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Research Paper

A mixture of Trachelospermi caulis and Moutan cortex radicis extracts suppresses collagen-induced arthritis in mice by inhibiting NF-κB and AP-1

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Keywords
collagen-induced arthritis; *Moutan cortex radicis*; osteoclasts; rheumatoid arthritis; *Trachelospermi caulis*

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Abstract

Objectives We aimed to determine the anti-arthritis effect and its mechanism of a combination of herbal extracts from *Trachelospermi caulis* (TC) and *Moutan cortex radicis* (MC) (TCMC).

Methods The anti-arthritis activity of TCMC was assessed using a mouse model of type II collagen-induced arthritis (CIA). Reverse transcriptase-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), electrophoretic mobility shift assay (EMSA) and other biological assays were performed.

Key findings TCMC significantly ameliorated various inflammatory parameters, such as clinical arthritis index, histological deformation of joints, serum levels of rheumatoid arthritis biomarkers (cartilage oligomeric matrix protein, serum amyloid P and anti-collagen type II IgG antibody), and Th1-related responses (T cell proliferation, and production of Interferon-γ and interleukin (IL)-2 in splenocytes isolated from CIA mice). The production of matrix metalloproteinases (MMPs), pro-inflammatory cytokines (tumour necrosis factor-α, IL-1β and IL-6) and chemokines (macrophage inflammatory protein-1, monocyte chemoattractant protein-1, and Regulated upon Activation, Normal T-cell Expressed, and Secreted) was suppressed by TCMC in CIA mice. In addition, the number of osteoclasts in the hind tibia was significantly decreased. With regard to the mechanism, TCMC suppressed the activation of the transcription factors nuclear factor (NF)-κB and activator protein (AP)-1.

Conclusions TCMC exerts an anti-arthritis effect in CIA mice by suppression of the production of various inflammatory factors and the formation of osteoclasts through the inhibition of NF-κB and AP-1 activation.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects joints, bones and other organs of the body. RA is closely associated with synovial hyperplasia and the destruction of cartilage and bone.[1] Accumulating evidence suggests that articular destruction, bone erosion, and functional disabilities in RA are caused by various inflammatory factors such as matrix metalloproteinases (MMPs), pro-inflammatory cytokines and chemokines.[2,3]

The cartilage destruction observed in RA patients is largely due to the activity of matrix metalloproteinases (MMPs).[4] Among these, MMP-1 and MMP-3 are the major isoforms produced in the RA synovium.[5] MMP-1 preferentially degrades fibrillar collagens and MMP-3 degrades a broad array of extracellular matrix substrates.[6–8] Tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 are highly expressed in the joints of RA patients and play a crucial role in the induction of inflammatory response.[9,10] In addition, osteoclasts play a central role in joint destruction and bone erosion in RA.[11] It is well-established that the activation of nuclear factor (NF)-κB and activator protein (AP)-1 is essential for the production of various cytokines and chemokines, and for the differentiation of osteoclasts in RA joints.[11–13]

Various types of drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) and biological response modifiers (BRMs) are currently used in the treatment of RA.[11] However, there is a need for more effective and safer drugs. Recently, complementary and alternative medicines have been considered as novel approaches in the development of such new therapies because of their low toxicity and long-recognized activity. Among these, many herbal extracts have been used as traditional folk remedies for treating various diseases in Asian countries, including Korea, Japan, and China.

Trachelospermi caulis (TC), the dried leafy stem of *Trachelospermum asiaticum* Nakai var. *intermedium*, has been used as an anti-inflammatory herbal medicine in east Asia, including Korea. *Moutan cortex radicis* (MC), the dried root cortex of *Paeonia suffruticosa* Andrews, has traditionally been used to treat atherosclerosis, allergic diseases and inflammatory diseases.[14,15] In our previous report, we investigated the anti-inflammatory, antinociceptive activity of TC extract and its mechanism of action.[16] Although the anti-inflammatory effect of both herbal extracts has long been recognized, the anti-arthritis effect and the mechanism of action of the combined herbal extracts of TC and MC (TCMC) are not known.

In this study, the anti-arthritis activity of TCMC in collagen-induced arthritis (CIA) and the mechanism underlying this activity were investigated.

Materials and Methods

Reagents

Bovine type II collagen and Freund's complete adjuvant were obtained from Chondrex (Redmond, WA, USA); indometacin from Sigma-Aldrich (St Louis, MO, USA); recombinant mouse macrophage colony-stimulating factor (M-CSF) and recombinant mouse receptor activator of nuclear factor kappa-B ligand (RANKL) from PeproTech Inc. (Rocky Hill, CT, USA); antibodies against NFATc1 and c-Fos from Santa Cruz Biotechnology (Santa Cruz, CA, USA); goat anti-mouse HRP-conjugated antibody from Cell Signaling Technology (Beverly, MA, USA); and transcription factor probes against AP-1 and NF-κB from Panomics (Fremont, CA, USA). Cell culture media and other culture reagents were obtained from Gibco RBL (Grand Island, NE, USA).

