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Pharmacological effects of tiludronate in horses after long-term immobilization ☆

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Abstract

Introduction: Tiludronate, a bisphosphonate, has recently been introduced in veterinary medicine to treat orthopedic conditions in the horse. This study was designed to evaluate its effects on biochemical biomarkers of bone metabolism and on bone density and structure in an experimental model of disuse osteoporosis induced by cast application in horses.
Methods: Two groups of eight horses were immobilized during 8 weeks. The first group (P-group) received a placebo, and the second group (T-group) received tiludronate 1 mg/kg by slow IV infusion. Both treatments were administered twice, 28 days apart. Immobilization consisted of stall rest with the left forelimb packed in a fiberglass cast. It was followed by a 4-week remobilization period and an 8-week standardized training protocol. One biomarker of bone resorption, the C-telopeptides of type I collagen cross-links (CTX-1) and one biomarker of bone formation, the bone isoenzyme of alkaline phosphatase (bone ALP), were assessed. Metacarpus III (MCIII) bone mineral density (BMD) and speed of sound (SOS) were evaluated respectively by dual energy X-ray absorptiometry (DEXA) and quantitative ultrasonography (QUS). Lameness was regularly assessed during the remobilization and training periods. Group- and time-related effects were tested by analysis of variance on repeated measurements.
Results: A rapid, transient and significant decrease in CTX-1 concentration was seen after each treatment in the T-group only. No significant differences between groups were seen in the evolution of bone ALP activity. At the end of the experiment, the loss of MCIII BMD measured by DEXA in the immobilized limb was significantly less in the T-group than in the P-group. The MCIII SOS measured by QUS did not significantly vary within or between groups throughout the study.
Discussion and conclusions: Tiludronate was found to significantly reduce bone resorption during immobilization, as well as to prevent long-term osteopenia in the immobilized limb. Disuse osteopenia did not affect the lateral superficial cortex of MCIII.

Keywords: Horse; Bisphosphonate; Bone densitometry; Bone turnover; Immobilization model

Introduction

Bisphosphonates are widely used in human medicine to reduce the rate of osteoclastic bone resorption, thereby increasing bone mineral density (BMD) and decreasing osteoporotic fracture incidence in post-menopausal women [1] or in patients suffering from osteogenesis imperfecta [2]. They are also particularly interesting compounds for the treatment of other pathologies involving excessive bone remodeling, like malignant hypercalcemia or Paget's disease [3]. In horses, increased remodeling is involved in a number of clinical orthopedic conditions such as degenerative joint disease, navicular syndrome, stress fractures or immobilization-induced osteopenia.

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The first bisphosphonate studied in horses was pamidronate [4,5], one of the first aminobisphosphonates authorized for use in humans. However, tiludronate was the first bisphosphonate to be licensed for use in the equine species to treat navicular disease and bone spavin [6]. Tiludronate is a non-aminobisphosphonate. Its primary pharmacological target is the osteoclast, but its effects are mostly mediated through the intra-cellular production of potentially cytotoxic ATP metabolites, while aminobisphosphonates inhibit the mevalonate pathway [7]. The pharmacological properties of tiludronate in laboratory animals and in humans have been extensively reported [3]. However, data on its effects on the equine bone are still lacking.

As in other species, non-invasive techniques to assess the equine bone include dosage of blood biochemical biomarkers and evaluation of BMD and bone properties by imaging techniques [8]. Several blood biomarkers of bone metabolism have been successfully tested in the horse, including the C-telopeptide of type I collagen cross-links (CTX-1) as a marker of bone resorption [9,10] and the bone isoenzyme of alkaline phosphatase (bone ALP) as a marker of bone formation [11]. Biomechanical properties and density of the equine bone can be assessed by quantitative ultrasonography (QUS) [12], or by dual energy X-ray absorptiometry (DEXA) [13]. With QUS, the time of propagation or speed of sound (SOS) of an ultrasound wave across a fixed distance in the bone is measured. It allows an evaluation of the superficial cortex properties [12] and estimates a combination of BMD and bone structure. SOS values are not influenced by soft tissue. With DEXA, bone evaluation is obtained via the attenuation it induces on a crossing bundle of photons coming from two different X-ray tubes of two energy levels. This attenuation is proportional to the calcium density of the bone, which is processed to obtain a value for BMD. In human medicine, DEXA is thought to be the fastest, most sensitive and most precise method to assess BMD in post-menopausal women, with a very low level of irradiation [14].

