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Original Article

Effect of caffeic acid phenethyl ester on cartilage in experimental osteoarthritis

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Abstract. Activation of nuclear factor kappa B (NF-κB) in synovial cells is seen in RA and OA patients. Caffeic acid phenethyl ester (CAPE) is a specific and potent inhibitor of NF-κB. We aimed to determine the in vivo effects of intra-articular injections of CAPE on cartilage in an experimental rabbit osteoarthritis (OA) model. Two groups of six New Zealand white rabbits underwent unilateral anterior cruciate ligament transection (ACLT). Four weeks after ACLT, the test group was injected with 150 μg/kg CAPE in 0.5% ethanol once daily for 2 weeks and the control group was injected the same amount of 0.5% ethanol intra-articularly. All rabbits were killed 2 weeks after the last injection, and cartilage tissue was evaluated morphologically. A histological score totaling 7 points was determined for each knee. The CAPE group showed significantly decreased cartilage destruction and reduced loss of matrix proteoglycans. The histological score for cartilage tissue was significantly better in the CAPE group than in the control group (3.0±0.25 vs 5.3±0.55, P=0.005). This study suggests that intra-articular injection of CAPE may protect cartilage against the development of experimentally induced OA.

Keywords. Caffeic acid phenethyl ester - Nuclear factor kappa B - Osteoarthritis

Introduction

Osteoarthritis (OA) is a degenerative disease characterized by progressive cartilage destruction and a variable degree of synovial inflammation. Proinflammatory cytokines are believed to play a pivotal role in the initiation and development of the osteoarthritis process, among which interleukin-1 β (IL-1 β) and tumor necrosis factor-α (TNF-α) appear prominently [*1*, *2*, *3*]. Interleukin-1 β is extremely important to cartilage destruction, while TNF-α appears to be a key mediator at onset of matrix degradation and to drive the inflammatory process [*4*, *5*]. These two cytokines appear to be produced first by the synovial membrane and diffused into the cartilage through the synovial fluid. They activate the chondrocytes, which in turn could produce catabolic factors such as matrix metalloproteases (MMP), nitric oxide (NO), and the other proinflammatory cytokines such as IL-8, IL-6, IL-11. Expression of the catabolic process in chondrocytes is responsible for the loss of extracellular matrix in OA [*1*, *6*, *7*, *8*, *9*, *10*, *11*]. Cytokine and enzyme expression is regulated by a variety of transcription factors. One of these, nuclear factor kappa B (NF-κB), is a key transcription factor involved in the activation of the TNF-α and IL-1 β genes. Nuclear factor κB also can induce activation of MMPs, cyclo-oxygenase-2 (COX-2), and inducible NO synthase (iNOS), the enzyme responsible for NO production [*12*]. Activation of NF-κB in synovial cells is a feature seen in both RA and OA patients [*13*]. The NF-κB is therefore an obvious target for new types of anti-inflammatory treatment. A variety of anti-inflammatory agents are currently being used. However, adverse drug effects, particularly gastrointestinal ulceration, are commonly associated with these agents. Therefore, currently there is much interest for more effective and physiologic approaches such as therapeutic use of biological agents that block the activity of NF-κB. Among the possibilities for such an agent is caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE), a flavonoid and an active component of propolis from honeybee hives which has been shown to be a potent and specific inhibitor of NF-κB [*14*]. This CAPE is a pharmacologically safe compound with known anti-inflammatory, immunomodulatory, anticarcinogenic, and antioxidant properties [*15*, *16*, *17*, *18*].

We aimed to investigate the in vivo effects following intra-articular injection of CAPE on the course of disease progression in an experimental osteoarthritis model in rabbits.

