Tiludronate is a non–nitrogen-containing bisphosphonate used to treat osteoporosis, Paget's disease, and other skeletal disorders in humans. It affects the bone remodeling process and results in increased bone mass and mechanical strength. In Europe, tiludronate is approved for the treatment of navicular disease and osteoarthritis of the distal intertarsal and tarsometatarsal joints in horses; however, in the United States, FDA approval of tiludronate for veterinary use is pending. After systemic administration, bisphosphonates have a high affinity for and bind to hydroxyapatite in bone. Bisphosphonates prevent bone resorption by chelation of the calcium ions of hydroxyapatite and the induction of osteoclast apoptosis via inhibition of important cellular processes. Specifically, after absorption by osteoclasts, non–nitrogen-containing bisphosphonates, such as tiludronate, form nonhydrolyzable ATP analogues, which inhibit ATP-dependent cellular processes and result in apoptosis. Alternatively, after nitrogen-containing bisphosphonates are absorbed by osteoclasts, the mevalonate pathway is inhibited, which prevents posttranslational modification of proteins, such as the

### Objective
To determine concentration-dependent effects of tiludronate on cartilage explants incubated with or without recombinant equine interleukin-1β (rEq IL-1).

### Sample
Articular cartilage explants from the femorotibial joints of 3 young adult horses.

### Procedures
Cartilage explants were incubated with 1 of 6 concentrations (0, 0.19, 1.9, 19, 190, or 1,900 mg/L) of tiludronate and with or without rEq IL-1 (0.01 ng/mL) for 96 hours. Prostaglandin E2 (PGE2) concentrations in culture medium and explant digests were analyzed via PGE2 enzyme immunoassay. Sulfated glycosaminoglycan (sGAG) concentrations in culture medium were quantified via 1,9-dimethylmethylene blue assay. Chondrocyte apoptosis in paraffin embedded explant sections was measured via terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay. Relative gene expression of matrix metalloproteinases (MMPs), interleukin (IL)-6, and IL-8 was determined via the comparative cycle threshold method.

### Results
rEq IL-1 increased PGE2 concentration, sGAG release from explants, chondrocyte apoptosis, and MMP gene expression. Lower tiludronate concentrations reduced rEq IL-1–induced sGAG release and chondrocyte apoptosis, whereas the higher tiludronate concentrations increased sGAG release and chondrocyte apoptosis. At the highest tiludronate concentration evaluated, IL-8 gene expression was increased independent of whether rEq IL-1 was present.

### Conclusions and Clinical Relevance
Tiludronate had biphasic concentration-dependent effects on cartilage explants that were independent of PGE2 secretion or MMP gene expression. Low tiludronate concentrations had some chondroprotective effects, whereas high tiludronate concentrations were detrimental to equine articular cartilage. Administration of tiludronate intra-articularly to horses may be detrimental, dependent on the dose used. In vivo studies are needed before intra-articular tiludronate administration to horses can be recommended. (Am J Vet Res 2012;73:1530–1539)
prenylation of small GTPases, which in turn interferes with the regulation of essential cellular activities and results in apoptosis.  

For the treatment of conditions in which there is an abnormal increase in bone resorption in relation to the rate of bone formation, the administration of bisphosphonates at recommended doses generally decreases bone resorption without negatively influencing normal bone formation.  

In horses, IV administration of tiludronate resulted in significantly greater clinical improvement, compared with that achieved with IV administration of a placebo for the treatment of naturally occurring navicular disease, osteoarthritis within the thoracolumbar vertebral column, and osteoarthrosis of the distal tarsal joint.  

Anecdotally, veterinarians have been administering tiludronate intra-articularly to horses for the treatment of osteoarthritis, despite a lack of data regarding the safety or efficacy of this practice. On the basis of these anecdotal reports, 50 mg of tiludronate administered intra-articularly into a joint (eg, the tibiotarsal joint) with a synovial fluid volume of approximately 25 mL will result in a tiludronate concentration in the synovial fluid of approximately 26 mL. In comparison, following IV administration of tiludronate (1 mg/kg) to horses, the mean peak plasma concentration of tiludronate was 9 mg/L.  

Although the peak synovial fluid concentration of tiludronate was not determined in that study, it was likely lower than the plasma concentration of tiludronate.  

The mechanism by which tiludronate contributes to the clinical improvement of horses with osteoarthritis is unknown. Tiludronate most likely alters subchondral bone turnover and also may modulate the metabolism of articular cartilage. The purpose of the study reported here was to determine the concentration-dependent effects of tiludronate on equine articular cartilage explants. We hypothesized that tiludronate would decrease proteoglycan loss from the cartilage matrix via downregulation of MMP expression and protect articular chondrocytes in vitro.

