CARTILAGE
2022, Vol. 13(4) 157–170
© The Author(s) 2022
DOI: 10.1177/19476035221129573
journals.sagepub.com/home/CAR
SAGE

Anti-Inflammatory and Chondro-Protective Effects of Acidic Polysaccharide from Enteromorpha Prolifera in Experimental Models of Osteoarthritis *In-Vitro* and *In-Vivo*

Chih-Chien Wang[1], Jeng-Wei Lu[2], Kuang-Hsing Chiang[3,4,5,6], Yu-Shuan Cheng[7], You-Hsiang Chu[8], Yi-Jen Peng[9], Chia-Hui Cheng[7], Chia-Yu Chang[10,11], and Jiunn-Jye Chuu[7,12]

Abstract

Objective. Osteoarthritis (OA) progression has been shown to increase the expression of inflammatory cytokines in joints, leading to the destruction of cartilage matrix. Interleukin (IL)-1β is a potent inflammatory cytokine associated with osteoarthritic synovial fluid. The protective effects of polysaccharides from Enteromorpha prolifera against acute hepatic injury was reported. *Design.* In this study, we examined the effects of Enteromorpha polysaccharide extracts (EPEs) in the treatment of OA. The effects of the EPEs were assessed using an IL-1β-stimulated SW1353 and SW982 cells. The expression levels of specific mRNA and proteins were evaluated using semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR) and western immunoblotting. An OA animal study involving C57BL/6J mice was also conducted to assess the effects on tactile sensitivity and anterior cruciate ligament transection (ACLT). *Results.* Acidic polysaccharide extract (APE) was shown to significantly reduce cytokine and chemokine mRNA levels in IL-1β-stimulated SW1353 and SW982 cells and attenuate the expression of proinflammatory cytokines and p38/AP-1 in SW1353 cells. APE was also shown to minimize the effect of osteolytic lesions in the knee joints of ACLT-induced osteoarthritic mice. *Conclusions*. APE is a potent inhibitor of joint degeneration associated with OA.

Keywords

osteoarthritis, enteromorpha prolifera, oxidative stress, chondrocyte, interleukin-1β

Introduction

Osteoarthritis (OA) is a chronic disease characterized by articular cartilage degeneration and joint inflammation, which leads to chronic disability.[1-3] It has been estimated that 18% of females and 9.6% of males beyond the age of 40 have symptomatic OA. Increased expression of inflammatory mediators in the joints has been found to lead to the destruction of the cartilage matrix in the progression of OA.[3-5] Current treatments for OA are meant to alleviate pain, delay or prevent disease progression, and maintain the quality of life.[6] However, most treatment regimens focused on relieving pain through the use of systemic or local drugs, physical therapy, or surgery.[7,8]

Chondrocyte function has been found to be associated with inflammatory cytokines production and catabolic factors that contribute to the promote the development of OA.[4] In many cases, this leads to the transient accumulation and activation of innate immune cells. Interleukin (IL)-1β is a potent inflammatory cytokine identified in osteoarthritic synovial fluid.[9] Cartilage catabolic effects, cell proliferation, and regeneration can be triggered by IL-1β expression. IL-1β expression can also activate the mitogen-activated protein (MAP) kinase signaling pathway and enhance the expression of cartilage matrix-degrading enzymes, such as cyclooxygenase 2 (COX2), IL-6, tumor necrosis factor alpha (TNF-α), and matrix metalloproteinases (MMPs).[3] The expression of chemokines, such as chemokine (C-C motif) ligand 1 (CCL-1), in OA also plays an important role.[10] Elevated cytokine expression has been linked to joint disorders and other pathological conditions. Note that the use of non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of such disorders can have many adverse effects.[11,12]

Numerous plant extracts possess anti-inflammatory and antioxidant effects. Enteromorpha prolifera is a form of the green algae *Ulva* widely distributed along seashores around the world.[13,14] Polysaccharides are the major active components isolated from Enteromorpha prolifera. They have been shown to have multiple beneficial effects, including anti-oxidation, anti-microbial actions, anti-hyperlipidemia, immunomodulation, and glucose metabolism. Recent studies have shown that Enteromorpha prolifera accelerates the metabolism of fat and decreases plasma lipoproteins levels[15]; however, the protective effects of Enteromorpha prolifera against OA have not been widely studied. In this study, Enteromorpha polysaccharide extracts (EPEs) were obtained using different extraction methods. Our objective was to investigate the role of Enteromorpha prolifera on chondrocytes, macrophages, and synovium cells in OA models, and thereby assess the clinical applicability of Enteromorpha prolifera in OA treatment.

Materials and Methods

Materials and Reagents

Celecoxib (Pfizer Inc., Manhattan, NY), Ketoprofen (Swiss Co., Ltd., Tainan, Taiwan), Zoletil (Virbac., Grasse, Carros, France), and recombinant mouse IL-1β (ACROBiosystems., Newark, DE) were purchased from commercial companies. p38 (MAPK) beta and AP1/JUN antibodies were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Rabbit polyclonal COX-2 and TNF-α, horseradish peroxidase (HRP)-anti-rabbit, or mouse IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Delaware, CA). Entermorpha prolifera was used to prepare EPEs included hot water polysaccharide extract (HPE), basic polysaccharide extract (BPE), and acidic polysaccharide extract (APE), provided by the Kinmen Fisheries Research Institute, Taiwan. Pulverized EPEs were extracted at a vehicle to raw material ratio of 50 mL/g via ultrasonic-assisted extraction using a frequency of 40 kHz at 100 °C over a period of 1.5 h. Neutralized water-soluble EPEs were precipitated by the addition of 95% EtOH (v/v) over a period of 48 h, followed by protein depletion. Finally, the extracts were concentrated using a rotary evaporator and powdered using a freeze-drying system (Kingmech, FD 20L-6S, Taipei, Taiwan). The individual total polysaccharide yield was determined via the phenol-sulphuric acid method using glucose as a standard reference.

Determination of Cell Viability

SW1353 and SW982 cells were seeded in 96-well flat-bottomed microtiter plates were maintained in an incubator at 37 °C. After incubation for 24 h with the HPE (0.04 mg/mL), BPE (0.13 mg/mL), APE (0.4 mg/mL), or IL-1β (5 ng/mL), the *in vitro* cytotoxicity of each EPE was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays.[16]

Inhibition of Nitric Oxide (NO) Production

All measurements of nitrite (a stable metabolite of NO) using a commercial NO detection kit (Griess Reagents System, Promega, Madison, WI) and according to manufacturer's instructions. Briefly, each supernatant was combined with an equal volume of Griess reagent in a 96-well plate and incubated for 10 min. Absorbance was then measured using an enzyme linked immunosorbent assay (ELISA) reader at a wavelength of 540 nm (Tecan). A sodium nitrite standard curve was used to calculate the NO level.[17]

[1]Department of Orthopedics, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[2]Antimicrobial Resistance Interdisciplinary Research Group, Singapore-MIT Alliance for Research and Technology, Singapore
[3]Taipei Heart Institute, Taipei Medical University, Taipei, Taiwan
[4]Division of Cardiology and Cardiovascular Research Center, Taipei Medical University Hospital, Taipei, Taiwan
[5]Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[6]Graduate Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, Taipei, Taiwan
[7]Department of Biotechnology and Food Technology, College of Engineering, Southern Taiwan University of Science and Technology, Tainan, Taiwan
[8]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan
[9]Department of Pathology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[10]Department of Neurology, Chi Mei Medical Center, Tainan, Taiwan
[11]Center for General Education, Southern Taiwan University of Science and Technology, Tainan, Taiwan
[12]Pharmacy Department, Wei Gong Memorial Hospital, Miaoli, Taiwan

Corresponding Authors:
Chia-Yu Chang, Department of Neurology, Chi-Mei Medical Center, No. 901, Zhonghua Rd., Yongkang Dist., Tainan 71004, Taiwan.
Email: chiayu.chang7@msa.hinet.net

Jiunn-Jye Chuu, Department of Biotechnology and Food Technology, College of Engineering, Southern Taiwan University of Science and Technology, No. 1, Nan-Tai Street, Yungkang Dist., Tainan 710301, Taiwan.
Email: jjchuu@stust.edu.tw

Table 1. Oligonucleotides Primers Used in This Study.

Gene Name	Primer Name	Sequence (5' to 3')
TNF-α	TNF-α-F	CGCGGATCATGC TTTCTGTG
	TNF-α-R	GGACTAGCCAGGAGGGAGAA
IL-1β	IL-1β-F	TCA AAGCAATGTGCTGGTGC-3
	IL-1β-R	ACCTAGCTGTCAACGTGTGG
IL-6	IL-6-F	CAAATTCGGTACATCCTCGAC
	IL-6-R	CTACGTTATTGGTGGGGACTG
MMP-9	MMP-9-F	ATGAGTTCGGCCACGCGCTGGGCTT
	MMP-9-R	GACTCAAAGCAGTAGCCGT
MMP-13	MMP-13-F	CATTTGATGGGCCCTCTGGCCTGC
	MMP-13-R	GTTTAG GGTTGGGGTCTTCATCTC
CCL-11	CCL11-F	CCCAACCACCTGCTGCTTTAACCTG
	CCL11-R	TGGCTTTGGAGTTGGAGAGATTTTTGG
CCR-3	CCR3-F	CCACACTCTGAGAATGACCATC
	CCR3-R	GCCCAGGTGCATGAGCAAGTGCC

TNF-α = tumor necrosis factor alpha; IL = Interleukin; MMP = matrix metalloproteinases.

