Equine Veterinary Journal

Equine Veterinary Journal ISSN 0425-1644
DOI: 10.1111/evj.13011

Clodronate improves lameness in horses without changing bone turnover markers

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Summary

Background: Clodronate is prescribed to performance horses with lameness. Despite its clinical popularity, little research has been done to understand the effects of clodronate in the horse.
Objectives: Our objective was to determine if a single treatment with clodronate at the clinically approved dose altered bone remodelling, bone cell recruitment or lameness in the horse.
Study design: Twelve university-owned equestrian team competition horses with a history of forelimb lameness due to navicular syndrome were randomised to receive either 1.4 mg/kg clodronate (CLOD n = 6) or an equivalent volume of LRS (CONT; n = 6) in a blinded manner.
Methods: Blood was evaluated weekly for 8 weeks before and after drug administration (clodronate or placebo) for bone turnover markers CTX-I and osteocalcin. Lameness evaluations were performed to assess for change in lameness 1 week before and 1, 2, 3 and 8 weeks after drug administration. Coach questionnaires were performed to assess for change in ridden performance 1, 2, 3 and 8 weeks after drug administration. Bone cell recruitment was evaluated in vitro 2 weeks before and after drug administration.
Results: There were no differences in in vitro bone cell recruitment from whole bone marrow or in bone turnover markers CTX-I or osteocalcin. A small but significant decrease in forelimb lameness was detected in CLOD treated horses 1 week after treatment (P = 0.005). There were no significant differences in hindlimb lameness. Coaches identified an improvement in performance significantly more often in CLOD vs. CONT (P = 0.01) at week 8.
Main limitations: Two CONT horses received intra-articular anti-inflammatory medication after treatment, which may have altered lameness results.
Conclusions: A single dose of clodronate appears to reduce lameness without producing detectable effects on bone turnover markers. Due to the long half-life of a bisphosphonate drug, the effect of multiple doses on bone remodelling and lameness should be investigated.

The Summary is available in Portuguese – see Supporting Information

Keywords: horse; clodronate; OSPHOS; bisphosphonate; bone turnover; lameness

Introduction

Bisphosphonates inhibit osteoclast function and are used in medicine for their resulting antiresorptive effect on bone. A variety of bisphosphonates have been prescribed to human patients for several diseases of bone turnover, and clodronate more recently has been investigated for osteoarthritis [1–4]. Mechanisms of improved function in osteoarthritis patients may be due to clodronate's antiresorptive effect on chondrocytes and subchondral bone, or off-target mechanisms leading to a reduction in inflammatory cytokines and macrophages, or other mechanisms for antinociceptive activity [3–12]. Although the mechanism has not been investigated in the horse, reduced lameness in horses following bisphosphonate use has been demonstrated [13–15].

After decades of bisphosphonate use in human patients, adverse drug reactions from bisphosphonate therapy were recognised including atypical femoral fracture and osteonecrosis of the jaw [16–18]. Although the pathophysiology of these complications is not fully understood, regular assessment of bone turnover markers, bone density, and duration of bisphosphonate therapy are used to determine the need for a drug holiday, which appears to minimise the risk of a serious adverse event [1,19].

C-terminal telopeptide of type I collagen (CTX-I) is a breakdown product of the most abundant protein in bone matrix, collagen type I, which is enzymatically released from bone by osteoclast mediated cathepsin K secretion. The concentration of serum CTX-I is considered one of the most relevant bone turnover markers when monitoring bisphosphonates in human subjects and has been used successfully in the horse to show antiresorptive efficacy of bisphosphonates [20] and cathepsin K inhibitors [21]. Significant reductions in CTX-I were demonstrated after tiludronate administration to horses at 1 mg/kg i.v. or 0.1 mg/kg i.v. once daily for 10 days. The same investigators then gave tiludronate at 1 mg/kg i.v. once on day 1 and again 28 days later, demonstrating a similar reduction in CTX-I after the second dose both in magnitude and duration [15,20,22]. To the best of our knowledge, there have been no reports on the antiresorptive effects of clodronate in the horse.

Given the rare but potentially disastrous bone metabolism-related complications in human patients following bisphosphonate usage, our objective was to understand clodronate effects on bone turnover in the horse after administration of the clinically approved dose. We administered clodronate vs. placebo and assayed for markers of bone remodelling and for effects on in vitro bone cell recruitment and mesenchymal stem cell (MSC) characterisation. Because lameness is a key determinant of clinical efficacy, we also assessed for a change in lameness. We found there was no change in markers of bone remodelling in vivo, in osteoclast and osteoblast recruitment in vitro or MSC characterisation in vitro. However, we found significant improvements in lameness as determined by an inertial sensor system and by coach evaluation.

