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# Zoledronic Acid Effect on Chondrocyte Apoptosis in Degenerative Osteoarthritis Model

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**Apoptosis refers to cell death without inflammatory process, and is related to degenerative changes in joints. We hypothesized that zoledronic acid (ZA) would have a positive effect on chondrocyte viability and decreases in chondrocyte loss, which are important for the progression of degeneration. This study aimed to reveal the difference in time-dependent apoptotic changes in cartilage tissue in the anterior cruciate ligament (ACL) transection model of osteoarthritis (OA) in rat knees after treatment with zoledronic acid. We randomly divided 48 male Wistar albino rats into 6 groups. The knees of all rats except those in the control group underwent the operation for ACL transection. ZA for half of the rats and saline solution for the others was injected weekly into knees. Animals were killed at 0, 3, and 6 weeks after surgery. Apoptosis of chondrocytes were analyzed using the terminal deoxynucleotidyl transferase dUTP nick end labeling method. Comparison of groups was performed using Kruskal Wallis analysis and the Mann Whitney *U* test. Significant differences were observed between the groups treated with ZA and saline. ZA treatment significantly decreased the number of apoptotic cells in chondral tissue. ZA prevents time-dependent degenerative changes in chondral tissue by decreasing chondrocyte death. Intra-articular ZA may have the potential to treat and conserve chondral viability. ZA prevents chondrocyte loss and may play a therapeutic role in OA and conserving joint health. Further studies are needed to evaluate the potential of intra-articular ZA for the prevention or treatment of age-related degenerative changes.**

*Key words:* Apoptosis – Osteoarthritis – Cartilage – Zoledronic acid – Chondrocyte

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Osteoarthritis (OA) has been considered to be, primarily, a cartilage disorder resulting from the changes in matrix composition of cartilage and the reduction of tissue cellularity. OA cartilage was initially thought to occur through loss of chondrocytes.[1] The factors that trigger the initial chondrocyte destruction still are not sufficiently apparent. OA is also associated with subchondral and periarticular bone changes.[2] Increased subchondral turnover leads to changes in subchondral bone architecture.[3]

Apoptosis or programmed cell death represents a physiologic form of cell death that does not induce an inflammatory response. Recent studies have reported that apoptosis plays a major role in cell death in cartilage tissue.[4–8] In some studies, it was also suggested that osteoarthritic cartilage has shown that apoptosis has also been positively correlated with the severity of cartilage destruction and matrix depletion.[4–6,9]

Bisphosphonates (BPs), which are the inhibitors of osteoclastic activity, show their therapeutic action by inhibition of bone remodeling. Higher rates of bone turnover is associated with progression of OA.[10] BP has a protective effect against the development of osteoarthritic changes.[11,12] Zoledronic acid (ZA), a third-generation BP, is the most potent form of its kind, so only small doses are required for therapeutic purposes.[9] It was shown that zoledronate partially protected the articular cartilage degeneration in an experimental arthritis model.[12] In an in vitro study, it was shown that BPs are harmless for articular chondrocytes.[13] However, there are no prior studies testing intraarticular administration of BP in an experimental osteoarthritis animal model.

It was hypothesized that BP would not only have an effect on subchondral bone, but would also have a direct effect on articular cartilage. The aim of this study was to observe the effects of ZA on articular cartilage after intra-articular administration in an experimental rat osteoarthritis model by detecting apoptotic changes in chondrocytes. To our knowledge, this is the first study of this kind in English scientific literature.

## Method

Forty-eight male Wistar albino rats (mean body weight 264 ± 21 g) were used in this study, obtained from the Baskent University Laboratory Animal Center. All operative procedures were performed in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals," and with the approval of the Baskent University Animal Care Committee and World Medical Association Declaration of Helsinki. The conditions of feeding and accommodating animals was standardized in the laboratory from 1 week before the experiment to the end of the study. Rats were randomly allocated to 6 groups of 8 animals each and separately housed in plastic cages (1 for each group). Selection of groups was performed randomly.