The mRNA Isolation and Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (sqRT-PCR)

We isolated mRNA and synthesized cDNA from SW1353 and SW982 cells after administering EPEs (0.01 or 0.04 mg/mL) plus IL-1β (5 ng/mL). The mRNA expression level was quantified using sqRT-PCR as previously described,[18] and each experiment was performed in triplicate. All primers are listed in **Table 1.**

Western Blotting Analysis

The protein extraction and western blot analysis method was performed in accordance with methods described previously.[19] Briefly, protein samples were transferred to a nitrocellulose membrane (NC) via a 4% stacking gel and 10% resolving gel electrophoresis for analysis. After blocking with non-fat milk buffer, the membranes were then incubated with anti-TNF-α (1:500) or anti-COX-2 (1:2,000) antibodies for 1 h. The NC membranes were incubated with the appropriate secondary antibodies diluted 1:5,000 for 1 h. In measuring the immunoreactive proteins expression of TNF-α and COX-2, followed by development using a cold-light image analyzer (FUJIFILM LAS-3000).[16]

Immunofluorescence Staining

To perform an immunofluorescence staining, SW1353 cells were fixed in 4% paraformaldehyde in PBS a for 30 min. Following this, the cells were stained with rabbit anti-p38 (MAPK) beta or anti-AP-1 (JUN) antibodies (1:200) for overnight. After washing with PBS, the cells were then incubated with anti-rabbit fluorescein-5-isothiocyanate (FITC) (1:500) at room temperature for 1 h. Finally, cells were stained using DAPI at room temperature for 10 min, after which the cells underwent imaging using an Olympus IX81 microscope (Olympus, Tokyo, Japan).

Molecular Weight Determination by Gel-Permeation Chromatography

A test sample of EPE was diluted to a concentration of 5 mg/mL in 20 mL of distilled water and utilized for injection. The samples were eluted using distilled water at a flow rate of 0.6 mL/min on a 45 °C column for gel-permeation chromatography (GPC). Dextran T10, T40, T70, and T500 were used to calibrate the polysaccharide molecular weight estimations. The regression line of the standard molecular weight versus elution volume plot was used to calculate the molecular weight of each fraction.

Animals and Treatment

Mice were purchased from the BioLASCO Taiwan Co., Ltd. All experimental protocols and procedures involving C57BL/6J mice (Gender: male; Age: 5 to 6 weeks old; Body weight: 25 to 30 g; Total numbers: $n = 35$) were approved by the Institutional Animal Care and Use Committee (IACUC) of the Southern Taiwan University of Science and Technology (approval no. STUST-IACUC-106-02), and they were conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. For the treatment of mice, BPE, APE or celecoxib (100, 300 or 30 mg/kg, respectively) was conducted over a period of 28 days by oral administration, and the effect of celecoxib is relative to the treated group as a positive control. Specifically, a single

experimenter (CPJ) with more than 1 year of experience in oral gavage of mice administered all treatments once daily for 28 consecutive days. The body weight of mice was assessed on a weekly basis during the tests.

Tactile Sensitivity Testing and Radiographic Assessments

Tactile sensitivity assay was performed as previously described.[20] Briefly, when the stimulus was administered, a positive reaction was defined as a quick withdrawal of the hind paw, and the number of positive responses for each stimulus was recorded. Tactile threshold was defined as a withdrawal reaction in 5 of 10 trials to a particular stimulus intensity, and thresholds were calculated once per mice. To understand changes in bone lesions and bone remodeling, and X-rays were obtained using a radiographic assessment method and Faxitron Specimen Radiography System (Field Emission Corp., McMinnville, OR).[20]

Histopathological Examination

Harvested samples were fixed in 4% paraformaldehyde, followed by dehydration and embedding into paraffin. 5-μm sections were made for hematoxylin and eosin (HE) staining with safranin-O/fast green counterstain (Sciencell Research Laboratories., Carlsbad, CA). Stained slides were mounted in Micromount (Leica) and Motic BA 400 microscope with Motic Advance 3.0 software (Motic Co., Fujian, China). Finally, the classification of OA cartilage in the grading of animal cartilage were based on established criteria as previously reported,[20] by two blinded, experienced pathologists.

Statistical Analysis

Data were presented as mean ± SEM and analyzed using Sigma Plot 10.0 software (SPSS Inc., Chicago, IL). All data were representative of at least three independent triplicate experiments. The statistical significance of mean values between groups was evaluated by *t* one-way analysis of variance (ANOVA) and Tukey's test. $^{*}P < 0.05$ and $^{**}P < 0.01$; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ was chosen to be statistically significant, respectively.[21]

Results

APE Significantly Inhibits NO Production with the Lowest Cytotoxicity in IL-1β-Stimulated SW1353 and SW982 Cells

HPE, BPE, and APE (0.04, 0.13 and 0.4 mg/mL, respectively) as well as IL-1β (5 ng/mL) were tested *in vitro* using MTT assays in SW1353 and SW982 cells over a period of 24 h. APE (at any dose) and IL-1β (at 5 ng/mL) were shown not significant effect on the growth of SW1353 (**Fig. 1A**) or SW982 cells (**Fig. 1B**). HPE at a high-dose (0.4 mg/mL) had a remarkable effect on both cell lines, reducing cell viability to 73% and 66% of the controls ($^{*}P < 0.05$ and $^{*}P < 0.05$). BPE at a high dose (0.4 mg/mL) had cytotoxic effects on SW982 cells (**Fig. 1B**) after 24 h of incubation. Following co-treatment with IL-1β and HPE, BPE, or APE for 24 h, we observed a marked increase in NO levels (measured as the stable non-volatile break-down product nitrite), compared to IL-1β treatment alone ($^{**}P < 0.01$). Only at a low dose (0.01 mg/mL) did APE significantly suppress NO production in IL-1β-stimulated SW1353 (**Fig. 1C**) or SW982 cells (**Fig. 1D**), compared to IL-1β treatment alone ($^{\#\#}P < 0.01$ and $^{\#\#}P < 0.01$, respectively).

APE Profoundly Reduces Cytokine and Chemokine mRNA in IL-1β-Stimulated SW1353 and SW982 Cells

Our objective in this study was to investigate the effects of EPEs (HPE, BPE, and APE) on cartilage integrity (joint wear and deformation) in the development of OA and to identify which EPE is the best candidate for development as a pharmacologic treatment aimed at preventing OA progression. TNF-α, IL-1β, and IL-6 are the main pro-inflammatory cytokines, and MMP-9 and MMP-13 are major enzymes targeting type II collagen, type IV collagen, osteonectin, and proteoglycan in cartilage. Chemokines (CCL-11 and CCR-3) induce the expression of MMP-13 and other catabolic mediators in articular chondrocytes. They promote IL-6 production from synovial fibroblasts, which are involved in the pathophysiology of OA. After a 24-h incubation with EPEs plus IL-1β, the expression of genes encoding proteins in terms of synovial joint and cartilage homeostasis was examined in SW1353 or SW982 cells.

sqRT-PCR results revealed significantly elevated mRNA levels of *IL-1*β, *IL-6*, *TNF*-α, *MMP-9*, *MMP-13*, *CCL-11*, and *CCR-3* in IL-1β-treatment group, compared with the vehicle control ($^{**}P < 0.01$). Low-dose (0.01 mg/mL) treatment using BPE or APE was shown to reduce the mRNA levels of *IL-1*β, *IL-6*, *TNF*-α, *MMP-9*, *CCL-11*, and *CCR-3* (but not *MMP-13*) in SW1353 (**Fig. 2A**) and SW982 cells (**Fig. 2B**), compared with the IL-1β-treatment group ($^{*}P < 0.05$, respectively). Interestingly, APE treatment at a high dose (0.04 mg/mL) significantly decreased the mRNA levels of all biomarkers in SW1353 cells ($^{**}P < 0.01$, respectively) but only decreased the mRNA levels of *IL-1*β, *IL-6*, *TNF*-α, *MMP-9*, *CCL-11*, and *CCR-3* in SW982 cells ($^{**}P < 0.01$), respectively, compared to IL-1β-treatment group. Overall, by decreasing upstream cytokine signaling and the gene expression of several proinflammatory cytokines, treatment with APE had anti-inflammatory effects *in vitro* in a dose-dependent manner.