Preliminary data obtained in adult healthy horses have shown that administration of tiludronate by slow infusion at a dosage rate of 1 mg/kg is well tolerated [10]. Over the first 24 h after administration, it induces a significant decrease in CTX-1, but not in the C-telopeptides of type I collagen cross-links generated by matrix metalloproteases (CTX-MMP). Levels of bone formation markers osteocalcin (OC) and bone ALP are not modified [10].

Cast immobilization has been shown to be an effective model to induce disuse osteopenia in horses [15,16] and could therefore be a suitable model to assess the effects of an anti-resorptive therapy in that species. The aim of this study was to evaluate the effects of tiludronate on bone metabolism, density and structure in healthy horses subjected to a long-term cast immobilization, followed by remobilization and a standardized training program. After cast removal, lameness was scored to assess the ability of the horse to cope with exercise after this long-term immobilization.

Material and methods

The experimental protocol was reviewed and approved by the Animal Care Committee of the ENV Lyon, which abides by the requirements of the directive 86/609 of the European Community Council. Experiments followed guidelines equivalent to those in "Guide to the care and use of experimental animals" published by the Laboratory Animal Resources Commission on Life Sciences National Research Council (National Academy press, Washington, DC, 1996)

Study design

This study was a double-blind, randomized, placebo-controlled trial. The experiment consisted of a unicentric study with two groups of horses: a placebo group (P-group, $n=8$) and a group treated with tiludronate (T-group, $n=8$). Placebo and tiludronate treatments were administered under blind conditions. Both groups underwent an 8-week immobilization period, followed by 4 weeks remobilization and 8 weeks standardized training.

Horses

Sixteen healthy 4- to 8-year old Standardbred geldings with a body weight of 498.6±38.7 kg were selected for the study. They were randomly allocated within groups according to age, in order to obtain a comparable mean age between groups. The mean age was 6.4±1.6 (mean±SD) and 6.0±1.1 years in P- and T-group, respectively. Horses were rested in pasture for 1 year before the experiment. To be included in the study, horses had to meet the following criteria: (1) to have a lameness score of 0 or 1 based on a scale from 0 to 5 (Table 1) [17]; (2) to have a normal serum creatinine concentration measured by a colorimetric method (as an indirect assessment of kidney function); and (3) to not have received any known treatment susceptible to affect bone metabolism, such as anti-inflammatory drugs, from 6 weeks prior to enrolment until the end of the study. Horses were fed good quality hay twice a day throughout the study.

Treatments

The test product was presented as a lyophilized powder extemporaneously reconstituted as a 2% injectable solution of tiludronate (expressed as acid). The volume of reconstituted solution necessary to treat each horse was calculated on the basis of body weight (1 mg/kg bwt). An equivalent volume of saline was removed from a 1-l bag (laboratories Aguettant, Lyon, France) before adding the volume of reconstituted solution, in order to obtain 1 l of solution to be administered by slow infusion over 30 min via a polyurethane catheter in the left jugular vein. Prepared and administered the same way as tiludronate, the placebo (vehicle of the test product without tiludronic acid) was presented as a lyophilized powder indistinguishable from the test product for blinding purposes. In each group, treatments were administered on study Days 0 and 28. The same vein was used for both treatments.

Experimental procedure

Immobilization and training schedule

All horses underwent an 8-week period of stall rest with cast immobilization of the left forelimb, from Days 0 to 56. The lightweight fiberglass cast was placed under sedation with detomidine (10 μg/kg IV, DOMOSEDAN™, Pfizer, New York, NY, USA), combined if needed with butorphanol (0.05 mg/kg IV, TORBUGESIC™, Fort-Dodge Animal Health, Overland Park, KS, USA) and/