Plant extracts and HPLC analysis

TCMC, the mixture of ethanol extracts of TC and MC, was supplied by the Life Science R&D Center, Sinil Pharm. Co., Ltd. (Chungju, Korea). Plant extracts were prepared using the dried plants according to the institute's standardized protocol. Briefly, TC and MC were separately extracted in 95% ethanol at 80°C for 2 h and then concentrated with a vacuum pump at 60°C. Finally, the two extracts were mixed in the weight ratio of 1 : 1. A voucher specimen (Sinil-000902) was retained at the Life Science R&D center, Sinil Pharm. Co., Ltd. To check reference compounds for quality control purposes, TCMC was dissolved in 20% acetonitrile (for arctigenin) or 10% acetonitrile (for paeoniflorin) and then analysed using an HPLC system equipped with a UV detector (Waters, Milford, MA, USA). TCMC was separated on an XBridge C18 5 um (4.6 × 250 mm) at a flow rate of 1.0 ml/min. Acetonitrile was used as the mobile phase with an injection volume of 20 µl. To detect arctigenin, the mobile phase consisted of water (solution A) and acetonitrile (solution B) with a gradient elution program: 20–40% acetonitrile in 30 min, 40–20% acetonitrile in 35 min, 20% acetonitrile isocratic in 40 min. To detect paeoniflorin, the mobile phase consisted of 0.1% phosphoric acid aqueous solution (solution A) and acetonitrile (solution B) with gradient elution program: 11% solution B isocratic in 25 min, 11–70% solution B in 30 min, 70% solution B isocratic in 35 min, 70–11% solution B in 40 min, 11% solution B isocratic in 45 min. The peaks were detected at 230 nm. TCMC was dissolved in dimethyl sulfoxide (DMSO) for cell culture assays and suspended in 5% Arabic gum for animal studies.

Induction of collagen-induced arthritis in mice

DBA/1J mice (males, 5–6 weeks old; 10 mice per group) were purchased from Charles River Breeding Laboratories (Kanagawa, Japan) and housed in a specific pathogen-free animal facility at Konkuk University (Seoul, Korea). After a one-week acclimation period, the animal study was performed in accordance with the institutional guidelines. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Konkuk University (Approval no. KU10012 on May 3rd, 2010). Collagen-induced arthritis (CIA) was induced as described previously.[17] Briefly, mice were intradermally injected in the tail with 100 µg of type II collagen emulsified in an equal volume of Freund's complete adjuvant. Twenty-three days after administering the first injection of collagen, the mice received an intraperitoneal booster injection of 100 µg of type II collagen. TCMC (50–200 mg/kg), bucillamine (300 mg/kg) as a reference drug, or a vehicle (5% Arabic gum) were orally administered once daily from the day the second injection of collagen was administered.

Evaluation of clinical arthritis score in collagen-induced arthritis

The arthritis severity was evaluated in all four paws of the mice by three blinded observers. The results were assessed according to a previously described method.[18] Briefly, the severity was scored as follows: 0, normal; 1, mild, apparent swelling limited to individual digits; 2, moderate, redness and swelling of the ankle; 3, redness and swelling in the paw as well

as in the digits; and 4, maximally inflamed leg with involvement of multiple joints. The arthritis score for each mouse was the sum of arthritis severity in all four paws, with the highest possible score being 16.

Histological analysis

On day 38 after the first immunization with collagen, the mice were euthanized by CO2. The right hind paws of all mice were harvested, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for three days, and then decalcified in 10% ethylene diamine tetraacetic acid in 4% paraformaldehyde for 30 days at 4°C. The decalcified paws were then dehydrated in a graded ethanol series (70–100%), washed three times with xylene for 3 min each, and then embedded in paraffin. Serial 5-μm-thick paraffin sections were stained with hematoxylin and eosin (H&E). Histopathological changes in joints were scored using the parameters described in a previous report.[19] Three pathologists who were blinded to the source of the tissues independently evaluated each section on a 5-point scale with the scores indicating the following findings: 0, normal; 1, minor destruction of the cartilage surface and infiltration of inflammatory cells; 2, mild hyperplasia of the synovial lining layer and localized cartilage destruction; 3, moderate inflammation and pannus formation; 4, moderate cartilage destruction and bone erosion; and 5, severe infiltration of inflammatory cells, bone erosion and severe destruction of cartilage.

Measurement of cartilage oligomeric matrix protein, serum amyloid P and anti-collagen type II IgG antibody

At the end of the experiments, serum samples were taken and levels of cartilage oligomeric matrix protein (COMP), serum amyloid P (SAP), and anti-collagen type II (CII) IgG antibody in serum were assessed using ELISA kits according to the manufacturers' instructions. Kits from Kamiya Biomedical Co. (Seattle, WA, USA) were used for COMP and SAP assessments, and the kit for anti-CII IgG antibody was obtained from Chondrex Inc. (Redmond, WA, USA).

Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. On day 38 after the first immunization with collagen, the mice were euthanized by CO2, their ankles were removed, minced in liquid nitrogen, and stored at −70°C. Total RNA was extracted from hind paws by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and was subjected to reverse transcription using the SuperScript first-strand synthesis system (Invitrogen) according to the manufacturer's protocol. The following primers were used: mouse MMP-1 (forward, 5′-CCAGGTGTGGGGTGCCTGAT-3′; reverse, 5′-CAAA CCTGGGCCTGGCTGGA-3′); MMP-3 (forward, 5′-GAACATCGATGCAGCCATTT-3′; reverse, 5′-AGGAGAA AACGAACATTTCA-3′); TNF-α (forward, 5′-GGCAGGTC TACTTTAGAGTCATTGC-3′; reverse, 5′-ACATTCGAGGCT CCAGTGAATTCGG-3′); IL-1β (forward, 5′-ATGGCAAC TGTTCCTGAACTCAAC-3′; reverse, 5′-CAGG ACAGGTA TAGATTCTTTCCTTT-3′); IL-6 (forward, 5′-ATGAAGT TCCTCTCTGCA AGAGAC T-3′; reverse, 5′-CACTAGGT TTGCCGAGTAGATCTC-3′); MIP-1 (forward, 5′-CCT CT TGCTCGTGGCTGCCT-3′; reverse, 5′-AGTGGCTCCTGC CCTGC-3′); MCP-1 (forward, 5′-TCCACCACCATGCA GGTCCC-3′; reverse, 5′-CCAGCAGGT GAGTGGGGCG TT-3′); RANTES (forward, 5′-CCTCACCATCATCCTCA CTGCA-3′; reverse, 5′-TCTTCTCTGGGTTGGCACACAC-3′); and glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-TTGGCCGTATTGGGCGCCTG-3′; reverse, 5′-ATCGGCA GAAGGGGCGGAGA-3′).