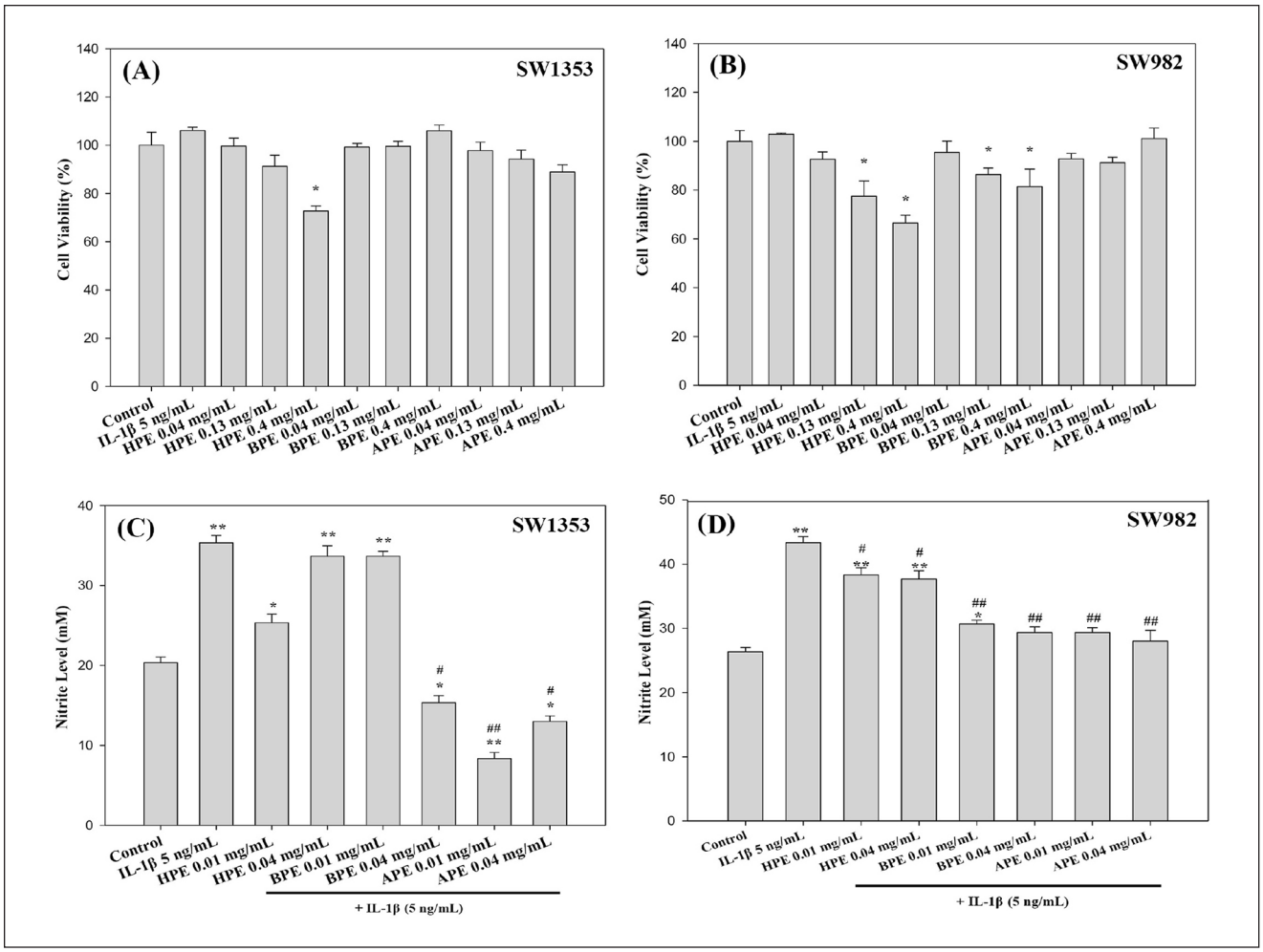


Figure 1. Cytotoxicity assay and effect of EPEs on NO production in IL-1β-stimulated SW1353 and SW982 cells. MTT assay results from SW1353 **(A)** and SW982 **(B)** cells after a 24-h incubation with either IL-1β or HPE, BPE, or APE. $^{*}P < 0.05$ indicates a significant difference compared to the control. At 24 h after IL-1β co-treatment with HPE, BPE, or APE, we estimated NO production in the cell culture medium using the Griess assay: SW1353 **(C)**; and SW982 **(D)**. Untreated cells served as nonstimulated controls. Cells treated with IL-1β served as stimulated controls. $^{*}p < 0.05$; $^{\#}p < 0.05$ and $^{**}p < 0.01$; $^{\#\#}p < 0.01$ indicates a significant difference compared to d.d H_2O or IL-1β treatment alone. EPE = Enteromorpha polysaccharide extracts; NO = Nitric Oxide; IL = Interleukin; HPE = hot water polysaccharide extract; BPE = basic polysaccharide extract; APE = acidic polysaccharide extract.

APE Attenuated the Expression of Proinflammatory Cytokines and p38/AP-1 in IL-1β-Stimulated SW1353 Cells

To elucidate the anti-OA effects of EPEs, we analyzed the expression of cellular transforming growth of TNF-α and COX-2 protein in SW1353 cells relative to β-actin after 24 h. We observed significantly lower HPE protein expression in SW1353 cells only at the high-dose (0.04 mg/mL) BPE and APE treatment groups ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, respectively), compared to IL-1β treatment alone (**Fig. 3A**). We observed lower COX-2 expression levels (**Fig. 3B**) in the low-dose (0.01 mg/mL) BPE and APE treatment groups, compared with the celecoxib (10 μM)-treated group ($^{\#}P < 0.05$, $^{\#}P < 0.05$, respectively). Interestingly, a high-dose (0.04 mg/mL) of HPE had no inhibitory effect on COX-2 expression but dramatically increased the expression of TNF-α, compared to the IL-1β treatment group ($^{***}P > 0.001$).

Proinflammatory genes encoding TNF-α and COX-2 are transcribed by these factors in injured chondrocytic cells. We investigated the inhibitory effects of EPEs (HPE, BPE, and APE) on the IL-1β-induced p38/AP-1 pathway via immunofluorescence of chondrocytes. Administering IL-1β (5 ng/mL) significantly elevated the fluorescence intensity of p38 (**Fig. 3C**) and AP-1 (**Fig. 3D**) after 24 h, whereas administering a low dose of APE (0.01 mg/mL) had a strong inhibitory effect on p38 (MAPK), FITC (+) cells; in green color, in SW1353 cells, compared to IL-1β treatment alone ($^{\#}P < 0.05$, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$, respectively, **Fig. 3E**). DAPI staining confirmed that IL-1β (5 ng/mL)