Materials and methods

Twelve New Zealand white rabbits, 7-8 months old and weighing 3.5-4.0 kg, were operated on for unilateral anterior cruciate ligament transection (ACLT) to create degenerative changes in the articular cartilage. These rabbits were skeletally mature, with closed epiphyses as seen by roentgenogram. Each animal was anesthetized with intramuscular injections of ketamine (80-100 mg/kg) and xylazine (7-10 mg/kg). The right hind limb was shaved and disinfected with a povidone-iodine solution. Medial parapatellar incision and arthrotomy were performed. The patella was dislocated laterally and the knee placed in full flexion. The ACL was visualized and transected with a no. 15 surgical blade. After ACLT, no bleeding and full range of motion of the joint were observed, and the joint was washed with sterile saline. The capsule was closed with 4-0 monofilament propylene sutures, and the skin was closed with 3-0 nylon sutures. After surgery, the rabbits were returned to cage activity (cage size 60 × 60 × 40 cm) and the limbs were not immobilized. The rabbits were given intramuscular

injections of analgesic (0.01-0.02 mg/kg buprenorphine) and antibiotic (1.0-1.3 mg/kg sefuroxim acetyl) for 3 days after surgery.

Four weeks after ACLT, the rabbits were divided into two groups of six animals each. The knees of the test group were injected intra-articularly with 150 μg/kg of CAPE in 0.5% ethanol once daily for 2 weeks, and the knees of the control group were injected with the same amount of 0.5% ethanol without CAPE. During intra-articular injection, the animals were anesthetized with small intramuscular doses of ketamine and xylasine. The CAPE was prepared as described in the literature [*18*]. Briefly, propolis (Aksu Bal Ltd) was extracted with 80% EtOH-H_2O for 24 h. This extract was filtered using a G4 porous filter, and the solvent was removed under vacuum. Finally, a golden-brown, solid extract was obtained. This ethanolic extract was dissolved in an 80% (400 ml) MeOH-H_2O mixture and sequentially extracted with hexane (6 × 80 ml), toluene (4 × 80 ml), and EtOAc (4 × 100 ml). The EtOAc extract showed best separation with a 4% i-PrOH-CH_2Cl_2 mixture in column chromatography. The obtained fractions were separated in terminal complement complex using 4% i-PrOH-CH_2Cl_2. The phase containing CAPE showed blue fluorescence illumination at 366 nm.

All rabbits were killed 2 weeks after the last injection with intracardiacal injection of a mixed solution of pentobarbital sodium, phenytoin sodium, ethyl alcohol, and propylene glycol. For histologic examination, the cartilage specimens of all knees for histologic examination were fixed in 10% neutral buffered formalin, decalcified with 10% Na_2 ethylenediaminetetra-acetate (EDTA) buffered at pH 7.4, and embedded in paraffin. Several sections were cut to 5-μm thickness from articular cartilage of medial and lateral condyles on a rotary microtome. For histological evaluation, sagittal sections derived from chondral tissues were stained with hematoxylin and eosin (H&E) and safranin O. Histological score was determined by an independent observer for each knee with a grading scale determined for H&E and safranin O. A characteristic parameter in OA is the progressive loss of articular cartilage. The depth of cartilage erosion was graded on a scale of 0-4 as follows: 0 no destruction of cartilage or bone (normal surface appearance), 1-2 localized cartilage erosions, 3 more extended erosions, and 4 general cartilage destruction and presence of bone erosions [*19*]. In addition, cartilage proteoglycan depletion was determined using safranin O staining. The loss of proteoglycans was scored from 0 to 3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage [*20*]. The final histological score for each rabbit knee was 7 points. This scoring system is based on the most severe histologic changes within each cartilage section.

Representative specimens of synovial membrane from the medial and lateral compartments of the knee were dissected from the underlying tissues, fixed in 10% buffered formalin, and embedded in paraffin, and 5-μm thick sections were cut and stained with H&E. Synovial inflammation was scored from 0 to 4 as follows: 0 no inflammation, 1-2 slight thickening of the lining layer and/or some infiltrating cells in the sublining layer, 3 thickening of lining layer and/or a more pronounced influx of cells in the sublining layer, and 4 thickening of lining layer and synovium highly infiltrated with numerous inflammatory cells [*19*].

