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ORIGINAL ARTICLE

Comparison of the protective effect of the upper zone of the growth plate and unique cartilage matrix-associated protein with hyaluronic acid and corticosteroids on an experimental rat osteoarthritis model

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ABSTRACT

Objectives: This study sought to compare the protective effect of the upper zone of the growth plate and unique cartilage matrix-associated protein (UCMA) with hyaluronic acid (HA) and corticosteroids (CS) in a rat model of osteoarthritis (OA).

Materials and methods: In the experimental animal study, 40 adult male rats were randomly assigned into five groups: control, monosodium iodoacetate (MIA) + vehicle (MIA+V), MIA+HA, MIA+CS, and MIA+UCMA. The OA model was induced by an intra-articular MIA injection to the right knee, and intra-articular injections into the right knee were performed on the treatment groups seven times every three days for 21 days. The knee joints were taken for histopathology and immunohistochemistry (IHC) analyses after the rats were sacrificed. All sections were stained with hematoxylin-eosin, safranin O and fast green FCF, and toluidine blue, and bone morphogenetic protein 2 (BMP-2) and nuclear factor-kappa B (NF-κB) expressions were analyzed with IHC. The Mankin scoring was utilized to determine the histopathological changes in the joint tissues.

Results: Mankin score was significantly higher in the MIA group compared to the control group. Histopathologically, in the UCMA-, HA-, and CS-treated groups, degenerations in the articular cartilage were milder than in the MIA+V group. Mankin score was found to be decreased significantly in the UCMA-, HA-, and CS-treated groups compared to the MIA group. Furthermore, IHC analyses revealed that NF-κB and BMP-2 expressions elevated in the MIA-induced OA model, while they were downregulated after UCMA, HA, and CS treatments.

Conclusion: Our data revealed that UCMA could be used as a potential protective molecule in the prevention and treatment of OA. Furthermore, the protective effect of UCMA was similar to HA and CS, and its possible beneficial roles against OA may be linked to the reduced BMP-2 and NF-κB levels. Further experimental research would make significant contributions to a better understanding of the therapeutic effect of UCMA on degenerative cartilage tissues.

Keywords: Corticosteroid, hyaluronic acid, monosodium iodoacetate, osteoarthritis, unique cartilage matrix-associated protein.

Osteoarthritis (OA) is the most prevalent progressive and irreversible musculoskeletal system disorder worldwide, which causes pain and movement restriction in the joints.[1] OA has an increasing prevalence due to aging and obesity and is a significant health problem that affects roughly 15% of the entire world population and is becoming increasingly common. Each year, almost 100,000 newly diagnosed OA patients cause notable health expenditures.[2,3] OA, the pathogenesis of which has not been fully elucidated, is mainly characterized by cartilage injury, inflammation in the synovial tissue, osteophyte formation, and subchondral sclerosis.[4] When treating OA, nonsteroidal drugs with anti-inflammatory effects and hyaluronic acid (HA) derivatives, which are found in the synovium and skeletal cartilage as the main component, are

widely used in intra-articular therapy.[5] However, while these methods contribute to alleviating the clinical manifestations of the disorder, they cannot effectively prevent cartilage destruction. Hence, it is of high priority to develop novel effective therapeutic agents that slow down the OA progression and prevent cartilage injury.[6,7]

The upper zone of the growth plate and unique cartilage matrix-associated protein (UCMA), a member of vitamin K-dependent proteins, has been reported to be associated with the pathogenesis of some diseases, including OA, vascular calcification, and kidney diseases.[7-11] UCMA, highly synthesized in osteoarthritic cartilage, might serve as an inhibitor for aggrecanase activity, which is involved in cartilage degeneration.[12] In a mouse model of OA induced by medial meniscus destabilization, proteoglycan loss, cartilage damage, and chondrocyte cell death have been shown to be increased in UCMA-deficient mice.[8] Furthermore, it has been revealed that UCMA triggers osteoblast differentiation and matrix mineralization in association with both Runx2 and Osterix genes.[13] UCMA has been shown to reduce the macrophagic THP-1 cell-tirggered proinflammatory response that is stimulated by lipopolysaccharides.[9,13-15] We have previously shown that the expression levels of UCMA increased in the synovial fluids of OA patients.[9] Moreover, in another study, we reported that intra-articular UCMA administration might have a protective effect on cartilage tissue in a rat OA model induced by monosodium iodoacetate (MIA).[7] The studies mentioned above have highlighted that UCMA is a promising therapeutic molecule that could be used in the treatment of OA. Yet, to our knowledge, no study in the literature has previously reported the comparative therapeutic efficacy of UCMA, HA, and corticosteroids (CS) in OA. Thus, we aimed to compare the protective effects of UCMA with HA and CS using a rat model of OA induced with MIA.