### Materials and Methods

**Animals**—All procedures were approved by the Institutional Animal Care and Use Committee of Oregon State University. Three horses (age, 2 to 6 years) were euthanized with an overdose of pentobarbital and phenytoin sodium administered IV for reasons unrelated to lameness. Following the euthanasia of each horse, near full-thickness cartilage (excluding calcified cartilage) was harvested aseptically from both femorotibial joints. No gross evidence of osteoarthritis or other joint disease was present in any of the femorotibial joints. Each femorotibial joint was used as a separate source for cartilage explants.

**Experimental design**—An in vitro method similar to that described in another study was used to simulate damage caused by osteoarthritis in the articular cartilage explants obtained from the study horses. Briefly, the harvested cartilage from each femorotibial joint was subdivided into 12 explants that were cultured in separate wells of 2 tissue culture plates, which resulted in 72 explants (12 explants from each joint 2 joints/horse × 3 horses). After an equilibration period in culture, 6 explants from each joint (n = 36) were incubated with rEq IL-1 to simulate cartilage damage caused by osteoarthritis while the remaining 6 explants from each joint (36) were not incubated with rEq IL-1. Each explant was then treated with 1 of 6 concentrations of tiludronate such that each tiludronate concentration was administered to an explant obtained from each femorotibial joint of the 3 horses within both the rEq IL-1–treated and untreated groups. Prostaglandin E2 and sGAG concentrations were determined, and chondrocyte apoptosis and gene expression of MMP-1, MMP-3, MMP-13, IL-6, and IL-8 were quantified.

**Cartilage explant cultures**—Each cartilage explant (size, approx 6 × 6 × 1 mm; wet weight, 100 mg) was placed in a well of a 6-well tissue culture plate. To each explant, 2 mL of Iscove modified Dulbecco medium supplemented with 5% fetal bovine serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and gentamicin (50 µg/mL; culture media) was added and then the plates were incubated in a humidified atmosphere of 5% CO2 at 37°C for a 48-hour equilibration period. Separate batches of treatment culture media were made that contained the respective combinations of rEq IL-1 and tiludronate in addition to the same culture media used during the equilibration period. At the end of the equilibration period (time 0), the culture media in each well was replaced with 2 mL of the assigned treatment culture media and the plates were incubated in a humidified atmosphere of 5% CO2 at 37°C for 48 hours (experimental period). After 48 hours of incubation during the experimental period, the treatment culture media was replaced with fresh treatment culture media. The plates were then incubated for another 48 hours. The experimental period ended 96 hours after initial administration of the assigned treatments.

**Experimental treatments**—To simulate cartilage damage caused by osteoarthritis, half of the explants (n = 36) were incubated with rEq IL-1 (0.01 ng/mL) that was added to the culture media at time 0 (end of the equilibration period) and again after the culture media was refreshed 48 hours later. The other half of the explants were not incubated with rEq IL-1 and served as the control group. Within both the rEq IL-1–incubated and control groups, each explant was also incubated with 1 of 6 concentrations of tiludronate (0, 0.19, 1.9, 19, 190, and 1,900 mg/L) that was added to the culture media at time 0 and again after the culture media was refreshed 48 hours later. The highest concentration (1,900 mg/L) of tiludronate evaluated was chosen to simulate the estimated concentration of tiludronate in synovial fluid after intra-articular administration of 50 mg tiludronate into a joint with a synovial fluid volume of approximately 26 mL. The lower concentrations of tiludronate evaluated were chosen on the basis of results of previous studies that evaluated the effects of bisphosphonates on chondrocytes in vitro.

**Sample collection and processing**—From each explant well, 2 mL of culture media was collected at 48 and 96 hours after initial treatment administration for determination of PGE2 and sGAG concentrations.
Culture of the cartilage explants ended 96 hours after initial treatment administration, and all cartilage explants were processed for quantification of sGAG content, chondrocyte apoptosis, and gene expression of MMP-1, MMP-3, MMP-13, IL-6, and IL-8. The wet weight of each cartilage explant was recorded. The explants designated for analysis of chondrocyte apoptosis were placed in neutral-buffered 10% formalin for 48 hours and then were stored in 70% ethanol until they were embedded in paraffin. All other explants were stored at –80°C until further processing. The explants designated for determination of sGAG concentration were papain digested. Each frozen cartilage explant was pulverized separately in a custom-made, liquid nitrogen-cooled stainless steel pulverizer. Then, 10 mg of the pulverized cartilage explant was mixed with 1 mL of papain digestion solution (0.5 mg/mL of papain in 3.45 g of monobasic sodium phosphate, 0.163 g of N-acetylcysteine, and 0.83 g of EDTA tetrasodium salt dissolved in H₂O to 500 mL; pH adjusted to 6.5) were mixed and placed into a shaker incubator at 65°C for 4 hours.

