Vigeo attenuates cartilage and bone destruction in a collagen-induced arthritis mouse model by reducing production of pro-inflammatory cytokines

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Abstract. Rheumatoid arthritis (RA) is an autoimmune and chronic inflammatory disease characterized by articular cartilage destruction, bone destruction and synovial hyperplasia. It has been suggested that Vigeo, a mixture of *Eleutherococcus senticosus*, *Achyranthes japonica and Atractylodes japonica* fermented with Korean *nuruk*, has an anti-osteoporotic effect in a mouse model of inflammation-mediated bone loss. The present study evaluated the therapeutic effects of Vigeo in RA using a collagen-induced arthritis (CIA) mouse model. DBA/1J mice were immunized with bovine type II collagen on days 0 and 21 and Vigeo was administered daily for 20 days beginning the day after the second type II collagen injection. The mice were sacrificed on day 42 and the joint tissues were anatomically separated and subjected to micro computed tomography and histological analyses. In addition, the serum levels of TNF-α, IL-6 and IL-1β were determined by enzyme-linked immunosorbent assays. CIA in DBA/1J mice caused symptoms of RA, such as joint inflammation, cartilage destruction and bone erosion. Treatment of CIA mice with Vigeo markedly decreased the symptoms and cartilage pathology. In addition, radiological and histological analyses showed that Vigeo attenuated bone and cartilage destruction. The serum TNF-α, IL-6 and IL-1β levels following oral Vigeo administration were also reduced when compared with those in CIA mice. The present study revealed that Vigeo suppressed arthritis symptoms in a CIA-RA mouse model, including bone loss and serum levels of TNF-α, IL-6 and IL-1β.

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Abbreviations: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; TV, total volume; BV, bone volume; VOI, volume of interest; PV, phalange-carpal joint; RC, radiocarpal; MCP, metacarpal; ELISA, enzyme-linked immunosorbent assay; OVX, ovariectomy; DW, distilled water; IGF, insulin-like growth factor; RANKL, receptor activator of the nuclear factor κB ligand; LPS, lipopolysaccharide; C II, type II collagen; CFA, C II mixed with complete Freund's adjuvant; IFA, C II mixed with incomplete Freund's adjuvant

Key words: collagen-induced arthritis, pro-inflammatory cytokine, rheumatoid arthritis, Vigeo

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by joint inflammation, excessive proliferation of synovial tissue and destruction of bone and cartilage, resulting in pain and loss of function (1). Under normal physiological conditions, bone remodeling by osteoblasts and osteoclasts occurs continuously, maintaining a balance between bone synthesis and degradation. However, in pathological conditions such as RA, this homeostasis is disrupted, resulting in uncontrolled formation of osteoclasts, which are highly specialized cells crucial for bone resorption (2).

Over the last 20 years, significant progress has been made in understanding the pathogenesis of RA. Autologous IgG antibodies, diagnostic markers of RA and anti-cyclic citrullinated peptide antibodies lead to hypertrophy and proliferation of the synovial membrane (3). Additionally, the involvement of fibroblasts, T cells, macrophages, mast cells, dendritic cells and B cells has been demonstrated (4). Macrophages activated by T cells secrete pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β. These cytokines promote tissue destruction through the activation of synovial fibroblasts and chondrocytes in the joint (5) and by inducing osteoclast differentiation. TNF-α is additionally produced by activated T cells and is involved in inflammation-induced bone resorption, which aids bone destruction by upregulating the expression

of receptor activator of the nuclear factor κB (RANK) and its ligand (RANKL) and the secretion of macrophage colony-stimulating factor in osteoblasts and stromal cells (6). IL-6 production is frequently dysregulated in RA patients and is essential for synovial inflammation. A novel immunotherapeutic agent that is an IL-6 receptor antagonist reduces bone remodeling and supports bone maintenance in patients with RA (7). IL-6 is also involved in other diseases associated with accelerated bone turnover, such as Paget's disease and multiple myeloma (8). IL-1β is a key pro-inflammatory cytokine that is highly expressed in patients with RA. IL-1 induces RANKL expression, which promotes osteoclast differentiation through the production of prostaglandin E in the bone tissue. It also affects bone resorption via an alternative pathway (9-11). Therefore, natural drugs that downregulate the production of inflammatory cytokines may be useful for the treatment of RA.

Vigeo is a natural product with anti-osteoporotic effects that contains a mixture of three substances fermented using Korean *nuruk*: *Eleutherococcus senticosus*, *Achyranthes japonica and Atractylodes japonica*. Vigeo has previously been shown to effectively inhibit RANKL-induced osteoclast differentiation by downregulating the phosphorylation activities of the Akt, IκB and MAPK signaling pathways. It also significantly suppresses bone loss in a lipopolysaccharide (LPS)-induced mouse model of osteoporosis. Furthermore, Vigeo suppresses the levels of the bone resorption marker C-terminal telopeptide-1 in the serum of these mice (12).

