ORIGINAL ARTICLE

Effect of An Herbal Mixture of *Cinnamon Cortex*, *Persicae Semen*, and *Natril Sulfas* on Collagen-Induced Arthritis and Lipopolysaccharides-Induced Nuclear Factor-κB Signaling

Ji-Won Lee, Jae-Hwan Lew, Tae-Woo Kim, and Hee Kang

ABSTRACT **Objective**: To investigate the anti-arthritic and anti-inflammatory effects of the mixture of three herbal agents, *Cinnamon Cortex*, *Persica Semen*, and *Natril Sulfas* (CPN), the major ingredients of Taoren Chengqi Decoction (桃仁承气汤). **Methods**: Collagen-induced arthritis (CIA) was induced by immunization with bovine type Ⅱ collagen on day 1 and 21. DBA/1J mice were orally administered the water extract of CPN (100 and 500 mg/kg) and indomethacin (1 mg/kg) or vehicle (water) 3 times per week for 6 weeks. Arthritic symptoms were recorded on day 29, 31, 33, 36 and 38. On sacrifice, serum was obtained for inflammatory markers and anti-collagen antibodies as well as arthritic joints were obtained for histologic analysis. For the evaluation of *in vitro* anti-inflammatory mechanism of CPN, peritoneal macrophages were isolated from thioglycollate injected C57BL/6 mice and stimulated with lipopolysaccharides (LPS) for 15 min in the presence of CPN extract. Levels of inhibitor of NF-κB α isoform (IκBα), phospho-p38, phospho-C-Jun N-terminal kinases (JNK) and phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) were detected by Western blot. **Results**: Compared with mice in CIA group, oral administration of CPN significantly reduced the clinical scores ($P<0.05$), histological analysis revealed the protective effect of CPN on inflamed joints. Serum levels of the pro-inflammatory markers tumor necrosis factor-α, interleukin-6 and prostaglandin E_2, but not anti-collagen antibodies, were significantly reduced ($P<0.05$). CPN did not affect the activation of p38, JNK and ERK1/2 but inhibited LPS-induced IκBα degradation, a required event prior to the translocation of NF-κB to the nucleus. **Conclusions**: The ameliorating effect of CPN on arthritis progression seems to be mediated by its anti-inflammatory effect, without affecting antibody response. As a supplementary agent, CPN could be beneficial for treatment of CIA.

KEYWORDS *Cinnamon Cortex*, *Persica Semen*, *Natril Sulfas*, rheumatoid arthritis, collagen-induced arthritis, inflammation

The prevalence of rheumatoid arthritis (RA) in different developed countries is estimated to affect 0.5%–1.0% of the adult population.[1] RA is characterized by significant pain, progressive joint inflammation, and gradual disability, leading to premature mortality.[2] Although the pathogenesis of RA is not fully understood, inflammatory and immunological mechanisms are implicated in the development and perpetuation of this disease. Activated T cells and B cells are responsible for immune responses and the generation of autoantibodies against joint tissue.[3] Synovial macrophages and fibroblast-like synoviocytes in the affected joint secret pro-inflammatory cytokines and mediators, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2), and matrix metalloproteinases (MMPs), which perpetuate the disease.[4-7]

The induction of pro-inflammatory cytokine genes in response to various stimuli including lipopolysaccharides (LPS), is generally regulated by nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways. These proteins have become the targets of novel anti-inflammatory drugs for RA and other chronic inflammatory disorders.[8-10] Under resting conditions, NF-κB is sequestered by inhibitor of NF-κB α isoform (IκBα) in the cytosol, but inflammatory stimuli, including cytokines and LPS, trigger the phosphorylation and subsequent degradation of a complexed IκBα protein by IκB kinase.[11] This allows NF-κB to migrate to the nucleus, where it activates many immune and inflammatory genes, including TNF-α, IL-6 and cyclooxygenase-2, the enzyme necessary for PGE_2 production.[8] Activation