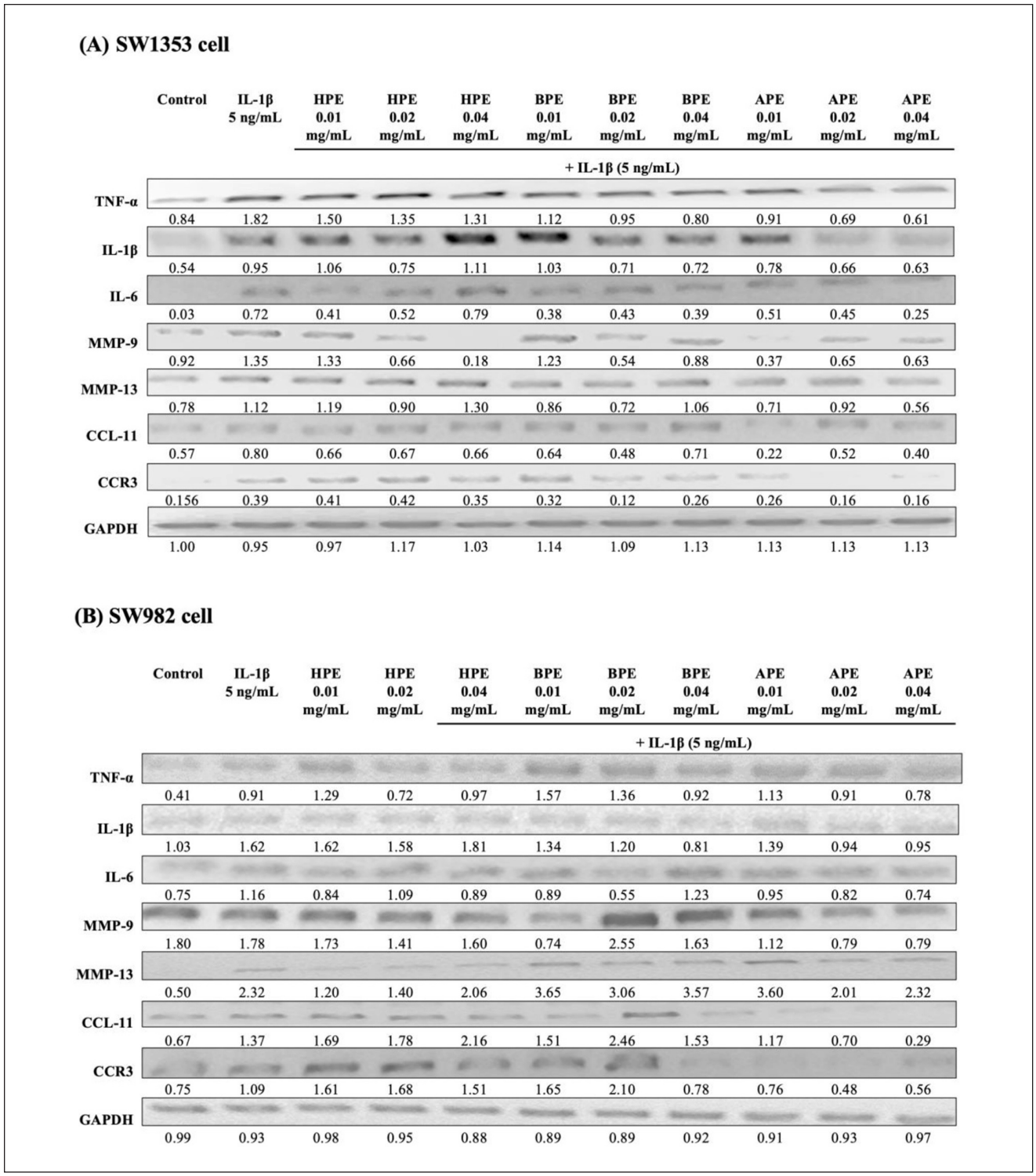


Figure 2. sqRT-PCR results indicating the effects of EPEs on the gene expression of cytokines and chemokines in IL-1β-induced SW1353 or SW982 cells. Cell lines SW1353 **(A)** and SW982 **(B)** were incubated in the presence of HPE, BPE, or APE followed by stimulation using IL-1β for 24 h. The treated cells were analyzed using sqRT-PCR to estimate the expression levels of *IL-1β*, *TNF-α*, *IL-6*, *MMP-9*, *MMP-13*) and *CCL-11* and *CCR-3*, compared to the vehicle control. GAPDH was used as an internal control. sqRT-PCR = semi-quantitative reverse transcription polymerase chain reaction; EPE = Enteromorpha polysaccharide extracts; IL = Interleukin; HPE = hot water polysaccharide extract; BPE = basic polysaccharide extract; APE = acidic polysaccharide extract; TNF-α = tumor necrosis factor alpha; MMP = matrix metalloproteinases.

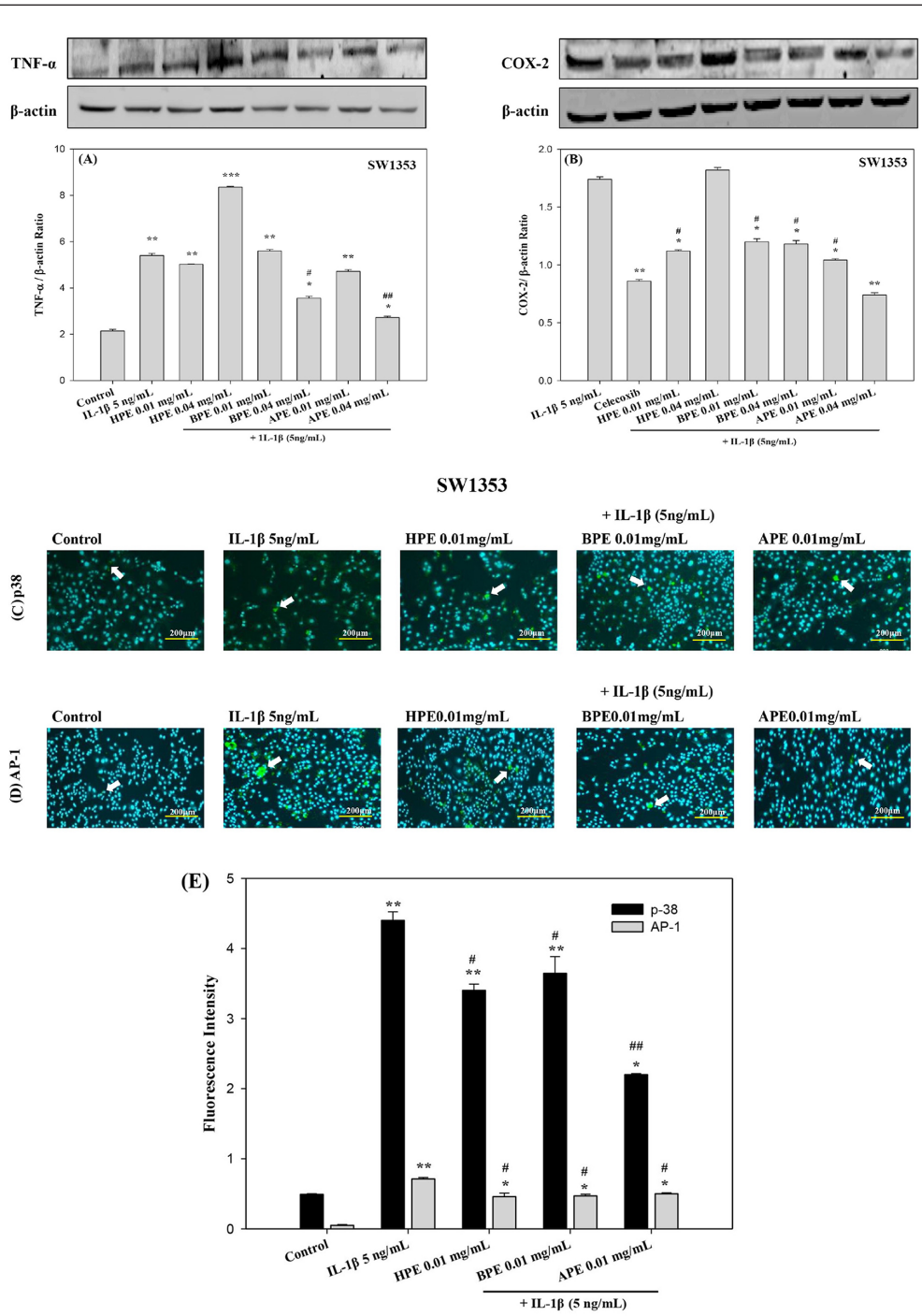


Figure 3. Protein expression of pro-inflammatory cytokines or p38 (MAPK) and AP-1 (JUN) in IL-1β-induced SW1353 cells using western blot analysis or immunofluorescence staining. Chondrosarcoma cell line SW1353 underwent co-stimulation with IL-1β and HPE, BPE, or APE for 24 h, after which the relative protein expression levels of COX-2 and TNF-αwere estimated using Western blot analysis; β-actin as a control. The data are respectively expressed as the COX-2/β-actin **(A)** and TNF-α/β-actin **(B)** ratios. Immunofluorescence staining was used to determine p38 (MAPK) **(C)** and AP-1 (JUN) **(D)** expression in IL-1β-induced SW1353 cells. Fluorescence images of p38 (MAPK) and AP-1 (JUN) FITC-conjugated goat anti-mouse IgG antibodies stained green (nucleolar), where the nuclei are counterlabeled using the DNA-selective dye DAPI (blue). Bars=20 μm; 40X magnification. **(E)** Quantification of fluorescence intensity p38 (MAPK) and AP-1 (JUN). Scale Bars =20 μm; 40X magnification. *$p < 0.05$; #$p < 0.05$ and **$p < 0.01$; ##$p < 0.01$ indicates a significant difference compared to d.d H_2O or IL-1β treatment alone. IL = Interleukin; HPE = hot water polysaccharide extract; BPE = basic polysaccharide extract; APE = acidic polysaccharide extract; COX-2 = cyclooxygenase 2; TNF-α = tumor necrosis factor alpha; FITC = fluorescein-5-isothiocyanate.

significantly suppressed SW1353 cell proliferation by decreasing the number of DAPI (+) cells at 24 h (in blue color), compared to the control ($^{*}P < 0.05$). Treatment with APE or BPE at a low dose (0.01 mg/mL) for 24 h led to an obvious decrease in the number of DAPI (+) cells at 24 h, compared to IL-1β (5 ng/mL) treatment alone (**Fig. 3C** and **3D**).