The present study aimed to determine the effect of Vigeo administration on bone resorption and the immune response in a mouse model of RA. It confirmed the anti-inflammatory effects of Vigeo in RA by evaluating bone loss in affected joints.

Materials and methods

Animals. A total of 20 healthy 8-week-old male DBA/1J mice weighing 19-23 g were purchased from Central Lab Animal Inc. The mice were randomly divided into four groups, each consisting of five mice: i) Control, ii) collagen-induced arthritis (CIA) group, iii) CIA + 200 mg/kg Vigeo (low dose) and iv) CIA + 500 mg/kg Vigeo (high dose). All animals were housed at a constant temperature (22±2˚C) and 50-55% humidity with a 12 h light/dark cycle. Animal experimental protocols were approved (approval no. WKU21-67) by the Institutional Animal Care and Use Committee (IACUC) of Wonkwang University (Iksan, Republic of Korea) and all experiments were conducted in accordance with the guidelines of the IACUC of Wonkwang University.

Reagents. Vigeo was purchased from Panax Bio Co. Ltd. and prepared and fermented as previously described (12) (Fig. 1A). Briefly, dried *Eleutherococcus senticosus* (135 g), *Achyranthes japonica* (78 g) and *Atractylodes japonica* (78 g) were boiled in hot water for 3 h in an extractor (Cosmos-660; Kyungseo E&P Co., Ltd.). Korean traditional fermentation starter, *nuruk* (1 kg), and fresh yeast (4 g) were mixed and fermented in 1.5 l distilled water (DW) for 96 h. The hot water extract was mixed with popped rice (3 kg) and fermented at 26˚C for 15 days. The resulting mixture of *nuruk*-fermented extract (Vigeo) was carefully freeze-dried and diluted using ultrapure water.

CIA-RA in vivo mouse model and Immunization of DBA/1J mice with type II collagen (CII). A solution to induce a primary immune response in DBA/1J mice was prepared by dissolving CII lyophilized powder (cat. no. 20022; lot no. 200297; Chondrex, Inc.) in acetic acid to a concentration of 2 mg/ml. Next, the collagen mixture was mixed with complete Freund's adjuvant (CFA; cat. no. 7001; lot. no. 200200; Chondrex, Inc.) in a 1:1 ratio and the final mixture was emulsified on ice using a homogenizer. The working solution (100 μl) was gently and slowly injected into the tail veins of the mice on day 0. The second booster injection consisted of CII mixed with an incomplete Freund's adjuvant (IFA; cat. no. 7002; lot no. 200341; Chondrex, Inc.) was injected as described above on day 21. Mice received either 200 or 500 mg/kg Vigeo or DW orally daily from days 22-41. All the mice were sacrificed on day 42.

Micro computed tomography (micro-CT) based quantification of paw-joint architecture. Micro-CT 3D images were obtained using a SkyScan 1173 high-energy micro-CT scanner (Bruker Corporation) on both the left and right hind paws. The scans were performed using source currents and voltages of 60 mA and 130 μV, respectively and the data acquired from the scanned paws were analyzed with Bruker CTAn software version 1.6 (build 22) by OBEN. Measured bone parameters included total volume (TV; mm^3), bone volume (BV; mm^3), bone volume/total volume (BV/TV; %) and total porosity (%). Parameter quantifications and assessments were performed as described previously by Perilli *et al* (13). Diameters of the volumes of interest (VOIs) were determined in three segments of the paw region: Region A, VOIs in the total paw volume (PV phalange-carpal joint); Region B, VOIs in the radiocarpal (RC) to metacarpal (MCP) joint (RC-MCP radiocarpal-metacarpal); and Region C, VOIs in the radiocarpal (RC) joint.

Histological analysis. Joint tissues were fixed in 10% neutral-buffered formalin for 24 h, washed in PBS, decalcified for 21 days in a 12% EDTA solution to remove the moisture remaining in the tissue, the alcohol concentration was adjusted step by step and the dehydration process was performed at 24-26˚C for 12 h each [70, 80, 90 and 100% EtOH (I), 100% EtOH (II) and 100% EtOH (III)]. Dehydrated tissues were immersed in xylene/EtOH (1:1) solution, followed by 100% xylene I, II, III solution for 3 h each. Subsequent to this clearing, the ankle tissue was infiltrated with 55˚C paraffin wax solution three times, each lasting 3 h, to facilitate paraffin permeation. To detect the cartilage in the paraffin-embedded tissue sections, hematoxylin (cat. no. 3540MIRA01; lot. no. 90104; BBC Biochemical) and eosin (cat. no. MA0101015MIRA01; lot. no. 89546; BBC Biochemical) (H&E), safranin O (cat. no. S8884; lot. no. MKBT3301V; MilliporeSigma), fast green (cat. no. F7258; lot. no. MKBR3297V; MilliporeSigma) and toluidine blue (cat. no. T3260; lot. no. MKBQ1663V; MilliporeSigma) staining methods were used. The sections were incubated with different staining reagents for varying