Measurement of pro-inflammatory cytokines in arthritic joints

The levels of TNF-α, IL-1β and IL-6 were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. Briefly, CIA mouse ankles were snap-frozen in liquid nitrogen. Approximately 1 g of fresh tissue was cut into small pieces. The tissue was ground a powder with liquid nitrogen in a mortar and pestle, and lysed with a lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1 mM phenylmethylsulfonyl fluoride) and homogenized on ice. The lysed extracts were then subjected to enzyme-linked immunosorbent assay (ELISA).

Splenocyte proliferation assay

Spleens removed from CIA mice on day 41 were treated with red blood cell lysis buffer, and the isolated splenocytes were cultured in a 96-well plate (5×10^5 cells/well) in supplemented RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. The cells were stimulated with 50 μg/ml inactivated type II collagen (heated for 10 min at 80°C) for 72 h at 37°C in a 5% CO_2 humidified atmosphere. Cell proliferation was measured using a BrdU cell proliferation ELISA kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

Measurement of Th1-type cytokines in splenocytes

Isolated splenocytes (5×10^6 cells/well in a 24-well culture plate) were cultured with 50 μg/ml inactivated type II collagen. After 48 h, culture supernatants were collected and the

levels of IFN-γ and IL-2 were measured using ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Electrophoresis mobility shift assay

Fifteen days after the mice received the booster dose of collagen in the CIA mice, their ankles were removed, minced in liquid nitrogen, and stored at −70°C. Nuclear extracts were made using a kit according to the manufacturer's instructions (Panomics, Fremont, CA, USA). Electrophoretic mobility shift assays (EMSA) were carried out using an EMSA 'Gel Shift' kit (Panomics) according to the manufacturer's instructions. Nuclear extracts were incubated with a biotin-labelled oligonucleotide, including the consensus binding sequence for NF-κB (5′-AGTTGAGGGGACTTTCCCA GGC-3′) or AP-1 (5′-CGCTTGATGACTCAGCCGGAA-3′) for 30 min at 15°C, and reactions were separated on a 6% non-denaturing polyacrylamide gel. After transfer to Biodyne B membrane (Pall Corporation, Ann Arbor, MI, USA) for 30 min at 300 mA, detection of biotinylated DNA was performed using horseradish peroxidase-based chemiluminescence according to the manufacturer's protocols.

Statistical analysis

The data are presented as the mean ± SEM from three or more independent experiments. Statistical analysis was performed by using *SigmaStat* Ver. 3.5 (SPSS Inc., Chicago, IL, USA). Parametric tests were used for the analysis of the data that passed the assumption tests of normality using a one-way analysis of variance. When the data did not pass the normality test of data distribution, the Kruskal–Wallis' one-way analysis of variance on ranks (for nonparametric data), followed by the All Pairwise Multiple Comparison Procedure using the Student–Newman–Keuls Method, were used. $P < 0.05$ was considered to be statistically significant.

Results

Effect of TCMC on collagen-induced arthritis in mice

In the preliminary study, we investigated the individual effect of TC or MC in CIA mice in the dose range 0–1000 mg/kg. Of note, a single treatment with TC or MC exerted a maximal effect at a dose of 100 mg/kg, and reached a plateau over a dose of 100 mg/kg (data not shown). Interestingly, the combined treatment of TC (100 mg/kg) with MC (100 mg/kg) showed an augmented effect compared with the individual treatment of TC or MC (Figure 1a). To further clarify the effect of the herbal mixture TCMC, we performed the experiments at a dose range 1–1000 mg/kg to determine the optimal doses for the main study. The anti-arthritis effect at 1 mg/kg was minimal, and the effect reached a plateau at doses above 200 mg/kg TCMC. On the basis of the dose-finding study, TCMC (50, 100 and 200 mg/kg) or a vehicle (5% Arabic gum) was administered once a day for 15 days, starting on the day of the second collagen injection. As the standard drugs, bucillamine (300 mg/kg) or meloxicam (4 mg/kg) were orally administered as was TCMC. The arthritis severity was evaluated daily over the same period (Figure 1b). The arthritis score was 13.5 ± 0.80 in the vehicle-treated control group. The scores showed a significant dose-dependent decrease in the TCMC treatment groups: 7.7 ± 1.50 at 50 mg/kg, 6.6 ± 2.19 at 100 mg/kg, 5.4 ± 1.47 at 200 mg/kg (Figure 1b). At 200 mg/kg of TCMC, the effect was equivalent to that of the reference drugs, bucillamine (300 mg/kg) or meloxicam (4 mg/kg) (data not shown).