Materials and methods

Study design

All animal procedures were approved by the university's Animal Care and Use Committee (IACUC 2016-0122). Twelve university-owned horses on the western horsemanship equestrian team were selected by the team coaches based on the following inclusion criteria: horses were expected to compete during the fall 2016 season, had a history of

lameness due to navicular syndrome, and had not previously received bisphosphonate treatment. Our university hospital and its veterinarians are responsible for the veterinary care of university-owned equestrian team horses. For each horse, the medical record was used to confirm that medical inclusion criteria were met: two horses had a presumptive diagnosis of navicular syndrome, two horses had a historical diagnosis of navicular syndrome (means of diagnosis was not recorded), and the remaining eight horses had a diagnosis of navicular syndrome based on nerve blocks and radiographic signs of navicular bone degeneration (Table 1); none of them had received bisphosphonate drugs previously. Whether horses had a diagnosis for hindlimb lameness in the medical record was also recorded. Treatment allocation was randomly assigned by the university pharmacist using an online program, www.random.org and remained unknown to all other parties until the project was completed and all clinical and laboratory data had been entered into spreadsheets. To ensure that no horses received greater than 900 mg per horse, one group received a single dose of 1.4 mg/kg clodronate[a] i.m. split between two injection sites (CLOD; n = 6), and the other received an equivalent volume of LRS i.m. split between two injection sites (CONT; n = 6). Treatments were administered and horses were monitored for 2 h post-treatment by a veterinarian who did not have contact with other study personnel, coaches, riders, or other horse care personnel. Treatment coincided with the start of the fall 2016 competition season. Jugular blood for bone turnover markers was collected weekly for 8 weeks prior and 8 weeks post-treatment. Bone marrow was aspirated 2 weeks prior and 2 weeks post-treatment. Lameness was evaluated 1 week prior to treatment and each week for 3 weeks and at week 8 post-treatment.

Bone cell recruitment

Bone marrow aspirate was collected as previously described [23]. Whole marrow underwent red blood cells lysis and was seeded into 12-well plates with or without the presence of 50 μmol/L clodronate for all culture types both before and after in vivo treatment administration [24,25].

For osteoblast differentiation, whole bone marrow was cultured in osteogenic differentiation medium (αMEM, 10% FBS, 50 umol/L ascorbic acid, and 10 mmol/L beta glycerolphosphate) [26]. For the osteoblastic lineage, cells were seeded at $1.5e^5$cells/cm^2 and grown for 10 days, formalin fixed and stained for alkaline phosphatase and counterstained with fast green. The number of colony-forming unit-fibroblasts (CFU-F) that were positive for alkaline phosphatase (AP+) activity was determined in four replicates[b] [26]. For mature osteoblast differentiation, cells were seeded at $2.5e^5$ cells/cm^2 in osteogenic differentiation medium for 28 days, followed by fixation and staining of mineral with alizarin red in four replicates [26]. For osteoclast differentiation, $1e^6$ cells/cm^2 cells were plated in osteogenic medium with the addition of 50 nmol/L 1,25 (OH) Vitamin D3. Cultures were maintained for 15 days before staining for tartrate resistant acid phosphatase (TRAP)[b] activity [26]. Osteoclasts (TRAP+ cells with 3 or more nuclei) were enumerated in 4 wells per treatment group.

Bone marrow derived MSC characterisation

MSCs were isolated from a portion of lysed bone marrow from each aspiration described above and maintained until passage 3 with or without the presence of 50 μmol/L clodronate for trilineage differentiation and immunophenotyping [27].

Bone remodelling markers – CTX-I and osteocalcin

Twelve millilitres of blood was collected from the jugular vein weekly for 8 weeks prior and 8 weeks post-treatment. Blood collection was performed at 10 am every Monday. Blood was placed in red top glass tubes and allowed to clot for a minimum of 30 min before centrifugation at 4000 ***g***. Serum was collected and stored in 1 mL aliquots at −80°C. Frozen aliquots were thawed and neat samples were used to assay for CTX-I and osteocalcin using commercially available ELISAs[c] per manufacturer instructions.