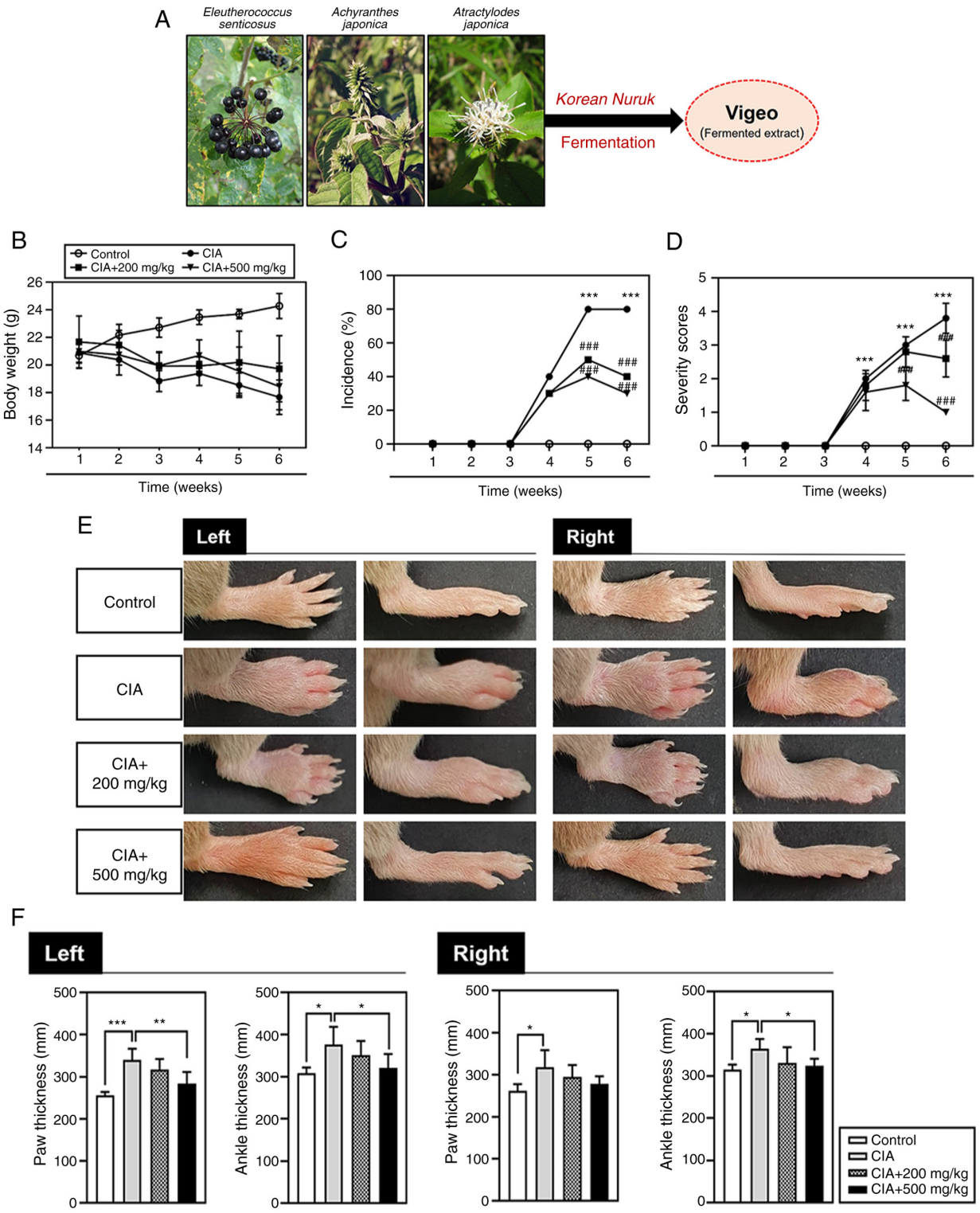

Figure 1. Evidence of the effects of Vigeo on paw edema and thickness (mm) in CIA-RA mice. DBA1/J mice were injected with an emulsion of type II collagen with CFA or IFA (first immune response: CII + CFA and second booster: CII + IFA). (A) Image of the three plants included in Vigeo (*Eleutherococcus senticosus*-https://commons.wikimedia.org/wiki/File: Eleutherococcus_senticosus_kz05.jpg, *Achyranthes japonica*-https://en.wikipedia.org/wiki/Achyranthes_japonica, *Atractylodes japonica*-https://www.wikidata.org/wiki/Q10893998). (B) Body weight was measured every week for 42 days. (C) Arthritic incidence and (D) severity score of each mouse were examined. (E) Images of joint destruction and (F) calculated paw thicknesses of the left and right paws on day 42. Data are presented as the mean ± standard deviation (n=5). *P<0.05, **P<0.01, ***P<0.001 vs. the control group or CIA control group and $^{\#\#\#}$P<0.001 vs. the CIA control group. CIA, collagen-induced arthritis; RA, rheumatoid arthritis; CII, type II collagen; CFA, CII mixed with complete Freund's adjuvant; IFA, CII mixed with incomplete Freund's adjuvant.

durations at 24-26˚C. The reaction times for each staining reagent are as follows: Hematoxylin-2 min; eosin-1 min; safranin O-5 min; fast green-5 min; toluidine blue-3 min.

Cytokine analysis. Blood samples were collected from mice by drawing 300-500 μl of blood from the orbital sinus after inhalation anesthesia with isoflurane and centrifuged at