All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health (NIH publication 85-23, revised 1985) and approved by the Committee on Animal Research at Inönü University, Malatya.

Statistical analysis

Histologic scores were expressed as mean±SEM. Differences between both groups were done using the Mann-Whitney U test on a microcomputer using SPSS software, version 9.05 (SPSS, Chicago, Ill., USA). *P* values lower than 0.05 were considered significant.

Results

All rabbits in each experimental group completed the study. No sign of CAPE toxicity was noted. The levels of daily activity were similar in all rabbits.

Histological analyses

In the CAPE group, significantly decreased cartilage destruction was determined by H&E staining. Loss of matrix proteoglycan content in the cartilage was also much lower, as determined by safranin O staining (Fig. 1). Specimens of cartilage from the controls showed morphologic changes characteristic of OA. These included fibrillation and fissures of the cartilage surface and loss of safranin O staining (Fig. 2). The total score of histologic lesions on femoral condyles confirmed the reduction in erosion scores resulting from CAPE treatment compared with the control group (3.0±0.25 vs 5.3±0.55, *P*=0.005) (Table 1). Although somewhat variable between rabbits within a group, synovial specimens from both groups showed similar findings for inflammation. There was a moderate inflammatory reaction including slightly thickened synovium associated with mild hyperplasia and some inflammatory cell infiltration (Fig. 3). Scores of synovial inflammation in rabbits that received CAPE treatment were similar to those of control rabbits (2.16±0.16 vs 2.5±0.2, respectively, *P*=0.241) (Fig. 4, Table 1).

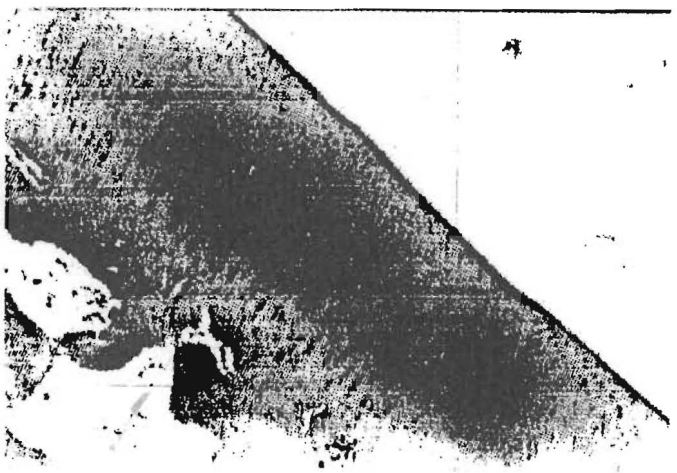

Fig. 1. Representative section of articular cartilage from the femoral condyles when administered 150 μg/kg caffeic acid phenethyl ester (CAPE) in 0.5% ethanol intra-articularly once daily for 2 weeks showed significantly decreased cartilage destruction and reduced loss of matrix proteoglycan content in the cartilage with safranin O staining. (Original magnification × 20)

Fig. 2. Specimen of cartilage tissue from the controls receiving 0.5% ethanol only showed severe cartilage destruction, with fibrillation and fissures of the cartilage surface and significant depletion of matrix proteoglycan, as evidenced by safranin O staining. (Original magnification × 20)

Table 1. Histological scores for cartilage erosions and the loss of matrix proteoglycans, staining with both hematoxylin-eosin (maximum 4 points) and safranin O in the groups of rabbits treated with CAPE and controls (maximum 3 points). Total score for cartilage lesions was 7 points. Also, scores of synovial inflammation (maximum 4 points) in both groups for hematoxylin-eosin staining are seen. Mann-Whitney U test. *H&E* hematoxylin-eosin staining, *S-O* safranin O staining