We used an anterior cruciate ligament (ACL) transection model of OA in rats. OA was induced in the right knees of rats using the procedure of ACL transection as described previously in the literature.[14] Rats with intact ACLs in group (G)1 served as controls and were killed at the beginning of the study, in week 0. All remaining rats in other groups received operations for ACL transection. Rats in G2 were killed after the surgical procedure in week 0.

### *Surgical procedure*

Rats were anesthetized by intraperitoneal administration of 7 mg/kg of xylazine (Rompun, Bayer, Istanbul, Turkey) and 60 mg/kg of ketamine hydrochloride (Ketalar, Parke-Davis, Istanbul, Turkey). The right knees of the rats were prepared for the operative procedure. After shaving the knee joint, the skin was disinfected with povidone iodine (Betadine, Eczacibasi, Turkey) and a parapatellar skin incision was made on the medial side of the joint. The approach on the medial side of the patellar tendon provided access to the joint space after which the patella was dislocated laterally with the leg in extension. The anterior cruciate ligament was transected using a #11 surgical blade. A positive anterior drawer test ensured complete transection of the ligament. The medial retinaculum was repaired with 5/0 polyglactin-910 absorbable sutures (Vicryl, Ethicon, Berkshire, UK) and the skin was closed separately with running 4/0 silk sutures (Mersilk, Ethicon). All operative procedures were performed using a loop magnification. Each animal was given free access to chow and water the morning after the procedure. A twice daily dose of 0.02 mg/kg fentanyl citrate (Fentanyl, Abbott, Chicago, Illinois) was administered subcutaneously from days 1 through 3 after surgery for postoperative analgesia.

### *Drug administration*

ZA (Zometa, Novartis Pharma AG, Basel, Switzerland) was used for treatment. The 2 groups of rats

(G4 and G6) were randomly chosen and treated with ZA. We injected 10 μg of ZA intra-articularly in 0.1 mL of sterile saline to the 16 animals of the 2 groups. Drug therapy began on the first day of the operation and was repeated weekly until the animals were killed. Animals in the other 2 groups (G3 and G5) were administered the same volume of sterile saline injected at the same periods.

### *Tissue preparation*

The rats were divided into groups according to ZA or saline therapy. Two groups consisted of ZA-treated rats (G4 and G6). The other rats, belonging to the saline-treated groups, were the comparison groups (G3 and G5). We killed the rats at 3 and 6 weeks after the operation in order to harvest the specimens. Rats in G3 and G4 were killed at postoperative week 3. Rats in G5 and G6 were killed at the end of the 6 weeks. After excision of the skin, the joint interval was reached by cutting the patellar tendon and joint capsule. Cartilage tissue samples were punched out on femoral condyle and embedded in paraffin. Biopsy sections were randomized and coded. An observer who did not know which group each specimen belonged to performed histologic grading.

### *Determination of apoptotic index*

The apoptotic cells were detected using a detection kit (ApopTag Plus Peroxidase In Situ Apoptosis, S7101, Chemicon International, Inc, Temecula, California). We applied the commercial reagent (Chemicon International, Inc) to 5 μm of histologic slides prepared from paraffin tissue blocks through an immunohistochemical method. The kit detects apoptotic cells by labeling and detecting DNA strand breaks using the indirect terminal deoxynucleotidtransferase-mediated dUTP nick-end labeling method.

Each slide was examined for morphological characteristics of apoptosis in meniscal chondroid tissue nuclei; specifically condense chromatin, disintegrated nuclei. In addition to recording the presence or absence of apoptosis for each meniscal specimen, we also determined its apoptotic index (AI).

AI was calculated as the percent of positively stained meniscal chondroid tissue nuclei, detected in a total of 1000 meniscal chondroid tissue nuclei, counted in 5 randomly selected fields at ×400 magnification.