Lameness evaluation

Lameness exams were performed 1 week prior to treatment and each week for 3 weeks and again at 8 weeks following treatment using three forms of evaluation: clinical evaluation, an inertial sensor system (Lameness Locator®)[d] and coach evaluations (only performed post-treatment), with all three forms of evaluation blinded to the others. For clinical evaluation, the horse was hand-walked in a 10 m straight line, hand-jogged in a 60 m straight line twice, and hand-jogged in two 20 m circles to the right and left on a concrete surface, and lameness was scored using the AAEP scoring system [28]. Following clinical evaluation, the same veterinarian supervised data collection for the inertial based sensor system (Lameness Locator®)[d]. Sensors were placed on the poll, right forelimb pastern, and croup, and horses were jogged in a straight 60 m line with 3 changes of direction in walk for data collection [29]. Coach questionnaires were administered at weeks 1, 2, 3 and 8 following treatment after lameness exams. One coach evaluated Horse 7 (CLOD) at all time points and the other coach evaluated all other horses at all time points. Coaches were not aware of the results of the lameness exams. The coaches were asked a series of questions to evaluate the horse's work performance based on their observations of the horse in western horsemanship practice in a sand arena: was the horse performing up to expectation (yes/no), was the horse in full work (yes/no), was the horse lame (yes/no), and compared to the prior evaluation (or prior to treatment for the first evaluation) was the horse's performance better, same, or worse. Any lameness therapies given during the trial including joint injections with intra-articular medications were documented.

TABLE 1: Navicular syndrome history. All horses in the study had previously received a diagnosis of forelimb lameness due to navicular syndrome. From the medical record, methods to diagnose navicular syndrome including radiographs demonstrating navicular bone degenerative changes, nerve blocks localising the lameness to the foot or historical diagnosis were recorded. When the medical record included a diagnosis for hindlimb lameness, this was also recorded

Horse	Group	Age (years)	Sex	Breed	Radiographs	Foot block	Hindlimb
1	CLOD	12	G	QH	Yes	Yes	Spavin
2	CONT	12	G	QH	Yes	Yes	Spavin
3	CLOD	11	M	QH	Historical	Historical	PSD
4	CONT	7	G	QH	Yes	Yes	Spavin
5	CONT	7	G	QH	No	Presumptive	None
6	CLOD	10	M	QH	Yes	Historical	Spavin
7	CLOD	7	G	Paint	Yes	Presumptive	Spavin, pastern OA
8	CLOD	13	G	QH	Yes	Yes	Spavin
9	CONT	15	G	QH	Yes	Yes	Spavin
10	CLOD	18	G	QH	Yes	Yes	Spavin
11	CONT	11	G	QH	Yes	Yes	None
12	CONT	11	M	QH	Yes	Yes	Spavin

Data analysis

Data were imported to a commercial statistical software program[e] for analysis. Data were tested for normality using the Shapiro-Wilk test. Differences between variables measured longitudinally over the entire study period were evaluated by repeated measures analysis of variance (ANOVA), with horse considered a random effect. Treatment group, time point, and their interaction were included as factors in the ANOVA. Analysis was performed using PROC MIXED, and an autoregressive correlation structure was specified. Differences at selected time points were further investigated by the student's *t*-test or Wilcoxon rank-sum test, depending on data distribution. Differences in bone marrow differentiation and recruitment were evaluated by two-way ANOVA with follow-up comparisons by in vitro clodronate treatment with Sidak's multiple comparison test. Coach evaluations were evaluated using a Fisher's exact test at each time point. Differences were considered significant when $P \leq 0.05$.

Results

All treatment injections were performed without incident, and no horses had signs of colic or other illness post-treatment. All horses were systemically healthy and remained on the equestrian team roster throughout the duration of the study.

Bone cell recruitment in vitro

In vitro treatment with clodronate had no effect on osteoblast or osteoclast development in bone marrow cultures from horses naive to clodronate treatment in vivo. However, there was a significant increase in osteoblast recruitment after in vivo clodronate treatment in cultures also treated in vitro with clodronate compared to in vitro control cultures ($P = 0.02$; Fig 1). There were no differences in mature osteoblast differentiation or osteoclastogenesis between CLOD or CONT groups either before or after in vivo treatment (Fig 1).

Bone marrow derived MSC characterisation in vitro

MSCs from CLOD and CONT groups before and after in vivo treatment with clodronate were able to undergo trilineage differentiation for osteoblasts, adipocytes and chondrocytes without differences between the groups. There was no difference in the positive expression of expressed CD29 and CD44 and lack of expression of MHCII and CD45 for control, ex vivo and in vivo treated cultures.

