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Original Article

# Effect of *Cinnamomum Cassia* on Cartilage Protection in Rabbit and Human Articular Cartilage

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**Background & Objective:** Articular cartilage is a potential target for drugs designed to inhibit the activity of matrix metalloproteinases (MMPs) to stop or slow the destruction of the proteoglycanand collagen in the cartilage extracellular matrix. The purpose of this study was to investigate the effects of *Cinnamomum cassia* in inhibiting the release of glycosaminoglycan (GAG), the degradation of collagen, and MMP activity in rabbit and human articular cartilage explants.

**Methods:** The cartilage-protective effects of *Cinnamomum cassia* were evaluated by using glycosaminoglycan degradation assay, collagen degradation assay, colorimetric analysis of MMP activity, measurement of lactate dehydrogenase activity and histological analysis in rabbit cartilage explants culture.

**Results:** Interleukin-1a (IL-1a) rapidly induced GAG, but collagen was much less readily released from cartilage explants. *Cinnamomum cassia* significantly inhibited GAG and collagen release in a concentration-dependent manner. *Cinnamomum cassia* dose-dependently inhibited MMP-1, MMP-3 and MMP-13 activities from IL-1a-treated cartilage explants culture when tested at concentrations ranging from 0.02 to 1 mg/ml.

**Conclusion :** These results indicate that *Cinnamomum cassia* inhibits the degradation of proteoglycan and collagen through the down regulation of MMP-1, MMP-3 and MMP-13 activities of IL-1a-stimulated rabbit and human articular cartilage explants.

***Key Words*** : *Cinnamomum cassia* articular cartilage; proteoglycan; collagen; matrix metalloproteinase Leave no space between the word and the colon that follows.

## Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by progressive loss of articular cartilage, subchondral bond remodeling, spur formation, synovial inflammation, and in particular, the degradation of proteo- glycan and collagen. The integrity of these macromolecules is vital to cartilage and joint function[1-3].

Proteoglycan is a component of the articular cartilage extracellular matrix, providing it with many of its characteristic physicochemical properties[4]. The carbohydrate component of aggrecan, which constitutes at least 90 % of its molecular mass, consistsof many long keratin sulfate, chondroitin sulfate, and glycosamino-glycan (GAG) chains covalently linked to a core protein[5]. Thus, the importance of these proteog-lycans is clear. However, neither their specific

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role nor the mechanisms regulating their synthesis are fully understood.

Collagen is another component of the articular cartilage, which consists primarily of type II collagen[6]. It plays a role in maintaining the integrity of the cartilage matrix and allows proteoglycan to be held in the matrix[7]. There is circumstantial *in vitro* and *in vivo* evidence indicating a significant role for matrix metalloproteinases (MMPs) in cartilage destruction in arthritis[8,9].

MMPs can be classified into four subgroups: collagenases (MMP-1, -8, -13), stromyelins (MMP-3, -10, -11), gelatinases (MMP-2, -9), and membrane-type MMPs.[10] MMPs are synthesized in response to cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-a, which is thought to be involved in the pathogenesis of arthritis[10-13]. MMPs are released as inactive proenzyme forms and are then activated by limited proteolysis of the propeptide domain by plasmin, kallikrein, or trypsin[10, 14]. MMP-3 is capable of cleaving the aggrecan core protein, as well as type II collagen (in the amino-terminal telopeptide) *in vitro*, but it is not clear if it is involved in the degradation of these proteins in cartilage[15,16]. MMP-13 is expressed by normal and osteoarthritic chondrocytes and has been localized to both rheumatoid and osteoarthritic cartilage[17]. MMP-13 is the most efficient collagenase against type II collagen, suggesting it has an important role in cartilage collagen turnover[18, 19].

Many cartilage protective agents have been developed from natural products, and they have resultedin the development of treatments for intractable diseases such as arthritis and inflammation[20,21]. Recently, the search for potential cartilage protective agents has been pursued intensively, with promising clinical results, because there is renewed interest in the treatment of OA in Korean medicine.

*Cinnamomum cassia* is one of the world's oldest spices that have been used in foods, beverages and the cosmetic industry. In traditional Korean medicine, *Cinnamomum cassia* is one of the medicinal plants that has been used to improve various diseases caused by insufficient blood microcirculation. This medicinal plant has also been often administered to patients suffering from women's diseases. *Cinnamomum cassia* is reported to reduce blood pressure in experimental rats[22] and possesses significant antiallergic, antiulcerogenic, antipyretic and anaesthetic activities[23]. However, relatively little work has been carried out on the OA. The present study therefore investigates the cartilage protective effects and mechanism of *Cinnamomum cassia* on rabbit and human articular cartilage.