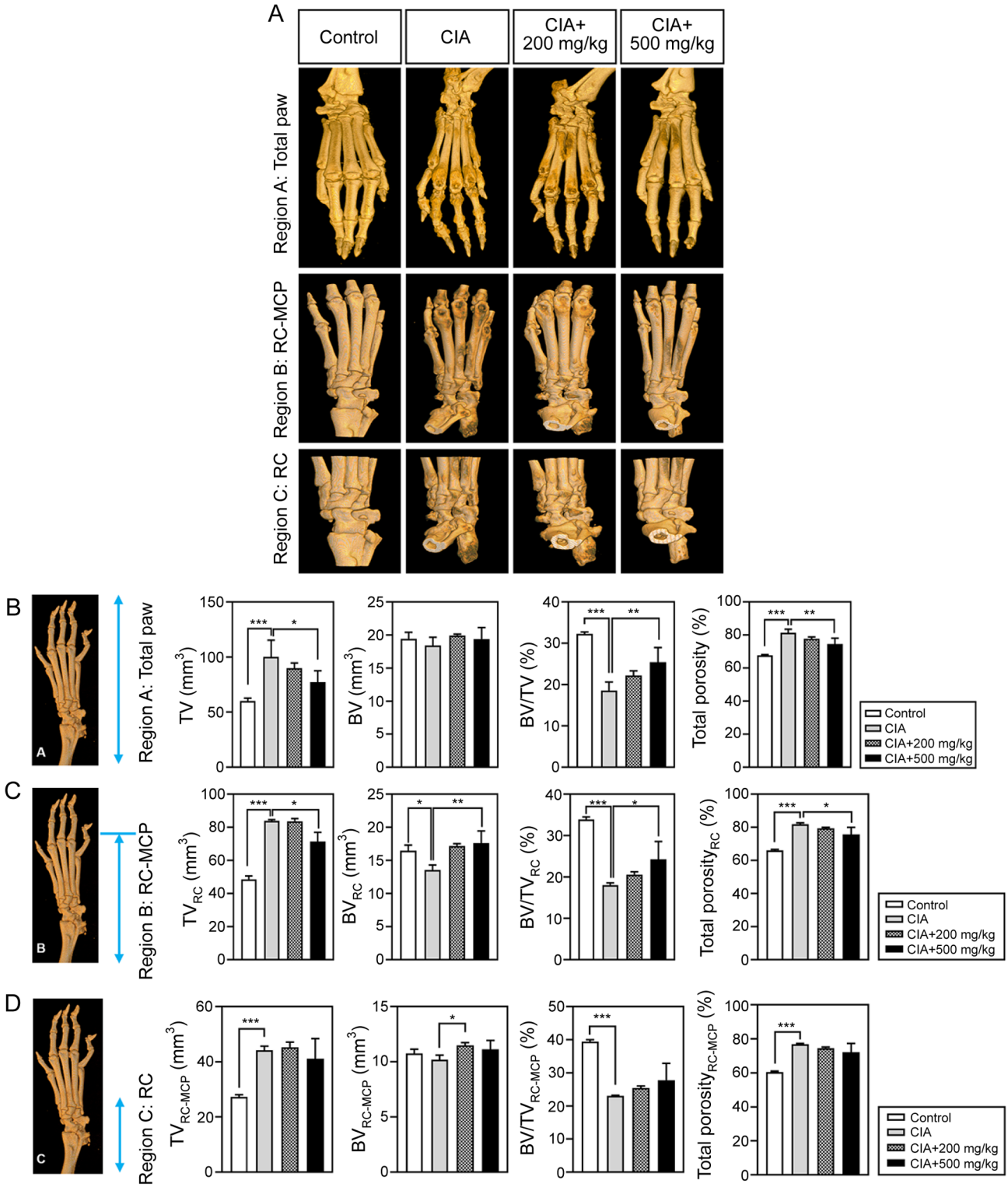

Figure 2. Evaluation of the anti-inflammatory effects of Vigeo in CIA-RA mice. Arthritis-induced joints were evaluated according to specific regions. The 3D images of each paw were captured and the paw VOIs were analyzed with a micro-CT analyzer in Regions (A-C). The images shown in B, C and D are presented to show the analysis regions in the same image. TV (mm^3), BV (mm^3), BV/TV (%) and total porosity (%) were determined in Region A from the phalanges-carpal to the RC joint, Region B from RC-MCP joint and Region C in the RC joint. (D) Data are represented as the mean ± standard deviation (n=5). *P<0.05, **P<0.01, ***P<0.001 vs. the control group or the CIA control group. CIA, collagen-induced arthritis; RA, rheumatoid arthritis; VOI, volume of interest; CT, computed tomography; TV, tissue volume; BV, bone volume; RC, radiocarpal; MCP, metacarpal.

