EXPERIMENTAL STUDY

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Protective effect of zoledronic acid on corticosteroid-induced chondrocyte apoptosis in rat articular cartilage

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Objective: The aim of this study was to assess the apoptotic effects of systemic corticosteroid application on the articular cartilage chondrocytes *in vivo* and to investigate the potential effects of zoledronic acid on corticosteroid-induced apoptosis.

Methods: Twenty-four Wistar rats were randomly divided into 3 groups. In the control group, intramuscular isotonic salt solution was injected weekly. In the second group, a dose of 10 mg/kg intramuscular corticosteroid (methylprednisolone) injection was applied weekly for 8 weeks. In the third group, a dose of 10 mg/kg intramuscular corticosteroid (methylprednisolone) injection was applied weekly for 8 weeks and 0.1 mg/kg zoledronic acid was injected subcutaneously on days 0, 21 and 42. Femoral head specimens from each group were obtained at the end of the treatment and the TUNEL method was applied to detect apoptotic chondrocytes. Comparison analyses were performed using the ANOVA method and Tukey's test.

Results: There was a significant difference between the corticosteroid group and two other groups (control group: p=0.005; corticosteroid + zoledronic acid group: p=0.047). Zoledronic acid treatment significantly decreased the number of corticosteroid-induced apoptotic chondrocytes in the joint cartilage ($p<0.05$).

Conclusion: Zoledronic acid may have the potential to prevent joint cartilage deterioration due to the corticosteroid-induced apoptosis of the chondrocytes.

Key words: Apoptosis; cartilage; chondrocyte; corticosteroid; zoledronic acid.

Although the effects of corticosteroids on articular cartilage after intra-articular application have been previously studied, only a few studies exist regarding the effects of systemic corticosteroid use on articular cartilage. In these animal experiments, ultrastructural changes of joint cartilage following systemic corticosteroid application have been investigated and deterioration in matrix synthesis, decreases in protein and proteoglycan synthesis and degenerative changes in cartilage surface may occur in a dose-dependent manner. Some studies have reported that corticosteroids can cause increased cell death, although specific methods to reveal apoptosis were not applied.[1-9]

Apoptosis refers to programmed cell death and describes a process which results in cell death due to various influences. A specific way to describe apoptosis is

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the TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) method, in which apoptosis is detected through enzymatic labeling of DNA strand breaks.[10,11] A limited number of studies have searched for chondrocyte apoptosis caused by corticosteroid treatment; intra-articular application,[12] cell culture models of corticosteroid treatment[13-17] and the effects of corticosteroids on the growth plate cartilage in renal failure model in rats.[18] Although all of these studies have reported chondrocyte apoptosis and detrimental effects on cartilage cells caused by corticosteroid treatment, to the best of our knowledge, there is no study in the literature seeking the apoptotic effects of systemic corticosteroid treatment on articular cartilage *in vivo*.

Corticosteroids are widely used to treat or control symptoms of various illnesses as well as following head traumas or organ transplantation. Due to a lack of sufficient scientific information on the fate of articular cartilage, we do not know how seriously systemic corticosteroid treatment affects articular cartilage, and precautions to prevent the detrimental effects of corticosteroids on articular cartilage have not been taken in the corticosteroid regimen.

It has been reported that bisphosphonates have antioxidant properties, thus inhibiting chondrocyte lipid peroxidation, and therapeutic potential in rheumatoid arthritis.[19] Additionally, the protective effect of bisphosphonates on corticosteroid-induced apoptosis of articular chondrocytes has been observed in chondrocyte cultures.[20]

The purpose of this study was to investigate the apoptotic effects of systemically applied corticosteroids *in vivo* and to investigate the potential effects of the bisphosphonates on corticosteroid induced apoptosis.

Materials and Methods

This study included 24 adult female Wistar rats, each weighing 200 to 250 g. The rats were randomly divided into 3 equal groups of 8 rats each and allowed free access to standard rat laboratory diet and tap water. The control group received injection of intramuscular isotonic salt solution once a week for 8 weeks. The corticosteroid group received a dose of 10 mg/kg intramuscular methylprednisolone (Prednol-L ampule; Mustafa Nevzat, İstanbul, Turkey) once a week for 8 weeks. High dose corticosteroid injections can cause the development of apoptosis in cartilage and bone cells[17] and low dose methylprednisolone application has not any major effect on calcium balance and bone composition in rats.[21] Therefore, we chose to apply a dose and practical way of application which could cause chondrocyte apoptosis depending on our pilot study.