Table 1
Definition of the lameness scores

Grade	Interpretation
0	Lameness not perceptible under any circumstances
1	Lameness difficult to observe; not consistently apparent regardless of circumstances
2	Lameness difficult to observe at a walk or trot in a straight line; consistently apparent under some circumstances
3	Lameness consistently observable at a trot under all circumstances
4	Lameness obvious; marked nodding hitching and/or shortened stride
5	Lameness obvious; minimal weight bearing in motion or rest; inability to move

or acepromazine (0.05 mg/kg IV, VETRANQUIL™ 1%, Ceva Santé Animale, Libourne, France) depending on the horse's behavior to avoid undesired movement of the horse's left forelimb during cast application. The cast included the foot and extended proximally to a point immediately distal to the carpus. Five rolls of 10 cm fiberglass material (3M) were applied over a standard padding. The two first rolls were placed with the limb bearing weight on the floor in order to have a physiological position. The foot was then lifted in order to include the entire foot using the remaining three rolls. A rubber pad was placed over the foot to improve grip and prevent wear. It was laced on and strapped with adhesive tape. Casts were replaced when necessary and at least once in the middle of the immobilization period. A new cast was applied after having cleaned the leg and pressure sores if present. Criteria for early change of the cast were: sudden horse discomfort evaluated by reluctance to bear weight on the immobilized limb, cast splitting or cast sores detected by the presence of heat or secretions during daily palpation of the cast. After definitive cast removal on Day 56, the horses underwent a 4 week-period of remobilization (15 min hand-walking 3 times a week for 2 weeks, and 2 more weeks in a grass paddock) and an 8-week period of standardized training. The standardized training consisted of 3 periods of exercise per week according to the following protocol:

- Week 1: acclimation to trainer and harness.
- Week 2: 30 min walk.
- Week 3: 10 min walk, 10 min trot, 10 min walk.
- Week 4: 5 min walk, 20 min trot, 5 min walk.
- Week 5: 5 min walk, 30 min trot, 5 min walk.
- At the end of the week, the mean distance covered by the horses of the same group when trotting during 30 min was calculated (D30).
- Weeks 6 to 8: 5 min walk, trot for the distance calculated at week 5 (D30), 5 min walk. During the training period, horses were alternatively trained clockwise and counter-clockwise every 2 weeks.

Bone biochemical biomarkers

Blood samples were collected on Days −5, −1, 7, 14, 27, 42, 56, 84, 112 and 140 for bone ALP, and on Days −5, −1, 1, 3, 7, 14, 27, 29, 31, 35, 42, 56, 84, 112 and 140 for CTX-1. After an overnight fast, samples were collected between 6:00 and 9:30 am from the right jugular vein into sterile Vacutainer glass tubes with no additives from Day −5 to Day 84, and with EDTA on Days 112 and 140. Sera or plasma was separated after centrifugation (3000 rpm at room temperature) and stored at −20 °C within 4 h after collection until analysis. Quantification of bone ALP activity was performed by immunochemiluminescence assay using the Ostase reagent on an automatic analyser (Ostase, Access, Beckman Coulter, Fullerton, CA, USA) while CTX-1 concentration assessment was performed by automated immunoenzymatic assay (Crosslaps, Elecsys, Roche Diagnostics, Basel, Switzerland).

Imaging techniques

Bone density assessments by DEXA and QUS were performed on Days 0, 56 and 140.

DEXA measurements. DEXA was performed using a PIXI device (Lunar, GE, Madison, WI, USA) on sedated horses (detomidine 10 μg/kg IV, combined with butorphanol 0.05 mg/kg and/or acepromazine 0.05 mg/kg IV when needed) on both left and right forelimbs, owing to the method described by Donabedian and collaborators [13]. The region of interest (ROI) was selected by default for the right forelimb but had to be manually adjusted for the left forelimb in order to measure BMD at the same site for both limbs. BMD was expressed in grams of calcium per cm^2 of section.

QUS measurements. QUS was performed using an Omnisense apparatus (Sunlight, Rehovot, Israel) on mildly sedated horses (detomidine 7 μg/kg IV) on both left and right forelimbs. The probe was placed vertically on the lateral aspect of metacarpus III (MCIII), 5 cm above the distal extremity of the fourth metacarpal bone. Three to five successive measures, depending on the coefficient of variation calculated by the computer, were performed, and the mean measure was recorded. SOS values were expressed in m/s.

Lameness examinations

Lameness examinations were conducted with the horse walking in a straight line, walking in small right and left circles and trotting in a straight line. These examinations were performed on Days 0, 56, 70, 84, 98, 112, 126 and 140. A lameness score (Table 1) was given to each horse at each examination time, and if different from 0, the lame limb was reported. A horse presenting a lameness score equal or superior to 3 in one limb was considered as severely lame. During the training period, severely lame horses were removed from training for 1 week before re-evaluation.