1,000 x g for 15 min at 4˚C. The inhalation of 4% isoflurane for 2 min in the anesthesia box induced sufficient anesthesia and was then maintained at 1.0-1.5% isoflurane, from which mice quickly recovered within ~50-80 sec after their removal from the anesthesia box under control conditions. Enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc.) were used to measure the serum levels of TNF-α (cat. no. MTA00B; lot. no. P362560), IL-6 (cat.

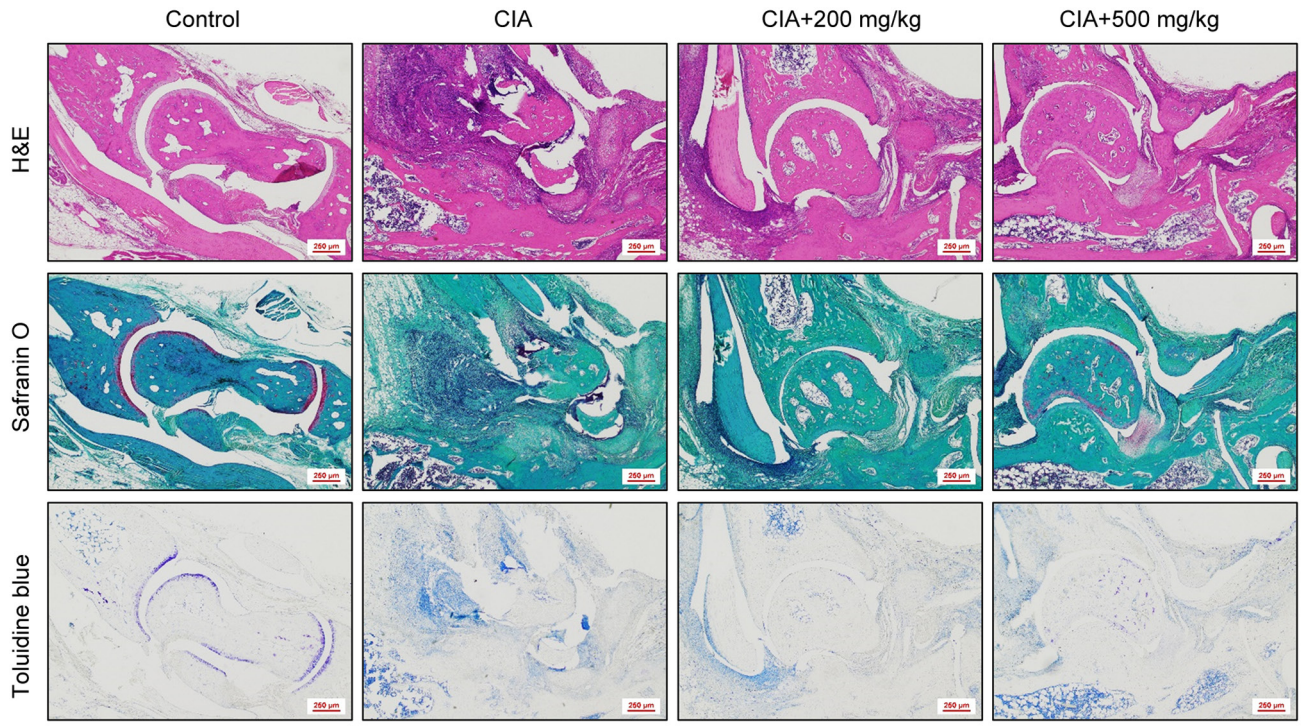

Figure 3. The anti-histopathological effects of Vigeo in CIA-RA mice illustrated by histological microphotography. Representative images of ankle joint histology sections stained with hematoxylin and eosin, safranin O and toluidine blue staining methods (magnification, x100; scale bars, 250 μm) are shown. CIA, collagen-induced arthritis; RA, rheumatoid arthritis.