HPLC analysis of TCMC

It is important to obtain complete chromatographic data on reference compounds from herbal extracts to minimize the possible variation in herbal components during the extraction process. Therefore, we analysed TCMC by two different conditions using HPLC as described in Materials and Methods. As the reference compounds, arctigenin (RT, 27 min) and paeoniflorin (RT, 22 min) were identified from the extract (Figure 1a).

Effect of TCMC on histological synovitis and cartilage destruction in collagen-induced arthritis mice

The above observations were further confirmed by histological analysis of the hind paws of CIA mice. Infiltration of immune cells, synovial hyperplasia, cartilage destruction and bone erosion were evident in the vehicle-treated CIA mice (Figure 2a). In contrast, minor damage was seen in the TCMC (200 mg/ml)-treated and bucillamine (300 mg/ml)-treated CIA mice (Figure 2a), and histological scores were significantly reduced by TCMC (Figure 2b). These results indicated that TCMC had an anti-arthritis effect in CIA mice.

Effect of TCMC on the levels of rheumatoid arthritis biomarkers in serum of collagen-induced arthritis mice

To assess the effect of TCMC on cartilage destruction, the level of serum COMP, a circulating marker of cartilage turnover, was analysed by ELISA. In normal mice, the serum COMP level was approximately 2.6 ± 0.03 μg/ml, which increased to 6.1 ± 0.4 μg/ml in vehicle-treated CIA mice (Figure 3a). However, TCMC markedly reduced serum COMP levels (4.4 ± 0.3 μg/ml, 29.1% inhibition), showing a similar potency to that of bucillamine (3.9 ± 0.2 μg/ml, 36.3% inhibition) (Figure 3a). SAP levels were barely detectable in normal mice, but were significantly elevated in

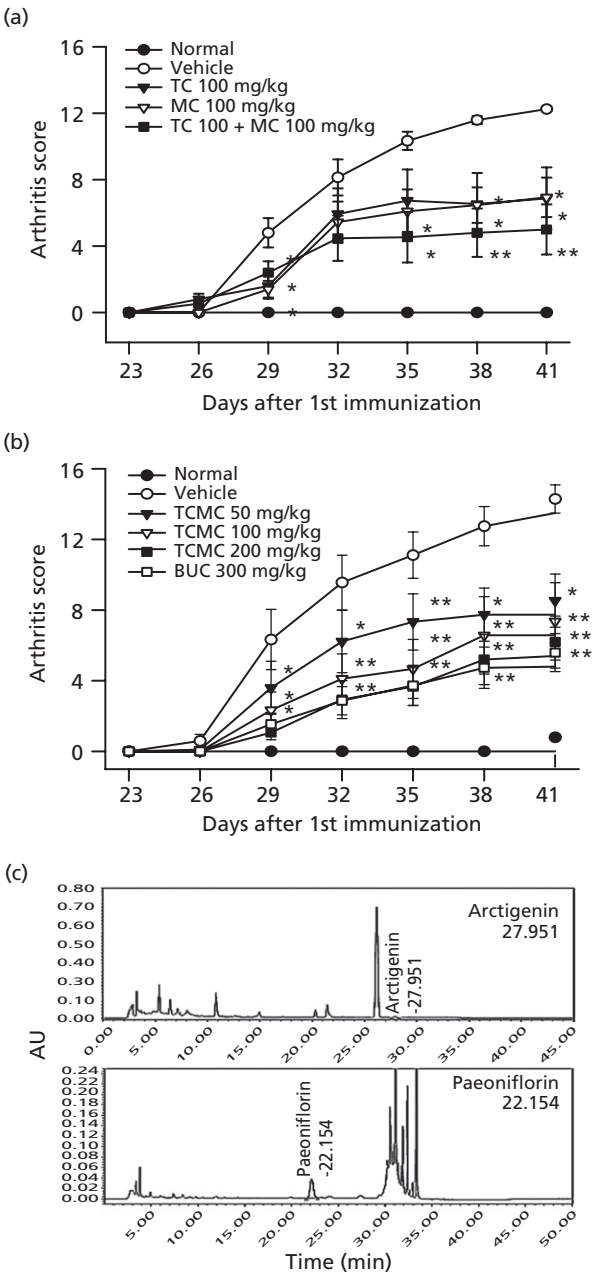

Figure 1 Effect of TCMC on collagen-induced arthritis in mice. (a,b) Collagen-induced arthritis (CIA) was induced in mice by injecting type II collagen. (a) For the preliminary study, TC (100 mg/kg), MC (100 mg/kg) or the combined mixture of TC (100 mg/kg) plus MC (100 mg/kg) were orally administered once daily after the mice were boosted with an injection of collagen. (b) TCMC (50–200 mg/kg), bucillamine (BUC; 300 mg/kg) or a vehicle was orally administered once daily after the mice were boosted with an injection of collagen. (a,b) Arthritis index scores are expressed as the mean ± SEM of values obtained from 10 mice in each group. $*P < 0.05$, $**P < 0.01$ compared with corresponding values from vehicle-treated mice with CIA. (c) TCMC was analysed by HPLC using XBridge C18 5 um (4.6 × 250 mm) column as described in Materials and Methods. Peaks were detected at 230 nm; arctigenin, RT 27.9 min; paeoniflorin, RT, 22.1 min.

