THE EFFECTS OF CLODRONATE DISODIUM ON EQUINE JOINT TISSUES AND OSSEOUS METABOLISM: FROM IN VITRO ANALYSIS TO A PRE-CLINICAL, OVINE MODEL UNDER EXERCISE

By

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ABSTRACT

Bisphosphonates (BPs) are commonly used drugs for managing bone loss or bone resorption in skeletal diseases, such as post-menopausal osteoporosis, Paget's disease, and bone cancer. In 2014, the FDA approved clodronate disodium (CLO) and tiludronate disodium for treating navicular syndrome in horses over 4 years old. However, concerns have arisen regarding the extra-label use of CLO, particularly in juvenile individuals subjected to exercise, such as racehorses, where BPs may impact bone metabolism by affecting bone modeling/remodeling. The overall objective of this dissertation was to determine the effects of CLO in equine joint tissues *in vitro* and CLO effects *in vivo* in juvenile, exercising sheep as a model for juvenile horses. A prior publication found that CLO reaches the synovial fluid when administered intramuscularly. Therefore, an initial *in vitro* study exposed equine cartilage explants, chondrocytes, and synoviocytes to recombinant equine interleukin-1β (reqIL-1β) to determine the effects of CLO in joint tissues. The results confirmed that reqIL-1β increased the release of inflammatory markers from joint tissues (e.g., GAG, IL-6, PGE_2, NO, $P < 0.05$), yet CLO did not reduce the inflammatory effects of reqIL-1β. Hence, this *in vitro* study established that CLO was neither cytotoxic nor cytoprotective to joint tissues. Sheep have served as models for assessing the impact of BPs in human medicine and have also been used as a suitable model for studying exercising horses. Before using sheep as a model for intramuscular CLO administration, we determined the pharmacokinetics (PK) and plasma protein binding (PPB) of CLO in sheep and compared them to horses. Sheep PK parameters were similar to those previously published for horses, particularly C_{max} and AUC_{all}. Unbound fractions of CLO differed by less than 1.4-fold between sheep and horses. Having established a dose that resulted in PK values similar to those in horses, we subjected the sheep to a novel exercise protocol, using a high-speed exerciser. The sheep adapted

quickly to the exercise protocol and tolerated it well with no evidence of significant lameness. Finally, the juvenile sheep were divided into four groups: CLO on day 0, CLO on day 84, CLO on days 0 and 84, and a control group (saline). Physical examinations and lameness evaluations were measured every 14 days and blood was collected every 28 days for the measurement of serum bone biomarkers. Bone was harvested from the tuber coxae halfway through the study and again at euthanasia. In addition, samples of lumbar vertebrae and fused metacarpal III+IV were obtained at euthanasia for bone microstructure analysis and biomechanical testing. No measurable effects of CLO on the sheep skeletons were detected. Serum bone formation markers, bone-specific alkaline phosphatase (BALP) and procollagen type I amino-terminal propeptide, increased over time. Serum bone resorption marker, carboxy-telopeptide of type I collagen cross-links (CTX-I), decreased at several time points consistent with the exercise stimulus. Male sheep had decreases in compressional stress and increases in modulus of elasticity of the fourth lumbar spine in comparison to female sheep, likely due the low levels of sex hormones in the castrated males. The relatively low dose of CLO used in large animals compared to humans may explain the lack of skeletal effects. Previous BPs studies on horses have reported improvements in lameness without evidence of reduced bone resorption, suggesting that analgesic effects may occur without significant changes in bone microstructure or serum markers. Future research should focus on the potential analgesic effects of CLO at low doses which could provide palliative care without significant effects on bone metabolism.

This dissertation work is dedicated to David’s Shepherd.

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CHAPTER 1: Is the use of bisphosphonates putting horses at risk? An osteoclast perspective

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SIMPLE SUMMARY

Bisphosphonates are a group of drugs that intervene in the bone resorption process, producing cellular death of osteoclasts. These drugs are used for skeletal conditions, such as osteoporosis in humans and are available for veterinary medical use. Clodronate and tiludronate are bisphosphonates approved for the treatment of navicular syndrome in horses greater than four years old. However, these drugs are sometimes used in juvenile animals under exercise, where osteoclast activity is higher. Bisphosphonate use in juvenile and/or exercising animals could have adverse effects, including maladaptation to exercise or accumulation of microdamage. Furthermore, bisphosphonates can be bound to the skeleton for several years, resulting in a prolonged effect with no pharmaceutical reversal available. This review presents an overview of osteoclast function and a review of bisphosphonate characteristics, mechanisms of action, and side effects in order to con-textualize the potential for adverse/side effects in young or exercising animals.

ABSTRACT

Osteoclasts are unique and vital bone cells involved in bone turnover. These cells are active throughout the individual's life and play an intricate role in growth and remodeling. However, extra-label bisphosphonate use may impair osteoclast function which could result in skeletal microdamage and impaired healing without commonly associated pain, affecting bone remodeling, fracture healing, and growth. These effects could be heightened when administered

to growing and exercising animals. Bisphosphonates (BPs) are unevenly distributed in the skeleton; blood supply and bone turnover rate determine BPs uptake in bone. Currently, there is a critical gap in scientific knowledge surrounding the biological impacts of BP use in exercising animals under two years old. This may have significant welfare ramifications for growing and exercising equids. Therefore, future research should investigate the effects of these drugs in skeletally immature horses.

INTRODUCTION

Since their discovery in 1873, osteoclasts have been recognized for their bone resorption ability [1]. Osteoclasts' resorption ability makes them a key cell for musculoskeletal development, bone metabolism, and bone repair throughout life [2]. Due to their intricate involvement in physiological processes, substantial pathologies are associated with overactive or impaired osteoclast function. In conditions where osteoclast resorption overcomes bone formation, it can be useful to decrease osteoclast activity through pharmaceutical interventions. Bisphosphonates (BPs), a class of drugs known to impair osteoclast function, have been available for human skeletal conditions for over 50 years and have been available in veterinary medicine for about 25 years [3].

In 2014, two bisphosphonates (Osphos® and Tildren®) were approved by the United States Food and Drug Administration (FDA) to treat navicular syndrome in horses over four years of age [4]. However, it is unknown how these drugs affect juvenile animals under exercise, where skeletal adaptations depend upon normal bone metabolism and normal osteoclast function [5]. The objective of this review is to explore the available scientific literature regarding the origin of osteoclasts and their functions, how BPs affect these cells, the current use and side effects of BPs in humans and other animal models, and the potential negative effects of BPs use

in the juvenile horses under exercise.

ORIGIN OF OSTEOCLASTS

Osteoclasts precursors are found in the bone marrow and originate from the myeloid lineage [6]. Key molecules involved in osteoclastogenesis include members of the tumor necrosis factor family α (TNFα) such as the receptor activator of nuclear factor (NF)-κB (RANK), RANK ligand (RANKL), osteoprotegerin (OPG), and the macrophage-colony stimulating factor (M-CSF) [7]. In the presence of M-CSF and other growth factors such as interleukin-3 (IL-3), osteoclast precursors proliferate and become preosteoclasts. These preosteoclasts fuse and generate mature multinucleated osteoclasts [8]. OPG serves as a negative feedback molecule for osteoclastogenesis, decreasing the RANKL function and osteoclast differentiation [6]. Osteoclastogenesis and subsequent osteoclast-mediated resorption are necessary for skeletal development, bone remodeling, and bone repair. Disruptions in osteoclastogenesis can lead to disease processes ranging from osteoporosis to osteopetrosis [8–10] A summary of the main molecules involved in osteoclastogenesis are presented in Table 1.

Table 1. Summary of osteoclastogenesis molecules, origin, and functions. Molecules essential for osteoclastogenesis include: receptor activator of nuclear factor (NF)-κB ligand (RANKL), osteoprotegerin (OPG), macrophage-colony stimulating factor (M-CSF).

Molecules	Origin	Function
RANKL	Bone marrow-derived stem cells, osteoblasts, osteocytes	Primary differentiation factor controlling gene expression binding to RANK [11,12]
OPG	Osteoblast and osteocytes	Decoy receptor for RANKL competing with RANK. Blocks RANKL-RANK interaction [11]
M-CSF	Bone marrow-derived stem cells, osteoblasts, osteocytes	Activates pathways stimulating proliferation and survival by binding to macrophage colony-stimulating factor 1 receptor (CSF-1 R/c-Fms)[11,12]

OSTEOCLASTS ARE CRITICAL FOR BONE MODELING AND REMODELING

Osteoclast morphology plays an important role in their function; osteoclasts are multinucleated cells with an active “ruffled membrane” where resorption occurs [13]. When adjacent to the bone, the finger-like extensions of the cytoplasm adjacent to the bone produce a

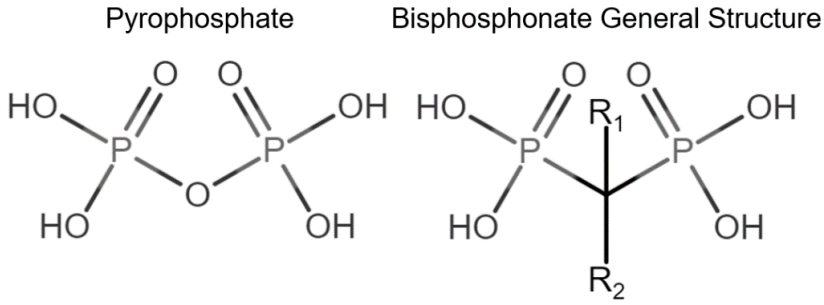
microenvironment through a proton pump, acidifying and demineralizing the bone matrix [14]. Alterations in osteoclast function can lead to critical bone diseases including osteopetrosis [15] but may also have more subtle effects on bone modeling and remodeling.