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of MAPK is involved in multiple steps of inflammation. There are three major groups of MAPKs: extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK) and p38. MAPKs phosphorylate many transcription factors, such as activator protein-1, or regulate inflammatory cytokine expression at both the transcriptional and post-transcriptional levels.[12]

Taoren Chengqi Decoction (桃仁承气汤) has been prescribed for patients who show the 'blood stasis' pattern characterized by acute lower abdominal pain, night fever, irritability and gynecological symptoms. This herbal complex, consisting of *Cinnamon cortex*, *Persica semen*, *Natril Sulfas*, *Rhei rhizoma*, and *Glycyrrhiza radix*, is recognized as removing heat and improving abnormal blood clotting. Based on the clinical observation that some characteristic patterns of blood stasis occur in RA patients, we attempted to make a shorter formula out of it. Combined with clinical experience, we selected three ingredients, *Cinnamon Cortex*, *Persica Semen*, and *Natril Sulfas* (CPN). Clinically, CPN together are often included in a variety of RA-targeted herbal formulations. Thus, we investigated the anti-arthritic and anti-inflammatory effects of CPN on the collagen-induced arthritis (CIA) animal model, the most widely used experimental tool for RA research.[13] To clarify its underlying anti-inflammatory mechanisms, the effects of CPN on NF-κB, JNK, p38, and ERK1/2 activation were tested in LPS-stimulated macrophages.

METHODS

Animals

Seven-week-old male specific pathogen-free (SPF) DBA/1J mice (*n*=50, SLC, Shizuoka, Japan) or 8-week-old male C57BL/6 mice (*n*=12 Tanonic, SamTaco, Osan, Republic of Korea) were housed in a temperature controlled room (at 23±1 ℃) with a 12-h light/dark cycle, in polystyrene cages at Kyung Hee University and fed standard rodent chow and water *ad libitum*. Mice were cared for according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., USA). All experiments were approved by Kyung Hee University Institutional Ethical Committee for animal welfare.

Preparation of CPN

Cinnamon Cortex (*Cinnamomum cassia* PresL), *Persica Semen* (*Prunus persica* L), and *Mirabilite* (*Natri sulfas*) were obtained from Omni Herb (Daegu, Republic of Korea). Voucher specimens (*Cinnamon Cortex*: HCC-002, *Persica Semen*: HPS-003, and *Natril Sulfas*: HM-004) were deposited at the Department of Herbology, Kyung Hee University. CPN consisted of *Cinnamon Cortex*, *Persica Semen*, and *Natril Sulfas* with a ratio of 2:2:1 (w/w). Dried samples were soaked in distilled water overnight, and further dissolved using sonication for 1 h. The extracts were filtered and evaporated using a –70 ℃ freeze dryer (EYELA, Japan). The yield of the extract was about 8.58%. The samples were resuspended in phosplate- buffered saline (PBS) and sterilized by passing through a 0.22 μm syringe filter.

CIA Induction and Treatment with CPN

Mice were divided into control (without CIA induction), CIA, CPN (100 and 500 mg/kg) and indomethacin groups. Each group consisted of 10 mice. Bovine type Ⅱ collagen (Chondrex, Redmond, WA, USA) was dissolved overnight at 4 ℃ in 0.05 mol/L acetic acid to 2 mg/mL, and emulsified in an equal volume of complete Freund's adjuvant (Chondrex).[14] On day 1, mice in CIA, CPN and indomethacin groups were intravenously injected at the base of the tail with 0.1 mL of the emulsion. On day 21, a booster injection with the same volume was given. Between day 1 and 38, mice were orally administered with CPN (100 and 500 mg/kg), indomethacin (1 mg/kg)[15] (Sigma, St. Louis, MO, USA), or vehicle only (water) 3 times a week.

Evaluation of Arthritis

On day 29, 31, 33, 36 and 38, each paw was scored individually as follows[16]: 0=normal, 1=swelling of one digit, 2=swelling of 2 or more digits, 3=swelling of heel, 4=joint deformity with ankylosis. Each mouse was assigned as arthritis score equaling the sum of the scores for each paw, so that the possible maximum score per mouse was 16.

