Treatment with Tiludronic Acid Helps Reduce the Development of Experimental Osteoarthritis Lesions in Dogs with Anterior Cruciate Ligament Transection Followed by Reconstructive Surgery: A 1-Year Study with Quantitative Magnetic Resonance Imaging

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ABSTRACT. ***Objective.*** To investigate over a 1-year period in dogs that underwent extracapsular stabilization surgery (ECS) following anterior cruciate ligament (ACL) transection: whether reconstructive surgery could prevent osteoarthritis (OA) progression and whether treatment with the bisphosphonate tiludronic acid (TA) could improve the chronic evolution of OA structural changes.

Methods. ACL transection was performed on dogs on Day 0 and ECS on Day 28. Dogs were randomly divided into 2 groups: 15 received placebo and 16 were treated with TA (2 mg/kg subcutaneous injection) on Days 14, 28, 56, and 84. Magnetic resonance images were acquired on Days –10, 26, 91, 210, and 357, and cartilage volume was quantified. At sacrifice (Day 364), cartilage from femoral condyles and tibial plateaus was macroscopically and histologically evaluated. Expression levels of MMP-1, -3, -13, ADAMTS-4, -5, BMP-2, FGF-2, IGF-1, TGF-ß1, collagen type II, and aggrecan were determined using real-time RT-PCR.

Results. The loss of cartilage volume observed after ACL transection stabilized following ECS. Thereafter, a gradual gain occurred, with the cartilage volume loss on the tibial plateaus reduced at Day 91 ($p < 0.02$) and Day 210 ($p < 0.001$) in the TA-treated dogs. At sacrifice, TA-treated dogs presented a reduction in the severity of macroscopic ($p = 0.03$ for plateaus) and histologic ($p = 0.07$ for plateaus) cartilage lesions, had a better preserved collagen network, and showed decreased MMP-13 ($p = 0.04$), MMP-1 and MMP-3 levels.

Conclusion. Our findings indicate that in dogs with ACL transection, ECS greatly prevents development of cartilage volume loss. Treatment with TA provided an additional benefit of reducing the development of OA lesions. (First Release Oct 15 2010; J Rheumatol 2011;38:118–28; doi:10.3899/jrheum.100642)

Key Indexing Terms:
EXPERIMENTAL OSTEOARTHRITIS — TILUDRONIC ACID
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Osteoarthritis (OA), the most common musculoskeletal disorder, affects the majority of people in the second half of their lifespan. The disease can induce a significant level of morbidity in patients and represents an increasing burden from a medical, social, and economic viewpoint. A number of risk factors that can induce or accelerate the progression

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of OA have been identified[1]. These include aging, genetics, high body mass index, joint injury, malalignment, and meniscal lesion/extrusion in the knee.

In humans, the development of joint structural changes in OA occurs over an extended period, making it very difficult to study the etiopathogenesis of the disease. Hence, experimental models that reproduce the disease alterations can be useful, especially for studying these changes over time with the possibility of sequential evaluation of the disease lesions. The majority of OA animal models have been the subject of very comprehensive reviews[2,3,4,5]. Among these, the dog anterior cruciate ligament (ACL) model has been extensively studied from a morphological and pathophysiological point of view and has demonstrated similarities to the human disease[5]. The traumatic rupture of the ACL is a frequent musculoskeletal pathology, both in dogs and in humans, and has been demonstrated to be a significant risk factor for the development of knee OA in many species[1,3,4,5,6].

Pond and Nuki were the first to introduce this dog model, created by experimentally transecting the ACL in normal dogs using a closed surgical procedure (stab incision)[7]. A variant of this model involves an open surgical procedure[8]. The histologic and biochemical changes in cartilage were reported to be analogous to those observed in the naturally occurring disorder[9], and include an increased level of proteoglycan and collagen type II synthesis with a breakdown of the collagen network and reduced aggrecan size[10,11]. Moreover, some proteases that degrade the extracellular matrix macromolecules were also found to be induced: matrix metalloproteases (MMP)-1, MMP-3 and MMP-13, cathepsin K, and a disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMTS)-4 and ADAMTS-5[12].

Progression of the disease over time has also been well documented in this model. Hence, the cartilage changes studied by different imaging technologies were found to evolve over a long period, and Brandt, *et al*[13] reported progression up to 4 years after the surgery. Recent studies using quantitative magnetic resonance imaging (MRI), in which progressive erosion and loss of cartilage were documented up to 48 weeks following the surgery, are also supportive of the previous findings[14,15]. The dog models also allow evaluation of the effect of disease-modifying OA drug (DMOAD) interventions on joint structure and disease pathways, and, in particular, the ACL model has been used to test numerous drugs, agents, and interventions that have the potential of modifying the progression of OA[2,4,16]. In this model, an MRI study showed, in addition to cartilage loss, the presence of bone marrow lesions (hypersignal)[17]. Interestingly, these were found to be topographically associated with cartilage lesions, suggesting that the changes in subchondral bone play a role in the genesis of cartilage lesions[14,17].

Moreover, the morphological changes that take place at the subchondral bone level in the early stages of the disease are predominantly resorptive[13,17,18]. Therefore, in choosing the subchondral bone as a therapeutic target, the selection of an antiresorptive compound is quite logical. In this line of thought, a study[19] conducted in the ACL dog model using NE-10035, a bisphosphonate administered via subcutaneous (SC) injection, showed that throughout the 3 months following surgery, the prophylactic treatment effectively reduced the turnover and resorption of the subchondral bone in the OA joint. However, treatment with this compound had no effect on osteophyte formation or the severity of cartilage changes. Other bisphosphonates have been studied as a strategy to reduce OA disease progression. One of them, tiludronic acid (TA), was shown in a pilot study in the ACL model[20] to be an interesting candidate. Tiludronate, a non-nitrogen-containing bisphosphonate, particularly indicated in the treatment of Paget's disease and osteoporosis, was shown to inhibit osteoclast-mediated bone resorption[21], to disrupt cytoskeleton resorption of osteoclasts[22] and inhibit activity of these cells on the proton pump[23], and to lead to osteoclast apoptosis[21,24].

In dogs that had spontaneous injury of the ACL, studies demonstrated that the stabilization of the knee by surgical procedure such as extracapsular stabilization (ECS) may positively alter the progression of joint functional changes[25,26]. Although the literature remains inconclusive about ACL repair and OA progression in both dogs and humans, a recent report[27] indicated that ECS can provide a positive clinical outcome at 6 months in stifle joints, with a reduction in progression to gonarthrosis in the majority of dogs.