Bone is a connective tissue with multiple roles, including mechanical support, protection, locomotion, mineral homeostasis, and endocrine functions [12,16,17]. Bones can be erroneously perceived as static tissue when, in fact, bones are constantly adapting to strain [14,18]. Bone adaptation is driven by two processes: bone modeling and remodeling [2]. Bone modeling is the process of mineral uptake and removal in growing organisms, leading to bone maturation. Meanwhile, bone remodeling is a process that takes place throughout the life of organisms as the bone adapts to new mechanical loads and repairs microdamage, allowing the bone to reach a proper geometry [19]. In bone remodeling, bone resorption and bone formation are tightly coupled. Osteoclasts are recruited and activated, resulting in bone resorption and then undergoing apoptosis. Then, osteoblasts produce a new organic bone matrix, followed by mineralization [19].

OSTEOCLAST-MODIFYING DRUGS: BISPHOSPHONATES

Bone modeling and remodeling can be affected by pharmacological interventions including bisphosphonates. Bisphosphonates (BPs) vary in their chemical structure, complexity, and binding capacity to bone tissue.[20] BPs are chemically stable analogs of inorganic pyrophosphates (PPi). BPs are not subject to enzymatic hydrolysis, are resistant to high temperatures, are not biodegradable [21,22], and have a high affinity to bone hydroxyapatite (HAP) [20] (Figure 1).

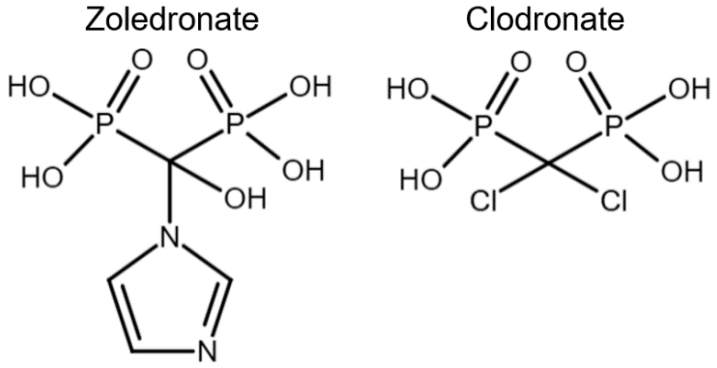
Figure 1. Comparison of pyrophosphate and basic bisphosphonate structures. Bisphosphonates differ from pyrophosphates primarily by the change of oxygen from their central atom to carbon, providing resistance to biological degradation.



Bisphosphonates have been classified into "generations" and mechanisms of action: first-generation BPs (i.e., clodronate), second-generation BPs (i.e., tiludronate and alendronate), and third-generation BPs (i.e., ibandronate, and zoledronate). Newer generations have greater antiresorptive capabilities, which allows for a lower dose administration [22]. BPs are also classified according to their mechanism of action: simple or non-nitrogen-containing BPs (sBPs) and nitrogen-containing BPs (nBPs). Both BP types interfere with the osteoclast activity but with different relative potency [23]. The R2 side chain of BP structure determines the biological activity (Figure 1), and the presence of nitrogen atoms in the R2 side chain has shown a larger antiresorptive effect (Figure 2) [24]. Simple BPs (i.e., clodronate and etidronate) are metabolized in the cytoplasm and generate cytotoxic ATP analogs (5- [β, γ-dichloromethylene] triphosphate) resulting in apoptosis due to a lack of free/functional ATP for cellular enzymatic function [20,23]. On the other hand, nBPs affect intracellular signaling, through farnesyl diphosphate synthase (FPPS) inhibition [20,23]. This interferes with the prenylation of GTPase proteins, vital for functions such as the formation of the ruffled membrane, vesicular transportation, or apoptosis [24]. FPPS impairment also produces an accumulation of isoprenyl pyrophosphate (IPP), producing another ATP analog (1-adenosine-5'-yl ester 3-[3-methylbut-3-enyl] ester), causing a similar effect to sBPs [20,22,23,25]. Bone resorption, in turn, affects the bone

formation phase due to the complex and coupled process of bone modeling and remodeling [26,27].

Figure 2. Chemical structure comparison between a third-generation nitrogen-containing BP (zoledronate) and a first-generation non-nitrogen-containing BP (clodronate).



The pharmacokinetics (PK) of BPs depends on the route of administration. BPs are poorly absorbed orally, attributed likely to their low lipophilicity [28]. Oral bioavailability in humans has been estimated between 0.3% for pamidronate [29], 0.7% for alendronate [30], and 1-2% for clodronate [31]. Parenteral routes provide better and nearly complete absorption of BPs [28]. Initial tissue distribution depends on the protein-biding properties and will vary depending on blood pH, serum calcium, drug dosage, and species [28]. Though there is evidence of non-calcified tissue retention of BPs at high dosages [32,33], the probability of this occurring at therapeutic dosages is minimal [28]. BP distribution is not homogeneous in the skeleton and may be affected by sex and age. BPs tend to bind to the trabecular bone, because of larger amounts of bone turnover and blood supply in comparison to cortical bone [34–36]. Young animals may have an increased absorption rate compared to adults, and females may absorb less BP than males [33]. Once in the bone, BPs are absorbed by osteoclasts via endocytosis.[23,25] As bone resorption continues, BPs reactivate, resulting in prolonged osteoclastic inhibition over time.[20] For this reason, BPs' half-life is difficult to define and may be up to 10 years depending on species, age, and the specific BP [4,28].

Clodronate and etidronate (sBPs) can undergo intracellular metabolization [37]; on the other hand, there is no evidence of metabolization of nBPs [36]. BPs not taken up by the bone are largely excreted unaltered by the kidneys [28,36]. In human and rat studies, BPs half-life ranges between 1 to 2 hours, a quick bloodstream elimination that depends on kidney excretion (renal clearance) and bone uptake (nonrenal clearance); this ratio varies among BPs. In humans, the clodronate renal/nonrenal clearance ratio is between 1.8 to 3 in comparison to pamidronate which is 0.18 [28]. Additionally, the same class of BPs may differ in some PK parameters. For example, clodronate and tiludronate are sBPs with similar potency [22], yet the half-life of clodronate is reported to be between 2 and 3 hours [38,39], and tiludronate's half-life is much longer (51 hours) [40]. Therefore, it is not accurate to extrapolate drug properties, even if BPs belong to the same class [41]. Because of this, studies must evaluate each BP and its individual effects on bone modeling and remodeling.

THERAPEUTIC EFFECTS OF BISPHOSPHONATES

Human conditions treated with BPs include postmenopausal osteoporosis, Paget's disease, osteogenesis imperfecta, and bone cancer/metastasis [42,43]. Bisphosphates are used to decrease bone resorption and increase bone mineral density (BMD) by decreasing bone catabolism.

Besides their main antiresorptive properties, BPs are believed to have anti-inflammatory and analgesic effects [4,44–48], which make them an attractive potential treatment for multiple diseases including osteoarthritis (OA). Meta-analyses and systematic reviews have concluded that BP studies have controversial results and BP effects may be more related to pain relief than disease modification [48–50]. These pain-relieving effects could be beneficial for some individuals, but in human or animal athletes, masking pain could also be dangerous and lead to

further deterioration of joint conditions.

BPs anti-inflammatory and pain-relieving effects may be due to a reduction in inflammatory mediators such as Prostaglandin E_2 (PGE_2). PGE_2 is considered one of the key inflammatory mediators, generating pain in OA [51], and is a critical outcome measure in equine OA studies [52,53]. There is evidence that people treated with neridronate, a nBPs, for osteogenesis imperfecta have reduced serum concentrations of PGE_2 and CTX-I/creatinine ratio [44]. Equine studies have demonstrated pain relief from BP administration in back OA [54], lower hock osteoarthritis [55], and navicular syndrome [56]. However, it is still unclear how BPs decrease pain in horses [53]. Additional pain-relieving mechanisms may be involved.

Recently, clodronate was identified as a selective and potent inhibitor of vesicular nucleotide transport (VNUT) [57]. Typically, this transporter is responsible for storing ATP in neurons. Research suggests clodronate may inhibit the release of ATP acting as a presynaptic blocker attenuating chronic neuropathic pain [57]. This activity may explain why in an equine study, clodronate did not change serum bone resorption markers but did significantly improve lameness [56]. Further investigation is warranted as masking pain could lead to adverse outcomes especially in exercising animals. This is especially important as bisphosphonates may remain active in the bone for months to years following administration.