no. M6000B; lot. no. P364710) and IL-1β (cat. no. MLB00C; lot. no. P361801).

Statistical analysis. Each experiment was performed at least three times and data were analyzed and expressed as the mean ± standard deviation. Statistical significance was determined using a one-way analysis of variance followed by Tukey's multiple-comparisons test using SPSS 14.0 (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Vigeo attenuates arthritic severity in CIA-DBA/1J mice. To determine the therapeutic effect of Vigeo treatment in mice with arthritis a CIA mouse model was selected, an *in vivo* experimental model of RA that is commonly used. CIA was induced in DBA/1J mice by primary immunization with CFA-emulsified CII (day 0) and secondary immunization with IFA-emulsified CII (day 21). The therapeutic effect of Vigeo in CIA mice was confirmed by oral administration of DW, or 200 or 500 mg/kg Vigeo daily, beginning on the day after the second immunization (day 22). The total duration of the experiment was 42 days, after which the mice were sacrificed. Visible symptoms of arthritis in the CIA mice gradually appeared after the second intravenous injection. Loss of appetite and body weight was observed on day 14. However, weight loss in the mice receiving Vigeo tended to be reduced when compared with that in the CIA group (Fig. 1B). In the CIA group, common arthritic symptoms such as joint edema began to appear 5 days after the second immunization on day 26 and tended to worsen from days 32 to 42. By contrast, the paw and ankle edema was significantly improved in the Vigeo oral administration group when compared with that in the CIA group (Fig. 1C and D). Moreover, the increase in paw and ankle thickness also improved with Vigeo treatment (Fig. 1E and F).

Vigeo alleviates bone and joint destruction in CIA-DBA/1J mice. The most severe effect of RA is bone erosion in the joints. Articular destruction and bone damage were confirmed in hind paws using micro computed tomography (micro-CT; Fig. 2A). Micro-CT images enabled quantification of bone changes in CIA mice (Fig. 2B and C). Furthermore, the analysis showed that due to edema and increased total porosity resulting from bone erosion, the TV was increased in CIA mice when compared with that in the control group. By contrast, the CIA group BV and BV/TV parameters were significantly lower than those in the control group. Following Vigeo treatment, the 200 and 500 mg/kg Vigeo group Larsen scores showed diminished TV and total porosity when compared with those in the CIA group. The high-dose Vigeo-treatment group also showed noticeably reduced TV and total porosity in the radio-carpal-metacarpophalangeal joint region (Fig. 2B and C).

Vigeo suppresses inflammation and joint destruction in CIA-DBA/1J mice. The present study conducted histological analyses of the mouse ankle joints to determine the degree of joint destruction and inflammation. H&E staining confirmed that the ankle joint region containing cartilage and bone was preserved in mice from the control group compared with that in the CIA group, which showed severe cartilage erosion. Joint destruction in mice from both the low- and high-dose Vigeo treatment groups was improved when compared with the CIA group. Safranin O/fast green staining was used as a visual