MATERIALS AND METHODS

This experimental study was conducted with 40 adult Wistar Albino male rats weighing between 400 and 500 g at the Research and Application Center for Experimental Researches of Hatay Mustafa Kemal University. Their food and water needs were controlled daily. The animals were housed in the optimal rat shelter, at an average temperature of 22±2°C, and in a 12-h light/dark cycle. The rats, which were expected to be compatible with each other after two weeks, were randomly divided into five groups: *(i)* control, *(ii)* MIA+vehicle (MIA+V), *(iii)* MIA+HA, *(iv)* MIA+CS, and *(v)* MIA+UCMA.

Animals were weighed regularly throughout the experimental protocol. The doses of anesthetic drugs were determined based on the weighing results. We induced the rat OA model with MIA as previously reported.[7,16] Except for the control group, all groups were administered with intraperitoneal injections of 10 mg/kg xylazine and 90 mg/kg ketamine hydrochloride, and 2.5 mg/rat MIA in 25 µL saline volume laterally into the shaved right knees of the rats after staining and sterile draping. The control group was not exposed to any treatment until the end of the experiment. The second group was MIA+V group; intra-articular MIA injection from the lateral side of the right knee to induce OA on the first day of the experiment, and after waiting for two weeks, an intra-articular saline injection as a vehicle was administered to the right knee lateral side every three days until the end of the experiment. The third group was the MIA+UCMA group; on the first day of the experiment, an intra-articular MIA injection was performed to the lateral side of the right knee to induce OA, and after waiting for two weeks, an intra-articular UCMA injection was administered to the lateral side of the right knee every three days until the end of the experiment. The fourth group was the MIA+HA group; intra-articular MIA injection was performed laterally to the right knee to create OA on the first day of the experiment, and after waiting two weeks, the intra-articular HA injection was performed laterally to the right knee every three days until the end of the experiment. The fifth group was the MIA+CS group (CS was composed of betamethasone acetate + betamethasone disodium phosphate); an intra-articular MIA injection was performed laterally to the right knee to create OA on the first day of the experiment, and after waiting for two weeks, intra-articular CS injection was performed laterally to the right knee every three days until the end of the experiment. Recombinant

UCMA (OriGene Technologies Inc., Rockville, MD, USA), HA (Inarthro, Regenyal Laboratories, San Benedetto del Tronto AP, Italy), and CS (Celestone, Sanofi Company, Kırklareli, Türkiye) were commercially purchased. On the 36th day of the experiment, high-dose anesthesia with ketamine-xylazine was administered to the rats, and the rats were sacrificed with blood taken by cardiac puncture. The right knee of the rats was entered with a medial parapatellar approach after an anterior skin incision. The femoral condyles, tibial plateaus, and patella were exposed by dissection; then, the knees were separated by cutting from the femoral and tibial shaft regions with bone scissors. The dissected tissues were fixed in a 10% formaldehyde solution.

Joint samples were handled and processed for histological examinations as described previously.[17] Briefly, the dissected tissues were fixed at room temperature with 10% neutral buffered formalin for three days and were kept for demineralization in 10% formic acid solution for 12 h after the fixation stage. Afterward, dehydration was performed with ascending percentage of ethanol and absolute xylene. Tissue blocks were obtained after embedding in hard paraffin. Sections of 7 µm were obtained from paraffin blocks via a semimotorized microtome (SLEE CUT-5062; SLEE Medical, Mainz, Germany). For histological analyses, the sections were stained with safranin O and fast green FCF, hematoxylin-eosin, and toluidine blue, and the expressions of bone morphogenetic protein 2 (BMP-2) and nuclear factor-kappa B (NF-κB) were analyzed with immunohistochemistry. Histological preparations were photographed with a light microscope (Olympus CX-41; Olympus Corp., Tokyo, Japan). The images were analyzed with software (Kameram Gen III; Argenit, Istanbul, Türkiye), and the cartilage degeneration in the medial tibial plateau was evaluated using the Mankin score model on a scale of 0 to 14 points.[18] In addition, we performed all data analyses in a randomized, blinded fashion to ensure no bias in the present study.