BISPHOSPHONATES' SIDE EFFECTS: HUMANS AND ANIMALS

Bisphosphonates have short and long-term side effects. In humans, the kidneys excrete about 50 to 60% of BPs without major biotransformation [21,22,25], and a rapid intravenous infusion can produce focal glomerulosclerosis [58]. Humans treated with BP can experience short-term adverse effects including fever, muscle aches, vomiting, and transient hypocalcemia [59]. Long-term exposure in humans can result in serious side effects including osteonecrosis of

the jaw (ONJ) [60] and atypical femur fracture (AFF) [61]. In horses, short-term side effects may include renal toxicity, especially when the animal has a history of renal disease or has been treated with nonsteroidal anti-inflammatory drugs [47]. Further, transient colic-like symptoms have been documented following intravenous infusion [22,38,40]. Other adverse side effects have not been well documented in horses, but a lack of documentation may be related to the scarcity of long-term studies currently available.

Between one and twelve percent of human oncologic patients experience ONJ after three years of IV treatment with BPs [62]. This condition has been also reported in several animal models, including sheep [63,64], mini-pigs [65], and dogs [66]. Recently, clinical reports showed symptoms compatible with ONJ in cats treated with long-term BPs for idiopathic hypercalcemia [67,68]. An additional serious adverse side effect, AFF, accounts for 1.1% of all femur fracture cases in humans [69]. Likewise, a bilateral patellar fracture has been reported in a cat after 8 years of alendronate treatment [70]. Changes in the femoral neck with increased bone brittleness have been found in mice using ibandronate [71]. Even though there is not currently a clear connection between equine stress fractures or catastrophic injuries and BPs, these drugs have shown the potential to produce severe adverse effects in multiple animal models and humans. In horses, clodronate has been isolated from bone samples 18 months following a single administration [72], and tiludronate has been found in low concentrations in plasma (0.05 – 1.0 ng/ml) and urine samples (0.03 – 1.5 ng/ml) after three years following administration [73]. Fluctuations in plasma and urine concentrations over time may have been influenced by activity level, health status, growth, and animal to animal variation [73]. BPs can be present in the skeleton of horses for long periods of time, potentially masking pain, and are documented to cause adverse bone effects in multiple species. Consequently, further investigation into the

relationship between BPs and bone injuries in horses is crucial for equine health.

BISPHOSPHONATE IN ADULT HORSES

Two BPs were approved for use in the horse by the FDA in 2014 for the treatment of navicular syndrome in horses over 4 years old [4]. Clodronate and tiludronate dosage characterizations are detailed in their respective FDA Freedom of Information Summary [38,74]. The lowest effective clodronate dosage found to decrease one grade in navicular syndrome-associated lameness was 1.8 mg/kg or 900 mg per horse [73]. For tiludronate, 1 mg/kg was found to alleviate symptoms associated with navicular syndrome [38,75]. BPs have resulted in reduced pain and lameness in other skeletal conditions, such as back pain, lower hock OA, and fetlock OA [54,55,76]. Additionally, BPs have been used to treat bone fragility disorder, an osteoclast-mediate osteoporosis [77,78]. Multiple publications have focused on short-term benefits of bisphosphonate use in horses. However, long term studies investigating potential long-term adverse effects are lacking.

During the 2019 American Association of Equine Practitioners convention, a roundtable discussion covered the extra-label use of BPs by equine practitioners. Participants indicated BPs were being used for various conditions with radiographic or nuclear scintigraphic abnormalities of the sacroiliac area, pelvis, or limb [79]. Participants described frequent BP administration (ex., three full doses in a month); despite the manufacturer's recommendation of a six-month separation between doses [38]. Researchers have raised concern about the extra-label use of BPs [5], especially in younger horses where bone turnover is significantly higher in individuals under 24 months of age [80].

THE USE OF BISPHOSPHONATES IN YOUNG/EXERCISING ANIMALS

Racehorses often start training and racing at 2 years of age. There is evidence that improper training and management is more of a factor in skeletal injury than age [81], as high-performance exercise may result in progressive microdamage accumulation, potentially leading to stress fractures (SF) [82]. Stress fractures have been associated with a high remodeling rate, leading to bone weakness and accumulation of microdamage over time [83]. It is believed BPs may be useful in preventing athletic SF due to their antiresorptive properties [84]. However, there is no conclusive evidence indicating SF healing by BPs [85], and their use in this condition is not recommended [86]. In truth, bone modeling and remodeling are complex processes especially when growth and exercise intersect. SF have been associated with normal remodeling and high strains, or normal strains with decreased remodeling [87]. Even though it is not clear what pathophysiological mechanism prevails in racehorses, any interruption in normal osteoclast resorption could be harmful and lead to damage accumulation over time.

In horses, common skeletal conditions, such as dorsal metacarpal disease, commonly known as bucked shins, and sesamoiditis, have been treated with BPs for their perceived skeletal and analgesic effects [4]. Dorsal metacarpal disease results from microfractures on the metacarpal cortical area, and sesamoiditis is the result of disease or osteolysis of the vascular channels of the proximal sesamoid bones [5]. It is believed that BPs may prevent pain and radiographic evidence of these pathologies [4,5]. However, the resolution of radiographic evidence of disease may be accompanied by detrimental effects in juvenile horses, where increasing bone density may not equate to increased bone strength. Further, impairing osteoclast function may harm normal bone remodeling and healing necessary for juvenile horses under high-performance exercise [5].

Bone turnover can be affected by exercise, and BPs can influence physiological adaptation to exercise. Bone resorption increases in response to acute exercise [88]. In long-term exercise, there is a BMD increase, indicating that prolonged exercise can be an osteogenic stimulus [88]. The increased mechanical load under exercising conditions induces osteoclast activation that can result in increasing serum markers of bone remodeling, such as CTX-I [88]. However, serum bone remodeling markers are not strong predictors of bone formation and/or resorption in human subjects [88]. For example, calves subjected to sprints 1 to 5 times per week have increased fracture force and dorsal width of their fused metacarpus compared to a non-exercise group, but no differences in CTX-I were detected between groups [89]. On the other hand, procollagen type II C-propeptide (CPII) and CTX-I increase as a response to exercise and bone turnover in foals [90]. In humans, BPs may reduce serum bone markers over time [91–93]. In horses, conflicting reports exist regarding the effect of BPs on CTX-I [40,56,76,94]. In conclusion, BPs may alter the normal skeletal adaptation to exercise, and assessment of the antiresorptive effects of BPs through serum bone markers is likely insufficient if performed alone. Future studies should consider new, comprehensive approaches to evaluate BP effects including measuring bone mineral density, fracture healing, and biomechanical testing whilst simultaneously determining BP concentration within the bone. In addition, advanced imaging such as micro-computed tomography (μCT) and Positron Emission Tomography (PET) CT may be warranted.

The use of BPs may have a greater impact on young horses due to their active growth, where osteoclasts play a significant role in the endochondral ossification process [13,19]. Osteoclasts are abundantly present in growth epiphyseal plates up to 2 years old [80]. The extra-label use of BPs in young animals could impair physiological bone development in this

population [80,95]. This has been demonstrated in a rabbit model where BP administration caused a 3% decrease in the length of the tibia [96]. Hence, BPs use in young animals could pose a significant risk to skeletal growth and/or adaptation to exercise, resulting in microdamage accumulation in juvenile horses without degenerative bone disorders.

BISPHOSPHONATES AND FUTURE STUDIES

Multiple animal models have already been used to investigate BP including mice, rabbits, mini-pigs, dogs, and sheep [63–66,71,96]. The authors recognize the ethical concerns around using animals for research purposes. However, some animal models may be particularly useful depending on the research goals and prior studies available. In particular, the sheep model has proven to be a reliable orthopedic model for human BP use. Sheep have a similar body weight and skeletal size to humans, procedures such as bone biopsies and blood sampling are simple, they are easy to handle, and large numbers of animals are usually available [97–100]. Furthermore, sheep can be trained to undergo forced exercise [101], making sheep a suitable animal model for investigating potential BP-associated bone changes under different exercise regimens. Although animal models have been used to investigate long and short-term BP effects with a focus on human health, few studies are available to guide equine use especially in juvenile and exercising populations. Future studies may include experimental large animal models of BP use which incorporate exercise to mimic athletic training. Specifically, terminal ovine models may allow for mechanical testing, advanced imaging, and analysis of long-term BP retention in bone and other organs. These studies, coupled with focused equine experimental trials, prospective and retrospective studies would provide a more comprehensive explanation of the benefits and risks of BP use in the horse.

To date, a single, large, retrospective study has evaluated the efficacy and safety of

tiludronate in 1,804 horses; 343 horses were followed for greater than 1 year [102]. The study revealed a low incidence of short-term adverse effects (1.3%), with colic-like symptoms being the most frequent. Less than 20% of horses were treated for navicular syndrome, confirming the extra-label use of BPs. Between one and nine doses of tiludronate were administered to horses included in the study [102]. Treated horses ranged in age from 2 years old to 26 years old. Future retrospective studies would ideally report diagnosis, age at administration, number and frequency of doses, long-term follow-up, concurrent treatments and evidence of disease progression.

Future prospective studies will ideally look beyond serum biomarkers and report multiple clinical and experimental parameters. These could include physical and lameness examinations coupled with bone biopsies, synovial fluid analysis, advanced imaging and biomechanical testing. Veterinarians, owners and researchers alike would benefit from a better understanding of the half-life of BPs within the skeleton and the physiologic factors such as age and exercise which may change the half-life of BPs. Tiludronate has been previously measured in tuber coxae biopsies, a relatively non-invasive location for bone biopsy [103]. Tiludronate can be detected with ultra-high-performance liquid chromatography-high-resolution mass spectrometry for up to three years in plasma and urine samples and clodronate was detected in bone 18 months following administration in a single horse in a single study [72,73]. However, long-term presence of BPs in bone in a large clinical population is currently unreported [72]. Little information is currently available to guide frequency of dosing to ensure clinical efficacy and safety.