The aim of our study was 2-fold. First, we wanted to determine, in dogs that underwent reconstructive ECS surgery following ACL transection, if ECS could slow or prevent OA progression; and second, to evaluate whether treatment with the bisphosphonate TA could, over a period of 1 year, improve the evolution of OA structural changes. The effects of treatment with TA on cartilage structural changes and metabolism were studied.

MATERIALS AND METHODS

Experimental groups and protocol. Thirty-one mature adult crossbred dogs (1–4 years old), each weighing 25 ± 5 kg, were used in this study. They were housed in a large kennel in individual galvanized-steel cages (1 m width × 1.75 m length × 2.4 m height), each separated by a panel. All cages were equipped with an automatic watering system. Dogs were selected following complete physical and musculoskeletal evaluation by a veterinarian. Clinical chemistry analysis and radiological examination of the right knee were performed before inclusion in the study to exclude any underlying pathology. The experimental timeline of the study is summarized in Figure 1. The study protocol was approved by the Institutional Ethics Committee and conducted according to the Institutional Animal Use and Care Committee and the Canadian Council on Animal Care regulations.

Surgical procedures; anesthesia. After acclimatizing for 4 weeks in the animal care facility, surgical sectioning of the ACL (Day 0) of the right knee was performed on all dogs, as described[28]. In brief, sectioning of the ACL

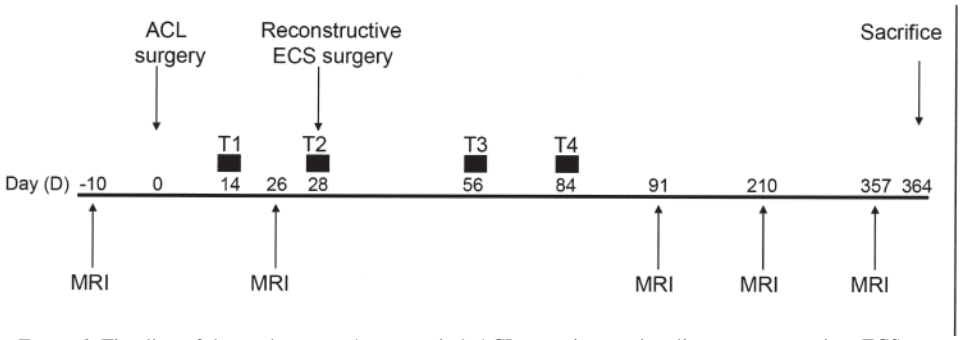

Figure 1. Timeline of the study over a 1-year period. ACL: anterior cruciate ligament transection; ECS: extracapsular stabilization; MRI: magnetic resonance imaging; T: treatment.

after joint capsule opening was done under general anesthesia with propofol (4 mg/kg given to effect, intravenous injection) and 2% isoflurane gas in oxygen, following intramuscular (IM) premedication (meperidine-acepromazine-glycopyrrolate). A multimodal and preemptive opioid-based analgesic protocol was initiated 18 h before surgery (transdermal fentanyl patch, Duragesic® 75 μg/h; Janssen-Ortho, Markham, ON, Canada), which also included an intraarticular block (5–8 ml bupivacaine, Marcaine® 0.5%; Hospira Inc., Lake Forest, IL, USA). During the postoperative period, if needed, dogs were treated with fentanyl patch, oxymorphone (0.1 mg/kg IM or SC injection; Sandoz, Boucherville, QC, Canada) or meperidine (4 mg/kg IM or SC injection; Sandoz), repeated as necessary.

The third day following the ACL surgery, the dogs were given free access to exercise in a large enclosure. They exercised and socialized in exterior runs (1.35 m width × 9.15 m length) for a 2 h period, 5 days a week under the supervision of an animal care technician.

Reconstructive ECS surgery. Twenty-eight days after ACL sectioning (Day 28), reconstructive surgery by ECS[26] was performed on the right knee under general anesthesia and analgesia, as described above. The subcutaneous tissues were dissected to the lateral retinaculum, which was entered via a stab incision. The lateral retinaculum was dissected from the lateral joint capsule. A 2 cm portion of the cranialis tibialis muscle was elevated from the proximal aspect of the tibia centered over the most prominent aspect of the tibial tuberosity. A hole was created in the tuberosity using a handchuck and a 5/32" Steinman pin. A double-strand monofilament nylon was passed around the lateral fabella then passed through the hole in the tibial tuberosity and under the patellar ligament. Both strands were tied independently using a sliding half-hitch. The lateral retinaculum was imbricated in a vest-over-pants pattern. The subcutaneous tissues were closed, then the skin. Following the surgery, the dogs had restricted exercise for a 20–30 min period twice a day from Week 5 to 16 and thereafter for 2 h 5 days a week until the end of the study.

Treatment. The OA dogs were randomly divided into 2 treatment groups, to which the animal care personnel were blinded. One group (n = 16) received 2 mg/kg TA (500 mg TA disodium salt as the active ingredient and 250 mg mannitol; CEVA Santé Animale, Libourne, France) administered by SC injection (0.2 ml/kg) in the interscapular space on Days 14, 28, 56, and 84 (Figure 1). The control group (n = 15) received a placebo consisting of 250 mg mannitol (CEVA Santé Animale) administered as above. The drug dosage was selected based on results from a pilot study[20].

MRI; sequence acquisition. MRI examinations of the right knee were performed before surgery (Day –10) and after ACL surgery at Days 26, 91, 210, and 357 with a 1.5 Tesla magnet (Signa HDx; General Electric Healthcare, Milwaukee, WI, USA) using a knee coil, as described[14]. All examinations were standardized by using a dedicated device allowing the dogs to be placed in supine position with the right leg fully extended and immobilized with custom-made cushions to guarantee consistent position within the imaging coil.

MRI acquisition was performed under general volatile anesthesia using isoflurane (2%–2.5%; Abbott, Chicago, IL, USA) mixed with oxygen (20–40 ml/kg/min). The dogs were appropriately premedicated with administration of buprenorphine (0.01 mg/kg IV), followed by induction with propofol (4 mg/kg IV). They were orotracheally intubated and hydrated with physiologic saline for the duration of the acquisition. The dogs were monitored under the supervision of a veterinarian.

The operated (right) knee was imaged with a sagittal 3D SPGR sequence with fat saturation (TR 42 ms, TE 6.6 ms, flip angle 20°, slice thickness/gap 1/0 mm, matrix size 384 × 384, field of view 10 cm, for a voxel dimension of 0.26 × 0.26 × 1.00 mm^3). This method of acquisition has been validated for cartilage volume measurement[14,29,30].