Histology

On day 38, the treatment was terminated and the mice were sacrificed by cervical dislocation. All joints were surgically removed and immediately fixed in 10% buffered formalin and decalcified in 9% formalin/10% formic acid (Sigma, USA) for 1 week. The tissue was then processed and embedded in paraffin. Five-micrometer tissue section was prepared and stained with hematoxylin and eosin (HE) using standard methods.

Determination of Serum Anti-Collagen Antibody Levels

Serum was collected on day 38. The level of serum anti-collagen antibody was measured by enzyme- linked immunosorbent assay (ELISA). Briefly, flat-bottom 96-well ELISA plates were coated overnight at 4 ℃ with 5 μg/mL ELISA grade bovine type Ⅱ collagen in dilution buffer (Chondrex, USA). Plates were blocked with 1% bovine serum albumin in PBS and incubated with diluted serum overnight at 4 ℃. The plates were washed and incubated with peroxidase-conjugated goat anti-mouse IgG (1:40,000, Sigma, USA) at room temperature for 30 min. Washed plates were treated with tetramethylbenzidine substrate solution (BD Pharmingen, San Diego, CA. USA) at room temperature for 30 min. Reactions were stopped with 0.2 mol/L H_2SO_4 and the absorbances were measured at 450–570 nm.

Measurement of Serum IL-6, TNF-α and PGE_2

Serum concentrations of IL-6, TNF-α, and PGE_2 were measured by ELISA, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Each sample was assayed in duplicate and concentrations were determined by standard curve.

Cell Culture

C57BL/6 mice were injected intraperitoneally with 2 mL of sterile thioglycollate medium (BD, Spark, MD, USA). Three days later, peritoneal macrophages were isolated by peritoneal lavage with cold Dulbecco's modified Eagle's medium (DMEM). Cells were suspended in DMEM with 10% fetal bovine serum (Welgene, Daegu, Republic of Korea) and 1% penicillin-streptomycin and incubated for 2 h in a humidified atmosphere of 5% CO_2 at 37 ℃. Non-adherent cells were removed. Cells were pretreated with CPN (50, 100, and 200 μg/mL), its 3 ingredients (200 μg/mL) or indomethacin (100 μmol/L) 1 h prior to the addition of 100 ng/mL LPS (Sigma, USA). After 15 min, cells were harvested for further assays.

Western Blot Analysis

Total cell extracts were prepared by resuspending the cells in lysis buffer (50 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1 mmol/L EDTA; 20 mmol/L NaF; 0.5% NP-40; and 1% Triton X-100) containing a phosphatase inhibitor (Sigma) and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using the Bradford assay. Cell extracts were run on a 10% sodiumdodecyl sulfate-polyacrylamide gel and were transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h. They were overnight at 4 ℃ incubated with IκBα, β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-JNK, JNK, phospho-p38, p38, phospho-ERK1/2, ERK1/2 (Cell Signaling Technology, Beverly, CA, USA) diluted 1/1000 in 5% skim milk in TBST. The blots were washed with TBST and incubated for 1 h with anti-rabbit or anti-mouse Horseradish Peroxidase-conjugated Ab (diluted 1:5000 in 5% skim milk in TBST). Immunoreactive bands were developed using an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Statistical Analysis

Data were expressed as mean ± standard error of mean ($\bar{x} \pm SEM$). The difference between the control and drug-treatment group was assessed using ANOVA followed by Dunnet's post *hoc* test (SPSS version 17). *P* values less than 0.05 were considered significant.

RESULTS

Inhibitory Effects of CPN on the Development of CIA

Upon the termination of treatment (on day 38), the clinical scores of the CIA mice reached 13.1 ± 4.1, while the scores were reduced to 8.0 ± 2.7 and 9.37 ± 2.9 in 100 and 500 mg/kg CPN groups, respectively (Figure 1). The lower dose group was more effective in ameliorating CIA by the end of the experiment. Indomethacin, used as a reference drug, recorded the lowest score. Histological evaluation of the hind paw in the CIA mice exhibited clear signs of widespread infiltration of inflammatory cells, extensive pannus formation into the articular cartilage, damaged joint space, cartilage destruction and bone erosion, as compared with control group (Figure 2). Treatment with CPN or indomethacin resulted in limited synovial infiltration, well-kept articular cartilage, discernible joint space and regeneration of chondrocytes.

