS family genes induced by IL-1β is also mediated by the transcriptional factor interferon regulatory factor-1 (IRF-1), which is expressed at a significantly higher level in OA chondrocytes compared to that in normal chondrocytes [17]. The NF-κB signaling pathway plays a crucial role in the regulation of inflammatory mediators related to the pathogenesis of OA [18]. NF-κB is localized in the cytoplasm as an inactive transcription factor that is associated with IκB. Upon phosphorylation to NF-κBp65, induced by IL-1β, NF-κB dissociates from IκB and is translocated to the nucleus as an active transcription factor to regulate the inflammatory response [19]. Besides, NF-κB inhibitors have been reported to reduce the expression of MMP-3 and MMP-13, which are overexpressed in response to IL-1β in human chondrocytes [20]. In addition, phosphorylation of extracellular signal-regulated kinase (ERK) −1/2, c-Jun N terminal kinase (JNK), and p38 is involved in catabolic and inflammatory responses [21].

Green algae provide a wide range of natural products with pharmacological activities, such as anti-inflammatory, anti-oxidative, anticancer, and anti-nociceptive effects [22]. *Codium fragile* (Suringar) Hariot (*C. fragile*) is an edible algae belonging to the Codiaceae that is widely distributed on the coasts of East Asia, Oceania, and Northern Europe. In Korea, *C. fragile* is a green algae used as a culinary ingredient and has been used in traditional medicine to treat enterobiasis, dropsy, and dysuria [23]. Several studies have reported that ethanol and methanol extracts of *C. fragile* have anti-inflammatory, anti-oxidative, and anti-cancer properties [24,25]. Recently, Lee et al. demonstrated that an aqueous extract of *C. fragile* (AECF) showed non-cytotoxic anti-inflammatory effects by inhibiting NF-kB activation in RAW264.7 cells [26]. Therefore, the anti-inflammatory effect of AECF could provide protection from the progression of OA. However, the protective effect and molecular mechanisms of AECF for articular cartilage are unknown. In this study, we investigated the cellular mechanism and anti-osteoarthritis activities of AECF both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Preparation of AECF

Fresh *C. fragile* (1 kg) was collected along the coast of Wando, Korea, in June 2016, washed three times with tap water to remove salt, epiphytes, and sand attached to the surface. The washed *C. fragile* is dried using an air supplied dryer oven at 50 °C for 48 h. The dried of *C. fragile* (100 g) sample was homogenized using a grinder and extracted with 50 volumes of distilled water at 90 °C for 4 h. This was followed by filtration utilizing a 55-μm bag filter, evaporation, and then lyophilization. The dry extract powder (8 g) was dissolved in distilled water (100 mg/mL), and the resulting solution was filtered using a 0.2-μm syringe filter before use.

2.2. Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulfanilamide, N-(1-naphthyl) ethylendiamine dihydrochloride, and phosphoric acid were purchased form Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's medium/Nutrient Mixture F-12, 1:1 Mixture (DMEM/F12) and penicillin-streptomycin solution were purchased from WelGene (Deagu, Republic of Korea). Fetal bovine serum (FBS) was purchased from Corning (Corning, NY, USA). Interleukin-1 beta (IL-1β) was purchased from Prospec (Prospec, Israel).

2.3. Cell culture and viability assay

The tibial and femoral cartilages of 5-day-old Sprague-Dawley (SD) rats were dissected, added to 10 mL of 3% (w/v) collagenase Type II and digested for 45 min at 37 °C, two times. The cartilage pieces were retrieved and placed in 10 mL of collagenase Type II solution at 0.5 mg/mL overnight at 37 °C. Chondrocytes were cultured at 37 °C in a 5% CO_2-humidified incubator in DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin. For the analysis of cell viability, chondrocytes were seeded at 2×10^5 cells/mL, incubated for 5 days, and treated with varying concentrations (0.5, 1, 2, and 3 mg/mL) of AECF for 24 h. Following incubation, cell viability was determined using the MTT assay.

2.4. Nitric oxide (NO) assay

Chondrocytes were seeded at 2×10^5 cells/mL in 12-well plates. Chondrocytes were pretreated with varying concentrations (0.5, 1, 2, and 3 mg/mL) of AECF for 1 h and subsequently cultured with IL-1β (20 ng/mL) for 24 h. Nitrite accumulation in the culture medium was measured as an indicator of NO production, based on the Griess reaction. In brief, 100 μL of each supernatant from AECF-treated samples was mixed with an equal volume of Griess reagent (1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine) in a dark room for 10 min. Absorbance was then measured at 540 nm on a microplate reader (Epoch Bioteck; Bio-Tek Instruments Inc., Winooski, VT, USA). The nitrite concentration was determined by comparison with a standard curve of sodium nitrite.

2.5. Isolation of total RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from chondrocytes using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using 1 μg of total RNA and a PrimeScript™ 1 st strand cDNA Synthesis kit (Takara Bio Inc., Otsu, Japan). cDNA was amplified via PCR using the following primers: rat *Mmp13* (562 bp) sense 5′- GGCAAAAGCCATTTCATGCTCC CA-3′ and antisense 5′- AGACAGCATCTACTTTGTCGCCA-3′, rat *Gapdh* (578 bp) sense 5′- TGGTGCTGAGTATGTCG TGGAGTC-3′ and antisense 5′- AGACAACCTGGTCCTCA GTGTAGC-3′. The PCR results were visualized via agarose gel electrophoresis. Deviations in the samples represent data from three independent experiments. *β-actin* was used as an internal control to evaluate relative expression of *Mmp13*.

2.6. Western blotting analysis

Cells were lysed using a protein extraction reagent (iNtRON Biotechnology, Seoul, Republic of Korea) for 30 min on ice. The