Properties of EPE Polysaccharides

Using the phenol/sulfuric acid method, we discovered that the three extracts of Entermorpha prolifera (HPE, BPE, and APE) respectively contained 21.3, 18.6, and 19.5 mg/mL of crude water-soluble polysaccharides (data not shown). Based on linear regression calculations of standard dextrans, the molecular weight of the polysaccharides, which is generally presented as a mean value distributed over a given range, is shown in **Fig. 4**. In this study, GPC revealed that the mean molecular weight distributions of the EPEs ranged from 0.590 kD to 27,584.667 kDa. GPC profiles revealed that the average molecular weights (Mws) were as follows: 7 HPE fractions (peak no.1: 27,584.667 kDa; peak no.2: 5,759.930 kDa; peak no.3: 4,835.963 kDa; peak no.4: 13.234 kDa; peak no.5: 3.514 kDa; peak no.6: 2.08 kDa; peak no.7: 0.612 kDa; **Fig. 4A**), 6 BPE fractions (peak no.1: 10,621.6 kDa; peak no.2: 198.917 kDa; peak no.3: 123.884 kDa; peak no.4: 5.810 kDa; peak no.5: 1.049 kDa; peak no.6: 0.861 kDa; **Fig. 4B**), and 5 APE fractions (peak no.1: 572.064 kDa; peak no.2: 4.310 kDa; peak no.3: 2.005 kDa; peak no.4: 0.590 kDa; peak no.5: N.A; **Fig. 4C**).

FTIR analysis of HPE, BPE, and APE revealed the transmittance at various wave numbers, to expose the key functional groups in the polysaccharides. Wide stretching peaks at 3356.40 cm^{-1}(HPE), 3350.26 cm^{-1}(BPE), and 3367.76 cm^{-1}(APE) can be attributed to hydroxyl functional groups. The peaks appearing at 1222.3 cm^{-1} (HPE), 1230.85 cm^{-1}(BPE), and 1201.3 cm^{-1}(APE) can be attributed to S=O bending vibrations. The absorption peaks at 846.35 cm^{-1} (HPE), 848.49 cm^{-1}(BPE), and 849.16 cm^{-1}(APE) can be attributed to the stretching and bending vibrations of COS. The weak bands at 1031.21 cm^{-1} (HPE), 1026.80 cm^{-1}(BPE), and 1032.05 cm^{-1}(APE) can be attributed to an α-glycosidic bond (COH and COC) in the polysaccharide framework (data not shown).

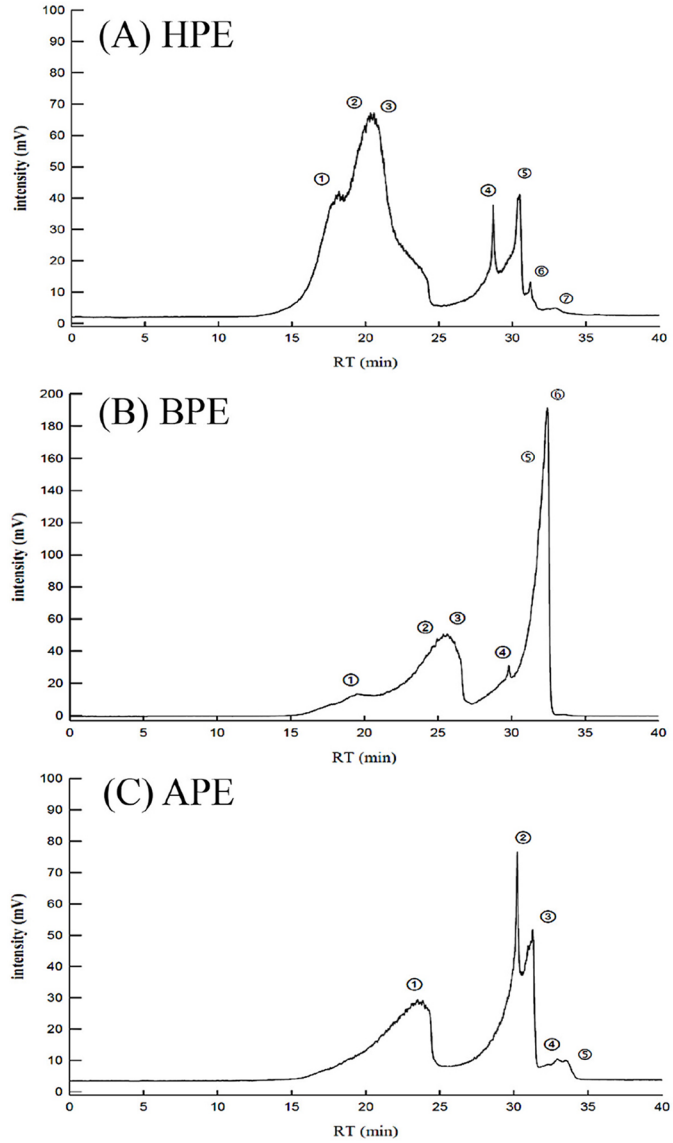
Alleviating the Effects of ACLT-induced Joint Pain and EPEs Reduced the Extent of Osteolytic Lesions in Knee Joints in ACLT-Induced OA Mice

PWT decreases via the progression of OA and corresponding secondary clinical symptoms, which suggests the occurrence of mechanical hyperalgesia. Thus, we used the von Frey test to examine the effects of HPE, BPE, and APE on pain in mice that underwent ACLT surgery (**Fig. 5A**). Baseline (BL) von frey testing was performed twice at an interval of 1 week, during which no repeated measure effects or interactions were noted. Thus, to facilitate analysis, we averaged the BL data. Relative to averaged BL values, ACLT surgery (saline-treatment alone) produced significant, progressive, and robust (almost 50%) decreases in withdrawal thresholds in both hind paws, compared with the sham-operation group ($^{**}P < 0.01$). At 28 days of EPEs treatment after ACLT, BPE (300 mg/kg/day) and APE (100 and 300 mg/kg/day) were shown to decrease secondary allodynia (i.e., a decrease in thresholds) similar to that obtained using celecoxib (10 mg/kg/day) ($^{\#}P < 0.05$), compared to the saline group ($^{\#}P < 0.05$ and $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$, respectively). These results indicate that the ACLT-induced mechanical hyperalgesia in OA mice was due primarily to tactile hypersensitivity of the paw, which means that BPE or APE treatment could theoretically reduce much of the nerve pain. Note that low-dose APE treatment (100 mg/kg/day) would be expected to have the strongest effect.

Abnormal bone architecture and soft bone loss in the subchondral bone of the femur and tibia observed using radiographic analysis. At 28 days post-sham surgery, the femur and tibia in sham-operated controls were relatively well preserved. (**Fig. 5B**); however, bone lesions and prominent bone formation extending into soft tissue were found in the saline-treated ACLT group (**Fig. 5C**). A high dose of BPE (300 mg/kg/day) (**Fig. 5E** and **5F**) and moderate to high doses of APE (100 or 300 mg/kg/day) (**Fig. 5G** and **5H**) significantly decreased the extent of osteolytic lesions and bone spurs, compared to the saline group (**Fig. 5C**). Compared to sham-operated controls, the similar results were not observed between the 10 mg/kg/day of celecoxib treatment (**Fig. 5D**) and the saline treatment (**Fig. 5C**). The appearance of the bones in the groups that received APE or BPE (100 or 300 mg/kg/day) was similar to that of the sham-operated control. Overall, the effects of APE treatment were more pronounced than that of BPE and celecoxib, both of which failed to prevent subchondral bone formation or moderate the loss of cartilage.

EPEs Moderated Cartilage Degeneration in the Knee Joints of ACLT-Induced OA Mice

The effect of EPEs on articular cartilage and subchondral bone in OA mice was analyzed by staining bone sections with HE (**Fig. 6A to 6G**). The population with a smooth surface and an intact superficial and upper zone of the cartilage of normal chondrocyte was found in sham-operated control (**Fig. 6A**). We also observed chondrocyte degeneration, erosion of articular cartilage, matrix changes, and typical chondrocyte clustering with apparent hypocellularity at saline group (**Fig. 6B**). Administering BPE or APE at a dose of 300 mg/kg/day (**Fig. 6E** and **6G**) outperformed celecoxib treatment (**Fig. 6C**) in maintaining a round chondrocyte



Peak N0.	Retention Time (Min)	MW (Da)
1	18.2	*27584667
2	20.35	*5759939
3	20.59	*4835963
4	28.69	13234
5	30.51	3514
6	31.23	2080
7	32.91	612

Peak N0.	Retention Time (Min)	MW (Da)
1	19.51	*10621600
2	24.97	198917
3	25.62	123884
4	29.82	5810
5	32.17	1049
6	32.44	861

Peak N0.	Retention Time (Min)	MW (Da)
1	23.52	*572064
2	30.23	4310
3	31.28	2005
4	32.96	590
5	33.45	/

Figure 4. Profile of the molecular weight distribution of EPEs using gel permeation chromatography analysis. We obtained the molecular weight distributions of HPE **(A)**, BPE **(B)**, and APE **(C)** from gel-permeation chromatography using the equations used to calculate standard dextran linear regression. The polysaccharides from the EPEs ranged from >5000 kDa to <10 kDa. The data represents the average molecular weight of the entire polymer distribution. EPE = Enteromorpha polysaccharide extracts; HPE = hot water polysaccharide extract; BPE = basic polysaccharide extract; APE = acidic polysaccharide extract; MW = molecular weights.

phenotype and smooth cartilaginous tissue. In histopathologically classifying the severity of osteoarthritic lesions (denudation or deformation) in the cartilage of ACLT-induced mice after 28 days, we employed the Mankin score based on OA grading of cartilage structure and cell distribution (**Fig. 6H**). Administering BPE (100 or 300 mg/kg/day) or APE (100 or 300 mg/kg/day) had effects similar to those observed in the celecoxib group ($^{\#}P < 0.05$) in terms of reducing cartilage destruction in a dose-dependent manner. These treatments resulted in uneven surfaces and chondrocyte degeneration in the superficial layer as well as significantly lower Mankin scores ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$ vs. the saline-treated group) ($^{***}P < 0.001$ vs. the sham-operated control).