Statistical analysis

The data were analyzed via the IBM SPSS version 21.0 software (IBM Corp., Armonk, NY, USA). All data were expressed with mean and standard deviation values. The nonparametric test Kruskal-Wallis H was used for multigroup comparisons. The Mann-Whitney U test was used for the comparison of two individual groups. A p-value <0.05 was considered statistically significant.

RESULTS

Histopathological examinations

In the general examination with hematoxylin-eosin staining (Figure 1a), it was observed that the joint structure was normal in the control group, the joint surface, cell morphology, and cell number were compatible with the healthy tissue structure, while the joint surface deteriorated, the cartilage layer was thinned, and the cellular density and the chondrocytes count was reduced in the MIA+V group. On the other hand, in the group treated with HA after MIA injections, the improvement in the joint surface was similar to the group in which UCMA was administered, while there was a general improvement of the tissue in the group treated with CS compared to the MIA+V group. In addition, it was determined that the tissue structure was less degenerated in the MIA+UCMA group compared to the MIA+V group, and the improvement in the joint surface and an increase in the cartilage cell density were remarkable.

In toluidine blue staining (Figure 1b), it was observed that the degenerations on the joint surface extended to the lower layers, there were changes in the distribution of cells and groupings. Meanwhile, in the UCMA-, HA-, and CS-treated groups, degenerations were detected in the articular cartilage, but a milder course was observed compared to the MIA+V group. In the MIA+V group, thinned and degenerated articular cartilage structure was detected, which was consistent with the other staining findings, while a significant improvement in the joint surface and a decrease in degenerations were detected in the HA-treated group compared to the MIA+V group. On the other hand, improvement in the joint surface was observed in the MIA+CS group compared to the MIA+V group, while improvements in the surface cartilage were observed in the MIA+UCMA group. Although thinning of the articular cartilage was more

obvious in MIA+V compared to the control group, it was determined that there was an increase both in the density of the cartilage matrix and the thickness of the articular cartilage compared to the MIA+V group.

Safranin O and Fast Green FCF staining results (Figure 1c) clearly reveal changes in the structure and thickness of articular cartilage and the density of cartilage cells. No degenerations were observed on the articular surface in the control group, whereas thinning of the cartilage layer on the articular surface and degenerations on the articular surface were observed in the MIA+V group. A significant improvement was observed in the cartilage matrix density and thickness and in the articular surface in the MIA+HA group. On the other hand, in the group treated with CS, there was an increase in cartilage thickness on the joint surface compared to the MIA+V group, while thickening of the surface cartilage and significant condensation in the cartilage matrix were observed in the group treated with UCMA.

Results of Mankin's scoring

Mankin score values were calculated based on the histological sections of the groups (Figure 2). The analyses have revealed a significant difference among the groups ($p<0.05$). It was determined that the Mankin score of the MIA+V group was significantly higher compared to the control group ($p<0.05$). It was found that the Mankin score was significantly lower in the UCMA-, HA-, and