## Materials and methods

### 1. Preparation of *Cinnamomum cassia* total extract and fraction

*Cinnamomum cassia* (CC) was obtained from Kyunghee Oriental Medical Center, following kindly confirmation of Nam-je Kim for quality control. The *Cinnamomum cassia* was incubated in 50 % (v/v) ethanol−water at room temperature for 24 h. The extract was then filtered and concentrated under low pressure using a vacuum rotary evaporator (Eyela, Japan). This material was successively suspended in H2O (CCT) and then partitioned with butanol (CCB). The partitioned portion was dry vacuumed to yield a butanol fraction and a residue fraction (CCR). The remaining residue was lyophilized in a freeze-dryer, and stored at −20 °C. The powder

was dissolved in dimethyl sulfoxide (DMSO) and diluted with Dulbecco's modified Eagle's medium (DMEM) to final concentrations of total extract ranging from 0.02 to 1 mg/ml.

### 2. Rabbit cartilage explants culture

Articular cartilages were obtained from the joints of five-week-old rabbits (Samtako Biokorea Co., Korea). In brief, the articular surfaces were surgically exposed under sterile conditions; approximately 200-220 mg of articular surface per joint was removed and steeped in complete medium (DMEM supplemented with heat-inactivated 5 % fetal bovine serum [FBS] and 100 unit/ml of penicillin-streptomycin [Gibco BRL, Maryland, USA]). The samples were then rinsed several times with complete medium and incubated for 1-2 days at 37 °C in a humidified CO2/95 % air incubator to stabilize them. The complete medium was replaced with basal medium (DMEM supplemented with heat-inactivated 1 % FBS, 10 mM HEPES, and 100 unit/ml penicillin-streptomycin).Approximately 30 mg of cartilage pieces were placed in 48-well plates. Cartilage tissues were cultured alone or in the prescence of 5 ng/ml IL-1 (R&D Systems, Minneapolis, USA). After 1 h, of CCT was added at the start of the culture at concentrations of 20, 100, 200, 400ug/ml. The supernatants were harvested and replaced with fresh media containing test reagents. These were incubated for a further 3 days, supernatant were collected and stored at -20 °C until assayed.

### 3. Human cartilage explants culture

OA human cartilage obtained from patients at knee replacement surgery with diagnosed OA was obtained postoperatively. The average age of patients from whom tissue was procured was 68 years (range, 49-80) and was evenly distributed between males and females. NSAID medication was stopped 7 days before surgery, thus no interference of previous medication use is to be expected. Cartilage that appeared to be full thickness with significant fibrillation was selected, so the entire joint had a worse appearance than represented by the cartilage used in the evaluation. Collection of cartilage was done according to the medical ethical regulations of the KyungHee University Medical Center. Slices of cartilage were cut aseptically as thick as possible from the articular bone surface, cut into square pieces, weighed aseptically (range 25 ±0.1 mg) and cultured individually in 48-well plates of 400 μl culture medium. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 mM HEPES, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 5 % fetal bovine serum (FBS). Cartilage was pre-cultured for 24 hours, after which culture medium was refreshed before the start of the experiment. Cartilage tissues were cultured alone or in the prescence of 5 ng/ml IL-1 (R&D Systems, Minneapolis, USA). After 1 h, of CCT, CCB, CCR were added at the start of the culture at concentrations of 100, 400, 1000 ug/ml. The supernatants were harvested and replaced with fresh media containing test reagents. These were incubated for a further 7 days and supernatant were collected and stored at -20 °C until assayed.

### 4. Glycosaminoglycan degradation assay

Glycosaminoglycan levels in the culture medium were determined by the amount of polyanionic material reacting with 1,9-dimethylmethylene blue, using shark chondroitin

sulfate as thestandard. Samples were examined spectrophotometrically at 540 nm (Spectramax, Molecular Devices, Sunnyvale, CA, USA). The percentage recovery was calculated from the peak height of the sample relative to that of the standard.

### 5. Collagen degradation assay

Type II collagen levels in the culture medium were determined using the Sircol Collagen Assay (Biocolor Ltd., Valley Business Center, Northern Ireland). Samples werereacted with Sirius red dye containing sulfonic acid for 30 min at room temperature. The reaction mixture measured optical density at 540 nm. The percentage of recovery was calculated from the peak height of the sample relative to that of the standard.