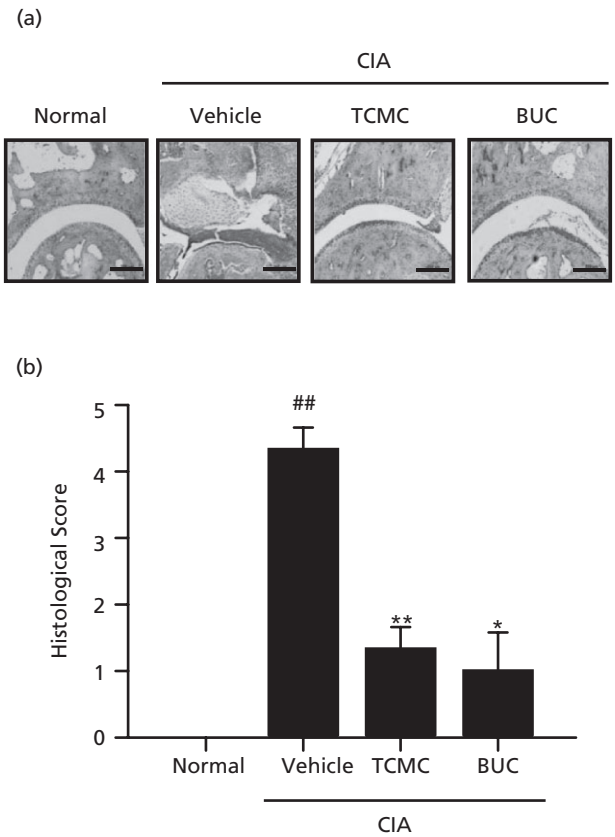

Figure 2 Histological effects of TCMC in collagen-induced arthritis (CIA) mice. Ankle tissue sections were prepared from normal or CIA mice with or without TCMC treatment, as described in the Figure 1 legend, and stained with hematoxylin and eosin (H&E) as described in Materials and Methods. (a) Representative histological images from three independent experiments are shown. Scale bar: 0.2 mm. (b) Values are represented as the mean ± SEM from three independent CIA experiments, $n = 5$ for each experiment. $^{\#\#}P < 0.01$, compared with values from normal mice; $**P < 0.01$ compared with values from the vehicle-treated group. Normal, without treatment of collagen; Vehicle, 5% Arabic gum; TCMC, 200 mg/kg; bucillamine (BUC), 300 mg/kg.

vehicle-treated CIA mice (1084.8 ± 31.8 μg/ml) (Figure 3b). TCMC and bucillamine significantly reduced the level of SAP by 44.4% and 66.2%, respectively, relative to levels in vehicle-treated CIA mice (Figure 3b). To study the effect of TCMC on the humoral immune response against type II collagen, the level of anti-CII IgG was analysed in sera of CIA mice. The anti-CII IgG antibody was minimally detectable in the sera of normal mice, but its levels were significantly elevated in vehicle-treated CIA mice (Figure 3c). The administration of TCMC significantly reduced the level of anti-CII IgG antibody by 76.6% relative to levels in vehicle-treated CIA mice, and so showed a similar potency to that of bucillamine (60.3% inhibition) (Figure 3c).

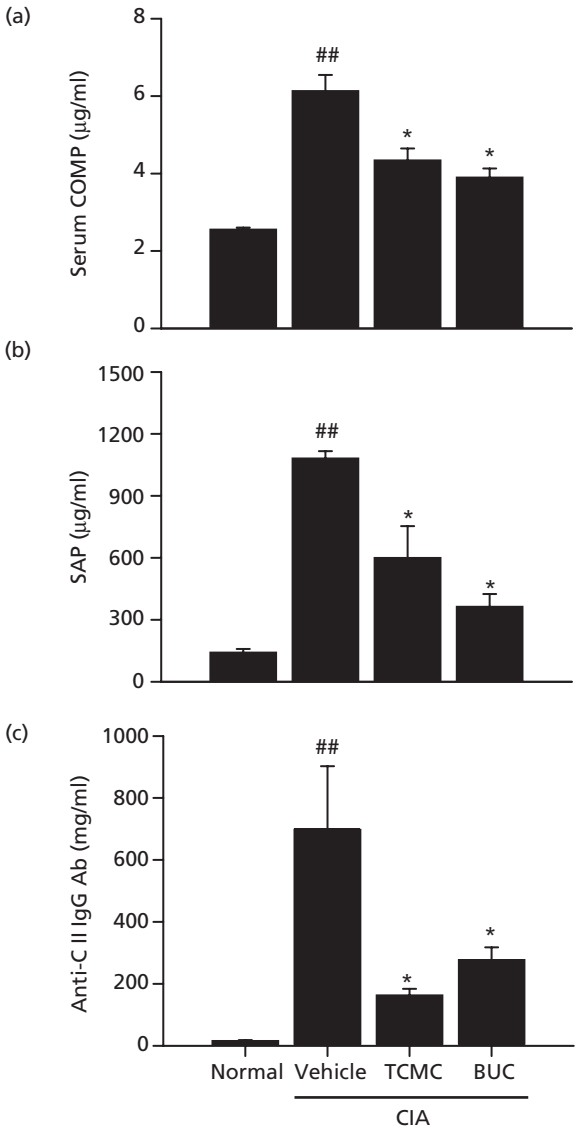

Figure 3 Effect of TCMC on the levels of serum rheumatoid arthritis (RA) markers in collagen-induced arthritis (CIA) mice. The level of serum cartilage oligomeric matrix protein (COMP) (a), serum amyloid P (SAP) (b), and anti-collagen type II (CII) IgG antibody (c) were analysed by ELISA according to the manufacturer's instructions. Values are expressed as the mean ± SEM from three independent CIA experiments; $n = 5$ for each experiment. $^{\#\#}P < 0.01$, compared with values from normal mice; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with values from vehicle-treated mice with CIA.