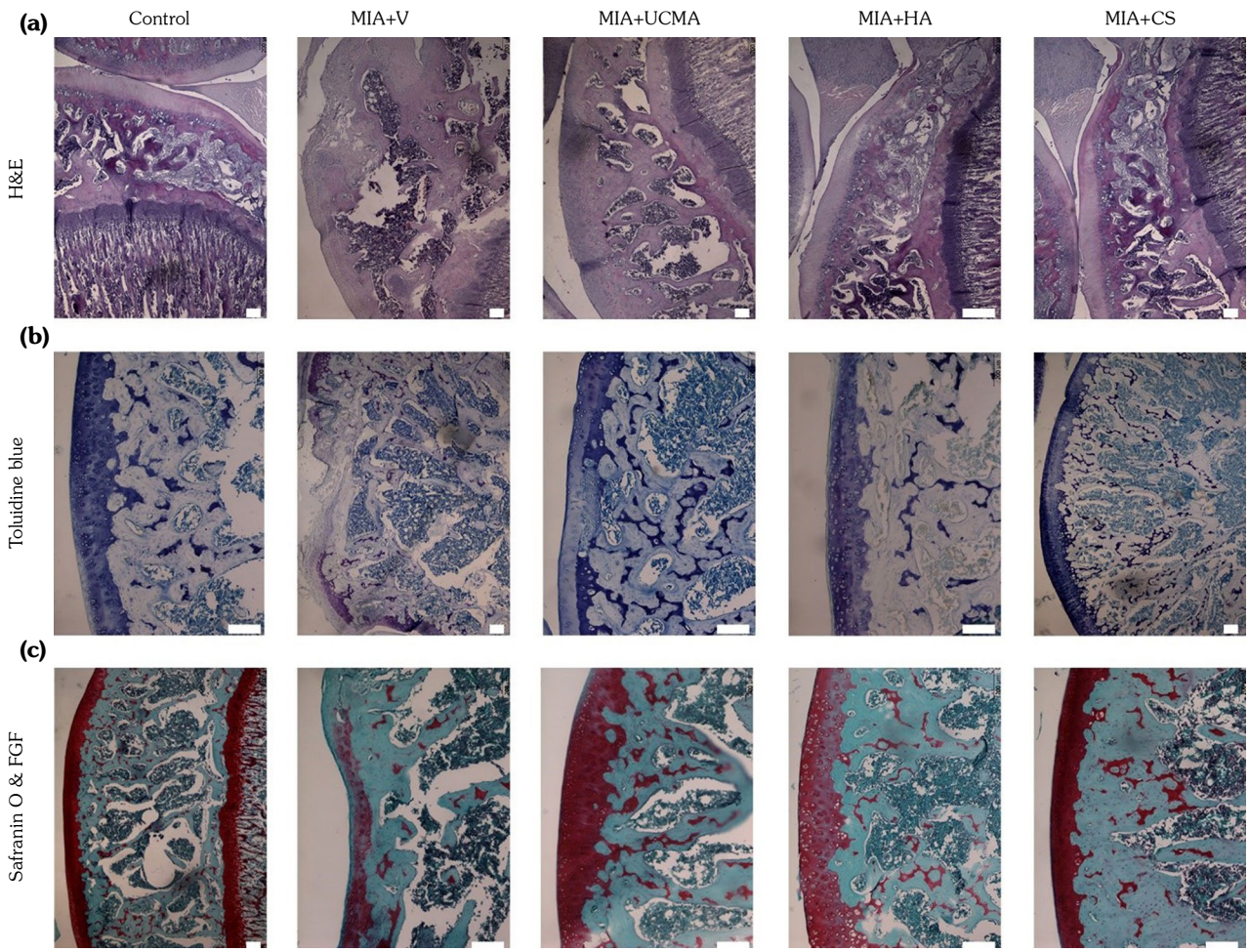

Figure 1. Histopathological stainings of the rat knees. **(a)** Hematoxylin-eosin, **(b)** Toluidine blue, and **(c)** Safranin O and fast green FCF stainings of the control, MIA+UCMA, MIA+HA, and MIA+CS groups.

MIA+V: Monosodium iodoacetate + vehicle; UCMA: Upper zone of growth plate and cartilage matrix-associated protein; HA: Hyaluronic acid; CS: Corticosteroid.

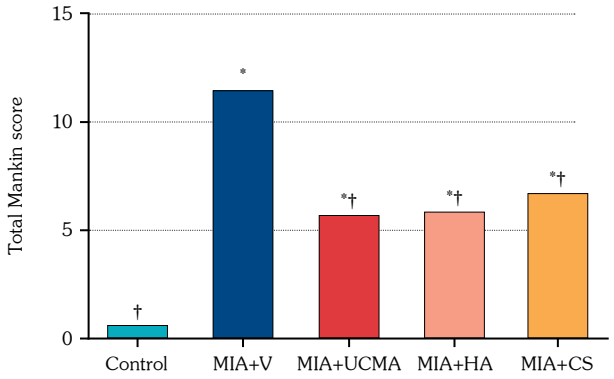

Figure 2. Mankin scores of the control, MIA+V, MIA+UCMA, MIA+HA, and MIA+CS groups.

MIA+V: Monosodium iodoacetate + vehicle; UCMA: Upper zone of growth plate and cartilage matrix-associated protein; HA: Hyaluronic acid; CS: Corticosteroid; *† p<0.05 compar.

CS-treated groups compared to the MIA+V group (p<0.05).

Immunohistochemical staining results

There was a significant difference when the immunoreactivity of NF-κB was compared among all groups (p<0.05; Figures 3a, c). It was found that the immunoreactivity of NF-κB in the MIA+V group was significantly higher compared to the control group (p<0.05). On the other hand, immunoreactivity of NF-κB was found to be significantly decreased in the UCMA-, HA-, and CS-administered groups compared to the MIA+V group (p<0.05).