Statistical analysis

Statistical analysis was run on SAS Institute Inc Software, version 8.2.

After normalization of the data, a linear mixed model was used to describe the experiment, as follows:

$$Y_{gijk} = \grave{\imath} + G_i + D_j + (GD)_{ij} + A_{ijg} + \mathring{a}_{gijk}$$

where Y is the measurement, ß is the grand mean, G_i and D_j are the main effects of groups and dates, respectively, and $(GD)_{ij}$ are the group–date interaction effects. Lower cases represent animal number (g), group number (i), date of measurement (j) and measurement number (k). The A_{ijg} represent the random effects of animals while $\mathring{a}_{gijk}$ are the random errors associated with the measurements. Standard stochastic assumptions are made that the random effects A_{ijg} and $\mathring{a}_{gijk}$ are normally distributed with zero means and variances σ_a^2 and $\sigma_{\mathring{a}}^2$, respectively. The A_{ijg} was assumed to be independent but we detected fluctuations in the variances over time so we modeled the covariance structure with a first-order auto-regressive structure (Proc Mixed, Type=AR(1), SAS Institute Inc., 1999) for which the co-variances between repeated measures on the same animal decreased exponentially with time. Finally, least-squares means were obtained for the main and interaction effects along with all pair-wise comparisons.

Percentages of severely lame horses (grade ≥ 3) at Day 56 were compared between groups by Fisher exact test.

The significance threshold was set at 5% (p-value < 0.05).

Results

Clinical events during the study

One horse of T-group was submitted to euthanasia on Day 10 because of severe digestive problems considered unrelated to the experiment. This horse was replaced by a new horse on Day 28, and data obtained from the euthanized horse were not included in the results.

At cast removal, most horses presented sores over the palmar fetlock and/or pastern region, ranging from mild to severe partial thickness dermal ulcerations. One horse of each group was excluded from the study because of full thickness dermal ulceration on Day 20. They were replaced by two new horses on Day 28, and data obtained from the excluded horses were not included in the results.

The three new horses which entered the study on Day 28 were selected according to the same criteria and underwent the same study protocol as the other horses of their respective group but 28 days apart. Because of moderate to important cast sores, six horses (two from P-group and four from T-group) received antimicrobial therapy (trimethoprim sulfamethoxypyridazine, 15 mg/kg PO BID) for 5 to 29 days, depending on the severity of the lesions.

Bone biochemical biomarkers

Results are expressed as percent of baseline values, which are represented by the mean of Days −5 and −1 values.

Serum CTX-1

Evolution of mean CTX-1 concentrations expressed as a percent change from baseline values is shown in Fig. 1.

While mean CTX-1 values of the P-group increased and remained above the baseline value from Days 0 to 42, values of the T-group sharply decreased after each tiludronate administration and remained under the baseline value at every sampling time of the study, except on Day 14. Changes in CTX-1 concentrations from baseline were statistically different between P- and T-groups ($p<0.05$) from Days 1 to 14 and from Days 29 to 35. The evolution of mean CTX-1 values was similar in both P- and T-groups from Day 56 (cast removal) to Day 140 (end of study), with a significant decrease ($p<0.05$) between Days 56 and 84 (i.e. remobilization period) followed by a significant increase between Days 84 and 140 (i.e. training period).

Serum bone ALP

Evolution of mean bone ALP concentrations expressed as a percent change from baseline values is shown in Fig. 2. Mean bone ALP values in P- and T-groups had a similar evolution over time. In both groups, they remained under baseline values for most of the immobilization and remobilization periods and then reached high values (two to three times as much as the baseline values on average). Overall, both groups did not statistically differ regarding evolution of bone ALP concentrations during the whole study.

Imaging techniques

Results are expressed as percent of baseline (Day 0) values for both BMD and SOS. All the horses of both P- and T-groups presented an important edema of the left forelimb after cast removal, when the assessment of the bone density and structure was performed on Day 56.