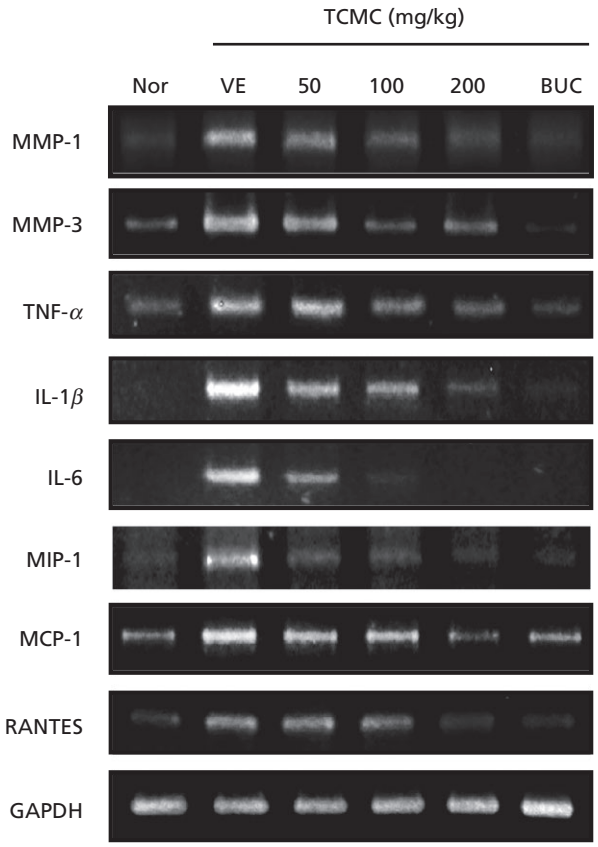

Figure 4 Effect of TCMC on the activation of arthritis-associated rheumatoid arthritis (RA) factors in collagen-induced arthritis (CIA) mice. Total RNA was extracted from normal or CIA mouse ankle tissues and was subjected to reverse transcription. The mRNA levels of matrix metalloproteinases (MMPs) (MMP-1 and MMP-3), cytokines (tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6) and chemokines (macrophage inflammatory protein-1, monocyte chemoattractant protein-1 and Regulated upon Activation, Normal T-cell Expressed, and Secreted) were determined by RT-PCR as described in Materials and Methods. Representative images from three independent experiments are shown.

Effect of TCMC on the expression of inflammation-associated factors in joints of collagen-induced arthritis mice

Many inflammatory factors are associated with RA. Thus, we determined the effect of TCMC on the production of critical factors in joints of CIA mice. First, we tested whether TCMC suppresses the expression of inflammatory proteinases (MMP-1 and MMP-3), cytokines (TNF-α, IL-1β and IL-6) or chemokines (MIP-1, MCP-1 and RANTES) in the joints of CIA mice. We found that the mRNA expression of most factors was suppressed by TCMC in a dose-dependent manner (Figure 4a). Consistently, the protein levels of TNF-α, IL-1β and IL-6 were significantly suppressed by TCMC (200 mg/kg) in the ankle joints of CIA mice (data not shown). These results show that the anti-arthritis effect of TCMC is accompanied by down-regulation of various inflammatory factors.

Effect of TCMC on type II collagen-specific T-cell proliferation and Th1-type cytokine production in splenocytes from collagen-induced arthritis mice

RA is characterized by chronic Th1-associated inflammation.[4,20] Thus, we investigated CII-specific proliferation of splenocytes isolated from CIA mice treated with vehicle, TCMC or bucillamine. The proliferation of splenocytes from vehicle-treated CIA mice was enhanced by stimulation with CII, in comparison with the proliferation of normal splenocytes. However, the proliferation of splenocytes from CIA mice treated with TCMC or bucillamine was significantly suppressed (Figure 5a). Furthermore, TCMC treatment significantly inhibited the production of CII-induced Th1 cytokines by 74.7% (for IFN-γ) and 36.8% (for IL-2), relative to that in vehicle-treated CIA mice (Figure 5b).

Effect of TCMC on osteoclast differentiation *in vitro* or in the joints of collagen-induced arthritis mice

Multinucleated osteoclasts play a pivotal role in bone destruction and erosion in RA.[11] We tested whether TCMC has any effect on the differentiation of osteoclasts. We found that TCMC significantly reduced the number of osteoclasts in the proximal tibia of CIA mice (Figure 6a and 6b). To confirm this data, we evaluated the differentiation of osteoclasts from bone marrow derived macrophages (BMMs) in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) with or without TCMC. As expected, the differentiation of osteoclasts from BMMs was inhibited by TCMC in a dose-dependent manner (data not shown). The expression of NFATc1 and c-Fos, typical osteoclast markers, was also inhibited by TCMC (100 μg/ml) (data not shown). These results suggested that TCMC could also suppress the differentiation of osteoclasts *in vitro* and *in vivo*.