The pain-relieving effects of BPs are still being investigated. Although pain relief may be a clinical benefit, it could also result in further injury especially in high performance athletes. BPs have been detected in the synovial fluid after systemic administration [39]. Further investigation is necessary to understand the potential anti-inflammatory effects of BPs

systemically and within in the joint environment. This can be accomplished through *in vitro* studies, *in vivo* animal models of pain and inflammation, and clinical studies [104].

CONCLUSION

Bisphosphonates are well-known for their antiresorptive properties, impairing osteoclast functionality. In 2014, two bisphosphonates (clodronate and tiludronate) were approved by the FDA to treat navicular syndrome in horses over four years of age. Several *in vitro,* animal models, and human studies indicate that bisphosphonates may have anti-inflammatory and pain-relieving effects, which has led to extra-label use of these drugs for other conditions and in juvenile horses. Although there may be therapeutic effects, there are concerns regarding impairment of normal physiological functions (growth, bone repair, and bone remodeling) especially in juvenile and exercising animals. Additional research must focus on the identifying the short-term and long-term effects of bisphosphonates in young and exercising animals to ensure the efficacious and judicious use of this powerful, long-lasting group of drugs.

CHAPTER 2: Clodronate disodium is neither cytotoxic nor cytoprotective to normal and recombinant equine interleukin-1β-treated joint tissues in vitro

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ABSTRACT

Objective: To determine the effects of clodronate disodium (CLO) on control and recombinant equine interleukin-1β (IL-1β)-treated equine joint tissues.

Study design: *In vitro* experimental study.

Sample population: Cartilage explants, chondrocytes, and synoviocytes (n = 3 horses).

Methods: Monolayer cultures of chondrocytes and synoviocytes from three horses were subjected to: control media (CON), 5 ng/ml CLO (C/low), 50 ng/ml CLO (C/med), 100 ng/ml CLO (C/high), with and without IL-1β, and 10 ng/ml IL-1β (IL) alone for 72 hours. Cartilage explants from three horses were subjected to CON, IL, C/low, and C/med with and without IL-1β for 72 hours. Culture media was analyzed for prostaglandin-E_2 (PGE_2), interleukin-6 (IL-6), and nitric oxide (NO). Explant media was analyzed for glycosaminoglycan (GAG) content and NO. At 72 hours, explant and monolayer culture viability were assessed, and explant GAG content was measured.

Results: IL-1β treatment resulted in higher media concentrations of GAG, NO, PGE_2, and IL-6 compared to the CON treatment ($P < 0.05$), demonstrating a catabolic effect of IL-1β on explants and monolayer cultures. CLO treatments did not increase media concentrations of GAG, NO, PGE_2, or IL-6 compared to CON, indicating no cytotoxic effect. Nevertheless, CLO

treatments administered to IL-1β-treated monolayer cultures and explants did not significantly reduce the inflammatory response regardless of concentration.

Conclusion: CLO did not demonstrate cytotoxic nor cytoprotective effects in normal and IL-1β-stimulated chondrocytes, synoviocytes or explants in culture.

Clinical significance: This study does not support the use of CLO as an anti-inflammatory treatment. Further research is necessary to confirm any anti-inflammatory effects of CLO on joint tissues.

CHAPTER 3: Pharmacokinetics and plasma protein binding of a single dose of clodronate disodium are similar for juvenile sheep and horses

F.B. Vergara-Hernandez, B.D. Nielsen, J.J. Kottwitz, C.L. Panek, C.I. Robison, B.L. Paris, T.H. Welsh Jr., A.N. Bradbery, J.L. Leatherwood, A.C. Colbath, Pharmacokinetics and plasma protein binding of a single dose of clodronate disodium are similar for juvenile sheep and horses, Amer J Vet Res. 84 (2023) 1–7. https://doi.org/10.2460/ajvr.23.03.0051.

ABSTRACT

Objective: To determine the single-dose pharmacokinetics of clodronate disodium (CLO) in juvenile sheep and the plasma protein binding (PPB) of CLO in juvenile sheep and horses.

Animals: Eleven juvenile crossbred sheep (252 ± 6 days) for the pharmacokinetic study. Three juvenile crossbred sheep (281 ± 4 days) and three juvenile Quarter Horses (599 ± 25 days) for PPB analysis.

Methods: CLO concentrations were determined using liquid chromatography-mass spectrometry. Pharmacokinetic parameters were calculated by noncompartmental analysis from plasma samples obtained at 0, 0.5, 1, 3, 6, 12, 24, 48, and 72 hours after CLO administered IM at 0.6 mg/kg. PPB was determined using equine and ovine plasma in a single-use rapid equilibrium dialysis system.

Results: The mean and range for maximum plasma concentration (C_{max}: 5,596; 2,396–8,613 ng/mL), time of maximal concentration (T_{max}: 0.5; 0.5–1.0 h), and area under the curve (AUC_{all}: 12,831; 7,590–17,593 h × ng/mL) were similar to those previously reported in horses. PPB in sheep and horses was moderate to high, with unbound fractions of 26.1 ± 5.1% in sheep and 18.7 ± 7.5% in horses, showing less than a 1.4-fold difference.

Clinical relevance: The pharmacokinetic parameters and PPB of CLO in juvenile sheep

were similar to those previously reported in horses. The results suggest sheep may be an appropriate animal model for studying the potential risks and/or benefits of bisphosphonate use in juvenile horses.

INTRODUCTION

Bisphosphonates (BPs) have been used for over 40 years in human medicine and their use in veterinary medicine has gained interest in the last 10–20 years due to their effects on osteoclast inhibition [5]. In 2014, two BPs, tiludronate disodium ($C_7H_9ClO_6P_2S$) and clodronate disodium ($CH_2Cl_2Na_2O_6P_2$), were approved by the Food and Drug Administration under the New Animal Drug Application (FDA NADA) 141-420 (tiludronate disodium) and 141-427 (clodronate disodium [CLO]) for the treatment of navicular syndrome in horses four years of age and older [38,74]. BPs' ability to impair osteoclast function [24] with possible concomitant pain-relieving effects [57] make them potentially useful for a myriad of musculoskeletal conditions, including fetlock and distal tarsal osteoarthritis [55,76] and thoracolumbar vertebral disease [54]. After administering BPs, the drug that is not absorbed by the skeleton is excreted unchanged by the kidneys [105]. In veterinary medicine, clinicians have raised concern over adverse effects from extra-label use in young and exercising horses including impediment of growth, masking of pain, and fracture predisposition, in addition to known potentially adverse effects such as renal toxicity and gastrointestinal discomfort [4,5,106].

Sheep have been used to assess the efficacy of BPs for osteoporosis and to investigate adverse effects experienced by humans such as necrosis of the jaw and atypical femoral fractures [64,107–110]. In addition, sheep have been used in exercise studies as a model for horses [101]. Therefore, juvenile sheep may be suitable for investigating the effects of BPs under exercise, providing a model to assess the effects of BPs in juvenile, exercising horses.

To use sheep as a model for juvenile, exercising horses, an appropriate dose of CLO must be determined. The ideal dose would result in pharmacokinetic and plasma protein binding profiles similar to those observed in horses. Pharmacokinetics (PK) focus on how drugs enter the body, travel to their site of action, and are removed from the organism [111]. Drug accumulation and elimination rates from an organism are determined by the absorption, distribution, metabolism, and excretion of a specific drug over time [112]. The quantification of these parameters help establish a safe and effective dosage of a drug [112]. Plasma protein binding (PPB) determines the fractions of drug bound (f_b) and unbound (f_u) to plasma proteins in the blood, which influence the volumes of distribution (Vd) [113] and clearance (CL) of drugs [114–116]. Not evaluating PPB leads to misinterpretations of the total drug plasma concentration, as f_u is directly associated with the CL of the total drug plasma concentration [117]. Hence, differences in PPB values between species may affect the safety margin of drugs, leading to potential incompatibility between species [118]. The PK parameters of a commercially-available, intramuscular (IM) formulation of CLO have been published for horses [38,39,119] but not sheep, and the authors are unaware of studies that describe the PPB of CLO in sheep or horses.

The current study compared the PK of a single dose of CLO (0.6 mg/kg IM) in juvenile sheep and determined the PPB of CLO in both sheep and horses. The CLO dose of 0.6 mg/kg IM was based on a preliminary study [120]. We hypothesized that a single 0.6 mg/kg dose of CLO administered IM in sheep would lead to similar PK parameters as a single dose of CLO administered at 1.8 mg/kg IM in adult horses. Furthermore, we hypothesized that there would be less than a 5-fold difference between the fu of CLO in sheep and horses.

METHODS

Sheep

The animal use experimental protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (202000264). All sheep were obtained from an established flock at the MSU Sheep Teaching and Research Center (Lansing, MI).