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**Assays**—A commercially available PGE_{2} enzyme immunoassay was used to determine PGE_{2} concentration in the culture media. The enzyme immunoassay was performed in accordance with the manufacturer’s instructions on samples of papain-digested culture media that had not been purified or diluted. For each sample, the PGE_{2} concentration was assayed in triplicate.

To evaluate proteoglycan degradation, the percentage of sGAG released from the cartilage explants was determined by use of a DMMB assay in a microplate format as described with some modifications. Briefly, 50 µL of sample (papain-digested culture media or cartilage explant), standard, or control was placed into assigned wells of a 96-well plate. A multichannel pipette was used to add 250 µL of DMMB working solution (8 mg of DMMB in 2.55 mL of ethanol [100%] in 1 g of sodium formate and 1 mL of formic acid [88%] dissolved in H₂O to 500 mL; pH adjusted to 3.5) to each well, and the light absorbance for each sample was immediately measured at 540 nm. All samples were assayed in triplicate, and for each sample, the concentration of sGAG was determined by comparison with a standard curve that was generated from serial dilutions (5 to 100 g/mL) of shark chondroitin-6-sulfate. The percentage of sGAG released from each explant into the culture media was determined as ([(sGAG in culture media collected 48 hours after initial treatment + sGAG in culture media collected 96 hours after initial treatment) × (sGAG in culture media collected 96 hours after initial treatment + sGAG in cartilage explant)] × 100).

Quantification of chondrocyte apoptosis in each cartilage explant was determined with a TUNEL method by use of a commercially available kit. Briefly, slides with formalin-fixed, paraffin embedded sections of cartilage explants were deparaffinized by incubating them at 53°C for 30 minutes, followed by washes with xylenes and dehydration with a dilution series of ethanol and a final rinse with water. Slides were then placed in citrate buffer (0.1M; pH 6.0), microwaved for 1 minute, cooled down with molecular grade water, and placed into PBS solution. The slides were treated with the TUNEL reaction mixture in accordance with the manufacturer’s instructions. Slides with cartilage explant sections treated with DNase I served as positive controls, and slides with cartilage explant sections treated with the label solution instead of the TUNEL reaction mixture served as negative controls. All slides were stored and protected from light until analysis, which was performed within 5 days after processing by an investigator (ND) who was unaware of the treatment assignment for the explants. To determine the percentage of apoptotic chondrocytes in cartilage explants, 1 slide with a representative section of 1 explant from each experimental group (combination of rEq IL-1–incubated group or control group with each concentration of tiludronate [0, 0.19, 1.9, 19, 190, and 1,900 mg/L]) was analyzed with a fluorescence microscope at an excitation wavelength of 450 to 500 nm and a filter cube to detect green fluorescent light (wavelength, 515 to 565 nm). All cells emitting a green signal within the section were counted as apoptotic chondrocytes. Then, the slide was evaluated with a bright light and all chondrocytes present in the section were counted. The percentage of apoptotic chondrocytes in each section was calculated as follows: (number of apoptotic chondrocytes/number of all chondrocytes) × 100.

Quantification of gene expression of MMP-1, MMP-3, MMP-13, IL-6, and IL-8 in cartilage explants was performed via reverse transcriptase and quantitative real-time PCR assays (Appendix). The RNA was isolated from the cartilage explant specimens in accordance with a modified RNA extraction protocol. Briefly, pulverized cartilage explant samples were homogenized twice in 0.038 mL of reagent/mg of cartilage (wt weight). Then, a chloroform extraction was performed twice with 0.3 mL of chloroform/mL of reagent. The RNA was precipitated with 100% isopropanol, washed in 70% ethanol, and dissolved in 100 µL of nuclelease-free water. An on-column clean-up and DNase digestion was performed in accordance with the manufacturer’s recommendations for first-strand cDNA synthesis to each experimental group (combination of rEq IL-1–incubated group or control group with each concentration of tiludronate) as an objective method for evaluation of mRNA quality. First-strand cDNA synthesis was performed in accordance with the manufacturer’s instructions for first-strand cDNA synthesis that used oligo-(dT)_{20} primers. Samples were also processed without the addition of the reverse transcriptase for first-strand cDNA synthesis to provide non–reverse-transcribed samples to control for genetic DNA contamination. Quantitative
real-time PCR assays were performed with a commercially available kit. Controls for each assay included template-free and non-reverse-transcribed negative controls. All samples were assayed in duplicate, and a final volume of 25 µL was used for all reactions. All reactions were performed for 40 cycles.