The difference among all groups was also statistically significant regarding BMP-2

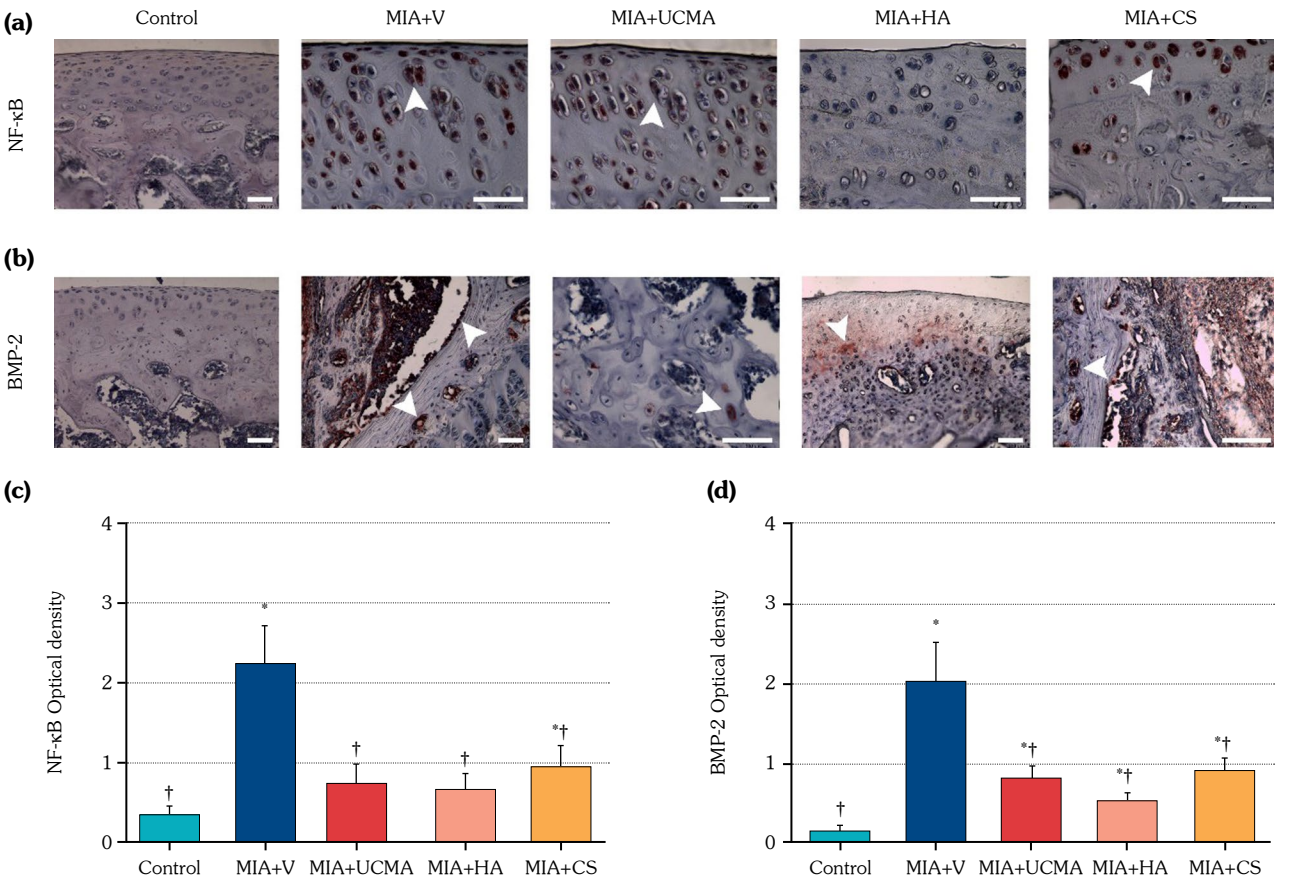

Figure 3. Immunoreactivity findings. Representative microscopic photos of **(a)** NF-κB, **(b)** BMP-2 immunohistochemical stainings, and **(c, d)** their corresponding optical density analyses in the control, MIA+V, MIA+UCMA, MIA+HA, and MIA+CS groups.