BMD evaluated by DEXA

A significant decrease ($p<0.05$) in left MCIII BMD between Days 0 and 140 was observed in the P-group but not in the T-group ($p=0.39$) (Fig. 3). Moreover, a significant increase ($p<0.05$) in left MCIII BMD was observed between Days 0 and 56 in the T-group, while a non-significant decrease ($p=0.72$) was observed in the P-group. Between Days 56 and 140, both P- and T-groups underwent a significant decrease ($p<0.05$) in left MCIII BMD. On Day 56, the difference in left forelimb BMD between P- and T-group was nearly significant

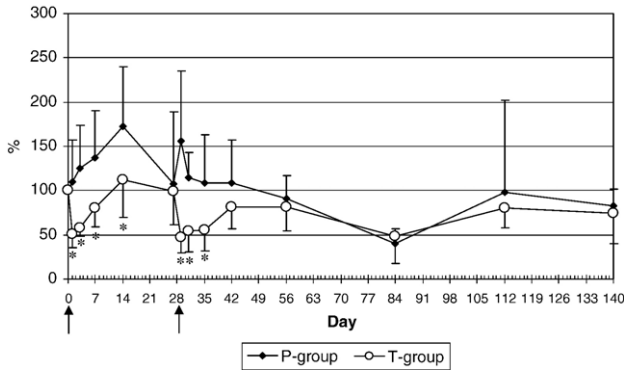

Fig. 1. Mean CTX-1 expressed as percent change from baseline values versus time for the P-group (black diamonds) and the T-group (grey circles). Black arrows indicate time of treatment administrations. *T-group values are significantly different from P-group values at $p<0.05$.

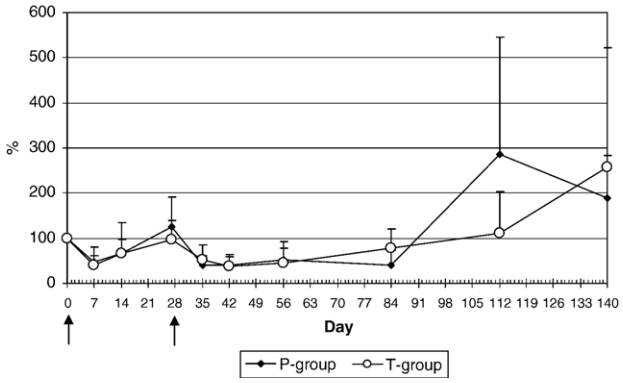

Fig. 2. Mean bone ALP expressed as percent change from baseline values versus time for the P-group (black diamonds) and the T-group (open circles). Black arrows indicate time of treatment administrations.

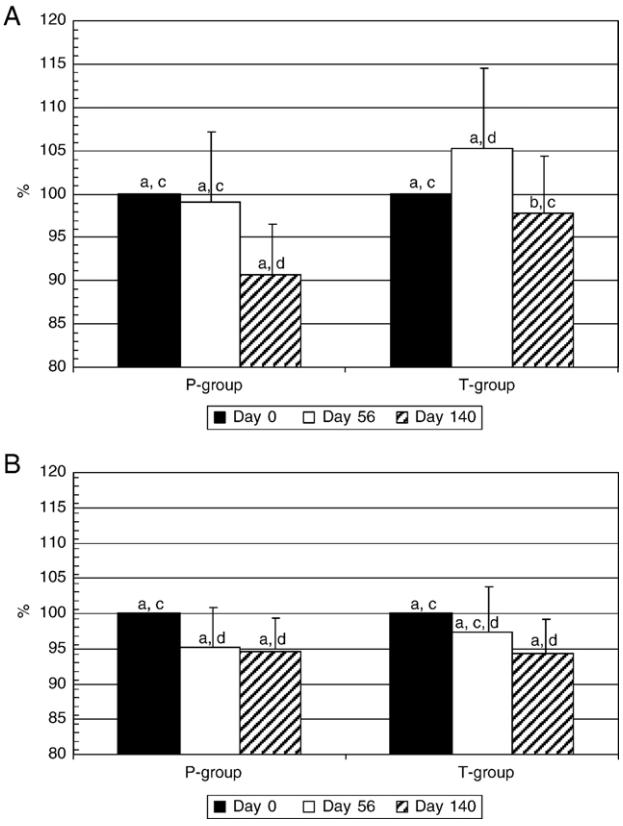

Fig. 3. Mean left (A) and right (B) MCIII BMD expressed as percent change from baseline values for P- and T-groups at Days 0, 56 and 140. (a, b) T-group values are significantly different from P-group values at $p<0.05$ when superscripts are different. (c, d) Days 56 and/or 140 values are significantly different from Day 0 (baseline) values at $p<0.05$ when superscripts are different for a given group.