The relative change in gene expression was determined via the comparative cycle threshold method. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control (housekeeping gene), and samples from the control (no rEq IL-1) group that were not incubated with tiludronate (0 mg/L) were used as reference samples. The ∆Ct for each gene was defined as the cycle number at which fluorescence reached the gene-specific threshold. The following formula was used to calculate the fold change in gene expression between each sample and the reference sample:

\[
\text{Fold change} = \frac{(\Delta C_t \text{ for gene of interest in sample} - \Delta C_t \text{ for housekeeping gene in sample})}{(\Delta C_t \text{ for gene of interest in reference sample} - \Delta C_t \text{ for housekeeping gene in reference sample})}
\]

**Statistical analysis**—Data were analyzed with a mixed-model ANOVA; horse and limb nested within horse were considered random effects, and rEq IL-1 and concentration of tiludronate were considered fixed effects. The Dunnett method was used to evaluate differences between explants incubated with and without rEq IL-1 and among explants treated with the various concentrations of tiludronate. The Dunnett method was also used to identify interactions between rEq IL-1 and tiludronate concentration. For all analyses, values of \(P < 0.05\) were considered significant.

**Results**

PGE2 concentration in culture media—The PGE2 concentration was significantly \((P < 0.001)\) higher in culture media collected from explants incubated with rEq IL-1, compared with the PGE2 concentration in culture media collected from explants incubated without rEq IL-1, and the effect of rEq IL-1 on PGE2 concentration was not associated with tiludronate concentration \((P = 0.63)\). Tiludronate concentration had no effect on PGE2 concentration in the culture media \((P = 0.28)\). However, PGE2 concentration in the culture media was associated \((P < 0.001)\) with the random effect of limb and horse from which the explant was obtained.

sGAGs released from cartilage explants—The percentage of sGAG lost from cartilage explants was significantly \((P < 0.001)\) associated with rEq IL-1 and tiludronate concentration (Figure 1). In the absence of tiludronate (tiludronate concentration, 0 mg/L), explants incubated with rEq IL-1 released a higher percentage of sGAG than did control explants \((P < 0.001)\).

For the explants incubated with rEq IL-1, treatment with the lower concentrations (0.19, 1.9, 19, and 190 mg/L) of tiludronate significantly reduced the release of sGAG, compared with the release of sGAG from untreated explants \((P < 0.001)\). For the cartilage explants incubated without rEq IL-1, treatment with the higher concentrations (19, 190, and 1,900 mg/L) of tiludronate significantly increased the loss of sGAG from explants, compared with the sGAG loss from untreated explants.

Chondrocyte apoptosis—The percentage of apoptotic chondrocytes in the cartilage explants was significantly \((P < 0.001)\) associated with rEq IL-1 and tiludronate concentration \((P < 0.001)\) greater in the explants incubated with rEq IL-1, compared with the percentage of apoptotic chondrocytes in the control explants. For the explants incubated with rEq IL-1, treatment with the lower concentrations (0.19, 1.9, 19, and 190 mg/L) of tiludronate resulted in a significantly
lower percentage of apoptotic chondrocytes, compared with the percentage of apoptotic chondrocytes in explants that were not treated with tiludronate. For the explants incubated without rEq IL-1, treatment with the lowest concentration (0.19 mg/L) of tiludronate resulted in a significantly (P = 0.003) lower percentage of apoptotic chondrocytes, compared with percentage of apoptotic chondrocytes in explants that were not treated with tiludronate. Conversely, for explants incubated without rEq IL-1 that were treated with the higher concentrations (19, 190, and 1,900 mg/L) of tiludronate, the percentage of apoptotic chondrocytes was significantly higher than that for the explants that were not treated with tiludronate.