*Table 1 Distribution of AI among groups*

| Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | Group 6 |
|---|---|---|---|---|---|
| 93 | 164 | 227 | 184 | 248 | 112 |
| 184 | 182 | 263 | 176 | 232 | 84 |
| 176 | 198 | 232 | 148 | 192 | 98 |
| 103 | 179 | 219 | 104 | 224 | 118 |
| 178 | 146 | 223 | 102 | 208 | 98 |
| 189 | 153 | 256 | 114 | 217 | 96 |
| 176 | 118 | 251 | 88 | 182 | 94 |
| 168 | 115 | 215 | 98 | 236 | 102 |

Groups were created according to parameters such as ACL transection and ZA administration. Animals were killed at the beginning in G1 and G2, after 3 weeks in G3 and G4, and after 6 weeks in G5 and G6. ACL of knees were transected in all groups except G1 (control group). ZA was administered to knees in G4 and G6.

### *Statistical analysis*

Kruskal Wallis analysis of variance was used to compare the AI among groups. For multiple comparisons, the Bonferroni Adjusted Mann Whitney *U* test was used. The AI was presented as the mean ± standard and median (interquartile range). A value of $P < 0.05$ was considered significant. Analyses were performed using commercial software (IBM SPSS Statistics, version 22.0., IBM Corp, Armonk, New York).

## Results

The distribution of AI results for 48 rats in all groups is presented in Table 1. The mean and median values of AI are described for each group in Fig. 1. The groups constituted according to ACL transection and ZA administration. Follow-up period was 3 and 6 weeks. The variation of mean AI of all groups is also shown in Fig. 1. There was a statistically significant difference among groups ($P < 0.001$). The differences between the pairs of groups were surveyed and the pair with statistically significant differences were revealed. Mean AI increased significantly (48.85%) 3 weeks after ACL transection (G3) related to control group (G1; $P = 0.048$). There was a significant decrease (46.23% and 57.47%) in mean AI 3 and 6 weeks after ZA administration (G3 versus G4, $P = 0.001$ and G3 versus G6, $P < 0.001$). Mean AI markedly decreased (53.38%) after 6 weeks of ZA administration (G5 versus G6 $P < 0.001$). There was a significant difference between G4 and G5 (71.50%, $P = 0.013$). There was no difference between G2 and the other groups.

In Figs. 2 and 3, comparisons of typical histologic changes between groups at 3 and 6 weeks were

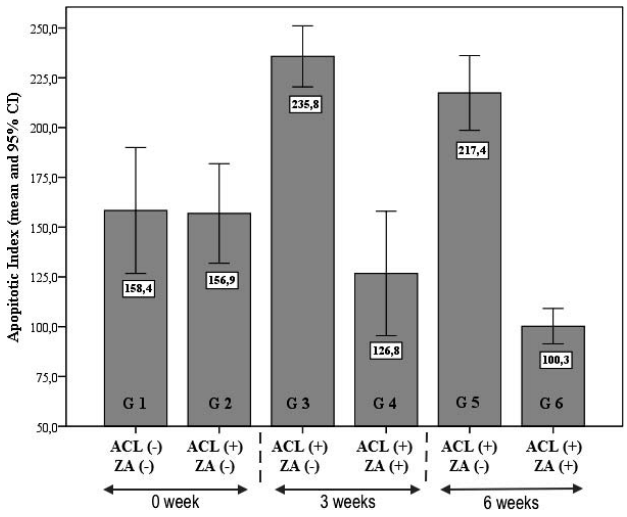


**Fig. 1** Comparison of mean AI of 6 groups (G1–G6) within 95% confidence interval. Groups were ordered according to ACL and ZA administration during the study period.

depicted. The decrease in the number of apoptotic chondrocytes was observed according to ZA therapy in both specimens at 3 (Fig. 2a and 2b) and 6 weeks (Fig. 3a and 3b).