Cartilage volume determination. Cartilage volume quantification was performed by 2 trained and blinded readers on the 3D SPGR images using a Cartiscope™ (ArthroVision Inc., Montreal, QC, Canada) as described[14,29,30]. Cartilage volume (mm^3) was assessed for the medial and lateral femoral condyle and the medial and lateral tibial plateau subregions. Between- and within-reader agreement of measurements was previously shown to be excellent[31]. Moreover, in addition to cartilage volume determination, the Cartiscope technology computes, as intermediate results, cartilage thickness/volume maps that provide the thickness/volume of each observation timepoint, enabling calculation of the percentage of cartilage loss or gain (separately) between baseline and followup visits[30].

Macroscopic measurement. At Day 364 the animals were sacrificed and the operated knee was dissected. The femoral condyle and the tibial plateau were separated. The extent of cartilage tissue damage was evaluated macroscopically on site by 2 independent observers blinded to the treatment groups. Cartilage lesion sizes of the condyles and plateaus for the medial and lateral regions were measured using a digital caliper according to a predefined procedure comprising surface (mm^2) and depth of erosion (grade 0–4)[32]. The latter was as follows: grade 0 = normal surface; 1 = minimal fibrillation or a slight yellowish discoloration of the surface; 2 = erosion extending into superficial or middle layers only; 3 = erosion extending into deep layers; and 4 = erosion extending to subchondral bone. The score was the sum of medial and lateral regions for the femoral condyles or tibial plateaus. An overall macroscopic score was obtained by analysis of surface × depth as described[32].

Histology. Sagittal sections of articular cartilage from the weight-bearing areas of each femoral condyle and tibial plateau were dissected as recommended by the OARSI guidelines[33]. Specimens were then fixed (TissuFix #2; Chaptec, Montreal, QC, Canada), embedded in paraffin, sectioned (5 μm thick), and stained with Fast Green/Safranin-O and hematoxylin.

All slides were randomly graded by 2 observers blinded to the treatment groups. Three sections per slide were evaluated using a scale slightly modified from Sakakibara, *et al*[34]. In brief, grade 0–4 = severity of the loss of Safranin-O staining; 0–2 = cellularity on tangential zone; 0–10 = cellularity on transitional and radial zone; 0–10 = structural changes (where 0 = normal cartilage structure and 10 = complete disorganization), and 0–3 = pannus. A total score of 87 corresponds to the sum of all scores from the 3 sections.

Each section of the articular cartilage was also processed to study the collagen infrastructure using the Picrosirius-polarization method[35,36]. Sections of the condyles and plateaus were stained with the Picrosirius and the loss of cartilage integrity ranked on a scale of 0–4 as follows: 0 = normal architecture; or loss of integrity in 1 = superficial zone; 2 = superficial and middle layers; 3 = down to the deep layer; and 4 = throughout the entire thickness.

Synovial membrane was removed and processed for histological analysis. Samples were stained with hematoxylin-phloxine-saffron. Severity of synovitis was graded on a scale of 0–10[28] by 2 blinded and independent observers (consensus score), who added the scores of histologic criteria: synovial cell hyperplasia (scale 0–2), villous hyperplasia (scale 0–3), and mononuclear (scale 0–4) and polymorphonuclear (scale 0–1) cell infiltration; 0 indicates normal structure.

Real-time polymerase chain reaction (PCR). Total RNA was extracted from cartilage samples using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) followed by RNeasy® Plant Mini Kit purification (Qiagen, Valencia, CA, USA). The RNA was then processed as described[37] and PCR performed with the Rotor-Gene RG-3000 (Qiagen) using the QuantiTect SYBR Green® PCR kit (Qiagen) following the manufacturer's specifications. Gene quantification was performed using primers for ADAMTS-4, -5, MMP-1, -3, -13, bone morphogenetic protein-2 (BMP-2), fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1), transforming growth factor-ß1 (TGF-ß1), collagen type IIα 1, and aggrecan (Table 1). The primer efficiency for the target gene was the same as for the housekeeping gene GAPDH. Plasmid DNA containing the gene of interest was used to generate a standard curve. The data were given as a threshold cycle (CT) and calculated as the ratio of the number of molecules of the target gene to GAPDH and expressed as arbitrary units. Reactions were performed in duplicate.

Statistical analysis. Data are presented as mean values ± SEM and analyses carried out using GraphPad Prism 5. The Mann-Whitney 2 tailed U test was used to evaluate significance between the placebo and TA samples, with significance set at $p \leq 0.05$.

Table 1. PCR primers.

ADAMTS-4	Sense	5'-TAC TAC TAT GTG CTG GAG CC-3'
	Anti-sense	5'-AGT GAC CAC ATT GTT GTA TCC-3'
ADAMTS-5	Sense	5'-GTC GGG ACC ATA TGT TCT C-3'
	Anti-sense	5'-TGA TGG TGG CTG AAG TAC AC-3'
MMP-1	Sense	5'-AAG ACA GGT TCT ACA TGC GC-3'
	Anti-sense	5'-AGT CAG CTG CTA TCA TCT GG-3'
MMP-3	Sense	5'-CAT CCA GTC CCT GTA TGG TG-3'
	Anti-sense	5'-TTC CTC TCA TGG CCC AGA AC-3'
MMP-13	Sense	5'-TTG GTC AGA TGT GAC ACC TC-3'
	Anti-sense	5'-ATC GGG AAG CAT AAA GTG GC-3'
BMP-2	Sense	5'-TGT ACG TGG ACT TCA GTG AC-3'
	Anti-sense	5'-CCA CAA CCA TGT CCT GAT AG-3'
FGF-2	Sense	5'-CTT CAA GGA CCC CAA GCG G-3'
	Anti-sense	5'-CCC AGT TCG TTT CAG TGC C-3'
IGF-1	Sense	5'-ATC TCT TCT ACC TGG CAC TG-3'
	Anti-sense	5'-TCC TGC ACT CCC TCT ACT TG-3'
TGF-ß1	Sense	5'-TTG ATG TCA CTG GAG TCG TG-3'
	Anti-sense	5'-CGG AAG TCA ATG TAG AGC TG-3'
Collagen type II α1	Sense	5'-ATC TGC TCA ACT GAC CTC G-3'
	Anti-sense	5'-GAT TTC CAG GGG TCC CAG-3'
Aggrecan	Sense	5'-ACA GTC ACA CCT GAG CAG C-3'
	Anti-sense	5'- ATT GCA AGG CAC GTC ATT C-3'
GAPDH	Sense	5'-AGG CTG TGG GCA AGG TCA TC-3'
	Anti-sense	5'- AAG GTG GAA GAG TGG GTG TC-3'

RESULTS

Characteristics of experimental animals. The body weight of the dogs remained stable and there were no signs of drug toxicity during the study period. A transient local inflammatory reaction at the site of SC injections was observed in a few dogs in both groups.