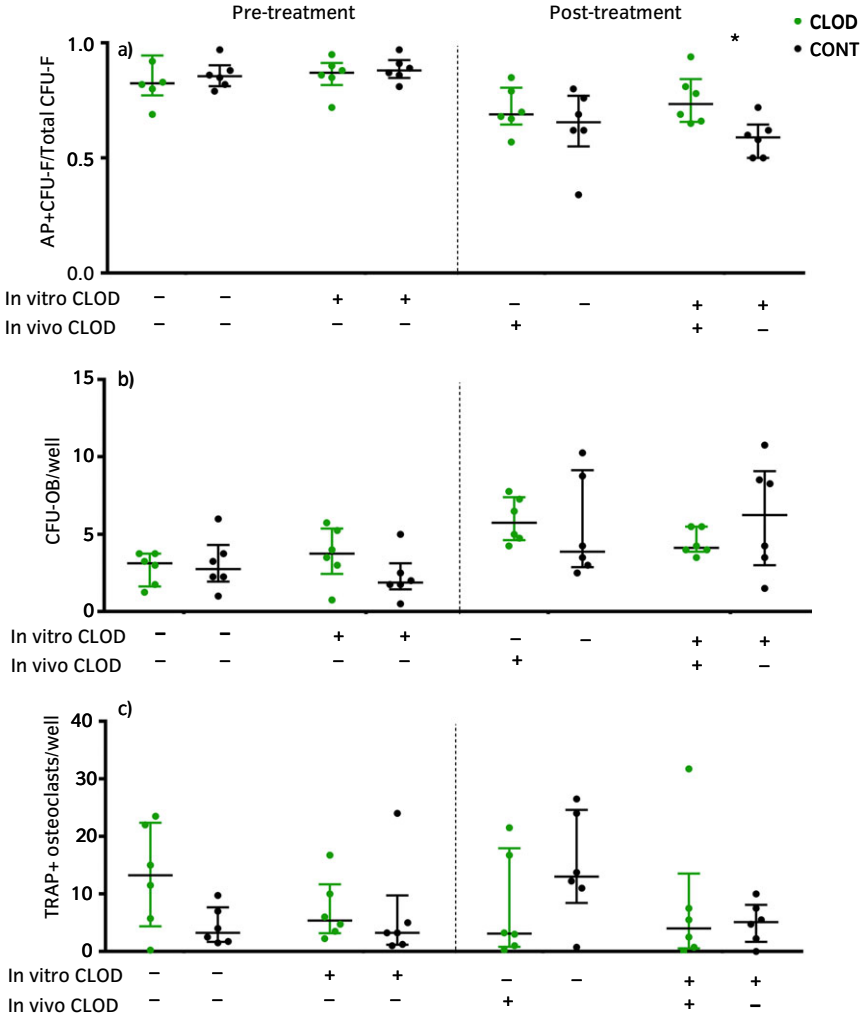

Fig 1: Cell recruitment and differentiation. Bone marrow was aspirated 2 weeks prior (Pre-treatment) and 2 weeks after (Post-treatment) drug administration (in vivo, placebo or clodronate) and was cultured with or without clodronate in vitro to evaluate a) osteoblast recruitment by the percentage of alkaline phosphatase positive colonies and b) osteoblast maturation capacity by the number of bone nodules, as well as c) osteoblastogenesis by the number of TRAP positive multinucleated osteoclast as described in methods (mean; standard deviation). There was significantly increased osteoblast recruitment when there had been both in vivo and ex vivo clodronate exposure.

CTX-I and osteocalcin levels in vivo

From the clodronate group, values were below CTX-I detection limit prior to treatment in Horse 1 (all 9 time points) and Horse 3 (2 time points) and after treatment in Horse 1 (4 time points). From the placebo group, values were below CTX-I detection limit prior to treatment in Horse 2 (7 time points), Horse 11 (3 time points), and Horse 12 (9 time points) and after treatment in Horse 2 (4 time points), Horse 11 (2 time points), and Horse 12 (8 time points). From the control group, values were below osteocalcin detection limit prior to treatment in Horse 5 (3 time points) and after treatment in Horse 2 (1 time point) and Horse 4 (4 time points). The remainder of the horses were above the detection limit at all time points. Only values above the limits of detection were used for analysis. There were no significant differences between CLOD and CONT groups in CTX-I or osteocalcin levels over time by repeated measures ANOVA (Fig 2a, b). To account for interindividual variation and the missing data due to levels below the detection limits at individual time points, we also analysed the data using an average value from all time points prior to treatment to calculate a percent change from baseline. There were no significant differences.

Lameness

By the inertial sensor system there were no differences in forelimb or hindlimb lameness over time between treatment groups. However, when we compared the two groups at individual time points, there was significantly decreased forelimb lameness ($P = 0.005$) in clodronate-treated horses at week 1 (Fig 3a) and no significant difference in hindlimb lameness (Fig 3b). There were no differences in subjective lameness grades (Table 2) over time or at individual time points (median forelimb and hindlimb score of 3 for both groups at weeks 1, 2 and 3 and median forelimb and hindlimb score of 2 for both groups at week 8; Table 2).

There were no significant differences in coach evaluations between groups for questions 1–3 at any time point (Table 3) but for question 4, there were proportionally more horses that the coaches assessed as "improved" in CLOD compared to CONT at the final time point of 8 weeks (week 1 $P = 0.06$; week 2 $P = 0.08$; week 3 $P = 0.2$; week 8 $P = 0.01$; Table 3).