Effect of TCMC on the activation of the transcription factors NF-κB and AP-1 in collagen-induced arthritis mice

NF-κB and AP-1 play a critical role in RA pathogenesis.[21] It is well-established that NF-κB and AP-1 regulate the production of MMPs and the expression of pro-inflammatory cytokines in RA joints.[22–24] It is also generally accepted that NF-κB and AP-1 are critical for OC differentiation.[11] These previous reports led us to investigate whether TCMC has an effect on the activation of NF-κB and AP-1 in CIA joints by using EMSA analysis. We found that the activity of NF-κB and AP-1 in vehicle-treated CIA mice was higher than that in normal mice. Interestingly, the activity of NF-κB and AP-1 was suppressed by TCMC, mostly in a dose-dependent manner (Figure 7). These results suggest that the inhibition of NF-κB and AP-1 was the pivotal mechanism for the anti-arthritis effect of TCMC in CIA mice.

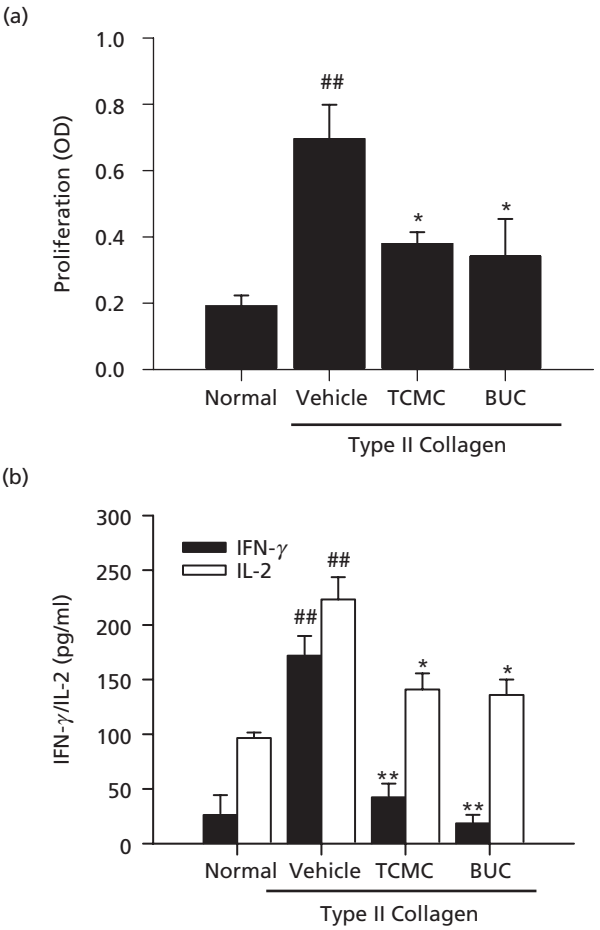

Figure 5 Effect of TCMC on collagen type II (CII)-specific T-cell proliferation and Th1-type cytokine production. Splenocytes were isolated from spleens of the mice described in the Figure 1 legend, as outlined in Materials and Methods. (a) Splenocytes were cultured in triplicate with 50 μg/ml CII for 72 h and cell proliferation was determined using BrdU cell proliferation ELISA kit. (b) Splenocytes were stimulated with 50 μg/ml CII for 48 h. The levels of IFN-γ and IL-2 in the culture media were determined by ELISA. Values are reported as the mean ± SEM from three independent CIA experiments; $n = 5$ for each experiment. $^{\#\#}P < 0.01$, compared with values from normal mice; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with values from vehicle-treated mice with CIA.

Discussion

The two herbal extracts that constitute TCMC are widely available in Asia, including Korea, and have been used in traditional oriental medicine. However, the pharmacological basis for the traditional usage of TC and MC extract for the treatment of various inflammatory diseases has not yet been established. A previous report investigated the use of TC as one component of a therapeutic formula for RA. The mixture

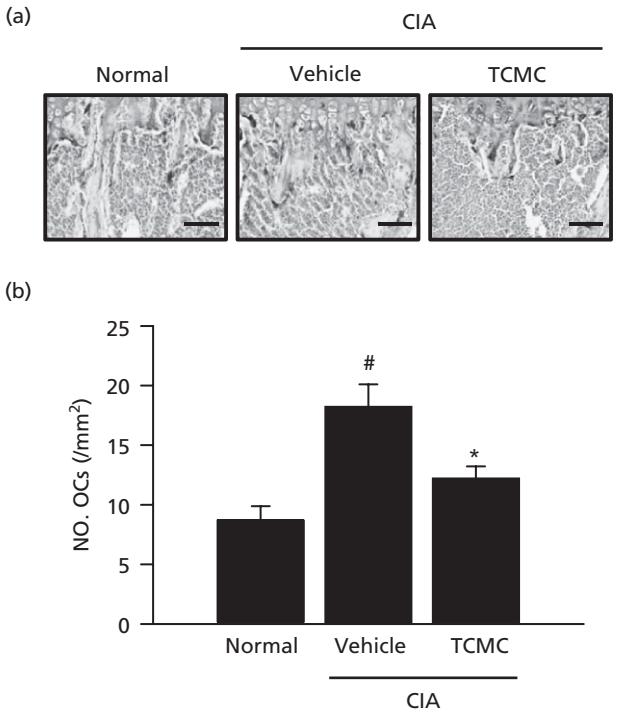

Figure 6 Effect of TCMC on osteoclast formation in the tibia of collagen-induced arthritis (CIA) mice. Paraffin sections of the proximal tibia were prepared from normal or CIA mice with or without TCMC treatment (200 mg/kg). The sections were examined after staining for tartrate-resistant acid phosphatase (TRAP). (a) Representative images are shown from three independent CIA experiments; $n = 5$ for each experiment. Scale bar: 0.2 mm. (b) The number of TRAP-positive osteoclasts per bone surface (mm^2) was counted. Values are presented as the mean ± SEM from three independent CIA experiments; $n = 5$ for each experiment. $^{\#\#}P < 0.001$, compared with numbers in normal mice; $^{*}P < 0.05$, compared with numbers in vehicle-treated mice with CIA.