Macroscopy. At the time of sacrifice, clinical examination showed the operated knee to be stable in both experimental groups. The cartilage lesions were found mainly in the weight-bearing areas, but also in the non-weight-bearing areas, on femoral condyles and tibial plateaus. In dogs treated with TA, the macroscopic scores on the tibial plateaus (180.7 ± 15.1) and femoral condyles (165.3 ± 16.5) were lower than those observed in the placebo group (228.4 ± 16.8, $p = 0.03$; 217.8 ± 25.0, $p = 0.1$ for trend, respectively). These differences were mainly related to a reduction in the size (surface) of lesions in the TA group.

Histology. The cartilage lesions on the condyles and plateaus were in general of mild to moderately severe grade (Table 2). Treatment with TA reduced the severity of lesions on the plateaus and condyles, but although showing a trend, this did not reach statistical significance ($p = 0.07$, $p = 0.1$, respectively). The TA effect was mainly related to a significant reduction in the loss of Safranin-O staining ($p = 0.002$), and pannus ($p = 0.003$) transitional cell scores on the tibial plateaus, and loss of Safranin-O staining ($p = 0.004$) on the condyles.

The effect in the collagen network disruption observed in the Picrosirius staining in the TA group was significantly reduced compared to placebo on the femoral condyles ($p = 0.01$), with a trend on the tibial plateaus ($p = 0.08$; Figure 2A). In addition, there was a decrease in number of non-aligned collagen fibers in the cartilage of dogs treated with TA compared to placebo (Figure 2B).

The synovial membranes from the placebo-treated dogs exhibited hyperplasia of the lining cells, villous hyperplasia, and mononuclear cellular infiltration. TA treatment induced a significant reduction ($p = 0.05$) in the total histological score (6.40 ± 0.31 for the placebo group and 5.38 ± 0.41 for the TA-treated group).

Polymerase chain reaction. The gene expression level of MMP-13 was significantly reduced ($p = 0.04$) in the dogs treated with TA compared to the placebo-treated group (Figure 3). TA treatment also decreased the levels of MMP-1 and MMP-3, but the difference did not reach statistical significance (Figure 3). However, no treatment-related difference was obtained with ADAMTS-4, -5, BMP-2, FGF-2, IGF-1, TGF-ß1, collagen type IIα 1, or aggrecan expression (data not shown).

Cartilage volume. Cartilage volume measurement as assessed by MRI showed that at Day 26, there was a loss (from baseline) of cartilage volume (5%–10%) observed in both the femoral condyles and tibial plateaus and for both placebo and TA (Table 3). There was no statistical difference between the 2 groups.

Table 2. Cartilage histological score in osteoarthritic dogs. Cartilage specimens of femoral condyles and tibial plateaus were processed and analyzed as described in Materials and Methods. Scores are expressed as mean ± SEM. Statistical analysis by Mann-Whitney test. p value compared to placebo.

	Treatment Group	Structure (0–30)	Tangential Cells (0–6)	Transitional Cells (0–30)	Safranin-O Staining (0–12)	Pannus (0–9)	Total (0–87)
Femoral condyles	Placebo	17.0 ± 0.8	4.8 ± 0.2	16.0 ± 0.6	5.7 ± 0.3	0.5 ± 0.2	44.0 ± 1.7
	Tiludronic acid	15.6 ± 0.8	4.5 ± 0.2	15.0 ± 0.5	4.3 ± 0.3 p = 0.004	0.3 ± 0.1	39.7 ± 1.5
Tibial plateaus	Placebo	18.1 ± 0.7	4.9 ± 0.2	16.0 ± 0.5	6.1 ± 0.3	3.0 ± 0.3	48.2 ± 1.5
	Tiludronic acid	17.0 ± 0.7	5.0 ± 0.2	14.8 ± 0.4 p = 0.07	4.8 ± 0.3 p = 0.002	2.0 ± 0.2 p = 0.003	43.5 ± 1.4 p = 0.07