As part of their normal care and not part of the study, two CONT horses were presented for lameness evaluation to the primary care veterinarian and treated with joint injections during the trial: Horse 4 received bilateral forelimb coffin joint injections with hyaluronate and triamcinolone and bilateral hock injections with methylprednisolone at week 2, and Horse 12 received bilateral forelimb coffin joint and bilateral hindlimb fetlock joint injections with hyaluronate and triamcinolone at week 5. No CLOD horses were evaluated for lameness as part of their normal care, and none received intra-articular medications.

Discussion

We found that a single administration of clodronate at the FDA and EMA-approved dose did not result in significant differences in measures of bone remodelling, overall bone cell recruitment, or MSC characterisation, but did result in improvements in forelimb lameness among clodronate-treated horses compared to placebo. We used serum CTX-I and osteocalcin as markers of bone turnover [30,31]. In human patients, measurable reductions in bone turnover using biomarkers including CTX-I are used to determine bisphosphonate efficacy in osteoclast inhibition and dosing regimens [32,33]. In contrast to what we expected, we did not find differences in bone turnover due to treatment with clodronate, even at the earliest time point. Although our group size was small, it is unlikely our study was underpowered because our sample size is similar to previous tiludronate equine studies where there were significant CTX-I reductions due to bisphosphonate administration [15,20] or cathepsin K inhibitors [21]. The lack of change in CTX-I could be because the approved dose for treating lameness is below the therapeutic threshold for measurable osteoclast inhibition in horses [15,20]. This seems likely because the i.m. clodronate dose in human patients for osteoclast inhibition is approximately double the equine dose on a mg/kg basis, is repeated daily for 10 days, and then repeated daily for 6 days every 3 months, whereas horses receive a single dose [3,4].

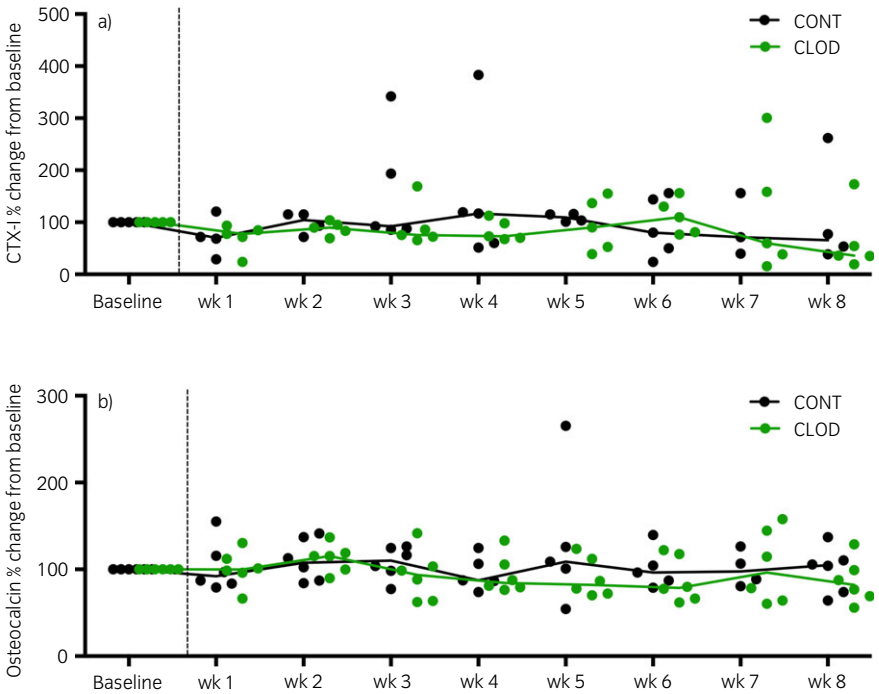

Fig 2: Percent change in baseline bone turnover levels after clodronate treatment. Blood was collected each week for 8 weeks before and after treatment and serum analysed for levels of bone resorption as indicated by a) CTX-I, and bone formation as indicated by b) Osteocalcin. Weeks -8 through week 0 were averaged for a baseline value. Time of treatment is represented by the dashed line. Medians at each time point are connected by solid line. CLOD and CONT groups were not significantly different by repeated measures ANOVA.