### 6. Colorimetric analysis of MMP activity

The levels of MMP activity in the conditioned media were evaluated using anenzyme-linked immunosorbent assay (ELISA) kit (Biomol Research Lab., Inc., PA, USA) according to the manufacturer's instructions. Briefly, a monoclonal antibody specific for pro-MMP-1 or MMP-3 or MMP-13 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells, and pro- MMP-1 or MMP-3 or MMP-13 is bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked monoclonal antibody specific for pro- MMP-1 or MMP-3 or MMP-13 bound in the initial step. The color development is stopped and the intensity of the color is measured.

### 7. Measurement of lactate dehydrogenase activity

As an indicator of cell viability, the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured in the culture medium. An optimized LDH test (Promega Corp., Madison, WI, USA) was used to quantify LDH activity in the medium of the cartilage explants cultures.

### 8. Statistical analysis

The results were expressed as means ± S.D. calculated from the specified numbers of determinations. Statistically significant differences relative to the untreated control group were calculated by Student's one-tailed paired t test. Differences with p values $< 0.05$ were deemed statistically significant.

## Results

### 1. Effect of *Cinnamomun cassia* on proteoglycan and collagen degradation

To study whether *Cinnamomun cassia* affects proteoglycan degradation in rabbit and human cartilage explants. Rabbit and human cartilage explants were cultured in the presence of 5 ng/ml IL-1a for 3 days (rabbit) or 7 days (human). In the rabbit cartilage explants, 20, 100, 200, 400 ug/ml concentration of CCT markedly reduced the IL-1a-mediated GAG release into the culture medium at 3 days. Moreover, CCT dose-dependently reduced IL-1a-mediated GAG release into the culture medium (Fig. 1). In the human cartilage explant, 400, 1000 ug/ml concentration of CCT and 400 ug/ml concentration of CCB reduced GAG degradation relative to that in the IL-1a-treated cultures (Fig. 2).

### 4. Effect of *Cinnamomun cassia* on collagen degradation

To study whether CCT, CCB, and CCR affects collagen degradation in human cartilage

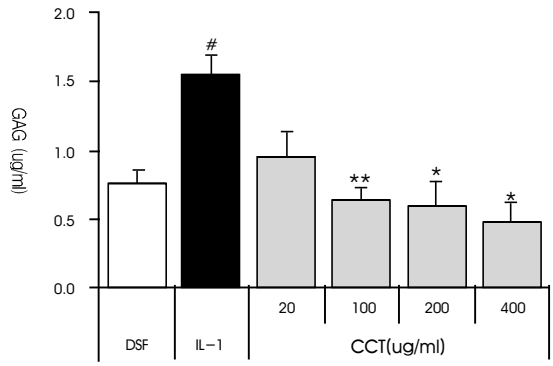


**Fig. 1.** Effect of *Cinnamomum cassia* on the dose response of proteoglycan degradation in rabbit cartilage explants cultures.
Cartilage was cultured in quadruplicate in 400 ㎕ of medium only, with 5 ng/ml IL-1a, or with 5 ng/ml IL-1a + 0.02, 0.1, 0.2, 0.4 mg/ml CCT for 3 days. GAG degradation is shown as the cumulative release into the medium, as a concentration of total GAG at different dose in the culture. Bars show the SDM of three experiments. #P < 0.05 compared with non-treated group, *P < 0.05, **P< 0.01 versus the respective controls (IL-1).

explants. Human cartilage explants were cltured in the presence of 5 ng/ml IL-1a for 28 days. At 100 ug/ml concentration of CCT, CCB and CCR reduced collagen degradation relative to that in the IL-1a-treated cultures, and significantly reduced at CCT and CCR (Fig. 3).

### 5. Effect of *Cinnamomun cassia* on MMP activity

We examined whether CCT, CCB, and CCR inhibited IL-1a-mediated MMP-1, MMP-3 and MMP-13 activities in human cartilage explant culture. We tested the levels of mediated MMP-1, MMP-3 and MMP-13 activity in the