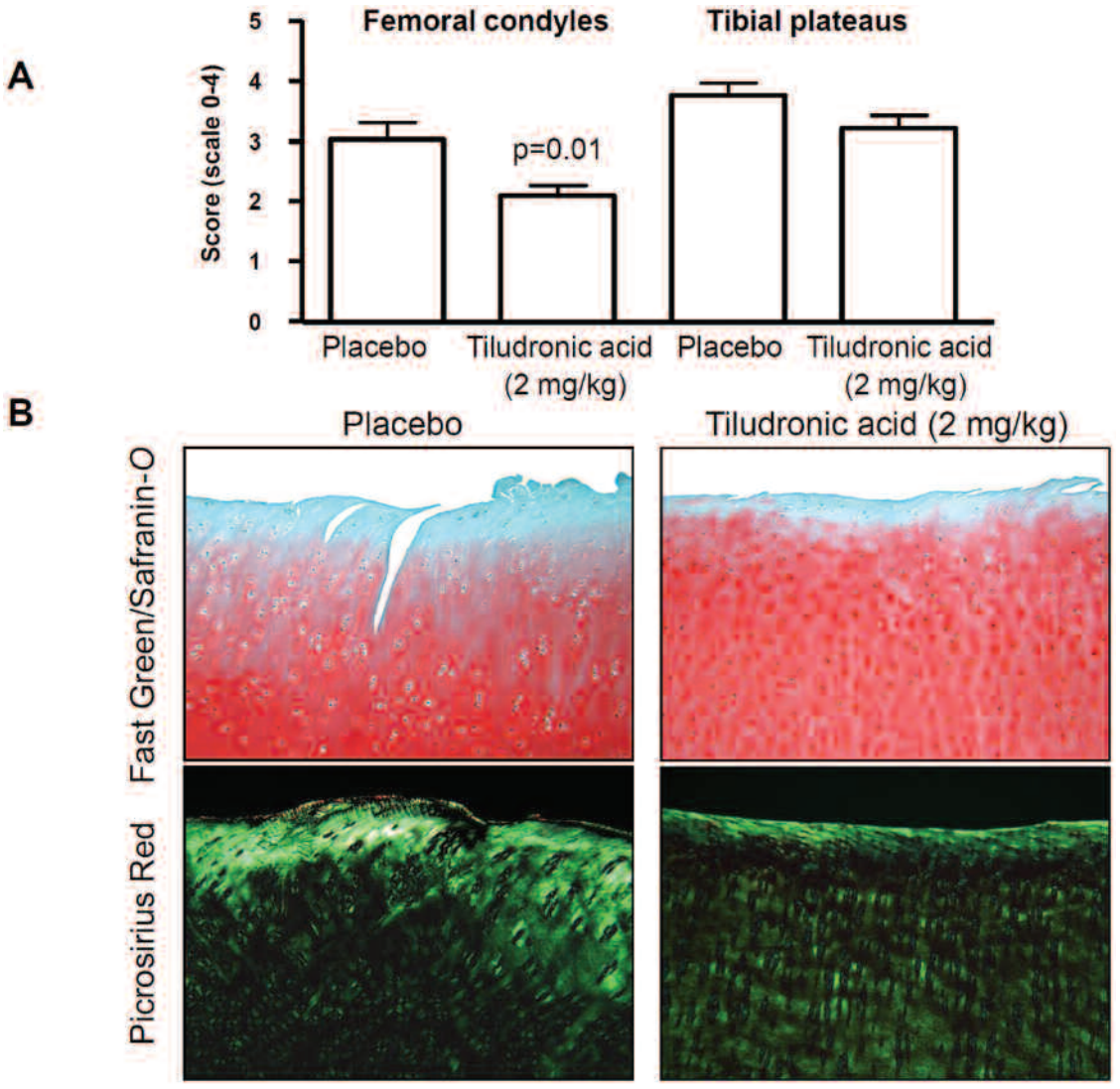

Figure 2. Histogram and representative histological sections of OA cartilage from placebo and tiludronic acid-treated dogs at Day 364 postsurgery. A. Data are expressed as mean ± SEM of the Picrosirius red staining score. Statistical analysis by Mann-Whitney 2-tailed U test; p value is compared to placebo. B. Sections were stained with Fast Green/Safranin-O (upper panels) or Picrosirius red (polarization; lower panels). Original magnification ×100.

After the ECS surgery (on Day 28), there was a gradual increase over time in the total cartilage volume until the end of the experiment in both experimental groups. This was found to be more pronounced in the femoral condyles than in the tibial plateaus. However, for both condyles and plateaus, similar findings were observed in the medial and

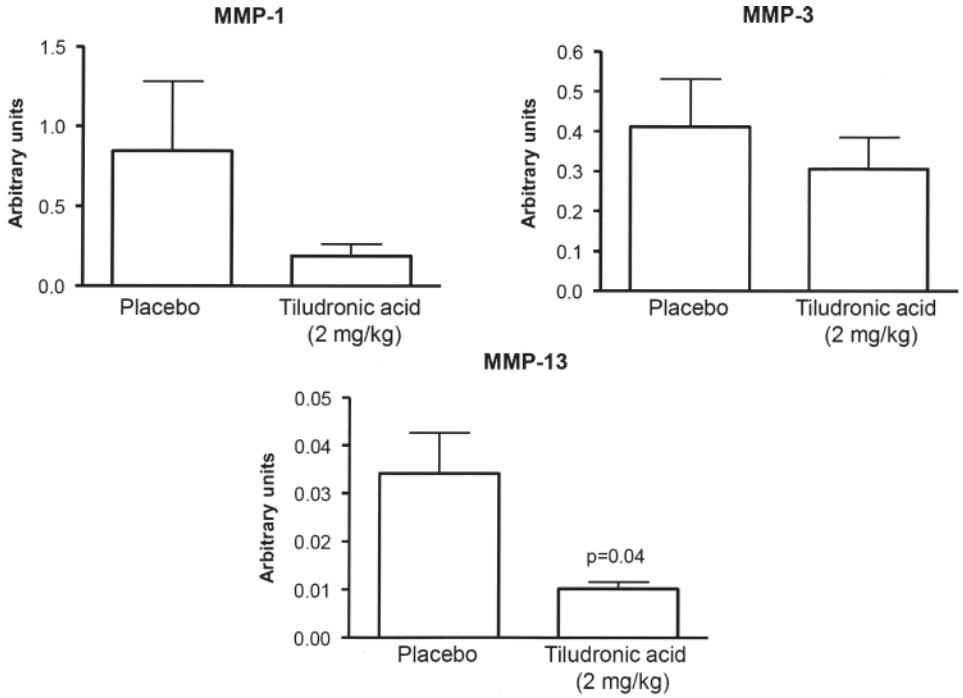

Figure 3. Expression of MMP-1, MMP-3, and MMP-13 in OA cartilage from placebo and tiludronic acid-treated dogs at Day 364 postsurgery. Data are mean ± SEM of the ratio of the number of molecules of the target gene over the GAPDH. Statistical analysis by Mann-Whitney 2-tailed U test; p value is versus placebo.

Table 3. Cange in cartilage volume in osteoarthritic dogs. Change in cartilage volume expressed as percentage from baseline was assessed as described in Materials and Methods. Data are mean ± SEM. Minus (–) indicates a decrease in cartilage volume.

	Medial		Lateral		Medial and Lateral	
	Placebo	Tiludronic Acid	Placebo	Tiludronic Acid	Placebo	Tiludronic Acid
Femoral condyles						
Day 26	–9.2 ± 3.3	–4.1 ± 4.7	–0.4 ± 3.1	–2.0 ± 1.8	–5.5 ± 1.8	–3.4 ± 3.1
Day 91	8.2 ± 5.9	19.1 ± 7.7	12.7 ± 4.9	15.3 ± 4.0	9.8 ± 4.8	16.9 ± 5.5
Day 210	44.5 ± 8.1	57.8 ± 12.6	43.1 ± 6.0	52.5 ± 6.4	42.6 ± 5.9	54.8 ± 9.1
Day 364	53.8 ± 10.1	61.7 ± 14.5	60.4 ± 8.4	64.1 ± 7.4	55.7 ± 8.6	62.2 ± 10.5
Tibial plateaus						
Day 26	–4.2 ± 2.6	–7.5 ± 2.0	–4.6 ± 2.1	–3.8 ± 2.5	–4.4 ± 2.1	–5.3 ± 2.2
Day 91	–0.8 ± 4.2	5.3 ± 3.6	–4.3 ± 3.3	1.7 ± 2.5	–2.9 ± 3.2	3.2 ± 2.7
Day 210	9.8 ± 5.0	14.5 ± 4.5	12.6 ± 3.9	11.7 ± 3.3	11.5 ± 3.3	12.9 ± 3.5
Day 364	13.8 ± 6.3	18.8 ± 4.9	12.6 ± 5.4	17.0 ± 4.2	13.1 ± 5.5	17.9 ± 4.0

lateral sides. Although the increase was slightly more pronounced in the group treated with TA (Table 3), there was no statistical difference between the 2 groups.