## Discussion

The most important finding of the present study is decreased chondrocyte apoptosis after intraarticular injection of ZA. All rats on ZA treatment had significant improvements on their AI in our study. Some recent studies have suggested that ZA has a partial chondroprotective effect and prevents the progression of OA in animal models.[13,15]

We hypothesized that intraarticular ZA administration would have a protective effect on chondrocyte degeneration. The results of this study proved our hypothesis and also showed compatibility with existing literature.[12,16]

Aside from the well-known effects of BP on bone tissue, its effects on cartilage tissue has not previously been clearly understood. The effect of BP on cartilage tissue was explained in two ways: (1) indirectly prevents focal destruction by inhibiting subchondral bone resorption, and (2) directly decreases cartilage matrix degeneration.[17] ZA had time-dependent effects on chondral tissue in the rat knee model. Compared with placebo and ZA treatment, ZA started to show positive effects on chondral tissue in knees after 3 weeks (G3 versus G4, $P = 0.001$). This positive effect continued until week 6 (G5 versus G6, $P < 0.001$). Our results show

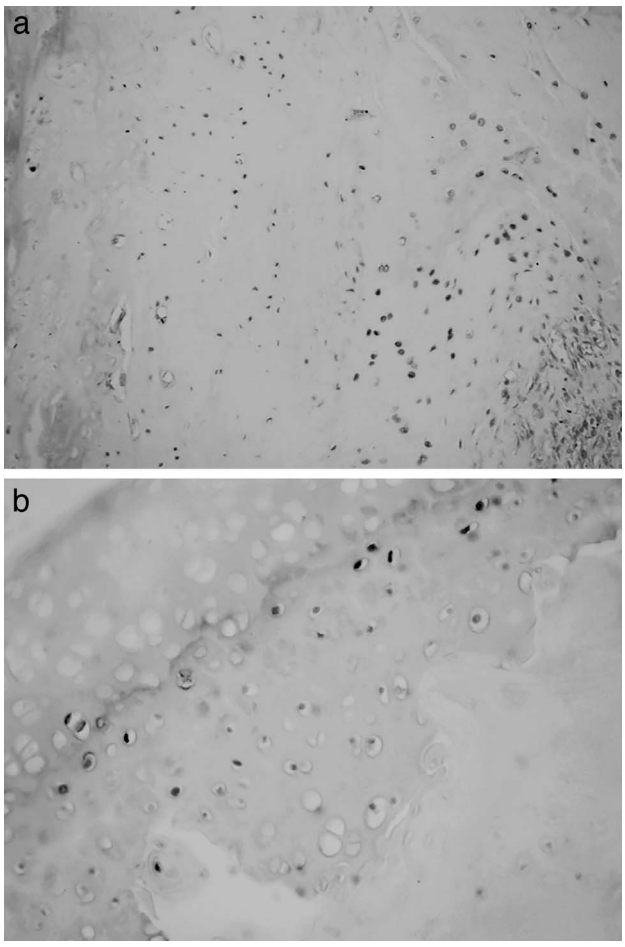


**Fig. 2** Difference in immunostaining of knee cartilage section of rats at 3 weeks. Dark staining nuclei of chondrocytes represent apoptotic chondrocytes. (a) Saline-injected knees (G3, AI = 251) contain more apoptotic cells than (b) ZA-injected knees (G4, AI = 114).

that intra-articular injection of ZA prevents chondrocyte death during the degeneration process.

Although intra-articular injection of ZA is not approved clinically, it was administered directly into the knee joint in our study. No side effects of ZA have been shown on cartilage. Subcutaneous injection of ZA has been shown to have direct effects on cartilage tissue,[18] but there is no study about intra-articular administration. Our goal for using intra-articular administration was to evaluate the direct effect of ZA, and minimize its systemic effect. It was revealed that intra-articular administration of ZA has a protective role on chondrocytes within cartilage tissue.