Gene expression by chondrocytes in cartilage explants—The RNA extracted from all cartilage explants was considered to be good quality (mean ± SEM RNA integrity number, 8.9 ± 0.08; range, 7 to 10). The gene expression data were characterized by high variability (Figure 3). Expression of genes MMP-1 (P < 0.001), MMP-3 (P < 0.001), and MMP-13 (P = 0.005) was significantly increased in the explants incubated with rEq IL-1, compared with the expression of those genes in the control explants. Within the explants incubated with rEq IL-1, gene expression of MMP-1 (P = 0.99) and MMP-3 (P = 0.95) did not vary significantly among the concentrations of tiludronate evaluated. Also, within the explants incubated with rEq IL-1, gene expression of MMP-13 decreased as the concentration of tiludronate increased, but this association was not significant (P = 0.49). Gene expression of IL-6 was significantly (P = 0.04) associated with rEq IL-1 and tiludronate concentration. At the lowest concentration (0.19 mg/L) of tiludronate, IL-6 gene expression in the explants incubated with rEq IL-1 was significantly higher, compared with the IL-6 gene expression in the explants incubated without rEq IL-1 as well as with the IL-6 gene expression in explants incubated with rEq IL-1 at each of the other concentrations (1.9, 19, 190, and 1,900 mg/L) of tiludronate. No other group comparisons were found to be significant. Thus, in the absence of tiludronate (0 mg/L), IL-6 gene expression did not significantly (P = 0.99) differ between explants incubated with and without rEq IL-1. Gene expression of IL-8 was significantly (P = 0.02) associated with tiludronate concentration. For explants in both the rEq IL-1 and control groups, at the highest concentration (1,900 mg/L) of tiludronate gene expression of IL-8 was significantly increased, compared with the gene expression of IL-8 at each of the other tiludronate concentrations evaluated.

Discussion

Results of the study reported here provide a better understanding of the mechanism by which tiludronate affects articular cartilage, which is the first step in determining a safe and effective intra-articular dose of tiludronate for the treatment of osteoarthritis in horses. The results indicated that tiludronate has biphasic concentration-dependent effects on cartilage explants that were not associated with PGE₂ secretion and MMP gene expression. High concentrations of tiludronate (≥ 19 mg/L) appeared to be detrimental to cartilage dependent on incubation with or without rEq IL-1, whereas lower concentrations of tiludronate appeared to have some protective effects for chondrocytes and cartilage matrix. However, in vivo studies are necessary before intra-articular administration of tiludronate to horses with osteoarthritis can be recommended or discouraged.

Addition of IL-1β to articular cartilage explants to simulate cartilage damage caused by osteoarthritis has been described, and this method allows evaluation of the articular cartilage matrix and chondrocyte apoptosis in situ. In the present study, we chose to use a lower concentration of rEq IL-1 than that used in other studies to simulate more closely the slow, gradual damage caused by osteoarthritis because investigators of another study reported that a rEq IL-1 concentration as low as 0.1 ng/mL induces near maximal proteoglycan degradation in articular cartilage explants obtained from horses. Additionally, evaluation of data obtained by our laboratory group from a pilot study that used a similar method to induce osteoarthritic changes in cartilage explants suggested that an rEq IL-1 concentration of 0.01 ng/mL would simulate the prolonged release of proteoglycans from damaged cartilage that has been described in horses that developed osteoarthritis naturally.

In the present study, incubation of cartilage explants with rEq IL-1 (0.01 ng/mL) induced chondrocyte and cartilage matrix damage, as evidenced by the significantly increased release of sGAG from cartilage matrix, apoptotic chondrocytes, and gene expressions of MMP-1, MMP-3, and MMP-13 compared with those variables for the control explants that were not treated with rEq IL-1. Incubation with rEq IL-1 did not upregulate gene expression of IL-6 and IL-8 in the present study, which was in contrast to the results of another study in which treatment of equine articular chondrocytes with recombinant human IL-1β resulted in a large upregulation of IL-6 and IL-8 gene expression. These contrasting results may be explained by the use of IL-1β proteins that were derived from different species or the higher concentration of recombinant human IL-1β (5 ng/mL) used in the other study compared with the concentration of rEq IL-1 (0.01 ng/mL).
used in the present study. However, the bioactivity of rEq IL-1 in equine articular cartilage is 40 to 100 times as great as that of recombinant human IL-1β.10

The mechanism by which tiludronate induces clinical improvement in horses with osteoarthritis is not completely understood. It has been hypothesized that after absorption into osteoclasts, bisphosphonates become incorporated into nonhydrolyzable ATP analogues, which impair the osteoclasts’ ability to perform ATP-dependent functions and result in apoptosis.2,3 That mechanism of action would explain the results of other studies,20–23 in which bisphosphonate treatment was associated with the partial reversal and prevention of further subchondral bone damage in animals with