Accordingly, we sought to determine the efficacy of BPE and APE on OA progression by examining the structural integrity of articular cartilage (AC)/Meniscus (M) via microscope analysis with safranin-O staining (orange) and a

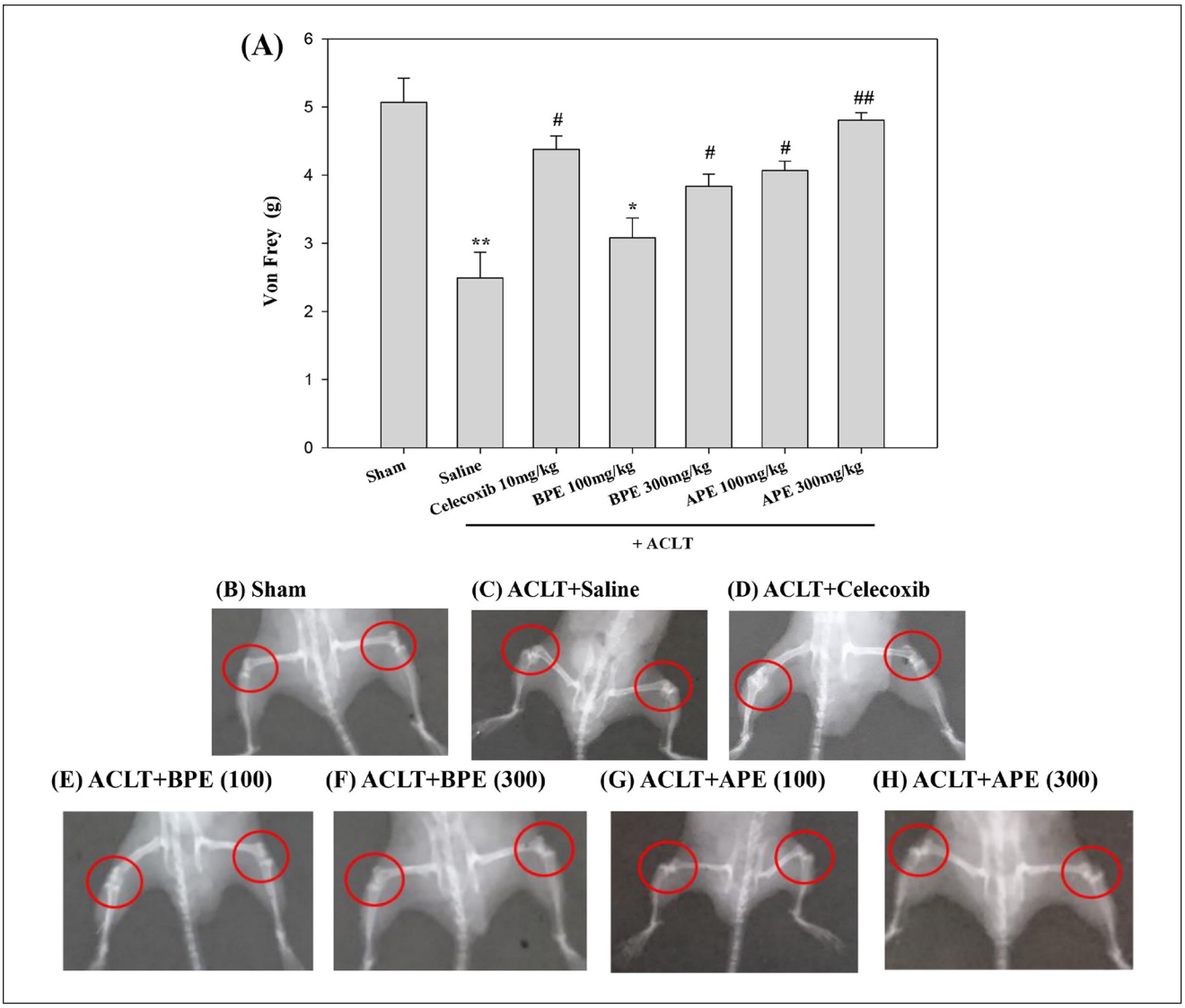


Figure 5. Influence of EPEs on tactile sensitivity in ACLT-induced OA-related allodynia, and radiographic results illustrating the effects of EPEs on ACLT-induced osteolytic bone lesions in OA mice. **(A)** Quantification of the tactile sensitivity of withdrawal threshold (PWT) values for ACLT-induced OA mice at each groups ($n = 5$). $^{*}p < 0.05$; $^{\#}p < 0.05$ and $^{**}p < 0.01$; $^{\#\#}p < 0.01$ indicates a significant difference compared to vehicle control (sham) or saline-treated (ACLT) mice. **(B to H)** Representative radiographic results from each groups in ACLT-induced osteolytic bone lesions in OA mice. EPE = Enteromorpha polysaccharide extracts; ACLT = anterior cruciate ligament transaction; OA = Osteoarthritis; BPE = basic polysaccharide extract; APE = acidic polysaccharide extract.

survey developed by Osteoarthritis Research Society International (OARSI) (**Fig. 6I to 6O**). In evaluating the symptoms of OA, severe OA damage to the cartilage and meniscus in the saline-treated group was detected 28 days after ACLT induction using safranin-O staining (a sensitive indicator of proteoglycan content). Note that at this point, moderate pathological osteoarthritis changes such as articular cartilage degeneration, proteoglycan loss, cartilage flutter, and cartilage erosion were found in mice. The average OARSI score in the saline-treated group was 13.7 ± 2.5 (**Fig. 6J** and **6P**), compared to sham-operated control, which obtained a score of 2.1 ± 0.6 (**Figs. 6I** and **6P**) ($^{**}P < 0.01$). Cartilage erosion and cartilage degradation in knee joints were less extensive in the APE treatment group (300 mg/kg/day, **Fig. 6O**) than in the BPE treatment group (300 mg/kg/day, **Fig. 6M**). Note that the OARSI score in the BPE group (**Fig. 6P**; 4.0 ± 0.8 and 7.4± 1.5, respectively; $^{\#\#\#}P < 0.001$ and $^{\#\#}P < 0.01$, respectively) was significantly lower than in the saline-treated group (**Fig. 6J** and **6O**). Overall, BPE or APE treatment (100 mg/kg/day) resulted in a suitable number of articular chondrocytes, the retention of proteoglycan, and a thinner calcified cartilage zone (**Fig. 6L** and **6N**) with a significantly lower OARSI score (**Fig. 6P**; 8.9 ± 1.3 and 5.2 ± 0.8, respectively; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$,