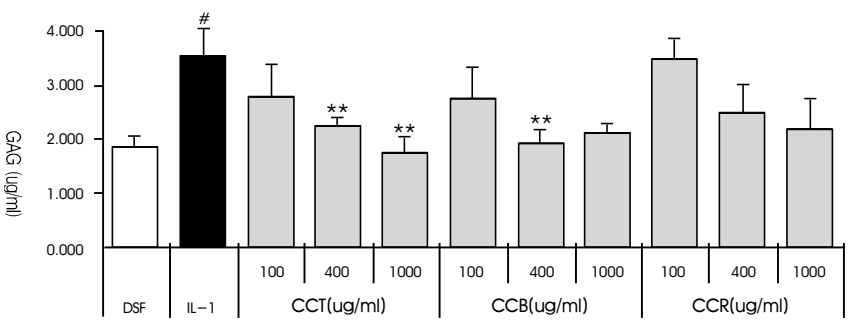


**Fig. 2.** Effect of *Cinnamomum cassia* on the dose response of proteoglycan degradation in human cartilage explants cultures.
Cartilage was cultured in quadruplicate in 400 ㎕ of medium only, with 5 ng/ml IL-1a, or with 5 ng/ml IL-1a + 0.1, 0.4, 1 mg/ml CCT, CCB and CCR for 7 days. Media with or without IL-1a were replenished once every 3 days. GAG degradation is shown as the cumulative release into the medium, as a concentration of total GAG at different dose in the culture. Bars show the SDM of three experiments. #P< 0.05 compared with non-treated group, **P < 0.01 versus the respective controls (IL-1).

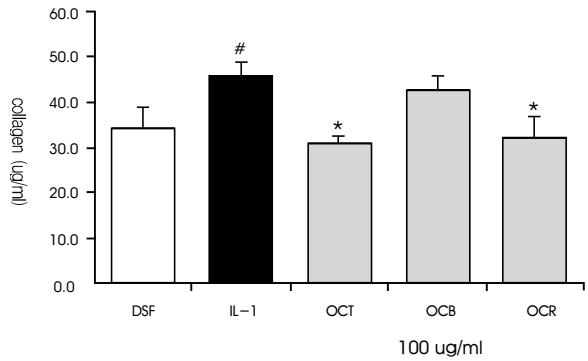


**Fig. 3.** Effect of *Cinnamomum cassia* on the dose response of collagen degradation in human cartilage explants cultures.
Cartilage was cultured in quadruplicate in 400 ㎕of medium only, with 5 ng/ml IL-1a, or with 5 ng/ml IL-1a + 0.1 mg/ml CCT, CCB and CCR for 28 days. Media with or without IL-1a were replenished once every 3 days. Collagen degradation is shown as the cumulative release into the medium, as a concentration of total collagen at different dose in the culture. Bars show the SDM of three experiments. #P < 0.05compared with non-treated group, *P < 0.05 versus the respective controls (IL-1).

medium from cultures after 7 days with or without CCT, CCB and CCR. MMP-1, MMP-3 and MMP-13 levels decreased dose-dependently in the culture media with CCT, CCB and CCR compared with the levels in IL-1a-treated cultures. In CCT and CCB-treated cultures, the levels of MMP-1, MMP-3 and MMP-13 activity were reduced more than in cultures treated with CCR (Fig. 4).

### 6. Effect of *Cinnamomun cassia* on the viability of cartilage explants

We examined whether *Cinnamomun cassia* affects chondrocytes viability in cartilage explants cultures. We were unable to detect any LDH activity in the incubation medium of cultures treated with the drug or with IL-1a alone, indicating that neither IL-1a nor *Cinnamomun cassia* have cytotoxic effects on chondrocytes cartilage explants during 3, 7, or 14 days of culture (Fig. 5).

## Discussion & Conclusion

In traditional Korean medicine, *Cinnamomum cassia* is reported to reduce blood pressure in experimental rats and possesses significant antiallergic, antiulcerogenic, antipyretic and anaesthetic activities. However, relatively little work has been carried out on the OA. The present study therefore investigates the cartilage-protective effects and mechanism of *Cinnamomum cassia* on rabbit and human articular cartilage.

In general, the destruction of cartilage in OA is initially caused by a decrease in its proteoglycan content, followed by the degradation of collagen fibers. Some studies have suggested that investigation into cartilage degradation should include an examination of both proteoglycan and the collagen matrix[1,23].

We investigated the protective effects of CCT, CCB and CCR on IL-1a-mediated proteoglycan and collagen release in rabbit or human cartilage explants. In this study, CCT dose-dependently

reduced IL-1a-mediated proteoglycan release into the culture medium in rabbit explants (Fig. 1). CCB and CCR reduced and IL-1a-mediated proteoglycan release into the culture medium in human explants (Fig. 2). CCT and CCR markedly reduced collagen degradation compared with that in IL-1a-treated cultures (Fig. 3). These results suggest that *Cinnamomum cassia* is effective for the reduction of proteoglycanand collagen degradation in rabbit and human cartilage explants .