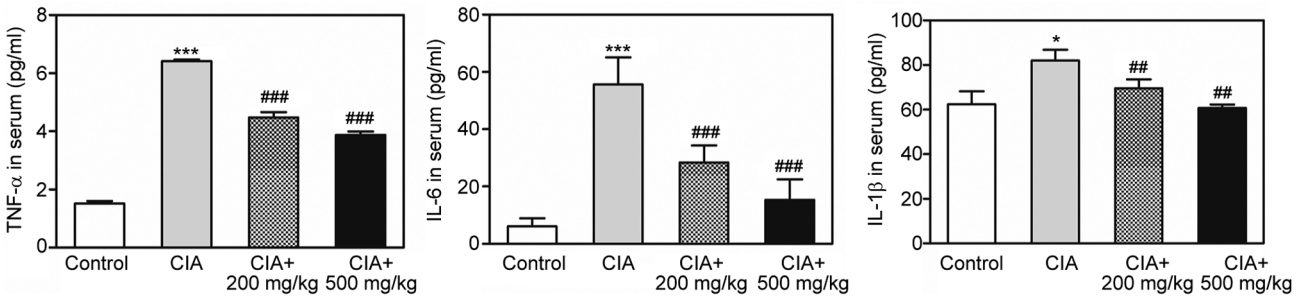

Figure 4. Evidence of the anti-arthritic and anti-inflammatory effects of Vigeo against CIA-induced production of pro-inflammatory cytokines in blood serum. The concentration values of cytokines including TNF-α, IL-6 and IL-1β were determined on day 42 by enzyme-linked immunosorbent assay. Elevated serum cytokine levels in CIA-RA mice were downregulated by Vigeo treatment with low (200 mg/kg) or high (500 mg/kg) concentrations. Data are represented as the mean ± standard deviation (n=5). *P<0.05, ***P<0.001 vs. the control group and $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the CIA control group. CIA, collagen-induced arthritis; RA, rheumatoid arthritis.

indicator of the extent of cartilage damage. Joints from control group mice showed well-maintained red cartilage tissue by safranin O staining, but joints from CIA mice had atypical red cartilage tissue in a disorganized shape due to significant cartilage destruction. By contrast, the preservation of joint cartilage in Vigeo-treated mice was superior compared with that in mice from the CIA group. Finally, toluidine blue staining was performed to evaluate the cartilage matrix. This test revealed that Vigeo treatment somewhat improved violet-stained cartilage regions by inhibiting the cartilage matrix destruction observed in the joints of CIA mice (Fig. 3).

Vigeo inhibits the production of pro-inflammatory cytokines in CIA-DBA/1J mice. RA is mainly driven by continuously high levels of pro-inflammatory factors, including TNF-α, IL-6 and IL-1β (5). To test whether Vigeo treatment affected the production of proinflammatory cytokines in CIA mice, the present study determined the serum levels of these cytokines using ELISA. Serum levels of TNF-α, IL-6 and IL-1β levels were all increased in mice from the CIA group when compared with the control group. However, the increase of cytokine secretion in CIA mice was significantly inhibited by both low and high concentrations of Vigeo when compared with the untreated CIA group (Fig. 4).

Discussion

For thousands of years, natural products have been regarded as therapeutic tools worldwide and have been developed alongside human culture. The production of substances with beneficial bioactivity has mainly been achieved through various traditional herbal separation and purification methods that were developed based on environmental, climatic and geographical factors (14,15). Fermentation is one of the main methods used by the food industry to manipulate and grow various types of bacteria, yeasts and fungi (16). However, in recent years, fermentation has been applied in various fields such as the mass production of highly complex substances including insulin, alternative proteins and antibiotics (17). In addition, the chemical and biological diversity of the natural products produced by microbial fermentation can improve quality and promote the absorption rate of active substances in the body. In particular, screening suitable disease targets by utilizing the secondary metabolites of microorganisms as new drugs has increasingly been attempted (18).

RA is a severe systemic and chronic autoimmune disease that is typically accompanied by the deformation and destruction of joint tissue and bone, with symptoms such as pain, stiffness and inflammation gradually appearing over several weeks (19,20). Despite successes in RA treatment with currently available therapeutics, these drugs are not always effective and concerns regarding adverse effects, including hepatotoxicity, primary gastrointestinal symptoms and respiratory symptoms, persist (21). Therefore, the present study chose a different approach through the application of safe and natural therapeutic agents (22). Previously, we reported a novel effect of Vigeo, a fermented extract of *Eleutherococcus senticosus*, *Achyranthes japonica and Atractylodes japonica* based on Korean *nuruk*, on osteoclast differentiation and function in LPS-induced inflammatory bone diseases, including osteoporosis. Vigeo strongly inhibited RANKL-induced Akt, IκB and MAPK signaling pathways and significantly suppressed mRNA and protein expression of c-Fos and nuclear factor of activated T cells 1 (downstream transcription factors in osteoclasts) and the expression of osteoclast differentiation marker genes (12). Previous studies (23-25) have demonstrated the efficacy of the three components of Vigeo in bone diseases, such as osteoporosis and RA. *Eleutherococcus senticosus* extracts have been shown to prevent ovariectomy (OVX)-induced osteoporosis in a rat model and to decrease the serum levels of factors that indicate high bone turnover, such as alkaline phosphatase, type I collagen and osteocalcin (23). *Achyranthes japonica* improves the serum levels of insulin-like growth factor (IGF) and IGF-binding protein 3, which are essential stimulators of bone formation in response to osteoporosis induced by OVX in rats (24). Extracts of *Atractylodes japonica* in 70% ethanol reduced osteoclast differentiation by decreasing NF-κB activation via RANKL in bone marrow-derived macrophages *in vitro* and protected against osteoporotic symptoms in a RANKL-injection mouse model of bone loss *in vivo* (25). The ability of each of the three plant extracts to inhibit osteoporosis in animal models suggests the possibility of preventing or treating osteoporosis using the newly produced Vigeo fermented with Korean *nuruk*.