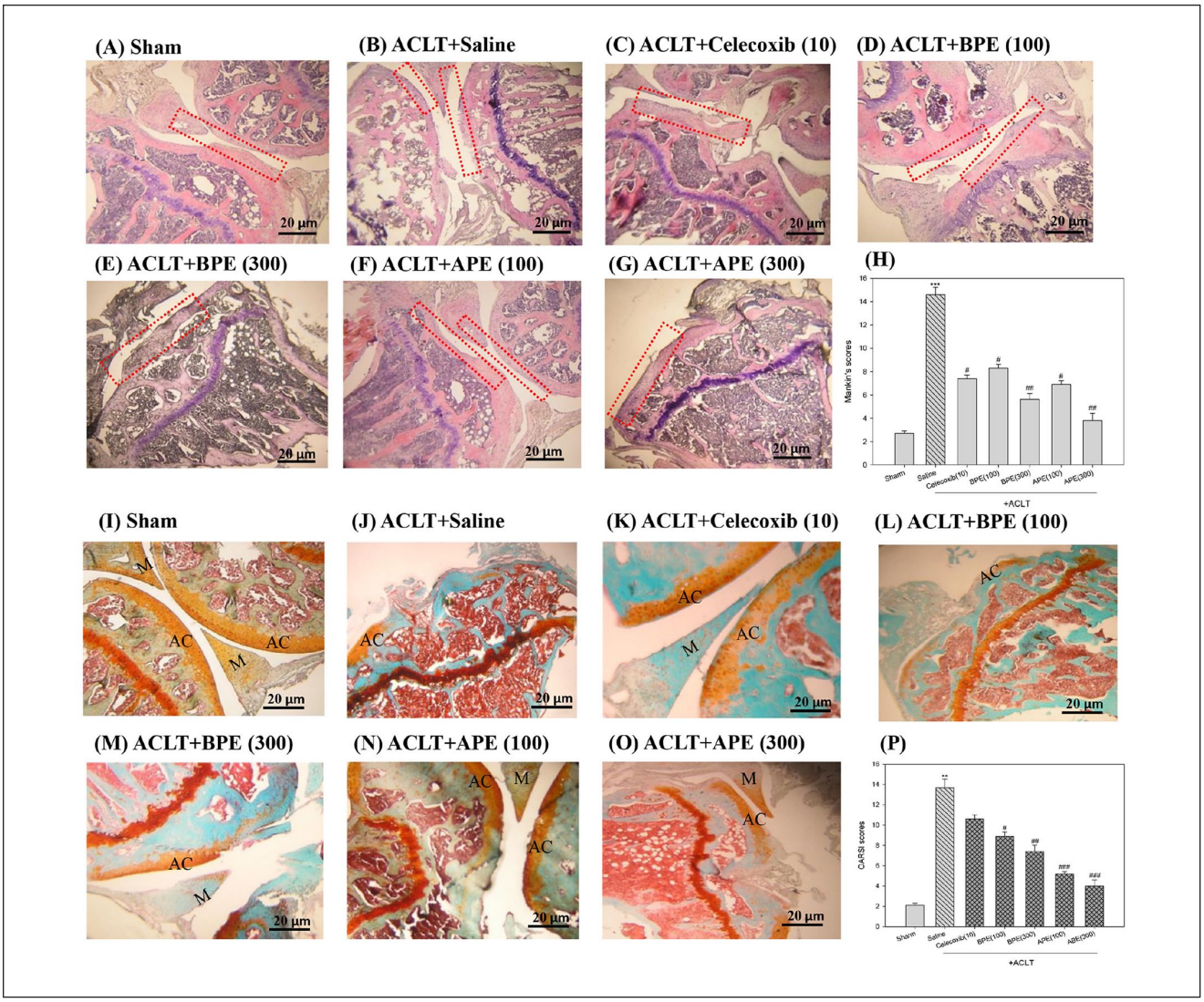


Figure 6. Morphological examination and histological changes of knee joints in ACLT-induced OA mice via HE or safranin-O/fast green staining. Representative joint sections from each groups in ACLT-induced OA mice. H&E staining showing signs of inflammation, synovial hyperplasia, and cartilage fissures along with disoriented and scattered dead chondrocytes **(A to H)**. Safranin-O/fast green staining (orange-red color indicates proteoglycan levels) showing degenerative changes in sagittal sections revealed the architecture (meniscus and cartilage) of the knee joint **(I to P)**. Scale Bars=20 μm; original magnification, × 400. M, meniscus; AC, articular cartilage. Quantification of the scores for all fields following staining at each groups ($n = 5$) (H and P). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, respectively. ACLT = anterior cruciate ligament transaction; OA = Osteoarthritis; HE = hematoxylin and eosin; BPE = basic polysaccharide extract; APE = acidic polysaccharide extract.

respectively) relative to the celecoxib group (**Fig. 6K** and **6P**; $^{\#}P > 0.05$). Taken together, these results indicate that APE significantly attenuated cartilage damage, making it a potent inhibitor of degenerative joint disease in cases of OA.

Discussion

OA is a discontinuous phasic disease with slow progression, which alters all tissue in the affected joint and leads to chronic disability. At present, evidence from a diabetic mice model suggests that polysaccharides, such as those extracted from Enteromorpha prolifera, play an important role in anti-inflammation.[22] In this study, we demonstrated that polysaccharides extracted from EPEs could attenuate the overexpression of cytokines and chemokines in IL-1β-insulted chondrocytes.

Previous studies have determined that IL-1β and TNF-α are secreted early in the progress of OA.[23] IL-1β is known to play a significant role in the pathogenesis of OA, *in vitro*. IL-1β insult stimulates COX-2 expression, which is responsible for PGE2 and has been implicated in bone resorption and joint pain in OA.[24] Researchers have also

reported on the upregulated production of MMPs and other inflammatory cytokines in cases of OA.[25] Inhibition of IL-1β-stimulated production of inflammatory mediators is a potential approach for the treatment of OA. In the present study, pretreatment with EPEs significantly reduced IL-1β-stimulated MMPs and COX-2 expression (**Figs. 3** and **4**).

MMPs as proteolytic enzymes, which play a key role in the degradation of articular cartilage,[26] making it a potential target for the OA treatment. The abundant expression of MMPs in joint disorders is largely due to their ability to degrade the extracellular matrix (ECM). The fact that MMP-1, MMP-3, and MMP-13 are mainly present in cartilage suggests that it is involved in OA progression.[27,28] Here, we sought to determine whether EPEs inhibit IL-1β-induced expression and/or activity of MMPs and inflammatory mediators in chondrocytes. IL-1β induced MMP-1, MMP-3, MMP-13, and a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) expression in human tendon cells was reported.[29] In this study, IL-1β was shown to induce the expression and activity of MMP-9 and MMP-13 in articular chondrocytes. Thus, it appears that EPEs exert chondroprotective effects, suggesting that EPEs could be a novel agent for the control of cartilage damage in OA **(Fig. 3)**.

The strong expression of inducible nitric oxide synthase (iNOS), COX2, and MMPs in arthritic joints can be attributed to a tightly regulated and synchronized signaling cascade involved in the MAPK signaling pathway.[30,31] NO generation suppresses iNOS phosphorylation, thereby delaying the activation of p38 and the extracellular signal-regulated kinase 1/2 (ERK1/2) MAP kinases. NO activates ERK signaling by downregulating MAP kinase phosphatase was reported.[32] MAPKs are activated via the phosphorylation of specific tyrosine and threonine residues in response to inflammatory signals. In our study, IL-1β was shown to enhance the p38 (MAPK) and ERK signaling pathways.

To further validate our *in-vitro* findings, we evaluated the potential of EPEs to alleviate ACLT-induced joint pain of osteolytic lesions or cartilage degeneration in knee joints at ACLT-induced OA mice **(Figs. 5** and **6)**. After 28 days of EPE treatment following ACLT, it was demonstrated that BPE (300 mg/kg/day) and APE (100 and 300 mg/kg/day) reduced secondary allodynia similar to that obtained using celecoxib (10 mg/kg/day). These results indicate that the BPE or APE treatment could theoretically reduce much of the nerve pain (**Fig. 5)**. Giving BPE (100 or 300 mg/kg/day) or APE (100 or 300 mg/kg/day) produced benefits similar to those seen in the celecoxib group in terms of lowering the cartilage damage when histopathologically grading the severity of osteoarthritic lesions in the cartilage of ACLT-induced mice after 28 days. In addition, safranin-O staining was used to find severe OA damage to the cartilage and meniscus in the saline-treated group at after 28 days ACLT induction. Treatment with BPE or APE (100 mg/kg/day) resulted in a suitable number of articular chondrocytes, the retention of proteoglycan, and a thinner calcified cartilage zone. Taken together, our findings show that APE significantly attenuated cartilage damage, making it a potent inhibitor of degenerative joint disease in cases of OA (**Fig. 6)**.

However, our data suggest that APE may be a potent inhibitor of joint degeneration associated with OA, in-vitro and in-vivo studies. Although the molecular mechanism of APE in OA is still not clear, it is possible that they reduce or protect the IL-1β-stimulated SW1353 cells from the expression of proinflammatory cytokines and p38/AP-1. In addition, the exact structure and molecular weight of effective small molecules in APE extracts still need to be further identified. Further research is required to test this hypothesis and to identify the exact effective small molecule structures.

In conclusion, the different methods used in EPE extraction were shown to affect the bioactivity of the resulting polysaccharide molecules in the extracts. Extraction via hot water produced EPEs with a larger number of high molecular weight polysaccharides. APE-produced EPEs proved particularly effective in attenuating the expression of cytokines and chemokines in the OA model. Thus, it appears that acid-phase extraction could be used to produce nutritional supplements for OA therapy. APE significantly reduced TNF-α expression and chemokine release, thereby reducing oxidative stress and also played an important role as an MMP activator against IL-1β-induced inflammation. Thus, it appears that APE could be used to produce nutritional supplements in the prevention and treatment of OA.

Author Contributions

Conceptualization, CCW, JWL, KHC, CYC and J JC; methodology, CCW, JWL, KHC, YSC, YHC, YJP, CHC, CYC and JJC; software, CCW, JWL, KHC. and YSC; validation, CCW, JWL, KHC, YSC, YHC, YJP and CHC; formal analysis, CCW, JWL, YHC, YJP and CHC.; investigation, CCW, JWL, KHC, YSC, YHC and CHC.; resources and data curation, CCW, JWL, KHC, YSC, YHC, YJP and CHC; writing—original draft preparation, CCW and JWL; writing—review and editing, CCW, JWL, CYC and JJC; visualization, CCW, JWL, KHC, YSC, YHC, YJP and CHC; supervision, CYC and JJC; project administration, CCW, CYC and JJC.