In our colorimetric analysis demonstrated that CCT, CCB and CCR markedly and dose-dependently down regulated MMP-1, MMP-3

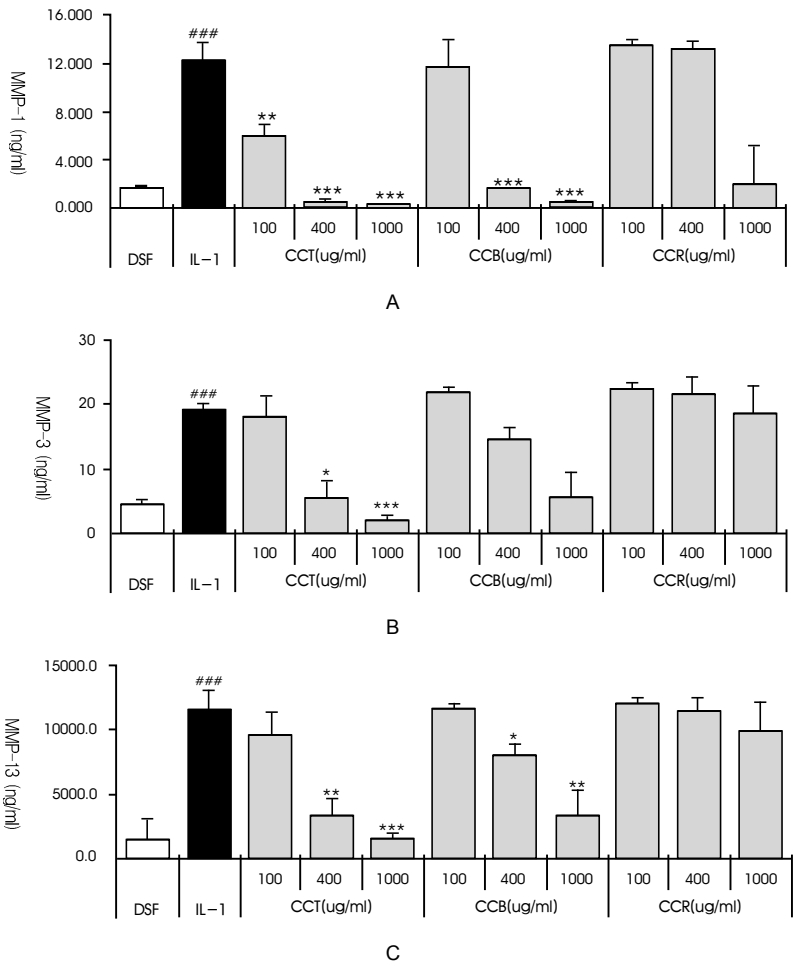


**Fig. 4.** Effect of *Cinnamomum cassia* on MMP activity in human cartilage explants cultures.
Cartilage was cultured in medium only, with 5 ng/ml IL-1a, with 5 ng/ml IL-1a + 0.1, 0.4, 1 mg/ml CCT, CCB and CCR for 7 days. Media with or without IL-1a were replenished once 3 days. (A) MMP-1 activity. (B) MMP-3 activity. (C) MMP-13 activity. Cumulative MMP-1, MMP-3 and MMP-13 activities were analyzed with a colorimetric substrate assay. Bars show the mean ± SD of three experiments. ### $P < 0.001$ compared with non-treated group, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with respective control (IL-1).

and MMP-13 expression at extract concentrations of 0.1−1 mg/ml (Fig.4). These results suggest that CCT, CCB and CCR is an effective inhibitor of cartilage loss. Proteoglycan is particularly vulnerable to proteinase attack and is therefore a sensitive indicator of proteolytic activity. Studies by Lin et al. have suggested that MMP-3 is the proteinase mainly responsible for the release of proteoglycan and collagen as fragments after cartilage resorption *in vitro* and *in vivo*, because they are produced by cleavage of the aggrecan molecule at the position cleaved by MMP-3[24,25]. Furthermore, Kozaci et al. suggested that MMP-13 plays a role in the cartilage destruction stimulated by IL-1a, and breaks down type II collagen breakdown in bovine nasal cartilage explants[26,27].

However, further investigation is required into the mechanism of action of *Cinnamomum cassia* in exerting its chondroprotective effect via aggrecanase expression, and to develop an effective regimen for the treatment of OA.

In summary, CCT, CCB and CCR have an inhibitory effect on the release of proteoglycan and collagen associated with the down-regulation of MMP-1, MMP-3 and MMP-13 activities in IL-1a-induced human articular cartilage explants. In CCT, CCB -treated cultures were reduced more than in cultures treated with CCR. We suggest that CCT and CCB could represent agent for pharmacological intervention in cartilage loss in the progress of OA.

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