Figure 3—Mean ± SEM fold change in gene expression of MMP-1 (A), MMP-3 (B), MMP-13 (C), IL6 (D), and IL8 (E) for chondrocytes from articular cartilage explants incubated with 1 of 6 concentrations (0, 0.19, 1.9, 19, 190, or 1,900 mg/L) of tiludronate and with (gray bars) or without (black bars) rEq IL-1 (0.01 ng/mL). Gene expression in each cartilage explant was quantified via reverse transcriptase and quantitative real-time PCR assays, and the fold change in gene expression was determined via the comparative cycle threshold method. The following formula was used to calculate the fold change in gene expression between each sample and the reference sample: fold change = (ΔCt for gene of interest in sample – ΔCt for housekeeping gene in sample) – (ΔCt for gene of interest in reference sample – ΔCt for housekeeping gene in reference sample). Glyceraldehyde 3-phosphate dehydrogenase gene was used as the housekeeping gene, and control explants that were not treated with tiludronate were used as the reference samples. Notice the y-axis scales differ among panels. See Figure 1 for remainder of key.
experimentally induced osteoarthritis. Bisphosphonate treatment has also been associated with the amelioration of cartilage damage in animals with experimentally induced osteoarthritis. The protective effect of bisphosphonates on articular cartilage may be an ancillary effect of the inhibition of osteoclast activity in subchondral bone, which maintains a more normal foundation for the articular cartilage and prevents cartilage degradation. Alternatively, or perhaps additionally, bisphosphonates may directly decrease articular cartilage degradation by inhibiting the activity of proteases that degrade proteoglycans or secretion of those proteases.

In a study in which osteoarthritis was experimentally induced in chondrocytes in vitro, tiludronate inhibited proteoglycan-degrading proteases at concentrations (0.05 to 0.5mM, which corresponds to 19 to 190 mg/L) similar to those used in the present study. Bisphosphonate treatment also has been associated with the downregulation of gene expression for proteolytic enzymes.

Results of the present study indicated that tiludronate directly affected cartilage degradation in vitro. However, the effect of tiludronate on cartilage degradation does not appear to be mediated via PGE2. In a previous study, there was a positive correlation between proteoglycan degradation in articular cartilage explants incubated with IL-1β and PGE2 concentration in the culture media, which suggested that PGE2 may modulate proteoglycan degradation. In the present study, there was no association between tiludronate concentration and PGE2 concentration in the culture media, which suggested that tiludronate had no effect on the synthesis and release of PGE2 from chondrocytes. Similarly, tiludronate did not have any effect on PGE2 synthesis in samples of whole blood and monocytes that had been stimulated with lipopolysaccharides.

In the cartilage explants of the present study, tiludronate had a biphasic concentration-dependent effect on proteoglycan degradation as determined by the percentage of gAG released from cartilage explants. Tiludronate provided protective effects (ie, decreased proteoglycan degradation, which resulted in a lower percentage of gAG lost) for cartilage matrix when administered at lower concentrations (0.19, 1.9, 19, and 190 mg/L for the explants incubated with rEq IL-1 and 0.19 mg/L for the control explants) but increased proteoglycan degradation in cartilage matrix when administered at higher concentrations (1,900 mg/L for the explants incubated with rEq IL-1 and 19, 190, and 1,900 mg/L for the explants not incubated with rEq IL-1).

Tiludronate had a similar biphasic concentration-dependent effect on chondrocyte apoptosis. When tiludronate was administered at the lower concentrations (0.19, 1.9, 19, and 190 mg/L for the explants incubated with rEq IL-1 and 0.19 mg/L for the control explants), the percentage of apoptotic chondrocytes decreased, compared with that for the explants that were not treated with tiludronate, whereas when tiludronate was administered at the higher concentrations, the percentage of apoptotic chondrocytes increased, compared with that for the explants that were not treated with tiludronate. These results are similar to those of another study in which concentrations of bisphosphonates comparable to those used in the present study inhibited dexamethasone-induced apoptosis in bovine chondrocytes.

The fact that low concentrations of tiludronate seemed to have an antiapoptotic effect for chondrocytes in the present study may seem counterintuitive given that administration of non–nitrogen-containing bisphosphonates causes apoptosis in osteoclasts by the disruption of ATP-dependent processes. However, results of other studies indicate that bisphosphonate administration decreases apoptosis in osteocytes and osteoblasts. Investigators of 1 study hypothesized that the pro- and antiapoptotic effects of bisphosphonates are concentration dependent. The results of the present study support this hypothesis, as do those of another study, in which low concentrations of a bisphosphonate prevented apoptosis in epiphyseal chondrocytes in vitro, whereas a bisphosphonate concentration 30 times greater resulted in apoptosis in osteoclasts. Whether bisphosphonates induce or prevent apoptosis appears to be also dependent on the type of cell evaluated. Cells such as osteoclasts that absorb bisphosphonates via endocytosis may have a resultant increase in intracellular bisphosphonate concentration sufficient to cause apoptosis. Conversely, bisphosphonate administration prevents apoptosis in cells such as osteocytes, osteoblasts, and hypertrophic epiphyseal chondrocytes that do not internalize bisphosphonates. Therefore, the mechanism by which bisphosphonates cause apoptosis in osteoclasts is different from the mechanism by which bisphosphonates prevent apoptosis in osteocytes and osteoblasts.