Eleven juvenile crossbred (Dorset × Polypay, 76 ± 8 kg, 252 ± 6 days of age, five castrated male and six female) sheep were used in the PK study. A juvenile sheep was defined as an animal that had reached approximately 80% of its adult weight.29,30 All sheep had a physical examination, including heart rate (HR), respiratory rate (RR), and rectal temperature (RT), prior to sample collection.

Three additional sheep (Dorset × Polypay, 68 ± 11 kg, 281 ± 4 days of age, one castrated male and two females) were used as PPB study subjects; these sheep had never received CLO and had a physical examination prior to the sample collection.

Three additional sheep (Dorset × Polypay, 68 ± 11 kg, 281 ± 4 days of age, one castrated male and two females) were used as PPB study subjects; these sheep had never received CLO and had a physical examination prior to the sample collection.

All sheep were housed in a 21.6 m^2 pen in an indoor facility at the MSU Bennett Road Farm. Sheep underwent a 2-week acclimation period prior to sampling. All sheep had free access to a 90% dry matter (DM) total mixed ration that contained chopped hay (82%), a corn/soybean blend (16.5%), and 1.5% of a mineral blend (Caledonia Farmers Elevator). The sheep ingested approximately 2.0-2.3% of their body weight (BW) in DM per day and had *ad libitum* access to water.

Horses

The animal use experimental protocols were approved by the Texas A&M Institutional Animal Care and Use Committee (2019-0325). All horses were obtained from Texas A&M Dick Freeman Arena (College Station, TX).

Horses were group housed in dry lots at the time of sample collection for PPB. Three juvenile horses (Quarter Horses, 411 ± 18 kg, 599 ± 25 days of age, one castrated male and two females) were used for the PPB. A juvenile horse was defined as an animal that had reached approximately 80% of its adult weight [121]. Horses were fed coastal Bermuda grass hay *ad libitum* and supplemented twice daily with 1.4 kg of a 12% protein, 8% fat commercially available concentrate. All animals were used as part of an equine behavior and training course and health status was monitored daily.

Pharmacokinetic study dose selection

The CLO dose was determined by a preliminary study in which twelve adult sheep were administered three different single doses of CLO (n = 4/treatment group: 0.6, 1.8, 3.0 mg/kg IM respectively) [120]. Preliminary study findings suggested a single dose of 0.6 mg/kg IM resulted in similar plasma concentrations of CLO as reported in mature horses for 48 hours following administration [38,120].

Pharmacokinetic study design

Sheep were moved through a chute system in a randomized order and restrained in a crate for sampling. Ten milliliters of blood were collected from the right jugular vein using an 18-gauge needle and vacutainer prior to single administration (hour 0) of CLO (OSPHOS®, Dechra Veterinary Products) at 0.6 mg/kg IM in the right side of the neck. An additional 10 mL of blood was collected from the right jugular vein at 0.5, 1, 3, 6, 12, 24, 48, and 72 h in the same

collection order established at hour 0. Blood was collected in a K_2 EDTA blood collection plastic tube and immediately placed on ice for transport followed by sample centrifugation at 2,000 × *g* for 10 min.

Plasma was aliquoted into 2.0 mL microcentrifuge tubes and frozen at -80 ºC until analysis. All samples were analyzed within four months of collection. Animals were monitored hourly during the first six hours and then daily following drug administration for acute adverse effects (e.g., syncope and sudden death), side effects described in other species (e.g., gastrointestinal discomfort and/or swelling at the injection site), and/or changes in normal behavior (e.g., agitation and depression) [22,38,59].

Bioanalytical methods

The liquid chromatography-mass spectrometry (LC-MS/MS) analysis was based on the methods of Hasan and colleagues.[122] Samples were removed from -80 ºC and thawed to room temperature. An internal standard of 20 µL (10 ng/µL of etidronate solution, Sigma-Aldrich) was added to 1.0 mL of plasma. Then, 200 µL of perchloric acid (10%) was added to precipitate proteins. The solution was thoroughly mixed using a vortex mixer and centrifuged at 17,000 × *g* for 10 min. The supernatant was evaporated until visibly dry under a stream of nitrogen at ~65 ºC, then dissolved in 150 µL glacial acetic acid and mixed with 500 µL trimethyl orthoacetate. The solution was incubated at 100 ºC for 30 min for derivatization. Samples were allowed to cool to room temperature and then 300 µL formic acid and 500 µL DI water were added. The solution was then transferred to a screw-top tube, followed by liquid-liquid extraction with methyl tert-butyl ether. The tube was capped, placed in a rotorack for 10 min, and centrifuged at 12,000 × *g* for 5 min. The bottom layer was removed by aspiration. The supernatant was transferred to a new glass tube and evaporated until visibly dry under a stream of nitrogen at ~65

ºC. The residue was reconstituted in 80 µL of a 1:1 methanol-water mixture and transferred to an autosampler vial (Zorian, Inc.) for the LC-MS/MS analysis. Plasma clodronate concentrations were quantified by LC-MS/MS analysis conducted in a Thermo TSQ Altis™ (ThermoFisher Scientific) triple quadrupole mass spectrometer with an electrospray ionization source, with a lower limit of detection (LOD) of 10 ng/mL for CLO.

Pharmacokinetics data analysis

Non-compartmental PK parameters were determined using commercially available software (Phoenix WinNonLin 8.3). The PK parameters included the time of maximum concentration (T_{max}), maximum concentration (C_{max}), the slope of the second phase (slow distribution phase) of the drug's concentration curve (λ_z), terminal half-life ($t_{1/2\lambda}$), area under the curve estimated to the last observation (AUC_{all}), area under the curve extrapolated to infinity ($AUC_{0\text{-}inf}$), and mean residence time (MRT).

Plasma protein binding assay

Twenty milliliters of blood were collected from the jugular vein of sheep and horses using an 18-gauge with a hub. Blood was immediately placed in a K_2 EDTA blood collection plastic tube and placed on ice for transport. Plasma was harvested and stored as previously described for no longer than three months. Plasma samples were thawed at room temperature and CLO (pharmaceutical grade, Millipore Sigma) was added to 2 mL of plasma to obtain the desired concentrations for sheep (0, 100, 1,000, 10,000, 20,000, 40,000 ng/mL) and horses (0, 100, 1,000, 7,500, 10,000, 15,000 ng/mL). These concentrations were selected based on our preliminary sheep study (C_{max} 11,605 ng/mL) [120] and a previously disclosed equine study (C_{max} 7,460 ng/mL) [38]. A single-use rapid equilibrium dialysis (RED®, ThermoFisher Scientific) system was used according to the manufacturer's recommendations.

In brief, 500 µL of the spiked samples were added to the plasma chamber and 750 µL of 1X PBS were added to the buffer chamber. The kit was covered with a sealing plate and incubated at 37 ºC for 6 hours on an orbital shaker at 250 rpm. The content of the plasma chamber and buffer chamber were then pipetted into separate microcentrifuge tubes and stored at -80 ºC. Samples were diluted with distilled water to increase the volume for laboratory analysis. Each biological replicate was measured in duplicate as a technical replicate. The CLO concentrations within both chambers were calculated using LC-MS/MS as described. The technical replicates for each biological replicate were averaged to create an individual data point for the analysis. The percentage of the unbound drug was calculated as follows: %free drug = (drug concentration buffer chamber) / (drug concentration plasma chamber) × 100.

Statistical analysis

Normality of the PK data was assessed by a Shapiro-Wilk test using Phoenix WinNonLin 8.3. Normally distributed data including physical examination, PPB, and PK parameters, except for Tmax, were reported as means and ranges. Non-normally distributed data (T_{max}) were reported as median and range.

RESULTS

Pharmacokinetics of clodronate in sheep

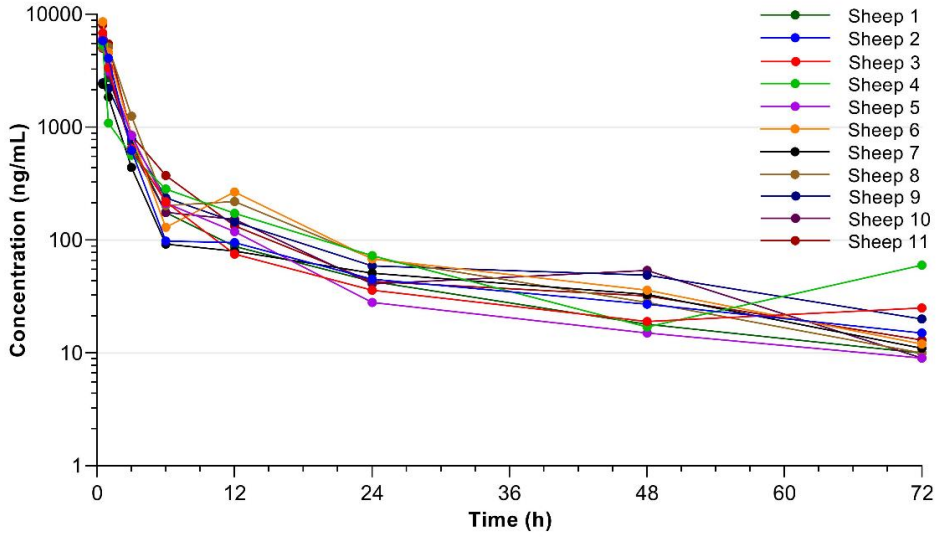
Sheep were determined to be bright and alert prior to PK sampling. Physical examination parameters reflected mild stress associated with temporary restraint: HR (138 [102-160] beats per min), RR (97 [60-132] breaths per min), and RT (39.5 [39.1-39.8] ºC]). No animals or data were excluded from the analyses. No adverse effects were detected following the administration of CLO to juvenile sheep (n = 11).