We used an ACL transection model of OA to create degenerative changes on cartilage and ob-

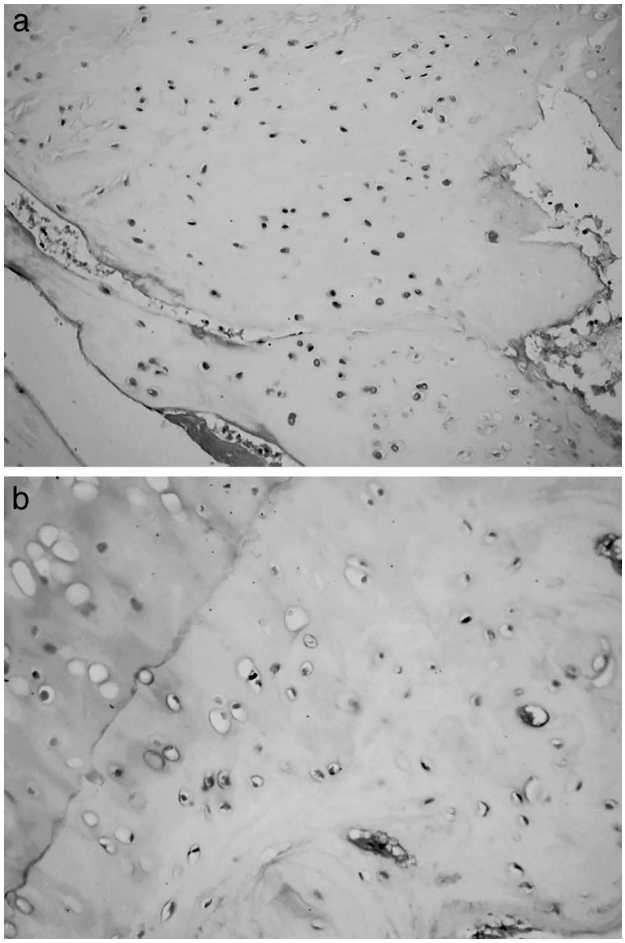


**Fig. 3** Apoptotic cells are represented as dark nuclei in samples of cartilage sections at 6 weeks. The number of apoptotic cells decreased with (a) saline therapy in (G5, AI = 98), compared with (b) ZA therapy in (G6, AI = 224) at 6 weeks.

serve the changes with ZA therapy on articular tissue. The AI of knees with transected ACL but no therapy was significantly higher than in healthy control groups (G3 versus G1, $P = 0.048$). The results reveal that the transection of ACL produced chondrocyte death resulting in chondral tissue degeneration. Time-dependent degenerative changes associated with apoptosis in the absence of anterior cruciate ligament have been shown in the literature.[19–21]

We used AI in our study to determine chondrocyte degeneration, and had comparable groups after ZA administration by detecting apoptosis levels in tissue ($P < 0.001$). Apoptosis refers to cell death without inflammatory process. It has been understood to be one of the precursors of chondral degeneration in several studies. These studies reported that the increased apoptosis chondral tissue was related to chondral degeneration.[19,22–25]

This study has some limitations. Six weeks of follow-up is a short-term period to observe a long-acting drug such as ZA. We are not aware of long-term effects of ZA on chondrocytes. The intra-articular effect of ZA is not fully understood so it is hard to differentiate exactly the direct and indirect effects of ZA on chondroid tissue. It is possible that ZA does so in both ways by preventing subchondral bone resorption and directly protecting chondral tissue. Preventing subchondral bone resorption may make an indirect contribution to chondral tissue protection. The mechanism of protective effects of ZA on chondral tissue is not certain. We also are not aware of any adverse effect of ZA when administered via the intra-articular route. Systemic and local effects need to be investigated in further studies.

We conclude that time-dependent degenerative changes occur in an ACL transection model of OA. Intra-articular administration of ZA prevents degenerative changes in cartilage tissue by decreasing chondrocyte death. Our study suggests that the intra-articular effect of ZA prevents chondrocyte loss and may play a therapeutic role to conserve joint health. Further studies are needed to evaluate the potential of intra-articular use of ZA for prevention or treatment of age-related degenerative changes.

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