Bisphosphonates bind to connexin 43 hemichannels in the cell membranes of osteocytes and osteoblasts, which activates a prosurvival signaling pathway (Src/ERK pathway). The cell membranes of articular chondrocytes also contain connexin 43 hemichannels. Results of another study indicate that incubation with IL-1β increases expression of the connexin 43 hemichannels in articular chondrocytes in vitro. This may explain why the antiapoptotic effect of tiludronate seemed to be amplified in the explants incubated with rEq IL-1, compared with that in the control explants in the present study.

Matrix metalloproteinases are associated with cartilage damage; MMP-1 degrades collagen, MMP-3 degrades noncollagenous matrix proteins such as proteoglycans, and MMP-13 degrades collagen and proteoglycans. In the present study, tiludronate was not associated with gene expression of MMP-1, MMP-3, and MMP-13. However, the wide range of variability for the expression of each gene at each concentration of tiludronate evaluated may have prevented an association from being detected.

Interleukin-6 has a proinflammatory role in the development of osteoarthritis via modulation of MMP expression. On the basis of results of a previous study, it was expected that tiludronate also would have no association with IL-6 expression. We believe that the significant increase in IL-6 gene expression detected in the explants incubated with rEq IL-1 that were treated with 0.19 mg/L of tiludronate was caused by 2 aberrantly high measurements, although no irregularities were detected during the processing of those 2 samples.
and no amplification of IL-6 was detected in the negative controls for the batch in which those 2 samples were assayed. Therefore, we subjectively concluded that tiludronate had no effect on IL-6 gene expression, a conclusion similar to that made by investigators of another study.11

Interleukin-8 is a potent neutrophil-attracting chemokine that plays an important role in acute inflammatory reactions in joints.11 In the present study, gene expression of IL-8 was significantly increased at the highest concentration (1,900 mg/L) of tiludronate, compared with that for each of the other concentrations of tiludronate evaluated for cartilage explants in both the rEq IL-1 and control groups. These results differ from those of another study,12 in which bisphosphonate administration had no effect on IL-8 gene expression. However, in that study,12 various other bisphosphonates (clodronate, pamidronate, and risedronate) were evaluated at lower concentrations than the concentrations of tiludronate used in the present study. For the study reported here, tiludronate at a concentration of 1,900 mg/L may have caused an acute inflammatory response in the chondrocytes, which resulted in upregulation in the expression of IL-8.

In the present study, gene expression data were characterized by high variability both between and within horses. The diverse genetic backgrounds of the 3 study horses may explain the variability between horses, whereas the variability between samples obtained from 2 joints within the same horse is more difficult to deduce. Grossly, none of the 6 joints had evidence of arthritis, such as cartilage fibrillation, score lines, or synovial hyperemia, hyperplasia, or fibrosis. However, the fact there was a significant association between PGE2 concentration and limb nested within horse may suggest that the joints from which the cartilage explants were harvested had various stages of subclinical arthritis.

Future studies with a similar design as that of the study reported here should be conducted with a larger sample size to decrease the amount of statistical variability and increase the power to detect relevant associations. Also, prior to study initiation, measurement of PGE2 concentration or concentrations of other proinflammatory mediators in the synovial fluid is suggested so that joints can be classified in accordance with prespecified cutoffs for proinflammatory mediators. This type of joint classification may allow for the enrollment of a more homogeneous group of cartilage explants into a study, which also should help ameliorate variability among samples.

Extrapolation of the results of the present study for in vivo application to horses with osteoarthritis warrants caution. It is unknown whether the tiludronate concentration in culture media is comparable to the tiludronate concentration in synovial fluid that is achieved after intra-articular administration. Tiludronate has a high affinity for calcium ions; therefore, after intra-articular administration, it is likely some tiludronate will be incorporated into the more calcium-rich subchondral bone and calcified cartilage, which will decrease the amount of tiludronate available to articular chondrocytes.