The 3D reconstruction map permits separation of the loss and gain in cartilage volume changes as well as identification of the areas of volume change. Indeed, our system allowed a 2D reconstruction of the cartilage, represented as colored images with a scale indicating the loss (Figure 4) or gain (Figure 5) of cartilage volume versus baseline, which was attributed a value of 0. Data showed that the cartilage volume loss was mainly in the central and medial regions of the tibial plateaus (Figure 4A, 4B) and the anterior and central areas of the condyles, as described[14]. Figure 4C-4E illustrates the percentage loss of cartilage volume comparing placebo to TA. There was a rapid loss of cartilage volume in both experimental groups until Day 26. After the ECS surgery, the placebo group demonstrated a decrease in cartilage volume with more or less stabilization in the cartilage volume loss at Day 91. In contrast to the placebo group, the TA-treated dogs had a slight but statistically significant increase in cartilage volume on the lateral tibial plateaus. The same trend was found for the femoral condyles; however, the differences in cartilage volume loss over time between the 2 groups did not reach statistical significance (data not shown).

Areas of cartilage volume gain were also observed in

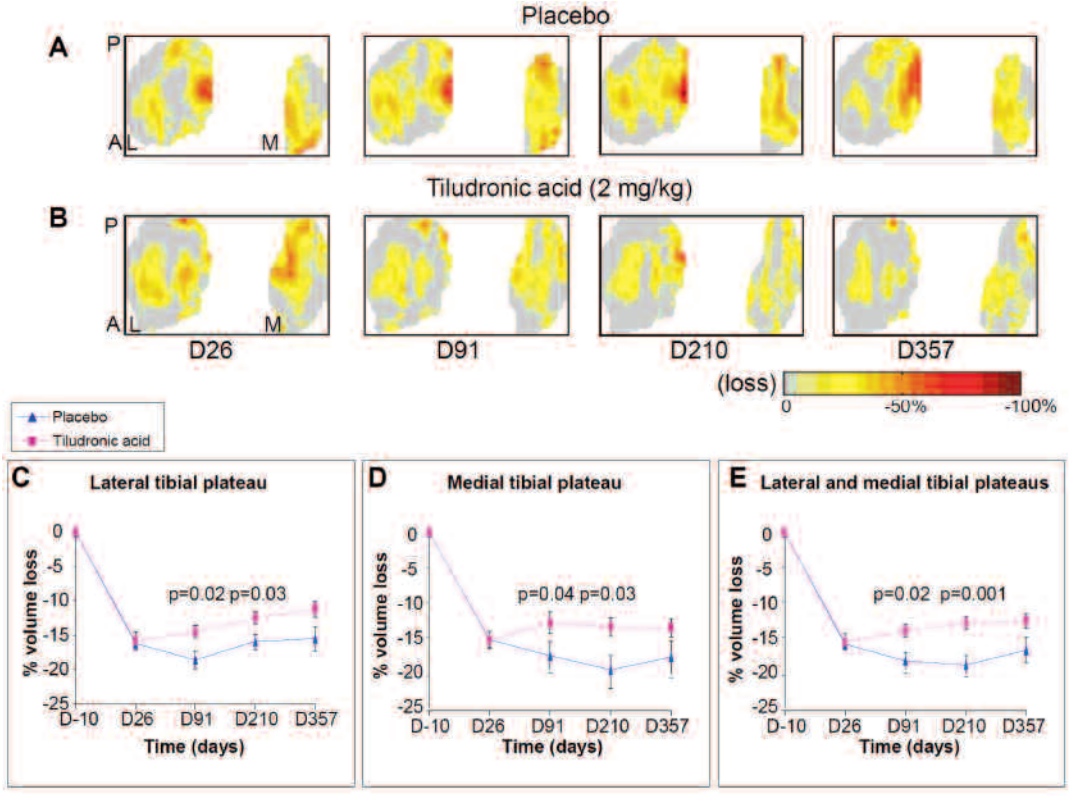

Figure 4. A and B. Cartilage volume loss map of representative lateral (L) and medial (M) tibial plateau cartilage loss at each observation timepoint expressed as percentage of baseline cartilage volume. Red represents areas of greatest cartilage loss. A: anterior; P: posterior. C, D, and E. Cartilage loss calculated as percentage from baseline in placebo and tiludronic acid-treated dogs for (C) lateral tibial plateau; (D) medial tibial plateau; and (E) lateral and medial tibial plateaus. Data are mean ± SEM. Statistical analysis by Mann-Whitney 2-tailed U test; p values are TA versus placebo.