Mean values of CLO plasma concentration in sheep over time are presented (Figure 3).

Following IM administration of CLO, the T_{max} was reached at 0.5 (0.5-1.0) h post-administration, with a C_{max} of 5,596 (2,396-8,613) ng/mL. The λ_z was 0.034 (0.023-0.042) 1/h, $t_{1/2\lambda}$ reached 21.2 (16.4-30.3) h with an AUC_{all} of 12,831 (7,590-17,593) × ng/mL, and $AUC_{0\text{-inf}}$ of 13,334 (7,947-17,973) × ng/mL (Table 2).

Figure 3. Plasma clodronate disodium concentration (ng/mL) time curves over time (72 h) after a single intramuscular administration of 0.6 mg/kg (OSPHOS®) in 11 juvenile sheep.

Table 2. Plasmatic pharmacokinetics (PK) parameters (mean or median [T_{max}] and range) for clodronate disodium (OSPHOS®) following single-intramuscular administration in juvenile sheep (0.6 mg/kg) determined through liquid chromatography-mass spectrometry and non-compartmental analysis compared to a previously published single dose (1.8 mg/kg) PK study in mature horses [119].



Parameter	Unit	Mean		Range	
		Sheep (n = 11)	Horse (n = 7) [119]	Sheep (n = 11)	Horse (n = 7) [119]
T_{max}	h	0.5	0.5	0.5-1.0	0.25-0.75
C_{max}	ng/mL	5,596	4,155	2,396-8,613	1,969-5,541
λ_z	1/h	0.034	0.002	0.023-0.042	0.0001-0.016
$t_{1/2\lambda}$ †	h	21.2	141.7	16.4-30.3	43.1-6,814.8
AUC_{all}	h × ng/mL	12,831	11,564	7,590-17,593	6,393-17,026
$AUC_{0\text{-inf}}$	h × ng/mL	13,334	11,912	7,947-17,973	6,507–17,606
$MRT_{0\text{-inf}}$	H	12.1	-	8.1-22.3	-

Abbreviations: $AUC_{0\text{-inf}}$ = area under the curve extrapolated to infinity. AUC_{all} = area under the curve to the last observation. C_{max} = maximal concentration. λ_z = slope of the distribution phase. $MRT_{0\text{-inf}}$ = mean resident time from 0 to infinity. $t_{1/2\lambda}$ = terminal half-life. T_{max} = Time of maximal concentration. †Harmonic mean

Plasma protein binding

The mean percentage of unbound CLO in sheep plasma was 26.1 ± 5.1% (n = 3 for each of 5 concentrations) from 100 to 40,000 ng/mL of CLO (Table 3). The mean percentage of unbound CLO in horse plasma was 18.7 ± 7.5% (n = 3 for each of 5 concentrations) from 100 to 15,000 ng/mL of CLO (Table 3).

Table 3. *In vitro* unbound fraction (f_u, mean ± SD) of clodronate disodium (ng/mL) in juvenile sheep and horses. The table indicates the unbound clodronate disodium fractions in sheep plasma (0, 100, 1,000, 10,000, 20,000, 40,000 ng/mL, n = 3) and horse plasma (0, 100, 1,000, 7,500, 15,000 ng/mL, n = 3), determined using single-use rapid equilibrium dialysis and liquid chromatography-mass spectrometry analysis. (ND, not determined, <LOD, below lower level of detection of 10 ng/mL).

	Clodronate disodium concentrations (ng/mL)								
	0	100	1,000	7,500	10,000	15,000	20,000	40,000	Average f_u (%)
Ovine (%)	<LOD	26.1 ± 0.1	34.5 ± 0.1	ND	25.5 ± 0.1	ND	22.7 ± 0.1	21.7 ± 0.1	26.1 ± 5.1
Equine (%)	<LOD	26.4 ± 0.1	21.9 ± 0.1	23.7 ± 0.1	11.8 ± 0.0	9.8 ± 0.0	ND	ND	18.7 ± 7.5

DISCUSSION

Sheep have been used as a model to evaluate potential adverse effects of BPs in humans [64,107–110] and exercise on horses [101], suggesting that sheep may be a useful model species to evaluate the effects of BPs in juvenile, exercising horses. The PK and PPB of CLO must be similar between sheep and horses for sheep to be a reasonable model for exercising horses. Therefore, this study sought to determine the PK of CLO in sheep and determine the PPB of CLO in horses and sheep. Sheep PK parameters, including C_{max}, T_{max}, AUC_{all}, and $AUC_{0\text{-}inf}$, were similar to the horse's PK parameters, and f_u in sheep was less than a 5-fold difference from the f_u of horses. These results support sheep as an appropriate model for CLO administration in horses [118].

This study measured the PK response in juvenile sheep over 72 hours in order to compare with recently available data in horses [119]. The PK parameters such as C_{max}, T_{max}, AUC_{all}, and

$AUC_{0\text{-}inf}$ were similar between sheep and recent findings by Knych and colleagues for horses [119], providing evidence that a 0.6 mg/kg IM dose in sheep may be appropriate to model a 1.8 mg/kg IM dose in horses. Despite the total duration of the PK study being longer (182 days) and the assay sensitivity being greater in the study by Knych and colleagues [119], our study reports sheep blood concentrations of CLO which appear to mirror what is reported in the horse for the initial 72 hours following administration. Although there are no clear criteria to define equivalent PK parameters between species, previous studies have suggested similar PK parameter values with up to a ~40% difference in AUC and C_{max} [123–125]. The current study indicates that sheep had 10% and 26% higher values for AUC_{all} and C_{max}, respectively, in comparison to published equine data [119], further supporting the validity of CLO administration in sheep as a model for the horse. A statistical analysis was not performed comparing sheep PK parameters with historical PK parameters in horses due to the multiple limitations of utilizing historical data. However, an a priori sample size analysis had indicated 6 animals would be needed in each group to achieve a power of 0.8 with a mean difference between groups of 4,600 h × ng/mL and SD of 2,500 h × ng/mL. A post-hoc power analysis comparing the AUC_{all} reported previously from 7 horses [119] to the AUC_{all} from the current study of 11 sheep, results in a power of 0.14, and a post hoc sample size analysis indicates that 81 animals would be required to obtain a power of 0.8 using the mean difference of 1,267 h × ng/mL between the AUC_{all} for sheep and that reported in horses [119]. The decreased post-hoc power and increased post-hoc sample size are reflective of the similarity found between the AUC_{all} for sheep and horses.

The majority of PK parameters between sheep and horses were similar, however, $t_{1/2\lambda}$ and λ_z were the exception. Differences in $t_{1/2\lambda}$ and λ_z between our study and the study by Knych and colleagues [119] may be explained by differences in sample analysis and study durations. The

study by Knych and colleagues had a more sensitive limit of quantification (0.1 ng/ml) than our study, allowing them to report the terminal phase of elimination of CLO [119]. Our analytical technique was not able to detect the terminal phase of elimination as reported by Knych et al. [119]. Instead, our study determined the slope of the drug slow distribution phase or second phase. Future studies should include additional sample times and more sensitive methods of BP detection in order to determine the terminal phase of elimination in sheep. This analysis would help to elucidate the reattachment and recirculation of CLO over time associated with bone turnover [126]. This would be particularly interesting to determine in exercising and growing animals which may experience frequent bone turnover and recirculation [119].

Physiologic differences between sheep and horses may provide an alternative explanation for the differences in λ_z and $t_{1/2\lambda}$ between studies [127]. These PK parameters have inverse proportionality ($t_{1/2\lambda} = 0.693 / \lambda_z$), where λ_z is dependent upon the drug elimination from the body, CL, and the ability of the drug to distribute to extravascular tissues, Vd ($\lambda_z = CL / Vd$) [113,127,128]. Vd can be considered a proportionality constant between the amount of drug in the body and plasma concentrations at a given time [113]. Different versions of Vd may be used as the proportionality ratio may have different values depending on the state of drug disposition [113] BPs largely bind to bone [129], and horses have significantly more bone mass than sheep (horse: 12-15% and sheep: 5.5% of BW) [130–132]. This increased bone mass may increase the terminal phase volume of distribution (Vd_{area}) in horses (compared to sheep) resulting in a lower λ_z and increased $t_{1/2\lambda}$ [113]. However, Vd_{area} of CLO cannot be measured in this study as this drug was not administered intravenously [113] in the previously published horse data [119] or the present sheep data. Therefore, the discussion of Vd must be theoretical in nature. This study illustrates that horses have a higher, although similar, PPB for CLO when compared to sheep.

Increased PPB should have the opposite effect on Vd, leading to decreased Vd and resulting in an increase in λ_z and lower $t_{1/2\lambda}$. Therefore, PPB is unable to explain the differences in λ_z and $t_{1/2\lambda}$ between sheep and horses. Although $t_{1/2\lambda}$ and λ_z are important PK parameters, they have the greatest influence on the dosing regimen of drugs [133]. As CLO is administered at large dosing intervals [38], these PK parameters may be less important for determining the viability of sheep as a model for horses. Similarities in the maximum concentration (C_{max}) and exposure to the drug (AUC_{all}) are arguably more relevant.