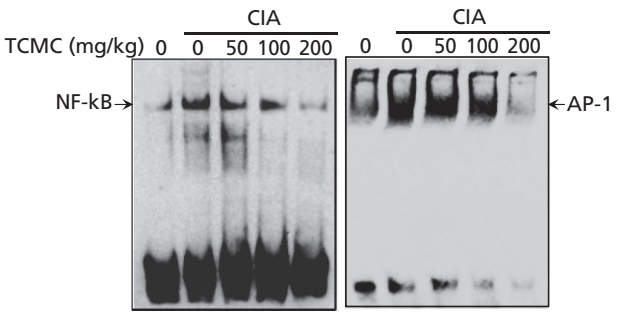

Figure 7 TCMC inhibits nuclear factor (NF)-κB and activator protein (AP)-1 DNA binding activity in CIA mice. Nuclear extracts were prepared from normal or collagen-induced arthritis (CIA) mouse ankle tissues with or without TCMC (50–200 mg/kg) and examined for NF-κB and AP-1 activity by EMSA as described in Materials and Methods. Representative images are shown for NF-κB and AP-1 from three independent experiments.

of TC and *Pyrolae herba* extract reduced arthritis macroscopic scores and histological changes.[25] However, the mechanism underlying the anti-arthritis effect of this formula, and of TC, was not clear. It was also previously reported that TC extract suppressed the expression of inducible nitric oxide synthase and nitric oxide production.[16] MC has been widely used as an oriental folk remedy for the treatment of atherosclerosis, infection and inflammation, including allergy.[14,15] In the course of our long-term efforts to find a good drug candidate to treat RA, we selected the mixture of TC and MC for analysis in this study.

RA is a chronic autoimmune inflammatory disease characterized by joint swelling, bone erosion and infiltration of immune cells into RA joints.[1] Because CIA in mice resembles human RA in both its clinical and histopathological characteristics, it is an ideal model to test the anti-arthritis effects of drug candidates.[26] TCMC significantly ameliorated CIA (Figure 1b and 1c) and histological changes (Figure 2) in mice, suggesting that TCMC could be used as a treatment for RA in humans.

An early study reported that MMP family members are key proteins involved in the remodelling of articular tissues in RA.[27] Among them, MMP-1 and MMP-3 are the major proteinases produced by fibroblast-like synoviocytes and chondrocytes in joints. The level of MMP-1 in serum is also correlated with the progression of joint destruction in RA patients.[5,28,29] In addition, it is known that pro-inflammatory cytokines, chemokines and growth factors play a central role in RA pathogenesis by inducing the infiltration of immune cells and stimulating the release of MMPs.[30–33] Our results showed that TCMC suppressed the expression of proteinases (MMP-1 and MMP-3), cytokines (TNF-α, IL-1β and IL-6) and chemokines (MIP-1, MCP-1 and RANTES) in CIA joints, indicating that TCMC is an effective inhibitor of joint pathogenesis in mice.

Several lines of evidence indicate that T cells are abnormally activated in human RA[1] and in CIA in mice.[34] In this study, we assessed whether TCMC treatment affected the proliferation of T cells in isolated splenocytes from CIA mice. The results showed that TCMC effectively inhibited the type II collagen-mediated proliferation of T cells (Figure 5a). Furthermore, TCMC suppressed the production of IFN-γ and IL-2 in type II collagen-stimulated splenocytes (Figure 5b). These results suggest that the Th1 response in CIA joints was suppressed by TCMC.

Osteoclasts are multinucleated cells formed by the fusion of mononuclear phagocyte precursors, and are unique in having bone resorption activity. They are associated with the pathogenesis of various bone diseases, including RA.[11] TCMC significantly reduced the number of osteoclasts in CIA mouse proximal tibiae (Figure 6a). Furthermore, the differentiation of osteoclasts and expression of the master differentiation factors, NFATc1 and AP-1, were inhibited by

TCMC (data not shown). These results demonstrate that TCMC may be useful to treat other bone-related diseases, including osteoporosis, in addition to RA.

The DNA binding activity of AP-1 and NF-κB is markedly increased in both CIA and RA. In CIA, activation of AP-1 and NF-κB precedes both clinical arthritis and metalloproteinase gene expression.[35,36] In the pathogenesis of RA, the production of MMPs, activation of Th1 responses and osteoclast formation are also regulated by transcriptional activation of NF-κB and AP-1.[11,23,37,38] The previous reports strongly suggest that the activation of NF-κB and AP-1 is critical for the pathogenesis of RA. In this study, the activation of NF-κB and AP-1 was inhibited by TCMC (Figure 7). Therefore, it is most likely that TCMC ameliorated CIA in mice by inhibiting activation of NF-κB and AP-1.

Conclusions

A combination extract of TC and MC exerts an anti-arthritis effect in CIA mice by suppression of the production of various inflammatory factors and the formation of osteoclasts through the inhibition of NF-κB and AP-1 activation. The data suggests that the herbal mixture TCMC might be useful in the treatment of RA. Further studies with TCMC should be carried out to assess its safety and characterize its active components for potential clinical application.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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