Consideration should also be given to the effect that route of administration has on tiludronate concentration in synovial fluid. In Europe, tiludronate is approved for the treatment of osteoarthritis of the distal intertarsal and tarsometatarsal joints in horses at a dose of 1 mg/kg, IV; however, the concentration of tiludronate in synovial fluid achieved after IV administration has not been determined. In our opinion, the concentration of tiludronate in synovial fluid after IV administration of 1 mg/kg of tiludronate will be much lower than the peak plasma tiludronate concentration, which is approximately 9 mg/L. It is possible that IV administration of tiludronate (1 mg/kg) is insufficient to achieve a clinically relevant concentration of tiludronate in the synovial fluid, although results of the present study indicated that a tiludronate concentration as low as 1.9 mg/L may ameliorate articular chondrocyte apoptosis and the release of proteoglycans into arthritic joints, thereby improving joint health. Anecdotally, veterinarians have administered tiludronate to horses via intra-articular injection and regional limb perfusion. On the basis of anecdotal reports of 50 mg of tiludronate administered intra-articularly into joints with a synovial fluid volume of 25 to 30 mL, a tiludronate concentration in the synovial fluid ranging from 1,666 to 2,000 mg/L should be expected. The concentration of tiludronate in the synovial fluid following regional limb perfusion of a similar (50 mg) amount of tiludronate is unknown. However, synovial fluid concentrations of antimicrobials achieved after regional limb perfusion are very high,12 and we assume that the distribution of tiludronate after regional limb perfusion would follow a similar pattern. Results of the present study indicate that exposure of osteoarthritic cartilage to concentrations of tiludronate ≥ 1.900 mg/L and normal articular cartilage to concentrations of ≥ 19 mg/L may be detrimental to joint health. Thus, the anecdotal dose of tiludronate that is currently administered via intra-articular injection or regional limb perfusion may be causing more harm than good to horses, and further investigation is necessary.

Current strategies for the management of horses with osteoarthritis focus on decreasing the severity of pain, improving joint function, and minimizing continued deterioration of joint tissues.9 Results of the present study indicated that concentrations of tiludronate ≤ 1.9 mg/L decreased proteoglycan degradation and chondrocyte apoptosis in articular cartilage independent of PGE2 production and MMP gene expression. Conversely, higher tiludronate concentrations (≥ 19 mg/L for explants representing healthy cartilage and ≥ 1,900 mg/L for explants representing osteoarthritic cartilage) increased proteoglycan degradation and chondrocyte apoptosis in articular cartilage. On the basis of these results, we consider a tiludronate concentration in synovial fluid between 0.19 and 1.9 mg/L to be safe and perhaps beneficial to joint health. A tiludronate concentration in synovial fluid ≥ 19 mg/L may be detrimental to articular cartilage, and this threshold is likely exceeded by the intra-articular administration of 50 mg of tiludronate (the dose that veterinarians have anecdotally administered intra-articularly to horses). In vivo studies are necessary before intra-articular administration of tiludronate to horses for the treatment of osteoarthritis can be recommended.
a. Beuthanasia, Schering-Plough Animal Health Corp, Union, NJ.
b. R&D Systems, Minneapolis, Minn.
c. Tilden, CEVA, Libourne, France.
d. PGE, EIA Kit, Monoclonal, Cayman Chemical, Ann Arbor, Mich.
e. nuc: Optical Bottom Plate nontreated, Thermo Fisher Scientific, Rochester, NY.
g. TaqMan assays, Real-time PCR Research and Diagnostics Core Facility, School of Veterinary Medicine, University of California-Davis, Davis, Calif.
h. Real-time PCR Research and Diagnostics Core Facility, School of Veterinary Medicine, University of California-Davis, Davis, Calif.
i. TRizol Reagent, Invitrogen Life Technologies, Carlsbad, Calif.
j. RNase-free DNase, Qiagen, Valencia, Calif.
k. RNase-free DNase, Qiagen, Valencia, Calif.
l. Tildren, CEVA, Libourne, France.
m. 2100 Expert software, Agilent Technologies, Palo Alto, Calif.

References


Appendix

Nucleotide sequences used in PCR assays to quantitatively determine gene expression of MMP-1, MMP-3, MMP-13, IL-6, and IL-8 in articular cartilage explants that were obtained from the femorotibial joints of 3 young adult horses and incubated with 1 of 6 concentrations (0, 0.19, 1.9, 19, 190, or 1,900 mg/L) of tiludronate and with or without rEq IL-1 (0.01 ng/mL) for 96 hours.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession No.</th>
<th>Forward primer</th>
<th>Probe</th>
<th>Reverse primer</th>
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<td>GAPDH</td>
<td>AF097179</td>
<td>AAGTG6AT7TGGTCGCCATC</td>
<td>AATGCTCCAACATCATGGTCACTATGTTCA</td>
<td>AACTG6C6ATG6GTGGAATC</td>
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<tr>
<td>IL-6</td>
<td>AF005227</td>
<td>AGGCTTGGTAAGGAGATGTTGACAA</td>
<td>ATTCAGGATCAGTTCTGTGTGGTCTA</td>
<td>CCAACTGTTAAGCTGGTC</td>
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<tr>
<td>MMP-3</td>
<td>AF18882</td>
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<tr>
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<td>IL-8</td>
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<td>TCAAGG6GTAA</td>
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</tr>
</tbody>
</table>

— = Not reported because sequences are proprietary. GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.