Based on the anti-osteoporotic effects of the three plant extracts, the present study was designed to determine the

synergistic effects of these different components in a CIA mouse model *in vivo*. It was hypothesized that Vigeo would have excellent therapeutic effects in reducing bone loss in a CIA mouse model of RA. RA is a systemic inflammatory disease mainly characterized by chronic inflammation in the joint synovium and infiltration of inflammatory factors that causes joint destruction and disability (1). The present study found that Vigeo significantly inhibited arthritic symptoms such as paw edema in CIA-RA mice. As shown in Fig. 2, micro-CT analysis revealed inhibition of joint damage and Fig. 3 shows that joint synovial hyperplasia and cartilage and bone erosion were attenuated by Vigeo administration at a dose of 500 mg/kg, as determined by histology. T cells, macrophages and synovial cells have crucial roles in RA pathogenesis (4). Various inflammatory cytokines, including TNF-α, IL-6 and IL-1β, are produced by these cells and high levels of these cytokines are observed in patients with RA (5). The regulation of cytokine levels is a potential therapeutic strategy for RA treatment (4,5). TNF-α is expressed primarily by macrophages and synovial cells as well as activated T cells within RA inflamed joints (26) and the involvement of TNF-α in arthritic bone destruction has been demonstrated in several studies (27-29). hTNF-tg mice develop chronic inflammatory arthritis with hyperplasia of the synovium, inflammatory infiltrates of the joint space, pannus formation and cartilage and bone destruction. Application of TNF-specific neutralizing antibodies in CIA mice reduces disease activity and bone damage (27). IL-1 is produced by activated macrophages and synovial fibroblasts in RA joints (30). In models of arthritis, overexpression of IL-1α or IL-1β, or deficiency of soluble IL-1Ra result in the development of a disease associated with bone and cartilage destruction (31). IL-6 is produced by various cell types in the inflamed RA bone microenvironment, including macrophages, fibroblast-like synovial cells and chondrocytes (32). Reports show that IL-6-deficient mice are protected from inflammation and bone destruction in an antigen-induced arthritis model (33-35).

The present study found that Vigeo significantly diminished the production of pro-inflammatory cytokines in CIA-RA mice. Therefore, the inhibitory effects of Vigeo on bone and cartilage loss in this mouse model may be attributed to the reduction in cytokine levels observed following treatment with Vigeo.

However, the present study had some limitations. As a study on signal transduction pathways related to synovial proliferation and angiogenesis during RA onset, the absence of experiments confirming changes in the VEGF and VEGFR/PI3K/AKT pathways in joint tissue and the results on the regulation of pro-inflammatory cytokines in patients remains a limitation. Also, the key targets focusing on the metabolism and potential compounds of Vigeo to treat RA still require further studies and verification and a detailed study of the appropriate dosing regimen of Vigeo is needed for clinical RA application.

In summary, the present study suggested the potential use of Vigeo in the treatment of inflammatory arthritis accompanied by pain, severe bone loss and joint deformity (Fig. S1). Vigeo significantly abrogated synovial inflammation and joint, cartilage and bone loss in a CIA-RA mouse model. The therapeutic properties of Vigeo may be attributed to the suppression of pro-inflammatory cytokine production. It is suggested that Vigeo may be an effective therapeutic agent for the treatment of RA.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JYK and MSL designed the study and revised the manuscript. YHC, CHL, SYE and GDP performed the experiments. YHC, CHL and CHC analyzed the data. YHC and CHL drafted the manuscript. JYK and MSL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. WKU21-67) and followed the guidelines of the Ethics Committee for Experimental Animals of Wonkwang University (Iksan, Republic of Korea). Among the medicinal ingredients of Vigeo used in the experimental study, *Eleutherococcus senticosus* belongs to Endangered wildlife level II and was not collected from the wild but purchased from Hwacheon Gasiogalpi Farm in Gandong-myeon, Hwacheon, Gangwon-do (Gangwon, Republic of Korea). It does not collect or damage internationally endangered species designated and announced by the Minister of Environment in accordance with the Convention on International Trade in Endangered Species of Wild Plants (CITES) (http://www.cites.org) and the United Nations. It complies with International Union for Conservation of Nature (IUCN) standards for natural conservation (https://s3.amazonaws.com/iucnredlist-newcms/staging/public/attachments/3154/reg_guidelines_en.pdf) and adheres to permitting procedures for protected species.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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