The corticosteroid + zoledronic acid group received a dose of 10 mg/kg intramuscular methylprednisolone once a week for 8 weeks and 0.1 mg/kg subcutaneous zoledronic acid (Zometa®; Novartis Pharma AG, Basel, Switzerland) on days 0, 21 and 42. Injections of 0.1 mg/kg zoledronic acid at 1 and 4 weeks postoperatively preserves femoral head architecture after surgically induced osteonecrosis of femoral head in rats.[22] Additionally, Zometa® is given every 3 to 4 weeks in clinical usage,[23] thus we chose to apply 0.1 mg/kg subcutaneous zoledronic acid injections on days 0, 21 and 42.

The experimental protocol was approved by the Animal Care and Usage Committee of our institution in accordance with The Declaration of Helsinki and The International Association for the Study of Pain guidelines.

At the end of the 8-week period, rats were sacrificed under high-dose anesthesia and their femoral heads were removed. Tissues were processed for embedment in paraffin wax for immunohistochemical investigations. Femoral head samples were fixed by immersion in 10% formalin for one week. Then, tissues were washed in tap water for 24 hrs, decalcified in 25% formic acid (Merck KGaA, Darmstadt, Germany) for 3 days, again washed in tap water for 24 hrs and neutralized in 0.35 M sodium sulfate (Lachema, Brno, Czech Republic) solution for an additional 3 days. Tissues were again washed in tap water for 24 hrs and dehydrated through a graded series of ethanol and embedded in paraffin wax. All samples were serially sectioned at 5 µm thickness and were stained for routine histological examination after hematoxylin-eosin (HE) staining or for immunohistochemical labeling.

Apoptosis in articular tissue was detected by enzymatic labeling of DNA strand breaks using the TUNEL method. Paraffin sections of 5 µm thickness from the femoral heads were cut and placed on slides covered with poly-L-lysine and left in the incubator at 45°C overnight and at 60°C for 1 hour after drying. After deparaffinization and rehydration, slides were washed twice in phosphate-buffered saline for 5 minutes. After incubation of slides with the permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 minutes at 4°C and washing twice with phosphate-buffered saline for 5 minutes, the labeling reaction was performed by using 50 µL of TUNEL reagent for each sample, except negative control, in which reagent without enzyme was added and incubated for 1 hour at 37°C. After phosphate-buffered saline washings, slides were incubated with converter reagent for 30 minutes at 37°C. After washing, color development for localization of cells containing labeled DNA strand breaks was performed by incubating the slides with Fast Red substrate

solution for 10 minutes. Labeling with TUNEL was conducted with a Cell Death Detection Kit (Roche, Mannheim, Germany) and was performed according to the manufacturer's instructions.

TUNEL-labeled cells in all groups were evaluated using an Axioplan microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. From three randomly selected slides, one from each group, five different fields at ×80 magnification were analyzed for TUNEL positive cells. Two observers blinded to the experimental groups performed the TUNEL positive cell counting. Average scores were used.

Data from the TUNEL positive cell counting were analyzed using the one-way ANOVA test. Intergroup significance was evaluated by post-hoc Tukey's test. Values are presented as mean±SD. Statistical calculations were performed using SigmaStat for Windows v.3.0 (Jandel Scientific Corp., San Rafael, CA, USA). Statistical significance was set at $p<0.05$.

Results

Apoptotic cell values (mean±SD) obtained from the control and experimental groups are presented in Table 1.

Apoptotic chondrocyte values increased significantly in the corticosteroid group compared to the control group and the corticosteroid + zoledronic acid treated groups ($p=0.005$ and $p=0.047$, respectively). There was no statistically significant difference between control and corticosteroid + zoledronic acid-treated group. Differences between groups in TUNEL staining can be seen clearly in Fig. 1. Intensive apoptosis was seen mostly in areas close to superficial zone in the corticosteroid treated group.

Discussion

Corticosteroids have been widely used to treat or control the symptoms of various illnesses as well as following head trauma and organ transplant. In these clinical settings, the long-term effect on joint cartilage is not currently known. Corticosteroid regimen is a standard protocol along with essential immunosuppressive therapy after organ transplantation. This regimen has been considered harmless to cartilage and progressive cartilage problems in major joints, with the exception of avascular necrosis (AVN)-related osteoarthritis (OA) in transplant patients, has not been determined.[24] In two recent publications of a total of 5 cases with rapid multifocal chondrolysis without AVN after liver transplan-

Table 1. Apoptotic chondrocyte counts in rat articular cartilage (mean±SD).