	Score (mean±SEM)
Erosion of cartilage in CAPE-treated group (H&E)	1.5±0.22
Erosion of cartilage in control group (H&E)*	3.0±0.89
Staining of matrix of cartilage in CAPE-treated group (S-O)	1.5±0.22
Staining of matrix of cartilage in control group (S-O)**	2.33±0.21
Total histological score in CAPE group	3.0±0.25
Total histological score in control group***	5.3±0.55
Histological score of synovial inflammation in CAPE group	2.16±0.16
Histological score of synovial inflammation in control group	2.5±0.22

*P=0.012

**P=0.03

***P=0.005

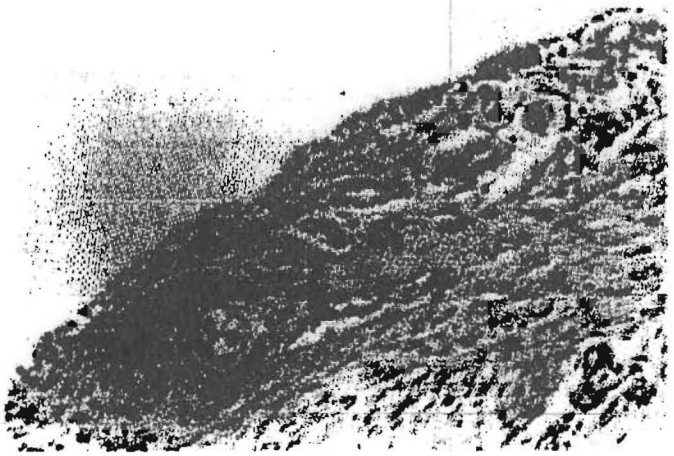

Fig. 3. Synovium recovered from rabbit knee receiving CAPE treatment. Specimens of synovium from both groups of rabbits showed mild-to-moderate inflammation, with slight thickening of the lining layer and some infiltration by inflammatory cells

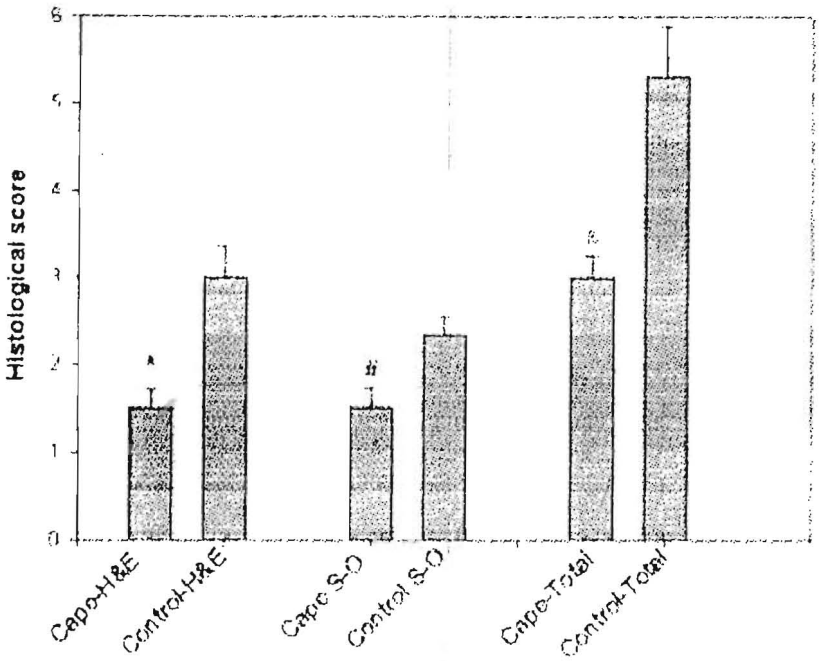

Fig. 4. A semiquantitave scoring for cartilage erosions and the loss of matrix proteoglycans with both hematoxylin-eosin (maximum 4 points) and safranin O staining in the groups of rabbits treated with CAPE and controls (maximum 3 points). Total score of cartilage lesions (maximum 7 points) confirmed that erosions were significantly reduced in the cartilage of rabbits that received in 0.5% ethanol with 10 μmol/kg CAPE compared with control rabbits receiving 0.5% ethanol only (P=0.005). Values are the total mean+SEM scores

Discussion

In the present study, treatment of experimental OA with intra-articular injection of 150 μg/kg of CAPE, a specific inhibitor of NF-κB, in 0.5% ethanol once daily for 2 weeks reduced cartilage pathology, as determined by histological examination.