Table 2
Mean left and right MCIII SOS expressed as percent change from baseline values (mean±SD) for P- and T-groups on Days 0, 56 and 140

		P-group	T-group
Left SOS	Day 56	99.5±2.2	97.8±4.7
	Day 140	100.0±4.8	100.5±4.5
Right SOS	Day 56	99.9±3.0	99.9±1.0
	Day 140	101.0±2.8	100.5±2.2

($p=0.06$), with mean values in the T-group higher than in the P-group. On Day 140, this difference was significant ($p<0.05$).

In the right forelimb, a significant decrease in mean MCIII BMD was observed between Days 0 and 140 in both P- and T-groups, but to a lesser extent than the decrease observed in the left forelimb MCIII BMD of the P-group. This decrease was significant between Days 0 and 56 in the P-group ($p<0.05$), but not in the T-group ($p=0.18$), and non-significant between Days 56 and 140 in both P- and T-groups ($p=0.76$ and 0.16, respectively). Differences between P- and T-groups were non-significant on both Days 56 and 140 ($p=0.36$ and 0.92, respectively).

SOS evaluated by QUS

No significant variations with time (within groups) or differences between groups in mean right and left MCIII SOS values were observed throughout the study (Table 2).

Lameness

In both groups, the highest lameness scores were recorded on Day 56, after cast removal. At this time, 75% and 50% of the horses in the P- and the T-group, respectively, presented a lameness score of ≥grade 3 (difference not statistically significant, $p=0.61$). No grade 5 lameness was recorded. In both groups, lameness scores tended to decrease from Day 56 to the end of the study, particularly during the remobilization period, so that most of the horses (5 in the P-group and 6 in the T-group) started the standardized training protocol with a lameness score of ≤1/5.

Discussion

To investigate bone metabolism in animal experimental models, it is critical to standardize as much as possible the tested subjects to reduce the individual variability known to be high on biomarkers of bone metabolism. Gender, age, breed and disease are known to influence biochemical biomarkers in the horse [8]. In this study, only clinically healthy 4- to 8-year-old Standardbred geldings were selected. Mares were not included to prevent variations in biomarkers due to hormonal cycle as was shown in other species [18]. Blood sampling was systematically performed in the morning on overnight-fasted horses to minimize circadian fluctuations [8] and horses were fed the same diet throughout the study in order to prevent the variations in bone density due to dietary intake [19].

Although three horses had to be excluded and were replaced 28 days later, the effect of this delay between the new subjects and the main study population was not deemed sufficient to cause seasonal variations in bone metabolism. Moreover, seasonal variations in bone ALP are known to be low in horses [20] and to occur as a slight rise between spring and summer in humans [21]. None of the observations in bone ALP evolution with time in this study is therefore thought to be due to a seasonal effect. Concentrations in CTX-1 are supposed to be higher, if different, in winter [22], what could have participated in the rise in CTX-1 observed in both P- and T-groups during the training period.

Consequently, remaining factors that could have influenced the evaluation of bone metabolism and bone density in this study were: immobilization, exercise and treatment.

It is known that the removal of regular weight-bearing activity generates a skeletal adaptive response in both humans and animals, resulting in a loss of bone mineral in the limbs, accompanied by alterations in biochemical markers of bone turnover. In horses, the period of limb immobilization in clinical situations ranges from a few weeks to a few months or more depending on the condition [23]. It is also known that a 7-week cast immobilization may induce osteopenia in subarticular regions and in cancellous and cortical bone tissues [16]. A 45% increase in serum CTX-1 on Day 7 in stall-rested horses, as well as elevated CTX-1 values during the five following weeks of stall rest, were previously described [9]. On Day 7 of the present study, mean CTX-1 values in the P-group were 36% above baseline, and rose until Day 14, with a peak value at 72% above baseline. Mean values remained above baseline until Day 42. The difference in peak CTX-1 percent values observed between the two studies can be explained by the difference between stall rest and cast immobilization, the latter leading to a more important total bone resorption phenomenon. Also, differences across models in the magnitude of the skeletal adaptive response are well described in human medicine [24]. During the remobilization period in the present study, CTX-1 values decreased in both groups, suggesting an inhibition of bone resorption in response to the biomechanical stress induced by a return to physical activity.