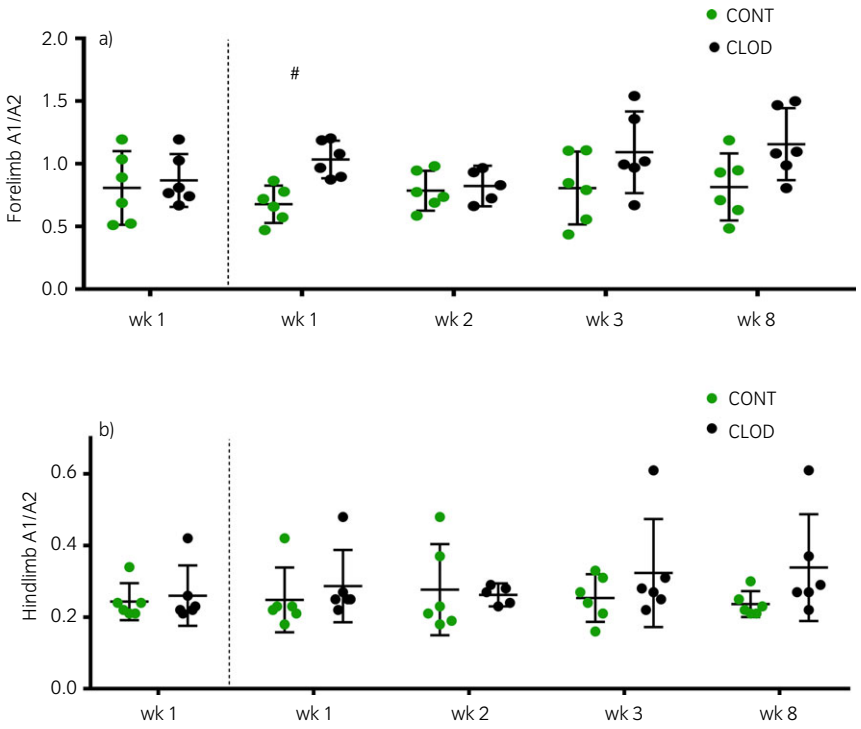

Fig 3: Lameness evaluations. Inertial sensor lameness evaluations were performed 1 week prior and each week after treatment for 3 weeks and at week 8 post-treatment and are represented by the mean and 95% confidence interval. Time of treatment (clodronate or placebo) is represented by the dashed line. There were significant improvements in the a) forelimb A1/A2 scores ($P<0.05$) at week 1 post-treatment.

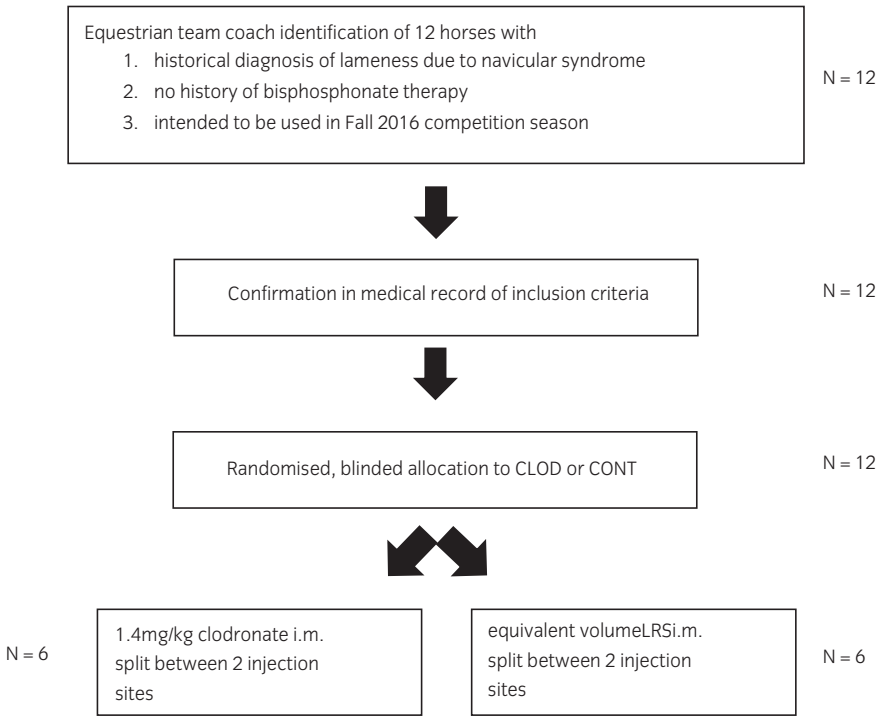

Fig 4: Flow chart of study. Twelve horses on the university's equestrian team roster and scheduled to compete in the fall 2016 season that had a history of navicular syndrome in the medical record and no historical bisphosphonate therapy were included in the study. Of these 12 horses, six were randomly allocated to receive placebo and six to receive clodronate.