Effect of CPN on Serum Anti-collagen Antibody and Pro-Inflammatory Markers in CIA Mice

CPN treatment did not affect the anti-collagen antibody response in CIA mice (Figure 3A). Indomethacin did not cause a significant change. Instead, 100 mg/kg CPN significantly reduced the serum levels of TNF-α, IL-6, and PGE_2, while the

inhibitory effects of indomethacin were restricted to only IL-6 and PGE_2 (Figure 3 B–D).

Effect of CPN on the Degradation of IκBα and Activation of p38, JNK, and ERK1/2 in LPS-Stimulated Macrophages

CPN suppressed degradation of IκBα in a concentration-dependent manner, but did not affect MAPK activity (Figure 4). Indomethacin did not influence any signaling molecules tested. *Cinnamon Cortex* strongly inhibited the degradation of IκBα and activation of p38, JNK and ERK1/2 altogether, and *Persica Semen* moderately inhibited the degradation of IκBα. *Mirabilite* affected none of these signaling molecules. Because *Cinnamon Cortex* accounts for approximately 40% of CPN, its anti-inflammatory effects within CPN became less potent than was used alone.

DISCUSSION

We investigated whether CPN could be a

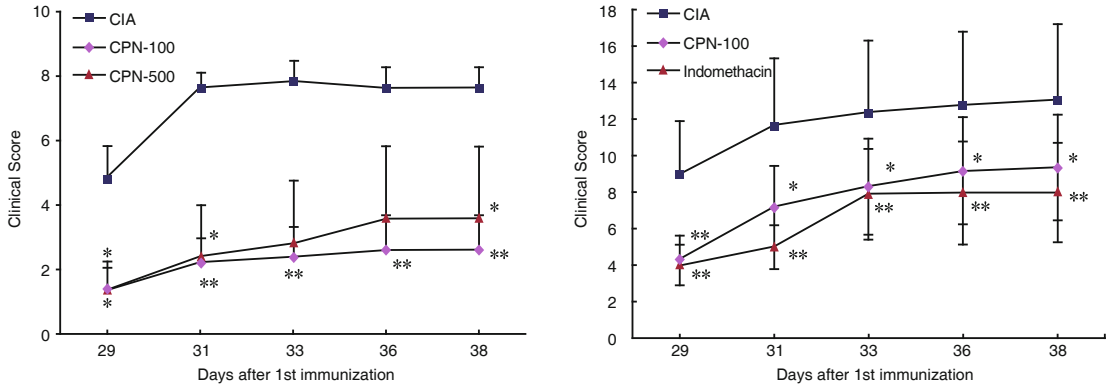

Figure 1. Inhibitory Effects of CPN on the Development of CIA ($\bar{x} \pm SEM$, n=10)

Notes: $^{*}P< 0.05$, $^{**}P<0.01$, compared with control group

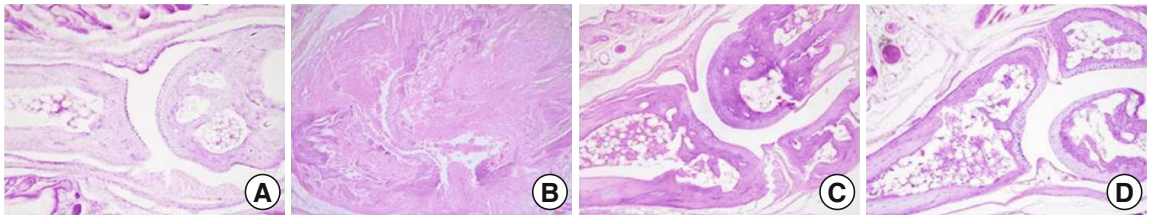

Figure 2. Histopathological Features of Representative Ankle Joints from DBA/1J Mice with CIA (HE Staining, × 100)

Notes: A: control, B: CIA, C: indomethacin, D: CPN (100 mg/kg)

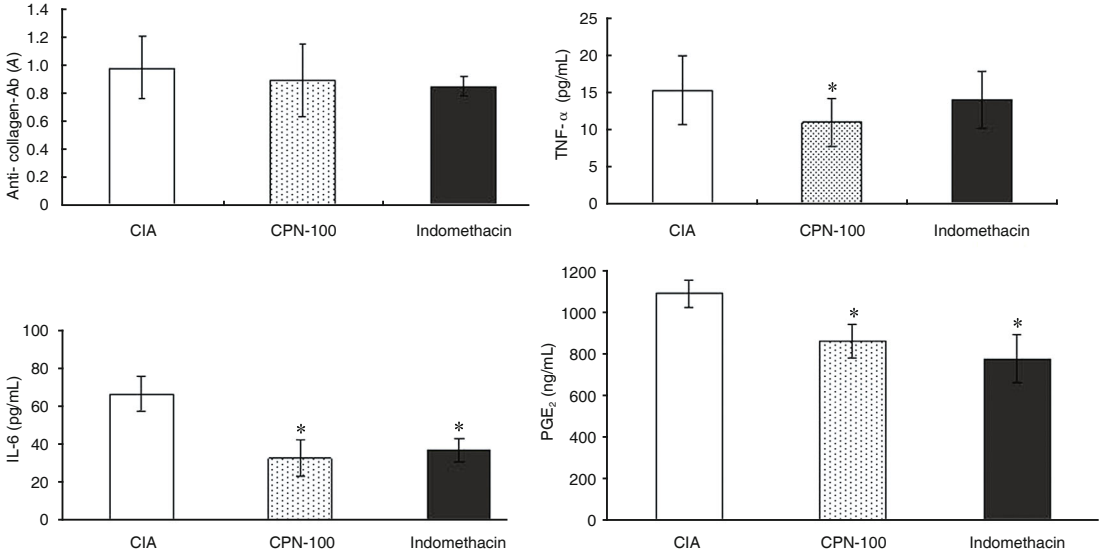

Figure 3. Effect of CPN on Serum Anti-collagen Antibody and Pro-inflammatory Markers in DBA/1J Mice with CIA ($\bar{x} \pm SEM$, n=10)

Notes: $^{*}P<0.05$, compared with CIA group

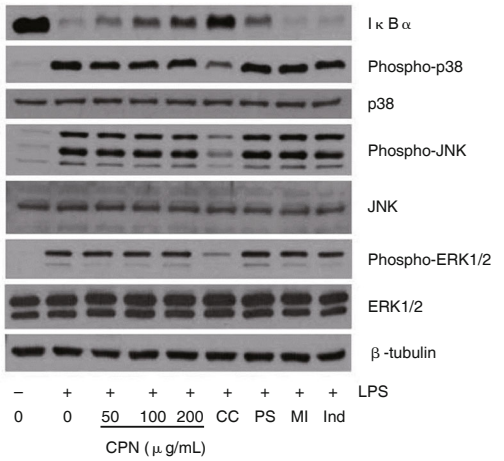

Figure 4. Effects of CPN on I κ B α Degradation and MAPK Activity in LPS-Stimulated Macrophages by Western Blot

Notes: CC: *Cinnamon Cortex*; PS: *Persica Semen*; MI: *Mirabilite*; Ind: Indomethacin; Mouse peritoneal macrophages were pre-treated with CPN, its ingredient herbs (0.2 mg/mL) or indomethacin (0.1 mmol/L) 1 h before stimulation with LPS (100 ng/mL) for 15 min.

potential anti-arthritic and anti-inflammatory agent using the *in vivo* CIA model and the *in vivo* LPS-stimulated macrophages. Oral administration of aqueous CPN extract to DBA/1J mice with CIA significantly reduced clinical symptoms and serum levels of TNF-α, IL-6, and PGE_2. The study also found that CPN negatively affected LPS-stimulated NF-κB pathway *via* inhibition of I κ B α degradation.

The CIA model involves the activation of the adaptive immune system. Type Ⅱ collagen-specific CD4 T cells are responsible for the development and regulation of the autoimmune response by modulating B cell responses, which include the generation of autoantibodies to type Ⅱ collagen.[17] Activated T cells migrate to the articular cartilage where type Ⅱ collagen is abundant, and induce the synovial macrophages and synoviocytes to release pro-inflammatory cytokines, such as TNF-α, IL-6, PGE_2, nitric oxide, and MMPs.[17] Although CIA is induced by exogenous and heterogenous antigen such as CFA and bovine type Ⅱ collagen, histological findings in the joint and the recognition of type Ⅱ collagen by T cells and B cells are almost identical to that of RA.[13] Anti-collagen antibodies play an important role in evoking arthritis following type Ⅱ collagen immunization.[18] The results show that CPN and indomethacin did not affect the generation of anti-collagen antibody in serum, indicating that the anti-arthritic effects of CPN and indomethacin are not directly mediated by the adaptive immune system.

CPN and indomethacin seem to be effective in modulating the inflammatory response induced by collagen immunization, based on the findings that oral administration of CPN and indomethacine systemically suppressed the serum levels of TNF-α, IL-6, and PGE_2 during the development of CIA. Although the etiology of RA has not yet been elucidated, high levels of TNF-α, IL-6, and PGE_2 are found in serum and synovial fluid from many RA patients and arthritic mice.[3,19] TNF-α and IL-6 clearly aggravate this disease, as monoclonal antibodies against these cytokines are effective in controlling RA symptoms.[20,21] These cytokines are largely regulated by NF-κB and, in turn, activate NF-κB itself through a positive feedback loop. In our study, CPN was able to inhibit the degradation of I κ B α, a step upstream of the translocation of NF-κB to the nucleus, in LPS stimulated macrophages. Since I κ B α degradation is a convergence point in LPS or TNF-α-mediated NF-κB activation, the results indicate that inhibition of I κ B α degradation may be one molecular mechanism for the anti-inflammatory actions of CPN. Further study is required to determine whether CPN reduces the local production of these pro-inflammatory cytokines in the joint.

Traditionally, *Cinnamon Cortex* has been applied for treatment of the common cold, arthritis, and gynecological diseases; *Natril Sulfas* has been used for stimulating bowel movements; *Persicae semen* is applied in blood stasis and constipation. Among the ingredients of CPN, *Cinnamon Cortex* might be more potent in suppressing inflammatory signaling pathways *in vitro* than the other ingredients or even CPN itself. The chronic use of a single agent may be less effective in treating disease than that of a combination of herbal agents because the latter can be more efficient in resisting the buffering effects of the biological system. Traditional herbal medicine has documented many pairs of herbal agents that can promote synergistic actions. Accordingly, *Persica Semen* and *Natril Sulfas* could be regarded as those partners that enhance the long-term effect of *Cinnamon Cortex* on ameliorating arthritic pains. In fact, the anti-inflammatory effects of the methanol extract of *Cinnamon cortex* alone were observed only in certain types of acute paw edema models *in vivo* but not in chronic ones such as adjuvant-induced arthritis in rats.[22]

Since we extracted CPN with water, cinnamaldehyde, the main essential oil of *Cinnamon Cortex*, and amygdalin, the well-known compound of *Persica semen* or others were not identified in

high performance liquid chromatography (data not shown). Up to now, many compounds utilized for standardization were lipid-soluble. Further study is expected to determine appropriate water-soluble components for quality control of CPN.

Taken together, we demonstrated the protective effect of CPN on the progression of CIA and its inhibition of NF-κB signaling in LPS-activated macrophages. CPN, a modified mixture of an existing herbal formula, can be beneficial for treatment of RA as a supplementary agent.

Conflict of Interests

The authors have no conflict of interest.

Author Contributions

Lee JW and Kang H contributed to the writing of the manuscript. Lee JW contributed to the literature survey. Kim TW and Kang H performed the experiments. All authors have read and approved the final manuscript.

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