During immobilization, values of bone ALP showed a significant decrease by 50 to 60% in both immobilized groups as soon as on Day 7. All values but one were under baseline in both groups until the end of remobilization. Altogether, these observations suggest that immobilization had a rapid inhibiting effect on bone formation which persisted at least until the beginning of training.

In this study, there is an overall trend to a decrease of MCIII BMD in both forelimbs, except in the left cast-immobilized forelimb on Day 56 where density is close to baseline value in the P-group and higher than baseline value in the T-group. Although DEXA should not be influenced by soft tissue, edema of the left forelimb could eventually have interfered with the accuracy of BMD measurements on Day 56. This could explain why left MCIII BMD values were still high at cast removal with a potential masking of the short-term effect of immobilization on BMD. If left MCIII BMD values observed at cast removal were accurate, it would suggest that this model of cast immobilization was ineffective, and that cast prevented the

significant bone loss observed in the contralateral limb in non-treated horses, which seems to be very unlikely. Moreover, it would be in disagreement with BMD values observed after remobilization and exercise, which were lower in the cast-immobilized limb than in the contralateral one in non-treated horses. It would also be very unlikely that remobilization and exercise, rather than cast immobilization, were responsible for the significant BMD decrease observed in the left forelimb of non-treated horses at the end of the experiment, especially if no further decrease was observed between cast removal and the end of experiment in their right forelimb. Finally, although tiludronic acid is thought to prevent bone resorption associated with immobilization, it seems also unlikely that it could induce such an important increase in BMD, in such a short time, in cast-immobilized limbs of healthy horses, and without concurrent effect on the contralateral limb. All together, these observations consolidate the assumption that BMD values measured at cast removal in left forelimbs of both groups were skewed to higher values, probably due to edema.

The long-term decrease in BMD observed in both forelimbs of non-treated horses and in the contralateral limb of treated horses is thought to be directly related to immobilization. Decreased values of bone mineral content (BMC) assessed by single photon absorptiometry have been observed after eight weeks of cast immobilization, with a higher decrease in the cast-immobilized limb [15], and this would be consistent with the present study's observations if it is considered that values of BMD measured on the cast-immobilized limbs at cast removal were indeed skewed by edema, while values observed at the end of the experiment were accurate. This also would suggest that the BMD changes of the right limb may reflect the effect of stall rest only while BMD changes in the left limb would be indicative of the cumulative effects of stall rest and cast immobilization. A decrease in BMD was also evidenced by DEXA in subarticular regions of the distal portion of the MCIII of a cast-immobilized limb after 7 weeks of immobilization and 8 weeks of remobilization when compared to the contralateral limb [16], suggesting that 8 weeks of remobilization were not sufficient to restore bone density after cast immobilization and supporting the hypothesis of a long-term negative effect of immobilization on BMD. In humans, restoration of bone mineral that has been lost because of a period of reduced weight bearing may be restored upon return to normal activity; however, the recovery may not be complete and/or may take longer than the time course of the original bone loss [24].

Lameness is a common observation after several weeks of immobilization and cast application in the horse but higher incidence of lameness was found in this study compared to the results reported by Harreveld and collaborators [25]. One possible explanation for this discrepancy could be that cast application on standing sedated horses in this study induced more dermal ulcerations compared to casts placed under general anesthesia. Although most of these lesions were superficial and easily treated by local care, the high incidence of this complication points out the difficulty of managing long-term casts in horses. Incidence and severity of sores were comparable between groups, while horses of T-group seemed to present decreased comfort on their casts when compared to horses of P-group during the second month of immobilization. Nevertheless, the degree of weight bearing while casted was not consistently predictive of the lameness score at cast removal. The lack of systematic scoring of the lesions observed at cast renewal impairs further analysis of the association between sores and lameness in this study.

Few data are available on the effects of training on bones of mature Standardbred horses. In a study, an 8-week lunging exercise program had no effect on ultrasound velocity or bone mineral content of MCIII [26]. Since exercise intensity was low in the present study and no contemporaneous control (rested) group of horses was available for comparison, interpretation of the results in terms of training effect is speculative and cannot be largely discussed. The only noticeable observation that could be related to a training effect was the rise in CTX-1 values in both groups during the training period, which is in accordance with a previous study evidencing higher values in trained foals than in controls during the first 2 months of training [27].