Two additional studies have analyzed the PK parameters of IM clodronate administration in horses [38,39], but neither provide the ideal comparison for the current study. The FDA NADA for OSPHOS® describes both a single-dose and multi-dose study [38]. Unfortunately, in the single-dose administration study, the maximal proposed dose (900 mg) was administered to each animal without consideration of the animal's weight. Further, the study reported only mean values for limited PK parameters [38]. A PK study was then conducted during the multi-dose safety assessment where CLO was administered every 28 days at 1.8 mg/kg IM; the PK study was conducted at day 84 following the fourth dose [38]. The PK parameters reported in this multidose study in horses were similar to our ovine study, including AUC_{all} (12,150 ± 1,830 h × ng/mL), C_{max} (5,360 ± 980 ng/mL), and T_{max} (0.33 ± 0 h), which could further support the use of sheep as a model for horses [38]. However, the multidose study FDA NADA for OSPHOS® had a shorter sample time (48 h), different methodology of CLO measurement (gas chromatography-mass spectrometry), and shorter $t_{1/2\lambda}$ in horses (1.7 ± 0.5 h) [38], making it difficult to propose a meaningful comparison to our study. The authors are aware of one additional PK study of CLO in horses [39]. However, that study returned markedly lower plasma CLO concentrations (e.g., AUC_{all}: 703 ± 158 h × ng/mL) in comparison to other published reports [39], possibly due to the

frequent administration of xylazine to facilitate arthrocentesis [39]. Xylazine increases osmotic diuresis, increasing CLO excretion [134,135]. Therefore, considering the multidose study used in the FDA NADA for OSPHOS® [38] and the administration of xylazine by Kreuger et al. [39], the authors recognize that the historical data obtained by Knych and colleagues [119] is most appropriate as a comparison to our ovine PK study.

The current study also presents novel information regarding the PPB of CLO in horses and sheep. The concentrations tested for the sheep (100-40,000 ng/mL) and horses (100-15,000 ng/mL) encompass the CLO concentrations observed after 0.6 mg/kg IM single administration in sheep and 1.8 mg/kg IM single administration in horses [119]. The PPB has been defined as high if less than 20% of the drug is unbound to plasmatic proteins, as moderate PPB if between 20-60% is unbound, and as low PPB if more than 40% is unbound [136]. Our results indicated a moderate to low f_u of CLO in both species. Although differences between sheep and horses were found, these differences were modest (not greater than 1.4-fold), suggesting that the plasmatic binding of CLO was similar between the two species. These findings further highlight the usefulness of sheep as an animal model for studying the effects of CLO and its potential implications for horses.

The main limitation of the present study is the lack of a parallel PK study in juvenile horses with an identical duration and assay sensitivity. In addition, measuring CLO excreted in urine would have helped to more accurately determine renal CL and Vd_{area} for CLO in sheep [113,137], as BPs are mainly excreted in urine [105]. Future studies could also include biochemical profiles, urinalysis, and kidney histological assessment to identify adverse renal effects of CLO administration in sheep [129]. Bone biopsy samples would be valuable for determining the concentration of CLO absorbed in cortical and trabecular bone at different

locations within the skeleton and could be performed under different physiologic conditions, such as routine exercise or growth.

Despite these limitations, this study provides valuable insights into the PK properties of CLO in juvenile sheep and its potential as an animal model for assessing the effects of BPs in horses. Our findings demonstrated that a 0.6 mg/kg IM administration of CLO (OSPHOS®) in sheep resulted in similar PK parameters (C_{max}, T_{max}, AUC_{all}, and $AUC_{0\text{-}inf}$) and PPB values when compared to 1.8 mg/kg IM single administration of CLO (OSPHOS®) in horses, without any measurable adverse effects. Therefore, an ovine model of CLO administration presents a promising and previously unexplored approach for assessing and translating the effects of CLO on juvenile exercising horses.

CHAPTER 4: Exercising sheep as a novel pre-clinical model for musculoskeletal research

ABSTRACT

Objective: To establish an orthopedic, pre-clinical, ovine model of controlled exercise using an equine walker.

Animals: 20 Dorset-Polypay sheep.

Procedures: Sheep underwent 11-weeks of group exercise, four days per week. Exercise duration and intensity increased until sheep performed 25 min walking (1.3 m/s) and 5 min trotting (2.0 m/s). Physical/lameness examinations were conducted every 14 days. Blood was collected every 28 days for analysis of serum bone biomarkers (SBB): bone alkaline phosphatase (BALP); procollagen type I amino-terminal propeptide (PINP); carboxy-telopeptide of type I collagen cross-Links (CTX-I); tartrate-resistant acid phosphatase 5b (TRAP5b); receptor activator of nuclear factor-κβ ligand (RANKL).

Results: Sheep adapted easily to group exercise with no significant lameness. Animals grew taller ($P = 0.006$) but had a 4% weight loss ($P = 0.003$). RANKL was reduced on days 28 and 84 compared to day 56 ($P < 0.05$), CTX-1 was reduced on days 28 and 84 compared to days 0 and 56 ($P < 0.05$), and TRAP5b was greater on day 28 compared to day 0 ($P = 0.009$). BALP and PINP did not change.

Clinical relevance: The described pre-clinical model of exercising sheep has distinct advantages including ease of handling, an established lameness scale, commercially available ovine SBB assays, and the ability to alter footing characteristics and complete circular exercise. Decreasing CTX-I and RANKL with no change in BALP and PINP, suggests reduced bone resorption over the study period. Future studies may include a sedentary group or utilize adult animals to alleviate any influence of growth on SBB.

INTRODUCTION

Pre-clinical models of orthopedic disease range from small animal models (e.g., mouse, rat, rabbit) to larger animal models (e.g., dog, sheep, horse). Exercise is a critical component of many musculoskeletal disease processes, making exercise models particularly important. By controlling exercise, musculoskeletal disease processes and therapeutic interventions can be evaluated in a more clinically-relevant and precise way.

Ovine and caprine models of musculoskeletal disease are common [138–140] and offer distinct advantages. Previous studies have utilized sheep as a model for exercising horses [101] and to determine the effects of musculoskeletal interventions such as bisphosphonates [141]. Despite anatomical differences between horses and sheep (e.g., hooves, body size, digestive system), sheep exhibit similar kinetic and kinematic characteristics to horses when scaled for size [142]. Additionally, during walking, sheep have a similar percentage distribution of body weight on their limbs as horses [142]. This makes sheep a valuable orthopedic animal model for studying musculoskeletal diseases in horses. Moreover, sheep and goats may be acquired from well managed breeding sources (e.g., university farms) with relatively uniform genetics and are easy to handle. Nevertheless, ovine exercise models have remained time-consuming, requiring several weeks of individual treadmill training and acclimation [143–145]. In addition, sheep may refuse to exercise on a treadmill [143–145]. Moreover, treadmill exercise may result in a different gait pattern than in overground outdoor conditions [146,147]. This may affect results, especially in studies assessing musculoskeletal injury and therapeutics. Both the animal model as well as the method of exercise are important factors to consider when performing musculoskeletal research.

Given the limitations of existing ovine exercise models, this study sought to assess the

feasibility of exercising a group of juvenile sheep using a high-speed walker in an exercise program of increasing duration and intensity over 84 days. Outcome measures included physical examinations, lameness evaluations, and serum bone biomarkers. The study hypothesized that sheep would complete an incremental exercise protocol with limited evidence of morbidity on physical or lameness examinations, and serum bone markers would reflect changes associated with physical activity.

MATERIALS AND METHODS

Animals and management

The animal use experimental protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (202000264). All sheep were obtained from an established flock at the MSU Sheep Teaching and Research Center (Lansing, MI).

Ten castrated male (wethers, 81 ± 5 kg) and ten female (ewes, 67 ± 5 kg) juvenile Dorset × Polypay sheep (254 ± 5 days of age) were used. Juvenile sheep were defined as an animal that had reached approximately 80% of their mature weight [148]. Sheep weight was monitored closely every 14 days, starting 20 weeks before the study began, with the goal of achieving 80% of their mature weight by the start of the study. Sheep were sheared 9 weeks prior to starting the exercise protocol. Two weeks prior to starting the study, animals were moved to the MSU Bennett Road Farm, where they were housed in an indoor pen of 21.6 m^2 with straw bedding, in similar housing conditions and managed by the same personnel as in their previous location. Sheep were fed a diet consisting of 90% dry matter total mixed ration (TMR) containing chopped hay (82%), a corn/soybean blend (16.5%), and a mineral blend (1.5%, Caledonia Farmers Elevator). Based on the average daily amount of TMR provided (33-37 kg), and sheep's

weights, animals consumed approximately 2.0-2.3% of their body weight (BW) in dry matter per day. The sheep had continuous access to water.