TABLE 2: Clinical evaluation of lameness. Lameness exams were performed 1 week prior to treatment and each week for 3 weeks and again at 8 weeks following treatment by clinical evaluation. The horse was hand-walked in a 10 m straight line, hand-jogged in a 60 m straight line twice and hand-jogged in two 20 m circles to the right and left on a concrete surface and lameness was scored using the AAEP scoring system. There were no significant differences

	Week 1	Week 1	Week 2	Week 3	Week 8
Forelimb					
CLOD					
Median	2	3	3	3	2
Confidence level	0.9688	0.9688	0.9688	0.9688	0.9688
CONT					
Median	3	3	3	3	2
Confidence level	0.9688	0.9688	0.9375	0.9688	0.9688
Hindlimb					
CLOD					
Median	3	3	3	3	2
Confidence level	0.9375	0.9688	0.9688	0.9688	0.9688
CONT					
Median	3	3	3	3	2
Confidence level	0.9688	0.9375	0.9375	0.9688	0.9688

TABLE 3: Coach evaluations. Coaches were asked a series of questions to evaluate the horse's work performance based on their observations of the horse in western horsemanship practice in a sand arena: Question 1) Was the horse performing up to expectation (yes/no); Question 2) Was the horse in full work (yes/no); Question 3) Was the horse lame (yes/no) and Question 4) Compared to the prior evaluation (or prior to treatment for the first evaluation) was the horse performance better, same or worse. A mark of 'improved' by the coaches was evaluated as an improvement in our data set. A mark of the same was considered 'improved' if the horse had been considered 'improved' the previous week. The percentage of horses in each treatment group scoring 'improved' or 'yes' was quantified at each time point, and the groups compared by Fisher's Exact test

		Week 1	Week 2	Week 3	Week 8
Q1. Up to expectation	CLOD	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)
	CONT	6/6 (100)	5/6 (83)	5/6 (83)	6/6 (100)
Q2. In full work	CLOD	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)
	CONT	6/6 (100)	5/6 (83)	4/6 (67)	6/6 (100)
Q3. Lame	CLOD	1/6 (17)	0/6 (0)	0/6 (0)	0/6 (0)
	CONT	4/6 (67)	3/6 (50)	4/6 (67)	2/6 (33)
Q 4. Improved	CLOD	4/6 (67)	5/6 (83)	5/6 (83)	5/6 (83)
	CONT	0/6 (0)	1/6 (17)	2/6 (33)	0/6 (0)

In human patients, lasting and marked reductions in bone turnover with resultant reductions in CTX-I increase the risk for the bisphosphonate-related complication of atypical fracture [1,34]. This complication is thought to occur because of accumulation of microdamage due to over-suppression of bone remodelling by bisphosphonates, even in patients without pre-existing bone turnover disease [35]. If fracture risk pathophysiology after bisphosphonate use in the horse is similar to that in human patients, then the lack of change in bone turnover that we found suggests that horses will not have increased fracture risk due to accumulation of microdamage after a single dose of clodronate.

Similar to in vivo bone turnover markers, there were minimal changes in osteoclast and osteoblast differentiation and MSC growth and differentiation in response to clodronate. There were only significant differences in osteoblast recruitment in cells that had been treated both in vivo and then also in vitro with clodronate. This is likely due to direct stimulation of osteoblastogenesis by clodronate [36]. Similar direct effects on osteoclastogenesis and MSC differentiation were expected but did not occur at the dose we used [37,38], suggesting either a species-specific or dosage-specific sensitivity to these effects in the horse.

Despite lack of change in bone turnover and bone cell recruitment and differentiation, we found a reduction in forelimb lameness and a reduction in lameness by coach evaluation of 'improved as compared to before treatment'. This was not surprising, as a reduction in forelimb lameness due to navicular syndrome was seen in horses treated with clodronate 56 days after treatment in the FDA approval study [13]. In addition, in human patients, reduction in pain after bisphosphonate administration is well known [5,39]. Reduction in pain after bisphosphonates could be due to osteoclast inhibition or it could be due to an effect not related to osteoclast function. Several off-target mechanisms for analgesia of bisphosphonates have been suggested over the past 20 years; however, a consensus has not been reached [4,39,40]. It is possible that the therapeutic dose for analgesia by bisphosphonates is different than for osteoclast inhibition or that analgesia is by a different mechanism of action, independent of antiresorptive effects. In support of an off-target, or non-osteoclast related mechanism for analgesia, non-nitrogenous bisphosphonates, such as clodronate, have exhibited more effective reductions in pain when directly compared to nitrogenous bisphosphonates that are more potent antiresorptives [5].

Although statistically significant in the forelimb, the changes found by the inertial sensor system were small and were not corroborated by similar changes in the subjective, AAEP scores. We think the lack of difference in AAEP scores is due to the wide range of lameness severity covered by each grade of lameness and thus lack of sensitivity for changes in lameness that are relevant to performance ability. For example, a horse could have a grade 3 lameness that was moderate and consistent when trotting in hand on concrete that improved to mild and consistent when trotting in hand on concrete, still receiving a grade of 3 despite a noticeable improvement in lameness. In fact, when using any grading or scoring system it is difficult to determine when small but significant improvements in measured lameness are relevant to horse performance. The clinical relevance of the small but significant inertial sensor system improvements in the clodronate-treated horses are supported by the blinded coach evaluations of 'improved as compared to before treatment'. These blinded coach evaluations are likely to better reflect relevant improvement, since coaches observed the horses under work throughout the week, whereas lameness evaluations were only conducted at designated evaluation time points. Importantly, the coaches were not assessing the presence, absence, or change in visible lameness when assessing performance improvements. Rather, they were assessing for improvements in ridden performance, which are assessed by gait quality and quality of required manoeuvres or movements during ridden work with bridle contact in an arena.