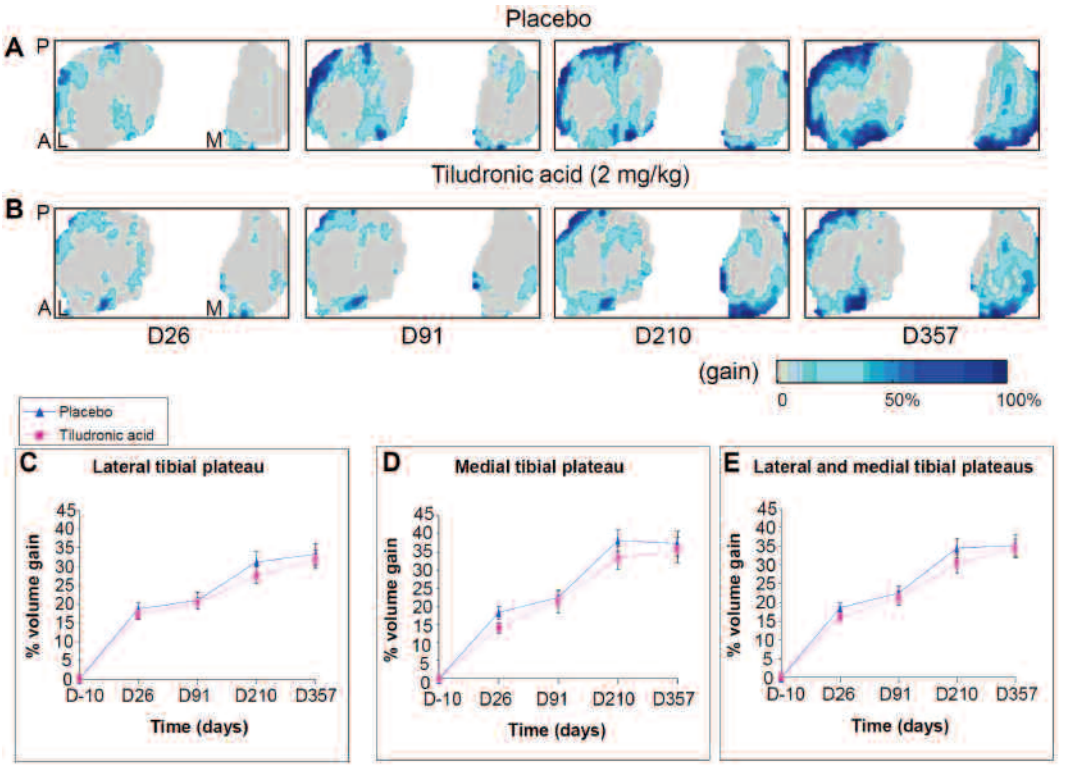

Figure 5. A and B. Cartilage volume gain map of representative lateral (L) and medial (M) tibial plateau gain at each observation timepoint expressed as percentage of the maximum gain in cartilage between visits. Blue represents areas of greatest cartilage gain. A: anterior; P: posterior. C, D, and E. Cartilage gain calculated as percentage from baseline in placebo and tiludronic acid-treated dogs for (C) lateral tibial plateau; (D) medial tibial plateau; and (E) lateral and medial tibial plateaus. Data are mean ± SEM. Statistical analysis by Mann-Whitney 2-tailed U test.

these OA dogs. They were found on both tibial plateaus (Figure 5) and femoral condyles. On the femoral condyles, the increase was fairly generalized (data not shown). On the tibial plateaus the gains were seen at the peripheral areas of the joint and, interestingly, also in the areas surrounding the cartilage lesions (Figure 5A, 5B). The percentage of cartilage volume gain on the tibial plateaus (Figure 5C, 5D, 5E) and on the femoral condyles (data not shown) was progressive over time, and a similar pattern was noted for placebo and TA-treated dogs.

At the time of sacrifice, the areas with macroscopic lesions or increased cartilage thickness corresponded well with the areas of cartilage volume changes detected by MRI (Figure 6).

DISCUSSION

This study provides interesting novel information on the longterm (1 year) development of cartilage lesions assessed by MRI in the ACL dog model of OA in which both reconstructive surgery to stabilize the joint and treatment with TA, a bisphosphonate, were initiated in the early stages of the disease (1 month after the ACL transection). Data indicate that following ECS surgery, the rapid and progressive loss of cartilage volume was reduced. Interestingly, over time, an increase in cartilage volume was found mainly in areas surrounding cartilage lesions and also in nonlesional areas. Further, concomitant treatment with TA at the time of the reconstructive surgery demonstrated an additional benefit by further reducing the cartilage matrix degradation and progression of lesions.

The ACL experimental dog OA model reproduces a number of structural changes observed in the natural disease[5,8] and the lesions are progressive over many years[8]. Recent studies using quantitative MRI[14,15,17] have shown the natural evolution of structural changes in this model, including those at the cartilage, bone, and osteophyte levels, to start early in the weeks following the surgery and increase steadily in severity over time, up to at least 48 weeks after surgery, which was the maximum observation time of our studies.

Data from quantitative MRI indicate that following the ACL and up to the time of ECS reconstructive surgery, there was a loss of cartilage volume on both femoral condyles and tibial plateaus. The rapid loss of cartilage in the early stages of the disease corroborates previous findings on clinical and functional outcomes in dogs with ACL injury[25,26,38,39,40,41].

Some data from dog[40,41,42] and human[43,44,45] studies are in support of the positive effect of ACL repair (joint stabilization) on the prevalence of knee OA. The protective effect of ECS on cartilage volume loss was shown in a recent study[27] in which 6 months after ECS the majority of dogs did