	Control	Corticosteroid	Corticosteroid + Zoledronic acid
TUNEL positive chondrocytes	18±14	77±40*	36±21

*$p<0.05$

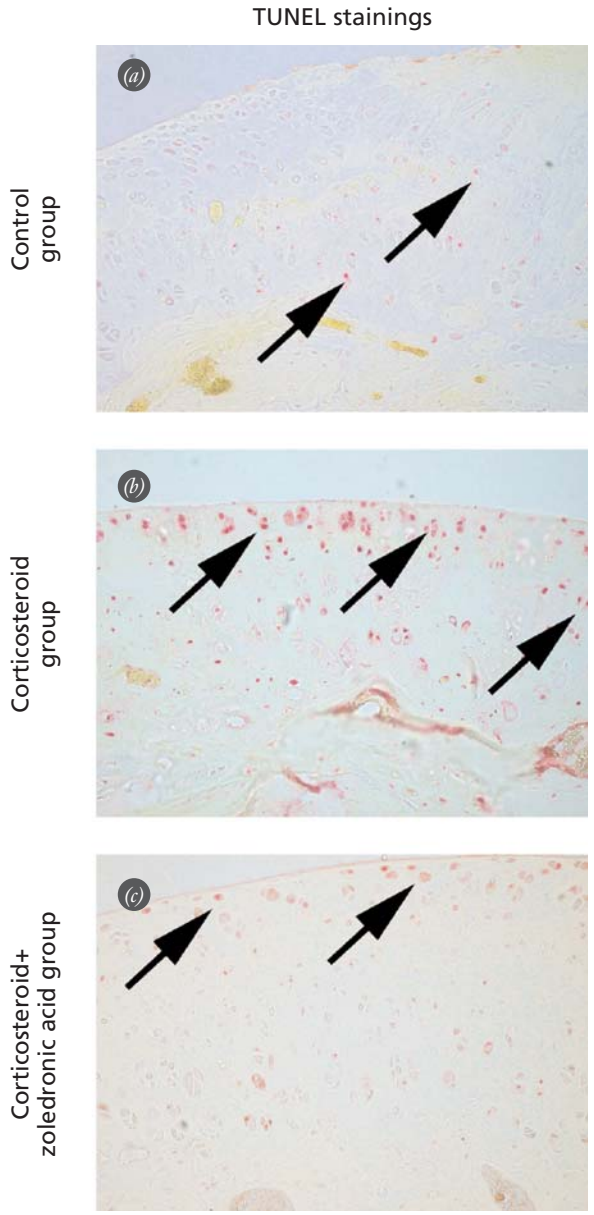

Fig. 1. TUNEL stainings of the groups. **(a)** In the control group, normal distribution of TUNEL positive cells is seen. Arrows show positive cells in the mid-zone of cartilage. **(b)** In the corticosteroid group, TUNEL positive cells significantly increased in number and intensity in the superficial zone of cartilage. Arrows show marked positive cells. **(c)** In the corticosteroid + zoledronic acid group, number and intensity of the TUNEL positive cells are decreased compared to the corticosteroid group. Arrows show positive cells (HE, ×80). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

tations,[2,24] corticosteroids were used as an adjunct to immunosuppressive therapy and no microfractures of the bone were detected. In both publications, multifactorial involvement was considered as the possible cause of chondrolysis, despite multiple potential additional factors that may lead to cartilage deterioration. Our study revealed that corticosteroids, one of the possible causative agents, may lead to cartilage break down by increased chondrocyte apoptosis after corticosteroid treatment. In this manner, corticosteroids should be taken into consideration in the future studies investigating cartilage deterioration after organ transplantation.