A limitation of the coach evaluations was that questionnaires were not filled out prior to treatment. Although this impacts questions 1–3 as there is no baseline value to compare to, it has minimal impact on question 4, which was to compare the horse's performance to before treatment or to the most recent evaluation. While analysis of coach evaluation is at risk for Type I error due to the number of comparisons made, clinical relevance of the statistical improvement is supported by the frequency of performance-limiting lameness that required therapy in the control group as compared to the clodronate-treated group.

Use of other therapies that may introduce bias are a potential risk of clinical trials where the horses enrolled are not managed primarily by the researchers. The administration of joint injections to horses that had performance limiting lameness during the trial may have skewed our lameness results due to reductions in lameness following intra-articular anti-inflammatory therapies in treated horses. Both horses that received these therapies during the trial were in the placebo-treated group, and both were treated after the first post-treatment evaluation time points (week 2 and 5). It is possible that had these horses not received the

therapies there would have been additional significant differences between the groups on the lameness data, with additional reduced lameness parameters in the clodronate-treated group.

A possible limitation when studying bone remodelling is that bone turnover is an incredibly dynamic process that is affected by age, sex, season, exercise and diet [31,41–43]. To minimise potential bias due to these factors, we used a placebo-controlled parallel design trial with adult horses that were current competition horses for our university's equestrian team. This group of horses was under the same housing, diet, exercise and turnout regimen for at least 1 year prior to and throughout our study.

A possible limitation of this study is the small sample size. However, we used a similar number of horses per group as was used for equine tiludronate studies and equine cathepsin K inhibitor studies, in which a decrease in bone turnover was found [20–22]. In addition, sample size recommendations for laboratory animal research suggest that group sizes of six animals is appropriate to account for between-subjects variability when investigating bisphosphonates for osteoclast suppression [44]. Finally, a 50% reduction in CTX-I is expected in human patients following clodronate vs. placebo treatment. Using these human data for our a priori power calculation, a sample size of 6 is appropriate [45,46]. An additional limitation is that our study was a parallel design rather than a crossover, which could help to minimise bias due to known and unknown horse factors. We did not use a crossover design because we could not determine an appropriate washout period for a bisphosphonate drug without some knowledge on the anti-resorptive function of that drug in the horse. In addition, there are clear seasonal and exercise effects on bone turnover, which would confound a crossover design [31,47]. Thus a randomised experimental study in which all horses were receiving the treatment (CLOD or CONT) at the same time while under the same exercise program was paramount.

Conclusions

Because of the long half-life of all the bisphosphonates, it is possible that clodronate will have a significant effect on bone turnover after re-dosing, and further dosing studies should therefore be conducted. Importantly, the findings herein demonstrate that a single dose of clodronate at the approved dose improved lameness, which might be independent of its antiresorptive actions. If this can be confirmed in a larger study, a single dose of clodronate should be safe to use without concern for adverse skeletal effects like those that occur in human patients when there are significant and lasting reductions in CTX-I.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

All animal procedures were approved by the Texas A&M University Institution's Animal Care and Use Committee (IACUC 2016-0122).

Sources of funding

This study was funded by internal grants awarded to Dr Watts: the Link Fund for Equine Research, the Linda and Dennis H. Clark '68 Chair in Equine Studies and a grant from the Department of Veterinary Large Animal Clinical Sciences at Texas A&M university.

Acknowledgements

We thank Dr Aileen Rowland and Hsing Fann for technical assistance. The study was supported by funding from the Texas A&M University, Department of Large Animal Clinical Sciences Faculty Grant, the Link Endowment for Equine Research at Texas A&M University and the Linda and Dennis H. Clark '68 Chair in Equine Studies.

Authorship

A. Mitchell participated in study design, performed laboratory work, performed statistical analysis and drafted the manuscript. G. Wright, M. Martin and S.N. Sampson performed laboratory work and assisted in drafting the manuscript. K. Cummings participated in drafting the manuscript and statistical analysis. D. Gaddy participated in study design and assisted in drafting the manuscript. A.E. Watts conceived, designed and coordinated the study, and assisted in drafting and revising the manuscript. All authors contributed to data interpretation and all authors read and approved the final manuscript.

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