Study design

Sheep were acclimated to the holding facility, sampling crate, and the circular 20-m diameter walker (Q-Line Horse Exerciser) over a 2-week period, between 07:00-10:00 h, prior to starting the study. Sheep were moved through the chute system twice a week to acclimate them to sample collections and physical examination. Thirty-two meters of fencing connected the outside walker to the interior pens so that sheep could be moved from the barn to the walker with minimal handling. The walker used in the study consisted of four bays, each separated by safety flex push panels equipped with electric shock (8.0 kV), and a 2NS sand surface with a depth of 5 cm on an 18 cm depth base of crushed asphalt. The exercise training commenced by setting the walker to a walking speed of 1.3 m/s. Under the guidance of one of the researchers, the 20 sheep were led to the walker with minimal encouragement, four days a week. During the initial acclimation period, each sheep walked clockwise and counterclockwise on alternating days for five minutes per day, respecting the designated divisions of each bay. The electric shock stimulus was employed to train the animals to remain in one bay of the walker at a time with no complications observed. Sheep predominantly positioned themselves in the middle and back of one bay.

Exercise protocol

The study took place between June 7 and August 30 of 2021. Sheep were subjected to an exercise protocol that was created based on previous treadmill studies [101,149]. Sheep initially walked briskly for 10 min/day. The duration of exercise was increased by 5 min each week, until reaching a maximum of 30 min of exercise per day at which time a strenuous pace (2.0 m/s)

[149] was included in the middle of the workout (Table 4). Sheep were exercised four times per week, alternating between exercising clockwise and counterclockwise each day.

Table 4. Exercise protocol and estimated distance traveled for juvenile sheep on a circular 20-m diameter high-speed walker.

Week	Exercise duration	Estimated distance traveled
1	10 min (WS)	780 m
2	15 min (WS)	1,170 m
3	20 min (WS)	1,560 m
4	25 min (WS)	1,950 m
5	30 min (WS)	2,340 m
6–8	13 ¾ min (WS) → 2 ½ min (TS) → 13 ¾ min (WS)	2,445 m
9–11	12 ½ min (WS) → 5 min (TS) → 12 ½ min (WS)	2,550 m

Abbreviations: WS = walking speed at 1.3 m/s. TS = trotting speed at 2.0 m/s. Sheep were exercised 4x/week alternating between clockwise and counterclockwise directions each day. The TS bout was incorporated in the middle of the 30 min exercise protocol, starting on week 6.

Physical examinations and lameness evaluation

A physical examination was performed between 07:00 and 09:00 h on day 0 and every 14 days for the duration of the study. The height, BW, resting heart rate (HR), respiratory rate (RR), and rectal temperature (RT) of the animals were recorded during the physical examinations, prior to exercising on that day. All physical examinations were conducted with minimal handling and minimal restraint using the indoor chute system and crate scale previously described during the acclimation period in order to reduce stress. Every 2 weeks for the duration of the study, all sheep were evaluated for lameness by two veterinarians (A.C.C., F.B.V.) using a sheep subjective lameness scoring system [150]. Each veterinarian assigned each sheep a score from 0 to 6. A score of 0 indicated no lameness, a 1 indicated an irregular posture without stride shortening, and a 2 was assigned to a noticeable head nod with a shortened stride. A score of 3 was assigned to an animal that displayed significant discomfort while moving indicated by excessive head flicking and stride shortening, a 4 indicated a reluctance to bear weight during

movement, a 5 indicated inability to stand up and reluctance to move and a 6 indicated an animal was unable to stand or move [150].

Blood collection

On days 0, 28, 56, and 84, 20-mL blood samples were collected from each sheep through jugular venipuncture using non-heparinized serum-separator blood between 7:00 and 9:00 h. Blood was stored on ice and allowed to coagulate for 1 h prior to centrifugation at 2,000 × *g* for 15 min. Serum was aliquoted into microcentrifuge tubes and frozen at -80 °C for future analysis.

Serum analysis

Serum samples were thawed before testing. Ovine-specific enzyme-linked immunoassays (Kendall Scientific) were performed following the manufacturer's recommendations for serum concentrations of amino-terminal bone-specific alkaline phosphatase (BALP), procollagen type I amino-terminal propeptide (PINP), receptor activator of nuclear factor-κβ ligand (RANKL), cross-linked C-terminal telopeptides of type I collagen (CTX-I), and tartrate-resistant acid phosphatase isoenzyme 5b (TRAP5b) with a SpectraMax ABS Microplate Reader (Molecular Devices, LLC) at 450 nm (Table 5).

Table 5. Summary of ovine-specific serum bone biomarkers and functions [151].

Biomarker	Abbreviation	Function [151]
Bone-specific alkaline phosphatase	BALP	Bone formation and mineralization
Procollagen type I amino-terminal propeptide	PINP	Bone formation and mineralization
Receptor activator of nuclear factor NF-κB ligand	RANKL	Bone resorption; osteoclast activity
Tartrate-resistant acid phosphatase isoenzyme 5b	TRAP5b	Bone resorption; osteoclast activity
Carboxy-telopeptide of type I collagen cross-links	CTX-I	Bone resorption

Statistical analysis

Sheep physical parameters and serum bone biomarkers were analyzed using a mixed-effects model that included the fixed effects of time, sex, and the interaction between time and sex, as well as repeated measures of time with individual sheep as the subject effect. The mixed-effects model was applied using the MIXED procedure of SAS 9.4. The normality of data was assessed by diagnostic plots of residuals for each independent variable. All data, except for serum biomarkers and lameness evaluations, were normally distributed. Serum bone biomarker data were log-transformed and followed a normal distribution after transformation. Height and BW were included as potential covariates in the model, but no significant correlations were detected between them and the independent variables. Therefore, they were removed from the final model. Post-hoc multiple comparisons of least-squares mean between different levels of sex and weeks were conducted using the Tukey test. Results are reported as means ± standard deviation (SD).

Lameness evaluations were analyzed as ordinal data. Each sheep had two sets of data, one from each veterinarian, and the lameness score for each individual was averaged and rounded to the nearest integer. Due to the low prevalence of lameness with only 6 out of 120 evaluations scoring 1 or 2, the data was transformed into binary format: 0 represented the absence of lameness and 1 represented the presence of lameness (average score of 1 or 2). A logistic regression model was applied to the binary data using RStudio v.2022.07.2. Post-hoc comparisons of the estimated marginal means between weeks were conducted using the Tukey method and the R package 'emmeans'. The significance level was set at $P \leq 0.05$ for all analyses.

RESULTS

Physical examinations and lameness evaluations

All sheep were able to use the walker without any issues and all individuals completed

the exercise protocol as outlined with no alterations. Interactions between sex and time were only detected for height and BW. Thus, males and females were grouped for the analysis of HR, RR, RT, lameness evaluation, and serum bone biomarkers. No significant differences were found between the initial and final BW of males or females and this similarity was kept until the end of the study. However, the overall average BW for both sexes decreased over time ($P < 0.001$) (Table 6). No significant differences were found in the initial and final height of males or females. However, the average height for both sexes increased over time ($P < 0.001$). At the beginning of the study, males and females had no differences in height and this similarity was kept until the end of the study (Table 6).

Table 6. Initial (d 0) and final (d 84) body weight (BW) and height (H) of 20 juvenile sheep.

Sex	BW (d 0)	BW (d 84)	P-value
Wethers (kg)	81 ± 5	78 ± 5	0.34†
Ewes (kg)	67 ± 5	64 ± 6	
Average BW (kg)	74 ± 9	71 ± 9	<0.001¥
Sex	H (d 0)	H (d 84)	P-value
Wethers (cm)	69 ± 3	73 ± 3	0.44†
Ewes (cm)	67 ± 3	69 ± 3	
Average H (cm)	68 ± 3	71 ± 3	<0.001¥

Values are expressed as mean ± SD.
†P-value represents the interaction between time and sex.
¥P-value represents the fixed effect of time.

Resting HR (measured in beats per minute, BPM) was higher on days 0 and 14 in comparison to days 28, 42, 56, 70, and 84 ($P < 0.001$). Resting RR was lower on day 28 in comparison to days 42, 70, and 84 ($P \leq 0.02$). RT was mildly but significantly decreased ($P < 0.001$) on day 70 when compared to days 0, 14, 28, and 84 (Table 7).

Table 7. Physical parameters in 20 juvenile sheep evaluated every 14 days for 84 days.

Day	HR (BPM)	RR (bpm)	RT (ºC)
0	138 ± 27^{a}	68 ± 10^{abc}	39.2 ± 0.5^{ab}
14	136 ± 16^{a}	61 ± 12^{bc}	39.3 ± 0.4^{a}
28	98 ± 11^{cd}	58 ± 11^{c}	39.4 ± 0.3^{a}
42	112 ± 15^{bc}	73 ± 10^{a}	39.0 ± 0.3^{bc}
56	89 ± 15^{d}	68 ± 7^{abc}	39.0 ± 0.1^{bc}
70	114 ± 13^{b}	70 ± 12^{ab}	38.7 ± 0.3^{c}
84	83 ± 10^{d}	76 ± 15^{a}	39.1 ± 0.4^{ab}
Reference values	65-80 [152]	20-38 [153]	38.3-39.9 [154]

Abbreviations: HR = resting heart rate in beats per minute (BPM). RR = respiratory rate in breaths per minute (bpm). RT = rectal temperature in Celsius (ºC). Means followed by a common letter are not significantly different by the Tukey-Kramer test at the 5% level of significance. Values are expressed as mean ± SD.

Veterinarians reported lameness in only five animals throughout the study, and all lamenesses were considered a grade 2 or below. