pISSN 1738-2696 · eISSN 2212-5469
http://dx.doi.org/10.1007/s13770-015-0028-0
ORIGINAL ARTICLE

TERM

The Chicken Combs Extract Alleviates Pain and Cartilage Degradation in Rat Model Osteoarthritis

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Osteoarthritis (OA) is a degenerative and inflammatory disorder on particular joint inducing annihilation of articular cartilage. This study is aimed to investigate the therapeutic effect of extract of chicken combs (CCE) on pain severity and cartilage degeneration in an experimental model of rat OA. OA was induced in rats by intra-articular injection of monosodium iodoacetate (MIA) to the right knee. CCE, hyaluronic acid and celecoxib were administrated orally every day after MIA injection. Pain severity was estimated by evaluation of secondary tactile allodynia using the von Frey assessment test. The severity of cartilage degradation was examined by histological analysis and Mankin scoring system. Protein expression was observed by immunohistochemistry. Real-time polymerase chain reaction was used to measure mRNA level. CCE decreased secondary tactile allodynia revealed by a promoted pain withdrawal latency and pain withdrawal threshold. Cartilage destruction in the osteoarthritic joints was improved by CCE treatment. CCE also suppressed the expression of metalloproteinase (MMP)-3, -13, interleukin-1β, inducible nitric oxide synthase and nitrotyrosine increased in osteoarthritic joints. The mRNA level of MMP-1, 3, and -13 was down-regulated by CCE treatment. On the other hand, CCE treatment induced the gene expression of tissue inhibitor of metalloproteinase-1 and -3. CCE treatment demonstrates the therapeutic effect of pain relief and attenuates cartilage degeneration through the suppression of inflammatory mediators and metalloproteinases performing a pivotal function in OA pathogenesis.

Tissue Eng Regen Med 2015;12(5):352-361

Key Words: Chicken combs extract; Monosodium iodoacetate; Osteoarthritis

INTRODUCTION

Osteoarthritis (OA) is a degenerative disorder up-regulating pressure on a particular joint or a destruction of articular cartilage matrix and characterized by increasing pain and movement limitations. It has been recognized that OA is the most common type of arthritis and recognized to interrupt quality of life [1,2]. Recently, the concept of OA has been changed as inflammatory disease [3]. The expression of proinflammatory cytokines, chemokines, matrix metalloproteinases (MMPs) and reactive oxygen species are increased in OA joint tissues inducing cartilage degradation [4,5].

Hyaluronic acid (HA) is a heteropolysaccharide shaped by various repeating unit of D-glucuronic acid and N-acetylglucosamine. As HA is constituent in synovial fluids and conducts biophysical and biochemical function in joint synovial tissues, it has been widely used to relive pain and articular destruction in knee of OA [6-8]. But, HA has several adverse effect in OA treatment. For example, HA injection results in harmful stimuli at injection area [9]. Moreover, it has been suggested that intra-articular injections of HA causes several adverse effect such as septic arthritis [10,11].

Celecoxib (CLX) is a nonsteroidal anti-inflammatory drug and works by suppression of hormones that lead to inflammation and pain. It is well documented that CLX is administered to various inflammatory diseases including OA [12]. However, CLX results in many side effects. It has been demonstrated that CLX leads to vascular related complication such as myocardial infarction, nonfatal stroke or problems with vision [13,14]. It

Received: April 28, 2015
Revised: May 28, 2015
Accepted: June 5, 2015
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is also reported that serious complications on the stomach or intestines including perforations and gastrointestinal bleeding are caused by CLX [14].

Chicken comb extract (CCE) used as a typical component in several recipes contains HA mainly [15]. It is well reported that CCE induces hyaluronic acid production by human synoviocytes [16]. As HA reveals anti-inflammatory effect, CCE has a potentiality of the therapeutic function in OA. It has been suggested that CCE attenuates several symptoms of OA patients such as pain and discomfort [1,17]. In addition, CCE may ameliorate the OA involved in biomarker such as the balance of cartilage type II collagen degradation/synthesis in knee of OA patients [17].

As OA is regarded as a disease of maintaing inflammation, we hypothesized that CCE has a therapeutic function on the inflammatory response in OA. Thus, it is plausible that CCE might have a therapeutic role in OA as well. The purpose of the present study was to determine whether CCE reduces pain and cartilage degradation in OA rat model.

MATERIALS AND METHODS

Animals

Male Wistar rats (Central Lab. Animal Inc., Seoul, Korea) weighing 140−230 g (6 weeks of age) at the start of the experiment were used. The animals were housed three per cage in a room with controlled temperature conditions (21−22°C) and lighting (12/12 h light/dark cycle), and had free access to sterile food and water. All of the animal procedures were approved by the Animal Research Ethics Committee at The Catholic University of Korea (Permit Number: 2014-0002-01).

Induction of OA in the rats and treatment with CCE, HA, and CLX

The animals were randomized and assigned to treatment groups before the study began. OA was induced by intra-articular injection of 50 μL containing 3 mg monosodium iodoacetate (MIA; Sigma, St. Louis, MO, USA) using a 26.5 G needle inserted through the patellar ligament into the intra-articular space of the right knee. The CCE (Api Co. Ltd., Gifu, Japan) and HA were kindly provided by Everlife. Co., Ltd. (Fukuoka, Japan). Animals with OA were treated with saline, CCE (200 mg/kg), HA (25 mg/kg), or CLX (5 mg/kg). CCE, HA, and CLX were administered orally each day.

Assessment of pain behavior

As described in detail in previously [4,18], the MIA-treated rats were randomized to each experimental group and mechanical sensitivity was assessed using a dynamic plantar aesthesiometer (UgoBasile, Comerio, Italy), which is essentially an automated version of the von Frey hair assessment procedure. The rats were placed on a metal mesh surface in an acrylic chamber in a temperature-controlled room (21−22°C), and were allowed to acclimatize for 15 min before the testing began. The touch stimulator unit was oriented under the animal. An adjustable angled mirror was used to place the stimulating microfilament (0.5 mm diameter) below the plantar surface of the hind paw. When the instrument was activated, a fine plastic monofilament was advanced at a constant speed and touched the paw in the proximal metatarsal region.

The filament exerted a gradually increasing force on the plantar surface, starting below the threshold of detection and increasing until the stimulus became painful, as indicated by removal of the paw. The force required to elicit a paw withdrawal reflex was recorded automatically and measured in grams. A maximum force of 50 g and a ramp speed of 20 s were used for all of the aesthesiometry tests. Pain behavioral tests of secondary tactile allodynia were conducted immediately before administering CCE, HA, and CLX.

Joint histology and immunohistochemical analyses

Histological changes were assessed to confirm the effects of CCE, HA, and CLX on cartilage degeneration in the knee joints of OA rats. The knee joints, including the patella and joint capsule, were resected and kept in the 10% neutral buffered formalin for an additional 48 h at 4°C. The fixed specimens were decalcified with 5% formic acid decalcifier for 6 days at 4°C. After decalcification, the specimens were embedded in paraffin. Standardized 7-μm serial sections were obtained at the medial and lateral mid condylar level in the sagittal plane and were stained with hematoxylin and eosin (H&E), Safranin O-fast green, and toluidine blue to enable evaluation of proteoglycan content. Slides for immunohistochemistry were deparaffinized and rehydrated using a graded ethanol series. The sections were depleted of endogenous peroxidase activity by adding methanolic H_2O_2 and then blocked with normal goat serum for 30 min. The samples were incubated overnight at 4°C with antibodies to interleukin (IL)-1β at a dilution of 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MMP-13 at 1:50 (Abcam, Cambridge, UK), MMP-3 at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), inducible nitric oxide synthase (iNOS) at 1:100 (Abcam), nitrotyrosine 1:100 (Santa Cruz). The samples were then incubated with the respective secondary antibodies, biotinylated anti-mouse IgG or rabbit IgG, for 20 min, conjugated to a streptavidine peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 1 h, and finally with 3,30-diaminobenzidine (Dako, Glostrup, Denmark). The sections were counterstained with Mayer's hema-

toxylin and photographed using an Olympus photomicroscope (Olympus, Tokyo, Japan).

A modified Mankin's histological score [19] (original score proposed by Mankin et al. [20]) was used to score histological injuries of the articular cartilage as follows. The structure was scored on a scale of 0−6, where 0=normal; 1=irregular surface, including fissures into the radial layer; 2=pannus; 3=absence of superficial cartilage layers; 4=slight disorganization (cellular row absent, some small superficial clusters); 5=fissure into the calcified cartilage layer; and 6=disorganization (chaotic structure, clusters, and osteoclasts activity). Cellular abnormalities were scored on a scale of 0−3, where 0=normal; 1=hypercellularity, including small superficial clusters; 2=clusters; and 3=hypocellularity. The matrix staining was scored on a scale of 0−4, where 0=normal/slight reduction in staining; 1=staining reduced in the radial layer; 2=staining reduced in the interterritorial matrix; 3=staining present only in the pericellular matrix; and 4=staining absent. Joint space width was estimated measuring the sum of the nearest distance of medial and lateral tibiofemoral joints. Histological evaluation was performed by two independent experienced researchers who were blinded to the treatment arm.

Microscopic imaging analysis of OA joints

Global joint pathology was evaluated by India ink. Femoral and tibial were carefully dissected separately without damping the cartilage surface, and were then stained with India ink to identify the location, size and severity of cartilage degradation. Digital images were taken using an Olympus photomicroscope (Olympus, Tokyo, Japan).

Chondrocyte isolation and expansion

Our study was approved by the Institutional Review Board of Bucheon St. Mary's Hospital (HC15TISI0002) and was performed in accordance with the Helsinki II Declaration. All patients were informed and gave their written consent. An informed consent was obtained from all patients with OA, who fulfilled the American College of Rheumatology criteria for this disease [21]. Cartilage samples were washed in calcium- and magnesium-free PBS and finely grounded. Chondrocytes were obtained by digesting the articular cartilage with 0.2% pronase (Sigma) for 1 h, followed by digestion with 0.2% Clostridia collagenase (Sigma) for 3 h at 37°C in high-glucose Dulbecco's modified Eagle medium (DMEM; Life Technologies, Gaithersburg, MD, USA) containing an antibiotic-antimycotic solution (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; Life Technologies). Undigested cartilage was removed with a 70-μg nylon mesh (Cell Strainer; Falcon), and the chondrocytes were collected by centrifugation, washed twice, followed by being resuspended in DMEM with 10% fetal bovine serum (FBS; Life Technologies). Finally, the cells were plated in 100-mm tissue culture dishes for expansion at 37°C in a 5% CO_2 humidified atmosphere for 10 days (Shel Lab). The medium of the suspension culture was changed every 2−3 days. In all experiments, chondrocytes were cultured under FBS-free DMEM conditions (5%, v/v) and were used in monolayers at confluence. Cells (1×10^5 cells/well) were placed in 24-well tissue culture plates, and the medium was replaced with serum-free DMEM on the next day. Twenty-four hours later, the cells were pretreated with eupatilin for 2 h and then stimulated with or without recombinant human IL-1β (20 ng/mL; R&D Systems, MN, USA) for 48 h.

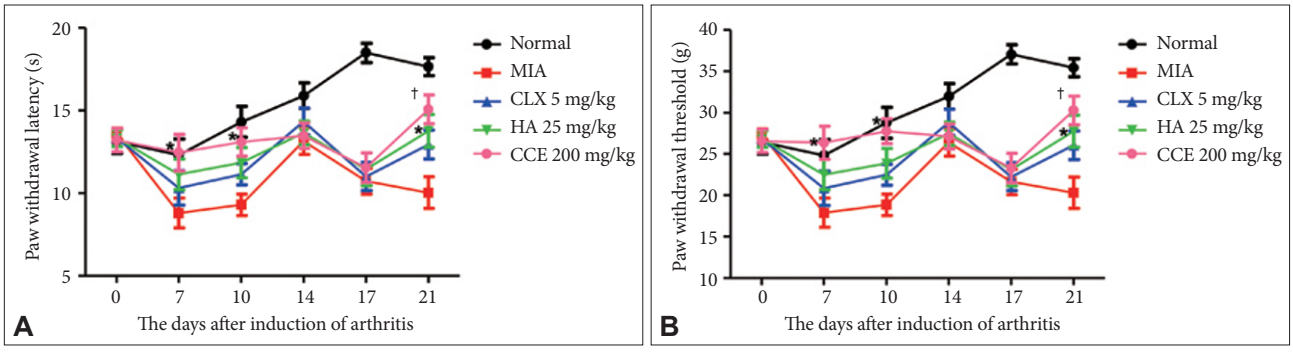

Figure 1. Therapeutic effect of chicken comb extract (CCE) in an early phase of monosodium iodoacetate (MIA) induced osteoarthritis (OA) in rats. CCE was administered orally every day from 4th day after MIA injection in the right knee of rats. Behavioral tests of secondary tactile allodynia in the right knee MIA injected rats treated with hyaluronic acid (HA), celecoxib (CLX) or CCE were measured using a dynamic plantar esthesiometer (n=10 on each day for each group). (A) Compared with vehicle-treated OA rats, OA animals treated with CCE at a dose of 100 mg/kg showed a significant increase in paw withdrawal latency (PWL). (B) Paw withdrawal threshold (PWT) was increased significantly in OA animals treated with CCE. The data are expressed as mean and error bars for three animals per group. PWL and PWT were conducted right before the administration of CCE. Significant differences between vehicle- and CCE-treated groups: *p<0.05 and †p<0.001 compared with the vehicle-treated OA group.

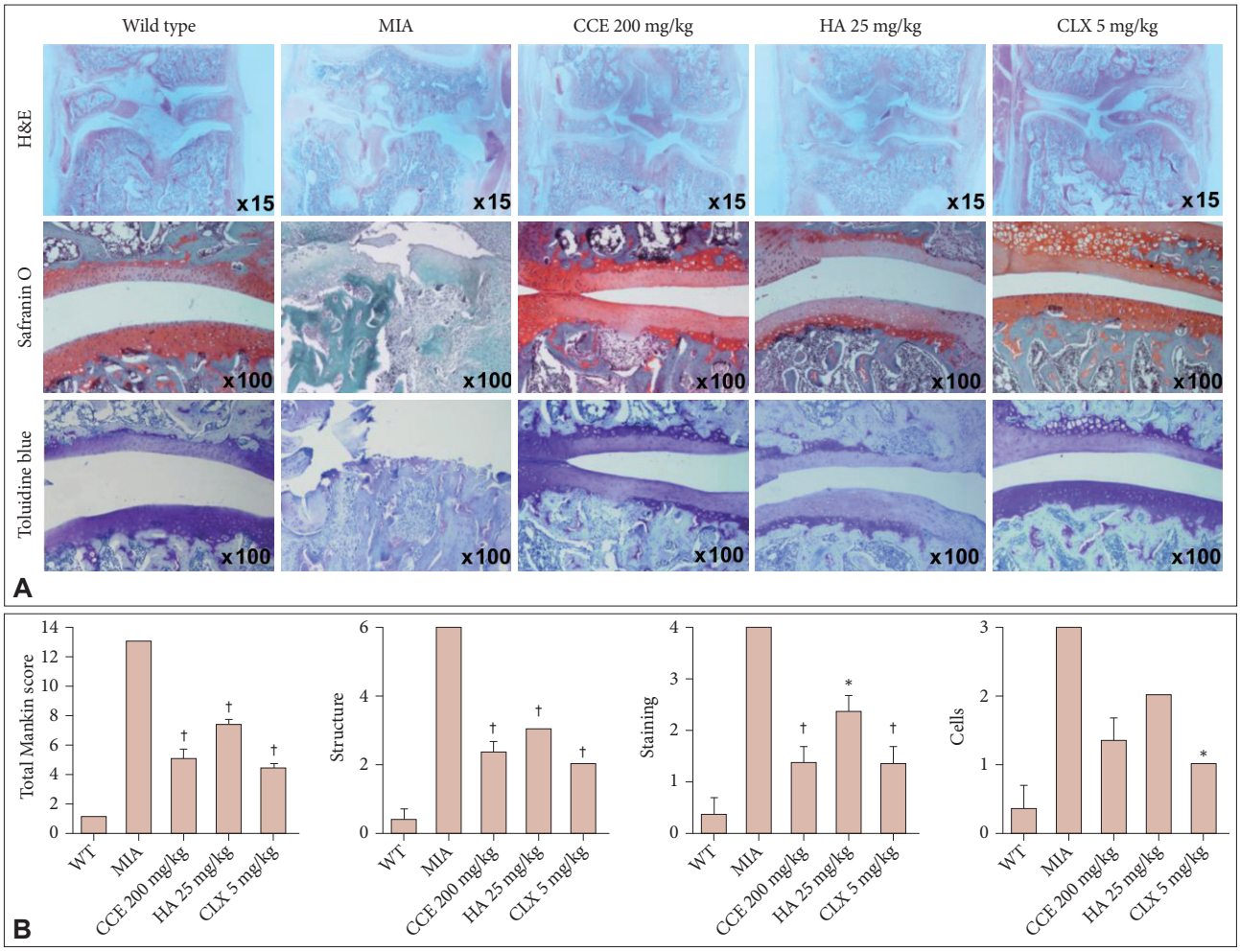

Figure 2. Histological evaluation of joints and osteoclastic activity after treatment with chicken comb extract (CCE) in monosodium iodoacetate (MIA)-induced osteoarthritis (OA). (A) The knee joints from the OA rats treated with hyaluronic acid (HA), celecoxib (CLX) or CCE were stained with hematoxylin and eosin (H&E), Safranin O-fast green, and toluidine blue. Compared with MIA group, comprehensive cartilage degradation, bone destruction, and fibrosis are suppressed in the HA, CLX, or CCE treated group. The joint lesions were graded on a scale of 0–13 using the modified Mankin scoring system. (B) Total Mankin score is a sum of the scores for cartilage structure, cellular abnormalities, and matrix staining. The data are expressed as mean±standard error of the mean for six animals per group. *p<0.05 and †p<0.001 compared with the MIA-injected group. WT: wild type.

Reverse transcription reaction and real-time polymerase chain reaction

Total RNA was isolated from human chondrocytes using the TRIzol method (Invitrogen). The complimentary DNA (cDNA) was prepared by reverse transcription of the single-stranded RNA according to the manufacturer's directions included in the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The levels of mRNA expression were estimated using real-time quantitative polymerase chain reaction (PCR) with LightCyclerFastStart DNA Master SYBR green I (Takara), according to the manufacturer's instructions. The primer pairs used in these reactions were as follows: for control human β-actin, forward 5'-GGA CTT CGA GCA AGA GAT GG-3', reverse 5'-TGT GTT GGC GTA CAG GTC TTT G-3'; for human tissue inhibitor of metalloproteinase (TIMP)-1, forward 5'-AAT TCC GAC CTC GTC ATC AG-3', reverse 5'-TGC AGT TTT CCA GCA ATG AG-3'; for human TIMP-3, forward 5'-CTG ACA GGT CGC GTC TAT GA-3', reverse 5'-GGC GTA GTG TTT CTG GT-3'; for MMP-1, forward 5'-CTG AAG GTG ATG AAG CAG CC-3', reverse 5'-AGT CCA AGA GAA TGG CCG AG-3'; for MMP-3, forward 5'-CTC ACA GAC CTG ACT CGG TT-3', reverse 5'-CAC GCC TGA AGG AAG AGA TG-3'; for MMP-13, forward 5'-CTA TGG TCC AGG AGA TGA AG-3', reverse 5'-AGA GTC TTG CCT GTA TCC TC-3'. The amplification reactions, data acquisition, and analysis were performed with the LightCycler Real-Time PCR system (Roche Diagnostics, San Francisco, CA, USA), and the relative levels of gene expression were normalized

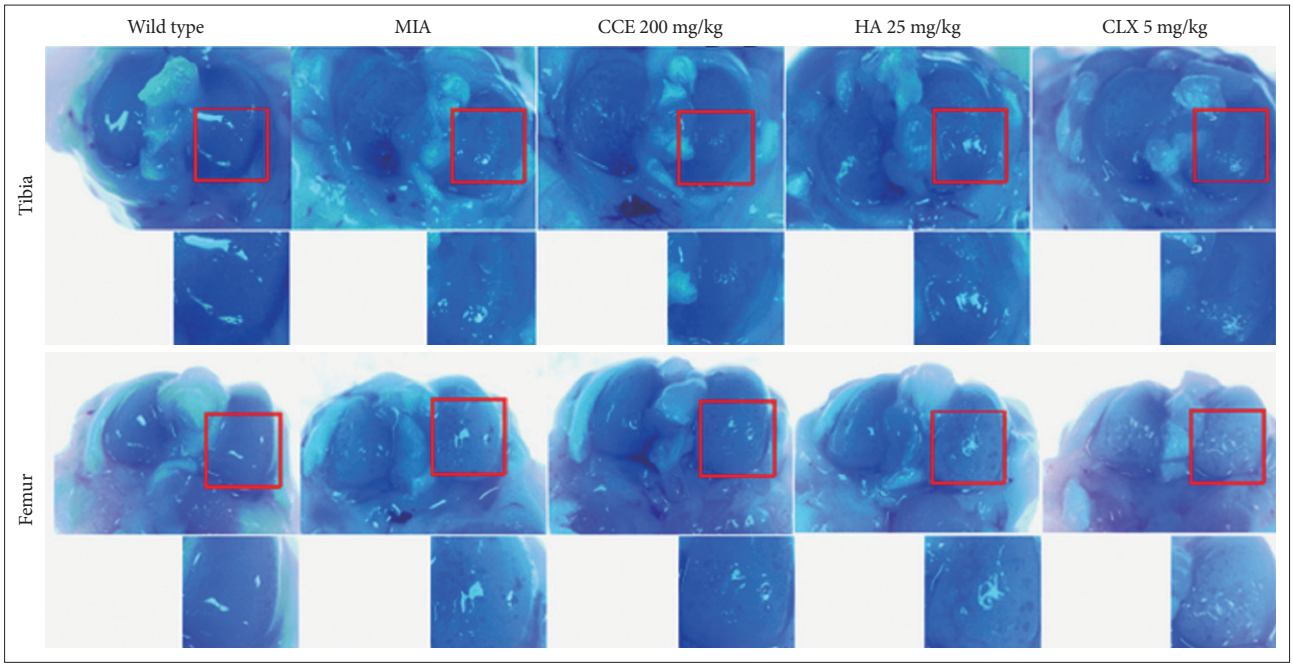

Figure 3. Microscope analysis of the damaged cartilage in the femoral and tibial articular cartilage of an osteoarthritis rat model using India ink staining at 4 weeks after monosodium iodoacetate (MIA) injection. The gross morphological changes of the femoral condyles and tibial plateau were photographed using a digital camera with application of India ink to contrast the cartilage lesions. CCE: chicken comb extract, HA: hyaluronic acid, CLX: celecoxib.

against β-actin.

Statistical analysis

The change of pain behavior was expressed as means±standard error of the mean. Each value of histological assessments and pain behaviors was represented as a dot plot. One-way analysis of variance followed by Bonferroni's post-hoc test was used to compare pain and histological scores. To assess the Gaussian distribution and the equality of variance, Shapiro-Wilk test and Levene's test were used, respectively. The program used for the statistical analysis was SPSS statistical software package standard version 16.0 (SPSS Inc., Chicago, IL, USA). *p* values less than 0.05 (two-tailed) were considered significant.

RESULTS

CCE revealed anti-nociceptive effects on MIA-induced OA rats

Secondary tactile allodynia in MIA-induced OA rats was performed to measure the ability of CCE to reduce the pain in OA compared to HA and CLX. In the automated von Frey hair assessment tests, the paw withdrawal latency (PWL) and the paw withdrawal threshold (PWT) were significantly enhanced in the group of OA rats treated with CCE (Fig. 1) versus the MIA group of OA rats. Treatment with HA and CLX also increased PWL and PWT, but CCE treatment most enhanced PWL and PWT in OA rats.

Cartilage damage was attenuated with CCE treatment in MIA-induced OA rats

The knees were excised, and the cartilage was stained with H&E, Safranin O-fast green, and toluidine blue to evaluate the ability of CCE to reduce cartilage damage in OA compared to HA and CLX. Compared to the MIA group, cartilage thickness and the proteoglycan content in joints of OA rats were reduced by treatment with CCE, HA, and CLX (Fig. 2A). The degree of cartilage degradation was evaluated using Mankin's score system. This system scores structural damage, cellular abnormalities, and matrix staining. The CCE-, HA-, and CLX-treated groups all showed significantly lower Mankin scores than the MIA group (Fig. 2B).

Reduced joint damage by oral administration of CCE in MIA-induced OA rats

We assessed damage to the articular cartilage surface using India ink 4 weeks after MIA injection. Grossly, the normal joints revealed smooth and shiny articular surfaces. The knees of the MIA-treated group showed irregular abrasions on the articular cartilage surfaces of the femoral condyle and the tibial plateau, but the CCE-, HA-, and CLX-treated animals had smoother articular surfaces than the MIA-treated group. The CCE treatment group was most similar to the normal group (Fig. 3).

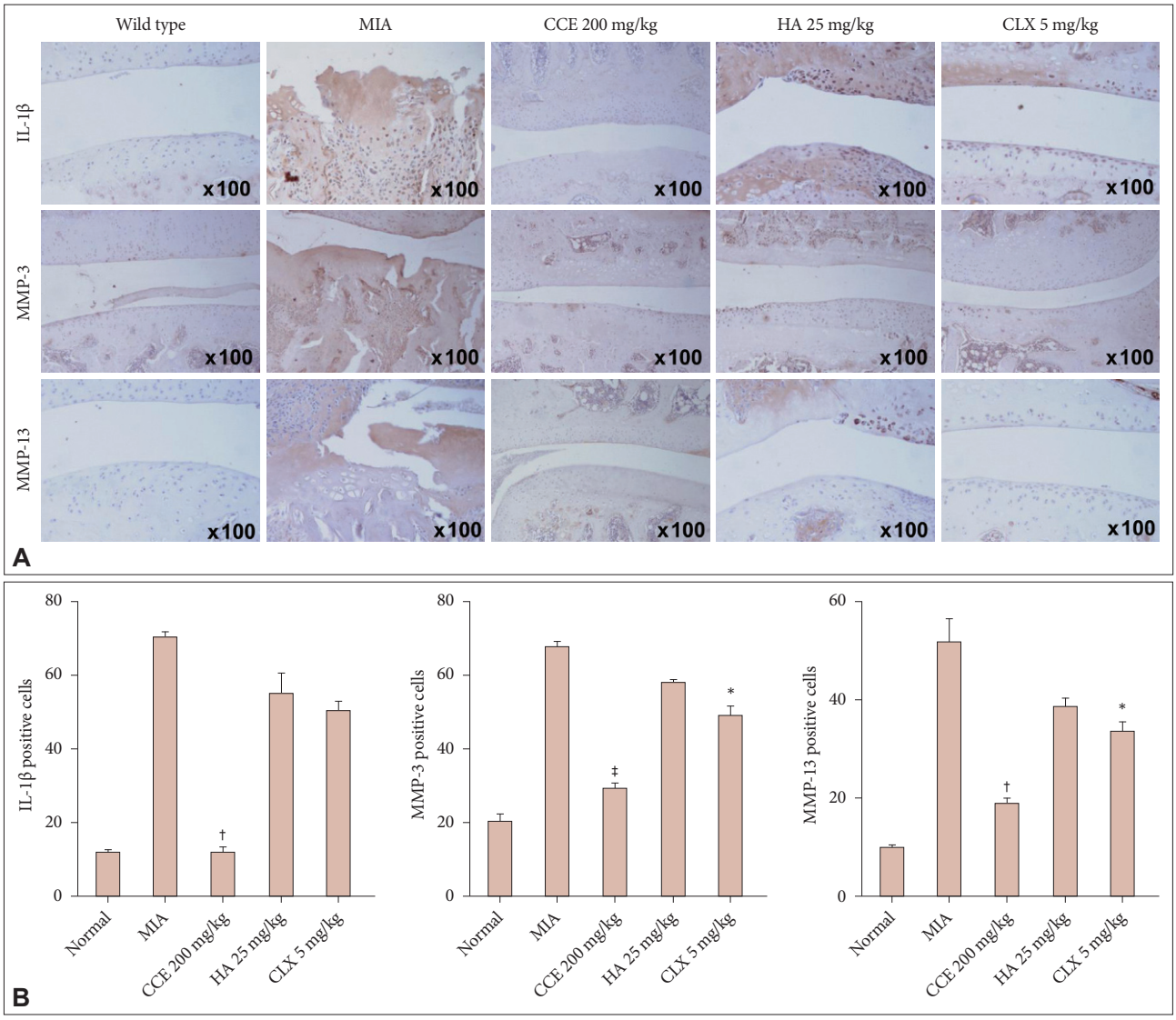

Figure 4. Effect of chicken comb extract (CCE) on the expression of interleukin (IL)-1β, matrix metalloproteinases (MMP)-3 and -13 in osteoarthritis joints. (A) Immunohistochemical staining was used to identify the expression of IL-1β, MMP-3, and -13 in the articular cartilage. (B) The cells expressing IL-1β, MMP-3, and -13 was reduced by hyaluronic acid (HA), celecoxib (CLX), or CCE in the articular cartilage of monosodium iodoacetate (MIA)-injected rats. The data are expressed as mean±standard error of the mean for six animals per group. *p<0.05, †p<0.01, and ‡p<0.001 compared with the MIA-injected group.

CCE treatment reduced expression of matrix MMP-3 and MMP-13 and IL-1β in OA rats

Expression of the matrix degrading enzymes, MMP-3 and MMP-13, was evaluated to assess the chondroprotective effect of CCE to suppress cartilage damage in OA compared to HA and CLX. MMP-3 and MMP-13 expression, upregulated by MIA injection, was downregulated after treatment with CCE, HA, and CLX. Expression of the proinflammatory cytokine IL-1β was also decreased by treatment with CCE, HA, and CLX. CCE treatment showed the largest decreases in IL-1β, MMP-3, and MMP-13 (Fig. 4).

Expression of inducible nitric oxide synthase and nitrotyrosine was decreased by treatment with CCE in OA rats

To measure the ability of CCE to inhibit oxidant factors related to OA's pathogenesis, compared to HA and CLX, the expression of iNOS and nitrotyrosine production in joints of OA rats were examined by immunohistochemistry. The expression of iNOS and nitrotyrosine production, induced by MIA injection, was suppressed after treatment with CCE, HA, and CLX. CCE treatment showed the largest decreases in IL-1β, MMP-3, and MMP-13 (Fig. 5).

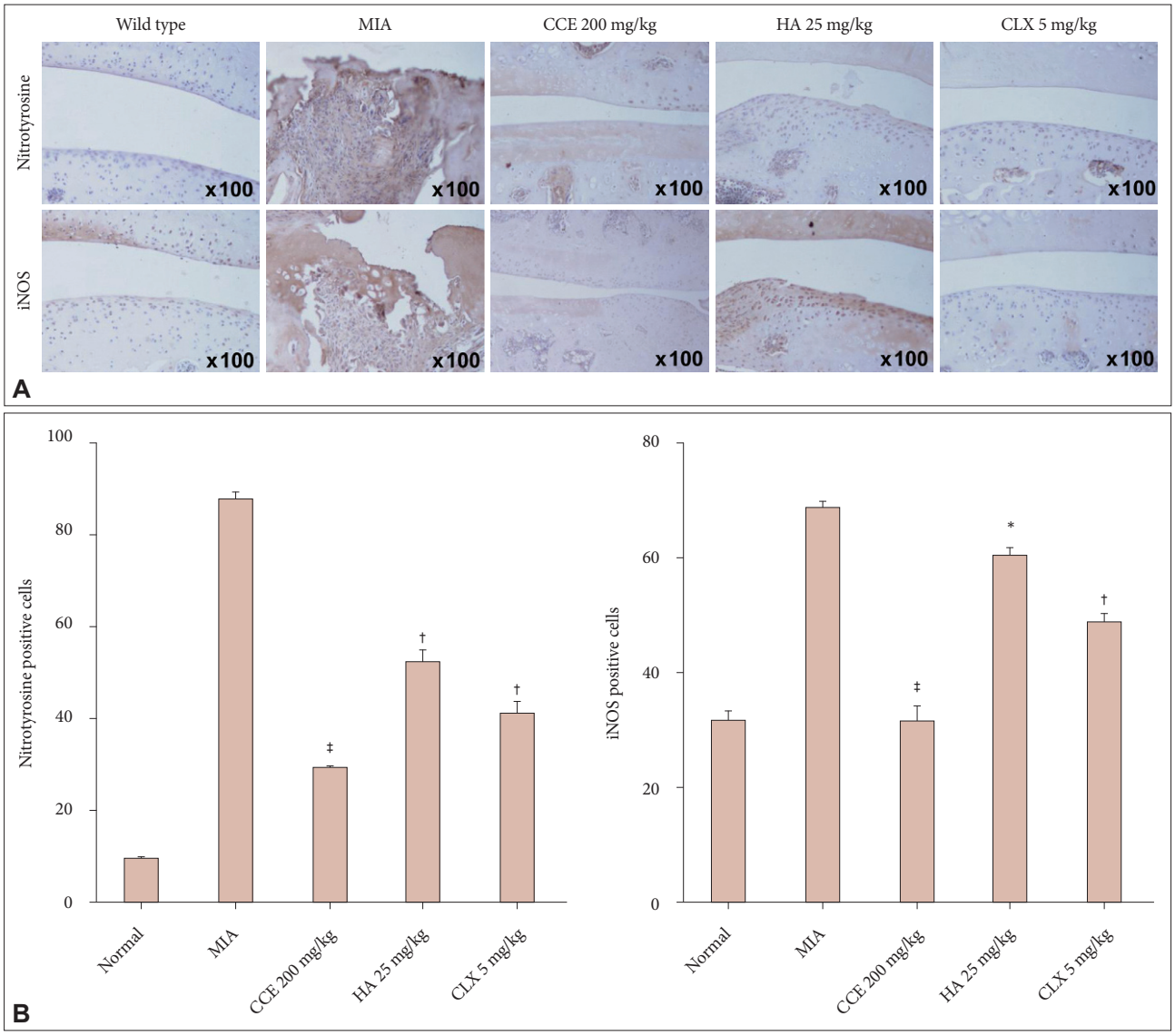

Figure 5. Attenuated induced nitric oxide synthase (iNOS) and nitrotyrosine in chicken comb extract (CCE)-treated osteoarthritis (OA) rats. (A) Immunohistochemical staining was used to identify the expression of iNOS and nitrotyrosine in the joint tissue. (B) The cells expressing iNOS and nitrotyrosine was suppressed in hyaluronic acid (HA), celecoxib (CLX) or CCE treated OA rats. The data are expressed as mean±S.E.M for three animals per group. *p<0.05, †p<0.01, and ‡p<0.001 compared with the monosodium iodoacetate (MIA)-injected group.

CCE treatment affected the balance between anabolic and catabolic mediators

The expression levels of anabolic and catabolic mediators were measured by real-time PCR to evaluate the ability of CCE to control the balance between anabolic and catabolic activity in OA, compared to HA and CLX. The relative mRNA expression levels of MMP-1, MMP-3, and MMP-13 were most significantly decreased by CCE treatment. Treatment with CCE most significantly increased the mRNA levels of TIMP-1 and TIMP-3 (Fig. 6).

DISCUSSION

Although CCE has been used as part of many dishes and recognized tissues containing HA, there is little evidence of the therapeutic role of CCE for OA related medical condition. In the present study, we showed the inhibitory cartilage degeneration and anti-inflammatory effect of CCE in MIA induced OA rat model.

The most important observation in this investigation is antioxidant function of CCE. It is well reported that nitric oxide (NO) is involved in the pathogenesis of OA and the expression

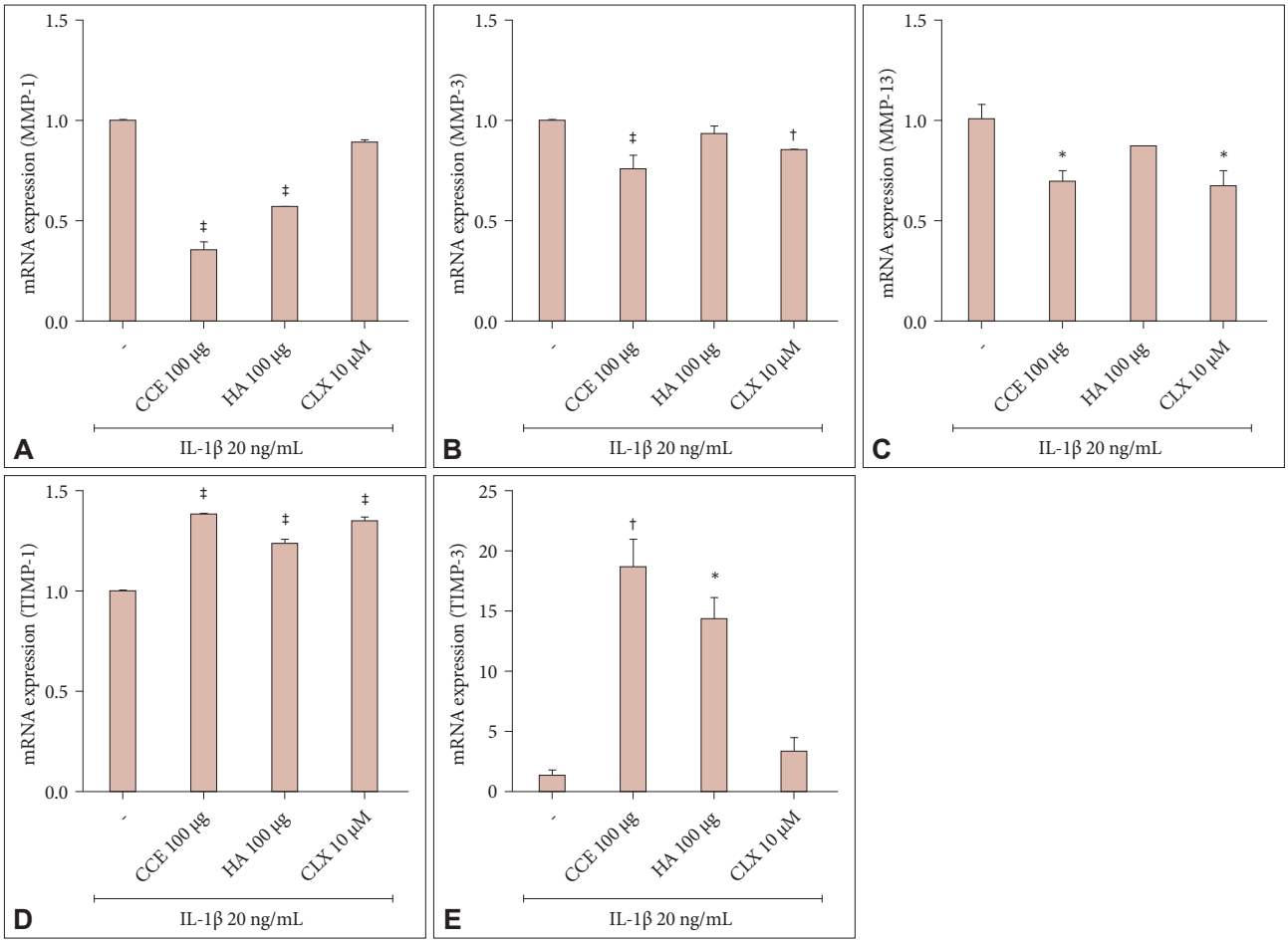

Figure 6. The regulation of anabolic and catabolic activity in human osteoarthritis (OA) chondrocytes by chicken comb extract (CCE) treatment. (A, B, and C) The mRNA level of catabolic mediators such as matrix metalloproteinases (MMP)-1, -3, and -13 was decreased in human OA chondrocytes by CCE treatment. (D and E) The mRNA level of MMPs inhibitors such as TIMP metallopeptidase inhibitor (TIMP)-1 and -3 was increased in human OA chondrocytes by CCE treatment. *p<0.05, †p<0.01, and ‡p<0.001 compared with the monosodium iodoacetate (MIA)-injected group. IL: interleukin, HA: hyaluronic acid, CLX: celecoxib.

of NO is promoted in OA reducing matrix synthesis and enhancing its degradation [22,23]. Additionally, the inhibition of iNOS suppresses the advancement of experimental OA reducing the expression of major catabolic factors such as MMP and IL-1β [24,25]. As NO and iNOS have been known as marker that is generated and increased in OA [26,27], the reduction of nitrotyrosine and iNOS expression is notable characteristic to attenuate OA. Therefore, our results have suggested that CCE can conduct therapeutic role in OA reducing MMP and IL-1β.

Anti-cartilage degenerative activity of CCE is an important finding in this study. It has been known that the degeneration of articular cartilage is the clinical syndrome of OA [28]. Cartilage degeneration is one of the most prevalent elements of joint pain and dysfunction [28,29]. Moreover, previous investigation has demonstrated that OA leads to deformation of tibia and femur [30]. CCE treatment decreases PWL and PWT indicating that CCE performed antinociceptive effects in pres,ent study. It also shows the inhibitory effect of cartilage degeneration, tibia and femur changes in joint of MIA induced OA rat like that of HA and CLX. Thus, CCE has a potential to improve joint disability and pain induced by OA.

As the imbalance between anabolic and catabolic factors results in cartilage degeneration, catabolic activity is significant in OA pathogenesis. There is evidence that several catabolic molecules such as MMP-3 and -13 are involved in OA pathogenesis and play a key role in advancement of OA [31,32]. The inducer of TIMPs reveals protective function in OA because TIMP-1 and -3 reduce MMPs activity [33,34]. Our observations show that CCE suppresses the mRNA expression of MMP-1, -3, and -13. On the other hands, TIMP-1 and -3 mRNA level is increased by CCE treatment. These results indicate that CCE may inhibit cartilage degradation and reveal therapeutic effect in OA.

As pain and inflammatory response in OA patients of the

knee was reduced by HA treatment, it has been recognized that HA is effective treatment for OA [35-37]. Based on our results, CCE has a therapeutic function in OA such as HA and the effect of CCE may be related with HA because CCE contains high amount of HA [38]. Moreover, HA was extracted from chicken comb and used to decrease the joint injury-associated cartilage degradation [38-40]. Thus, the therapeutic function of CCE may result from the therapeutic mechanism of HA.

There is little evidence in the study addressing inhibition of cartilage degeneration and pain relief by CCE. The present investigation suggests that CCE ameliorates MIA induced OA through the reduction of oxidative stress and MMPs. The function of CCE identified in this investigation demonstrates that it likely plays a significant role in suppression of OA. In conclusion, CCE can be candidate to therapeutic substance for OA.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

All of the animal procedures were approved by the Animal Research Ethics Committee at The Catholic University of Korea (Permit Number: 2014-0002-01).

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