Some studies have shown the apoptotic effects of corticosteroids on articular cartilage chondrocytes.[12-18] In these studies, it was reported that systemic corticosteroid treatment induces a decrease in growth cartilage thickness via a triggering of apoptosis in terminal hypertrophic chondrocytes and inhibiting chondrocyte proliferation[17] and increases apoptosis in proliferative and hypertrophic chondrocytes via a decrease in Bcl-2 and increase in Bax expression.[15,18] In our study, the apoptotic effects of systemic corticosteroids on articular cartilage chondrocytes was studied *in vivo* and apoptosis was observed to have increased. This is the first study that shows the apoptotic effects of corticosteroids on articular cartilage chondrocytes *in vivo*. Our results are consistent with studies showing the effects of corticosteroids on chondrocyte cultures. In these studies, the detrimental effects of corticosteroids were shown.[13-18]

The apoptotic pathway is a complex system with multiple constituents. Caspase enzymes, a family of proteins responsible for the degradation of targeted cells to undergo apoptosis, are important components in this pathway. Apoptosis can occur after the activation of the initiator caspase-8 or caspase-9.[25] In addition to these two caspase-dependent pathways, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is also considered as an important component in corticosteroid-induced apoptosis.[26] A recent study reported that corticosteroids increase apoptosis of chondrocytes through inhibition of Akt phosphorylation and therefore inhibition of the PI3K/Akt signaling pathway.[12]

A recent study of an OA model demonstrated that alendronate, a nitrogen-containing bisphosphonate, inhibits the expression of MMP13, IL-1b, RANKL, COLX, and VEGF in cartilage.[27] MMP13 and IL-1b are known to cause cartilage damage in OA, which is mediated by chondrocytes.[28] RANKL is known to regulate osteoclast differentiation, and is also present in the cartilage.[29] The inflammatory cells and cartilage of arthritic knee joints contain high levels of RANKL[30] and COLX.[31] In OA, the expression of the angiogenic factor VEGF is up-regulated in chondrocytes.[32] The authors concluded that alendronate conserves articular cartilage by inhibiting the expression of MMP13, IL-1b, COLX, VEGF, and RANKL.[27] In our study, zoledronic acid, a nitrogen-containing bisphosphonate, was used to prevent corticosteroid induced apoptosis.

In a study researching glucocorticoid-induced apoptosis in osteocytes and osteoblasts, bisphosphonates were shown to inhibit apoptosis regardless of the pro-apoptotic stimulus used, and the authors concluded that bisphosphonates inhibits cell death, independently of the pro-apoptotic pathway activated.[33] In another study,[34] it was shown that the anti-apoptotic effect of bisphosphonates on osteocytic cells and osteoblasts involved the rapid activation of extracellular signal-regulated kinases (ERKs). ERK-associated anti-apoptotic outcome induced by bisphosphonates requires the kinase activity of the cytoplasmic target of ERKs, which in turn phosphorylates the pro-apoptotic protein BAD and C/EBP (the CCAAT/enhancer binding protein).

In a recent study in mice, findings supported a role for activation of survival signaling by bisphosphonates in the glucocorticoid-augmented osteoblast apoptosis.[35]

Although we do not have detailed data to determine the exact mechanisms and pathways of the prevention of corticosteroid-induced apoptosis by zoledronic acid, our opinion is, as shown previously, that zoledronic acid may inhibit apoptosis regardless of the pro-apoptotic stimulus by mediating ERKs.

We tested our hypothesis using the TUNEL method solely, which may be considered a weakness of this study. Another accepted method to identify apoptotic chondrocytes in cultured cells is staining chondrocytes with FITC-labeled annexin-V and propidium-iodide (PI), in which flow cytometry is used.[36,37] Since chondrocyte culture or isolation is needed to perform flow cytometry, we did not have a chance to utilize it in our study.

A previous study reported a significant decrease in stem cell markers (CD105 and CD166) after systemic corticosteroid treatment in the articular cartilage of rats, and it was speculated that the decrease in these cell surface molecules may have affected the chondrocyte viability and overall articular cartilage structure.[38] The current study supports the hypothesis that chondrocyte apoptosis, which was marked in the superficial zone of articular cartilage, may potentially lead to cartilage deterioration in the long-term.

Our second result was the decrease of systemic corticosteroid induced apoptosis after zoledronic acid treatment. The only previous study on this subject

reported the decrease of corticosteroid-induced chondrocyte apoptosis after zoledronic acid treatment in chondrocyte cultures, and this *in vitro* study is consistent with our findings.[20]

In conclusion, patients should be informed that long-term treatment with corticosteroids may cause harmful effects on articular cartilage chondrocytes which may potentially lead to degenerative joint disease in the future. Further clinical studies to examine the effect of the bisphosphonates on patients with long-term systemic corticosteroid treatment are necessary. Additionally, future clinical studies are needed to determine the regenerative effect of bisphosphonates clinically on the deterioration of cartilage caused by corticosteroid treatment.

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Conflicts of Interest: No conflicts declared.

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