Since NF-κB likely plays a predominant role in perpetuating cartilage metabolism in OA [*13*, *21*], modulation of NF-κB activity may therefore be an important consideration in therapeutic interventions for OA or rheumatoid arthritis. Glucocorticosteroids are effective inhibitors of the DNA-binding activity of NF-κB, which may account for most of their anti-inflammatory actions, but they have endocrine and metabolic side effects when given systematically [*22*]. Aspirin and salicylates also inhibit the activation of NF-κB, albeit only in relatively high concentrations, which suggests that the anti-inflammatory effect of aspirin may be at least partly attributable to the inhibition of NF-κB [*23*]. Sulindac, a nonsteroidal anti-inflammatory agent, inhibits activation of the NF-κB pathway [*24*]. Because of known potential limitations of steroidal and nonsteroidal anti-inflammatory drug therapy, there is a need for further investigations to apply more effective and physiologic approaches.

Local delivery of anti-inflammatory cytokines or the in vivo induction of their expression using gene transfer may provide a novel approach for the treatment of osteoarthritis [*4*, *19*]. Some naturally occurring inhibitors of NF-κB have been identified, including CAPE, a phenolic compound derived from honeybee propolis [*13*, *14*]. Although the molecular basis for the multiple activities assigned to

CAPE have not been defined, most of the activities inhibited by CAPE require the activation of NF-κB. In the study of Natarajan et al., CAPE inhibited the TNF-α -dependent activation of NF-κB in a dose- and time-dependent manner, with maximum effect occurring at 25 μg/ml [*14*]. How CAPE inhibits the activation of NF-κB induced TNF is not clear. However, the inhibition with CAPE is dose-dependent and reversible [*17*]. To our knowledge, until now only one animal study was reported addressing artificially formed losses of the cartilaginous tissue with a preparation containing an ethanol extract of propolis, which caused acceleration of regenerating processes in the lesioned cartilage [*25*]. As an active component of propolis, CAPE may be responsible for this effect. We administered CAPE in 0.5% ethanol by intra-articular injection in order to access the joint directly, thereby maximizing the delivery of the lipofilic protein. Since this study is the first examining the effect of CAPE on the cartilage in an experimental OA model, administration of CAPE was continued intra-articularly once a day for 2 weeks. This time interval was selected to focus on the early phase of the disease process in this model. According to previous studies, a higher dose because of a large excess in the amount of NF-κB produced in OA knee joints inhibited the NF-κB in the cell culture.

Our results demonstrate that intra-articular injections of CAPE at this amount and interval after disease onset reduced the severity of cartilage lesions as determined by cartilage erosion scores in the experimental OA model.

In conclusion, CAPE may provide a novel and alternative approach as a disease-modifying agent in the progression of osteoarthritis. Although the specific mechanism(s) responsible for this effect are unknown, we believe that inhibition of the NF-κB pathway may possibly be responsible. The results of our work are preliminary, and further studies are necessary to evaluate more thoroughly the effects of CAPE in experimental models of OA.

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Your manuscript: Effect of caffeic acid phenethyl ester ...

Dear Dr. Elmali,

thank you very much for resubmission of your revised manuscript mentioned above.

I feel very happy to tell you that your revised version now has been accepted for publication in Rheumatology International and has already been sent to the publisher in Heidelberg. You may be contacted from there directly as process of publications proceeds.

Thankyou again for having submitted your data to Rheumatology International !

With my very best regards,

sincerely yours

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