Acknowledgments and Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was funded by Ministry of Science and Technology grant number MOST- 102-2320-B-218 -001 at Taiwan.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Not applicable.

ORCID iD

Jeng-Wei Lu https://orcid.org/0000-0003-2128-2046

References

1. Litwic A, Edwards MH, Dennison EM, Cooper C. Epidemiology and burden of osteoarthritis. Br Med Bull. 2013;105:185-99. doi:10.1093/bmb/lds038.
2. McAlindon TE, Cooper C, Kirwan JR, Dieppe PA. Knee pain and disability in the community. Br J Rheumatol. 1992;31:189-92. doi:10.1093/rheumatology/31.3.189.
3. Chow YY, Chin KY. The role of inflammation in the pathogenesis of osteoarthritis. Mediators Inflamm. 2020;2020:8293921. doi:10.1155/2020/8293921.
4. Hedbom E, Hauselmann HJ. Molecular aspects of pathogenesis in osteoarthritis: the role of inflammation. Cell Mol Life Sci. 2002;59(1):45-53. doi:10.1007/s00018-002-8404-z.
5. He Y, Li Z, Alexander PG, Ocasio-Nieves BD, Yocum L, Lin H, *et al*. Pathogenesis of osteoarthritis: risk factors, regulatory pathways in chondrocytes, and experimental models. Biology. 2020;9:194. doi:10.3390/biology9080194.
6. Grassel S, Muschter D. Recent advances in the treatment of osteoarthritis. 2020; F1000Res:9. doi:10.12688/f1000research.22115.1.
7. Mora JC, Przkora R, Cruz-Almeida Y. Knee osteoarthritis: pathophysiology and current treatment modalities. J Pain Res. 2018;11:2189-96. doi:10.2147/JPR.S154002.
8. Kong H, Wang XQ, Zhang XA. Exercise for osteoarthritis: a literature review of pathology and mechanism. Front Aging Neurosci. 2022;14:854026. doi:10.3389/fnagi.2022.854026.
9. Goldring MB. Osteoarthritis and cartilage: the role of cytokines. Curr Rheumatol Rep. 2000;2:459-65. doi:10.1007/s11926-000-0021-y.
10. Dawes JM, Kiesewetter H, Perkins JR, Bennett DLH, McMahon SB. Chemokine expression in peripheral tissues from the monosodium iodoacetate model of chronic joint pain. Mol Pain. 2013;9:57. doi:10.1186/1744-8069-9-57.
11. Amin AR, Vyas P, Attur M, Patel IR, Weissmann G, Abramson SB, *et al*. The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. Proc Natl Acad Sci U S A. 1995;92:7926-30. doi:10.1073/pnas.92.17.7926.
12. Abramson SB, Attur M, Amin AR, Clancy R. Nitric oxide and inflammatory mediators in the perpetuation of osteoarthritis. Curr Rheumatol Rep. 2001;3(6):535-41. doi:10.1007/s11926-001-0069-3.
13. Baek SY, Li FY, Kim DH, Kim SJ, Kim MR. Enteromorpha prolifera Extract Improves Memory in Scopolamine-Treated Mice via Downregulating Amyloid-beta Expression and Upregulating BDNF/TrkB pathway. Antioxidants. 2020;9:620. doi:10.3390/antiox9070620.
14. Cho M, Lee HS, Kang IJ, Won M-H, You S. Antioxidant properties of extract and fractions from Enteromorpha prolifera, a type of green seaweed. Food Chem. 2011;127:999-1006. doi:10.1016/j.foodchem.2011.01.072.
15. Teng Z, Qian L, Zhou Y. Hypolipidemic activity of the polysaccharides from Enteromorpha prolifera. Int J Biol Macromol. 2013;62:254-6. doi:10.1016/j.ijbiomac.2013.09.010.
16. Liu FC, Wang CC, Lu JW, Lee C-H, Chen S-C, Ho Y-J, *et al*. Chondroprotective effects of genistein against osteoarthritis induced joint inflammation. Nutrients. 2019;11:1180. doi:10.3390/nu11051180.
17. Lee YM, Son E, Kim SH, Kim OS, Kim DS. Anti-inflammatory and anti-osteoarthritis effect of Mollugo pentaphylla extract. Pharm Biol. 2019;57(1):74-81. doi:10.1080/13880209.2018.1557700.
18. Ho YJ, Lu JW, Ho LJ, Lai J-H, Huang H-S, Lee C-C, *et al*. Anti-inflammatory and anti-osteoarthritis effects of Cm-02 and Ck-02. Biochem Biophys Res Commun. 2019;517:155-63. doi:10.1016/j.bbrc.2019.07.036.
19. Peng YJ, Lu JW, Lee CH, Lee H-S, Chu Y-H, Ho Y-J, *et al*. Cardamonin attenuates inflammation and oxidative stress in interleukin-1beta-stimulated osteoarthritis chondrocyte through the Nrf2 pathway. Antioxidants. 2021;10:862. doi:10.3390/antiox10060862.
20. Chou YJ, Chuu JJ, Peng YJ, Cheng Y-H, Chang C-H, Chang C-M, *et al*. The potent anti-inflammatory effect of Guilu Erxian Glue extracts remedy joint pain and ameliorate the progression of osteoarthritis in mice. J Orthop Surg Res. 2018;13:259. doi:10.1186/s13018-018-0967-y.
21. Wang CC, Lu JW, Peng YJ, Lee C-H, Lee H-S, Chu Y-H, *et al*. Ameliorative effects of cardamonin on monosodium urate-induced gouty arthritis through inhibiting NLRP3 inflammasome mediation. Medicina. 2021;57:898. doi:10.3390/medicina57090898.
22. Lin G, Wan X, Liu D, Wen Y, Yang C, Zhao C. COL1A1 as a potential new biomarker and therapeutic target for type 2 diabetes. Pharmacol Res. 2021;165:105436. doi:10.1016/j.phrs.2021.105436.
23. Wojdasiewicz P, Poniatowski LA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. Mediators Inflamm. 2014;2014:561459. doi:10.1155/2014/561459.
24. Graham S, Gamie Z, Polyzois I, Narvani AA, Tzafetta K, Tsiridis E, *et al*. Prostaglandin EP2 and EP4 receptor agonists in bone formation and bone healing: in vivo and in vitro evidence. Expert Opin Investig Drugs. 2009;18(6):746-66. doi:10.1517/13543780902893051.
25. Molnar V, Matisic V, Kodvanj I, Bjelica R, Jeleč Ž, Hudetz D, *et al*. Cytokines and chemokines involved in osteoarthritis pathogenesis. Int J Mol Sci. 2021;22:9208. doi:10.3390/ijms22179208.
26. Mixon A, Savage A, Bahar-Moni AS, Adouni M, Faisal T. An in vitro investigation to understand the synergistic role of MMPs-1 and 9 on articular cartilage biomechanical properties. Sci Rep. 2021;11:14409. doi:10.1038/s41598-021-93744-1.
27. Hu Q, Ecker M. Overview of MMP-13 as a promising target for the treatment of osteoarthritis. Int J Mol Sci. 2021;22:1742. doi:10.3390/ijms22041742.
28. Zeng L, Rong XF, Li RH, Wu X-Y. Icariin inhibits MMP1, MMP3 and MMP13 expression through MAPK pathways in IL1betastimulated SW1353 chondrosarcoma cells. Mol Med Rep. 2017;15:2853-8. doi:10.3892/mmr.2017.6312.

29. Tsuzaki M, Guyton G, Garrett W, Archambault JM, Herzog W, Almekinders L, *et al*. IL-1 beta induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1 beta and IL-6 in human tendon cells. J Orthop Res. 2003;21(2):256-64. doi:10.1016/S0736-0266(02)00141-9.
30. Sondergaard BC, Schultz N, Madsen SH, Bay-Jensen AC, Kassem M, Karsdal MA. MAPKs are essential upstream signaling pathways in proteolytic cartilage degradation–divergence in pathways leading to aggrecanase and MMP-mediated articular cartilage degradation. Osteoarthritis Cartilage. 2010;18(3):279-88. doi:10.1016/j.joca.2009.11.005.
31. Liacini A, Sylvester J, Li WQ, Huang W, Dehnade F, Ahmad M, *et al*. Induction of matrix metalloproteinase-13 gene expression by TNF-alpha is mediated by MAP kinases, AP-1, and NF-kappaB transcription factors in articular chondrocytes. Exp Cell Res. 2003;288:208-17. doi:10.1016/s0014-4827(03)00180-0.
32. Castellano I, Ercolesi E, Palumbo A. Nitric oxide affects ERK signaling through down-regulation of MAP kinase phosphatase levels during larval development of the ascidian Ciona intestinalis. PLoS ONE. 2014;9(7):e102907. doi:10.1371/journal.pone.0102907.