Tiludronate induced a rapid and significant decrease of about 50% in mean CTX-1 values after each administration, while corresponding values in the P-group were largely increasing above baseline values. This suggests that CTX-1 is a rapid and sensitive marker of bone resorption rate and that it can be reliably used to follow-up a treatment with a bisphosphonate in the equine species, as it is already used in treated osteoporotic women [21,28]. In human medicine, intermittent administration of bisphosphonates, either cyclical oral etidronate and risedronate, or intravenous ibandronate and pamidronate, for the treatment of post-menopausal osteoporosis, also induces a rapid decrease in resorption markers followed within a few weeks by a slow increase that usually does not reach the baseline value at the time of the following administration [29,30]. Since mean CTX-1 values in the P- and T-groups remained similar from the time of cast removal to the end of the study, treatment may not have had further significant influence on overall bone resorption rate after remobilization.

No treatment effect was observed on bone ALP, suggesting an absence of deleterious effect of tiludronate at this dosage on bone formation rate. This is in agreement with histomorphometrical observations in human paraplegic patients treated with tiludronate [31].

In the present study, the tiludronate treatment also prevented long-term bone loss in the cast-immobilized limb. As previously mentioned, edema of the left forelimb may have interfered with the accuracy of the DEXA measurements on Day 56, but as edema was present in both groups, the net difference in MCIII BMD between the T- and the P-group was still considered significant. In the contralateral limb, the treatment prevented the decrease in MCIII BMD observed in the P-group at cast removal but this effect did not persist up to the end of the study. This supports the hypothesis that tiludronate exerts its inhibitory action on osteoclasts mostly in those bones with the highest resorption rates [32].

Descriptive comparisons suggest some consistency between variations in biochemical markers of bone metabolism during

the immobilization period and the general evolution of MCIII BMD. In the P-group, the increase of CTX-1 and decrease of bone ALP suggest an overall negative balance of bone turnover leading to a decreased BMD. In the T-group, the CTX-1 decrease combined with the decrease in bone ALP suggests a slow-down of bone turnover preserving BMD.

Metacarpal III SOS values did not change in any group, in any limb or at any time of the study, suggesting that in this model, training, immobilization and treatment did not affect the MCIII superficial cortex. This, in turn, suggests that the deeper layer of the cortical and/or the trabecular bone was the target for the remodeling processes, and that no deleterious effects of tiludronate could be observed on superficial cortical bone at the recommended dosage. Preservation of bone structure and integrity is important to maintain bone capacity to cope with mechanical stresses. Another explanation is that the lateral aspect of the MCIII was not the main site of remodeling in this model. This measurement site was chosen because it is the predilection location for bucked shins or dorsal metacarpal disease, a condition of fatigue failure and inadequacy of remodeling of MCIII in immature horses that undergo intense training. Incidence of the disease varies but affects up to 70% of young Thoroughbreds in training, which will have an increased risk of MCIII stress fracture within 6 to 12 months usually occurring on the dorsolateral aspect of the bone [33].

As BMD and mechanical strength are thought to be correlated [34], the prevention of MCIII BMD loss by tiludronate administration probably makes the metacarpal bone of treated horses more resistant to mechanical stresses during rehabilitation after immobilization than the metacarpal bone of non-treated horses.

No definitive conclusion can be drawn on the effects of tiludronate on lameness after cast removal. Differentiation between pain originating from soft tissue or from bone is not possible in this study. Bisphosphonates have been reported to reduce pain associated with extensive bone resorption [35], but not with pain associated with soft tissue injuries. Despite this, some beneficial analgesic effect of tiludronate could explain why there were less severely lame horses at removal of the casts in the T-group. Moreover, tiludronate is thought to have some potential anti-inflammatory properties [36].

In conclusion, this study demonstrates that cast-immobilization-induced osteopenia can be prevented by tiludronate administration, and that CTX-1 is an early and reliable bone biomarker to assess tiludronate treatment in horses. Preventive administration of tiludronate could be of clinical interest in long-term immobilized horses, by allowing a shorter rehabilitation time and a safer return to exercise after immobilization due to preserved bone density and mechanical bone strength.

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