NF-κB: Nuclear factor-kappa B; MIA+V: Monosodium iodoacetate + vehicle; UCMA: Upper zone of growth plate and cartilage matrix-associated protein; HA: Hyaluronic acid; CS: Corticosteroid.

immunohistochemical staining densities ($p<0.05$; Figures 3b, d). BMP-2 immunoreactivity was determined to be significantly higher in the MIA+V group compared to the control group ($p<0.05$). On the other hand, BMP-2 immunoreactivity was found to be significantly decreased in the UCMA-, HA-, and CS-administered groups compared to the MIA+V group ($p<0.05$).

DISCUSSION

Osteoarthritis is a degenerative, chronic, and progressive disorder of the entire joint and its prevalence and incidence are expected to increase due to the increasing obesity and the aging population.[19-21] Hence, identifying effective therapeutic targets for the treatment and prevention of OA has a high priority.[6,7] Among current therapeutic approaches, intra-articular injections of HA and CS are widely used for patients with knee OA. The intra-articular HA injection has beneficial effects against OA by increasing the shock-absorbing effect and improving knee joint viscoelasticity.[22] On the other hand, an intra-articular CS injection can provide analgesia to patients with OA from weeks to months. Although both methods are relatively safe, they have some adverse effects.[23] Increased OA patient population and treatment costs and lack of effective treatment and surgical approaches, which do not provide absolute patient satisfaction, have led the researchers to investigate various treatment modalities.

In the present study, we compared the protective effect of UCMA with HA and CS in an intra-articular MIA-induced rat OA model for the first time. The rat OA model used in our study is a rapid and minimally invasive method that provides joint cartilage and subchondral degeneration similar to OA.[24] UCMA, a vitamin K-dependent protein involved in bone and cartilage pathology, has been relatively documented.[7,8,12] However, there is no study comparing the cartilage-protective role of UCMA with HA and CS in the rat OA model. Previous studies have shown that UCMA might have diagnostic and therapeutic value in the prevention of OA.[7-9] In a surgical animal OA model induced by the medial meniscus destabilization, Stock et al.[8] reported that OA-related pathological collateral damages, including apoptosis, degeneration, and loss of matrix proteoglycan components, were more severe in UCMA deficient mice, suggesting that UCMA can have a protective role against OA. The results of the same study demonstrated that UCMA expressions were upregulated in osteoarthritic cartilage tissues. Moreover, we previously investigated the relationship of UCMA with symptomatic and radiographic severities of knee OA.[9] Our findings revealed that UCMA expressions increased in the synovial fluid of OA patients. Our data showed that UCMA expression is associated with severities and progression of OA, and UCMA may be used as a diagnostic marker for OA.[9]

Seuffert et al.[12] revealed that UCMA might act as an inhibitor for the aggrecanase activity, and thus, UCMA might have a protective role against cartilage degradation. Furthermore, in our previous study, we revealed that intra-articular UCMA injection reduced the cartilage degenerations in the rat OA model induced by MIA.[7] Consistent with these results, the present study showed that UCMA provides chondroprotective effects on cartilage degeneration in the rat OA model. More notably, our results demonstrated that UCMA exerts a similar protective effect to HA and CS administration. Moreover, intra-articular UCMA treatment diminished the expression of NF-κB and BMP-2 in joint tissues. Considering data obtained from the present study, we may speculate that the observed protective effects of UCMA may be attributed to its regulatory influence on NF-κB and BMP-2.

Emerging evidence has highlighted that NF-κB signalling is markedly involved in cartilage degeneration, subchondral alteration, and synovial inflammation, and its modulation might have therapeutic importance.[25] Therefore, our hypothesis was based on whether the protective effect of UCMA on knee OA is associated with NF-κB. Our results unveiled that UCMA administration decreased NF-κB expression levels, which is consistent with a previous study of our group.[7] Here, we can speculate that UCMA may have a protective effect by regulating NF-κB.

In conclusion, our data presented here suggest that UCMA could be an effective protective agent in the prevention of OA. Moreover, similar effects of UCMA have been observed in

HA- and CS-treated animals. As a result, UCMA is a promising molecule in the prevention of OA. Nonetheless, further studies with varying doses and durations are essential to confirm the potential therapeutic effect of UCMA against knee OA.

Ethics Committee Approval: The study protocol was approved by the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (date: 31.01.2019, no: 2018/01-1).

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: All authors contributed to the study design, material preparation, data collection, statistical analysis, supervision and writing of the manuscript and approved the final manuscript.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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