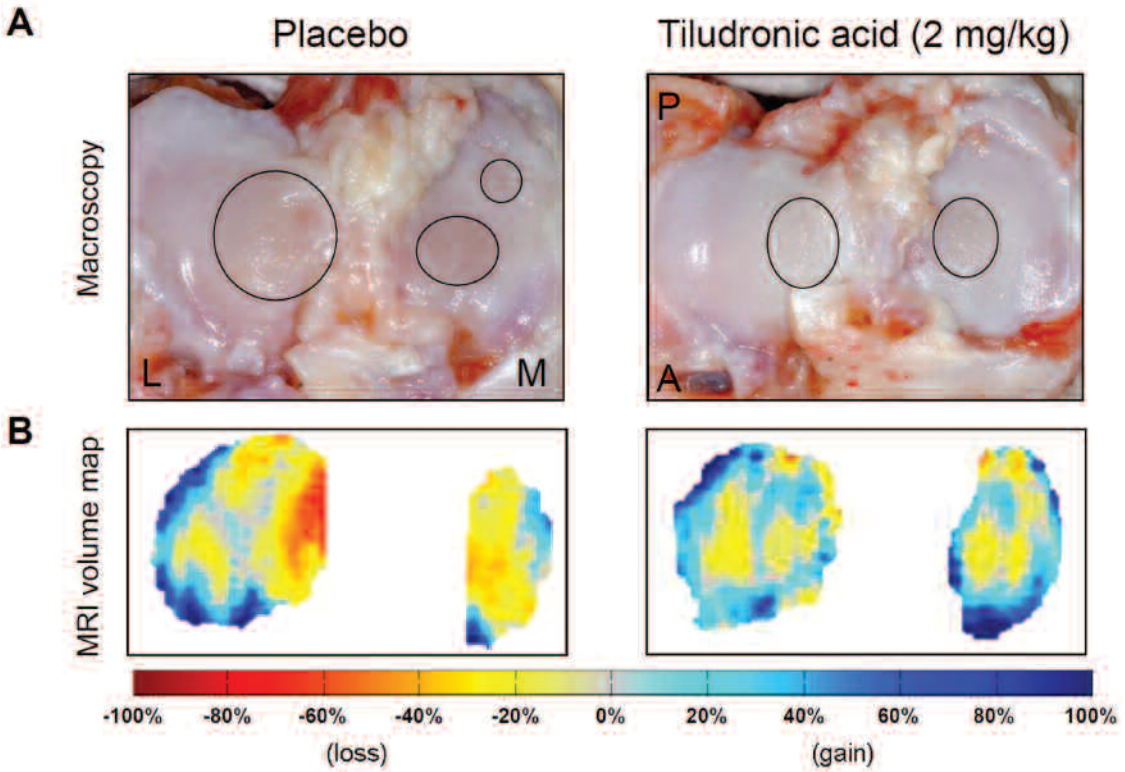

Figure 6. A. Representative macroscopic appearance of OA cartilage of the lateral (L) and medial (M) tibial plateau of placebo and tiludronic acid-treated dogs at Day 364 postsurgery. Circled areas indicate cartilage erosion. A: anterior; P: posterior. B. Cartilage volume loss/gain map of representative tibial plateaus; red represents loss, blue represents gain in cartilage volume between each observation timepoint and baseline.

not present radiographic signs of progression of OA. This agrees with our findings that knee stabilization prevents the loss of cartilage volume for up to 1 year (the duration of our study). However, one must be cautious about drawing conclusions from the results of the above studies, as some of those done in dogs[43,44,45] were limited by a lack of prospective objective evaluation, the use of various breeds, considerable heterogeneity in disease management, and a significant variation in the timing of the postoperative examination.

In our study we used MRI instead of radiographs, as it is now well known that radiographs have several significant limitations[46] in the assessment of cartilage loss. MRI, in turn, allows precise visualization of joint structures, including the cartilage, and the quantification of structural changes over time. In recent years, there have been a series of advances in the use of optimized MRI acquisition sequences to assess cartilage volume and thickness. In this context, the MRI reconstruction map allows precise identification throughout the course of the study of the anatomical regions of cartilage that are the sites of volume loss (lesions) or volume gain.

Using MRI, data showed that the lesional areas on femoral condyles and tibial plateaus were similar in appearance and location to those described in a previous study[14]. However, following ECS, the lesions partially regressed over time in both groups (Table 3). Interestingly, on the global evaluation there was a tendency toward a greater reduction in the TA group. The data regarding the loss (Figure 4) and gain (Figure 5) in cartilage volume clearly showed that the treatment with TA reduced cartilage degradation (lesions).

The rationale for the use of TA in this study was based on results from a pilot study in the ACL OA model showing functional improvement of the knee[20]. The treatment schedule was rationalized on the belief that after a traumatic rupture of the ACL, early (preventive) intervention is the most logical therapeutic option if one wishes to modify the course of the disease. Other studies using bisphosphonates or bone antiresorptive drugs[47,48,49,50,51] have also tested the hypothesis of the usefulness of preventing subchondral bone remodeling as a strategy to reduce OA disease progression. Indeed, some bisphosphonates were shown to be effective at reducing OA lesions in the rat ACL model[47] and the spontaneous guinea pig model[48]. In the ACL dog model, zoledronate was found to significantly impede the decrease in subchondral bone mineral density compared to control dogs and to improve the levels of bone metabolism markers[52]. Calcitonin, another antiresorptive agent, was found in the dog[49,50] and the rabbit[51] ACL models to have a protective effect on OA cartilage lesions. In humans, a phase III study in patients with knee OA with risedronate showed no effect on progression of the joint structural changes[53]. Of note, the latter study was performed using radiographs for assessing cartilage loss. The results from an ongoing clinical study with oral salmon calcitonin and using MRI in patients with knee OA should soon be available.

Not only data from MRI but also those from macroscopic and histologic evaluations of cartilage support the protective effect of TA at reducing the severity of OA cartilage lesions. Data on the aggrecan (Safranin-O staining) and collagen network (Picrosirius staining) clearly indicate a protective effect of TA on the cartilage matrix. An explanation for this finding could be the significant reduction in levels of MMP-13 expression in the TA-treated dogs. Bisphosphonates have been demonstrated to reduce MMP-13 synthesis in various cell types including rheumatoid pannus[54], endothelial cells[55], and rat OA cartilage[47]. Although the exact mechanism of action of bisphosphonates on MMP-13 has not been fully elucidated, it has been hypothesized to involve cation chelation. Of interest, the histologic findings of the significant decrease in the pannus score and synovitis in the TA group compared to placebo are also in accord with those from the 2-month pilot study using the ACL dog model, in which TA significantly decreased the synovial membrane lining score, the synovial effusion size, and the catabolic factors prostaglandin E_2 and nitric oxide in the synovial fluid[20].

We also investigated the possibility that TA could have exerted its positive action on cartilage by promoting tissue repair. Our data on the expression levels of aggrecan and collagen type II were negative, showing no difference between TA treatment and placebo. Similar findings were also observed for key growth factors known to be involved in cartilage repair. Therefore, a positive effect of TA treatment at improving the anabolism of OA cartilage seems unlikely. Thus, the gradual gain in cartilage volume over time following ECS in the TA group (Table 3) is likely linked to a reduction in cartilage volume loss by the inhibition of some catabolic factors.

Another finding from this study is that following the ECS reconstructive surgery, there was an increase in cartilage volume. Such a phenomenon has been previously reported[10] and was suggested to be related to cartilage swelling or edema in the early stage of the disease. Data revealed a continuous increase in cartilage volume over time suggestive of a repair phenomenon. Such a finding has never been reported from studies using the ACL or other surgically induced OA dog models. However, in a recent human clinical trial in patients who had an ACL injury, MRI evaluation showed that those patients who had an ACL reconstruction had an increase in knee cartilage volume compared to those that had received only rehabilitation[56]. The exact characteristics of this repair remain to be determined.

The main limitation of our study could be its length — 1 year. To obtain complete information about the longterm effects of TA treatment and in order to mimic the progression of the natural disease in humans, an extended observation time will be required.

In summary, the results of our study are 2-fold. First, they indicate that in the OA dog model created by ACL transection, reconstructive ECS surgery to stabilize the joint in the early stages is effective at preventing longterm progression of OA joint structural changes by reducing cartilage degradation, thus allowing cartilage repair. Second, treatment with TA, a bisphosphonate, concomitant with the time of the reconstructive surgery provided the additional benefit of further reducing cartilage matrix degradation and the progression of lesions, an effect that lasted at least 4 to 5 months after treatment was stopped.

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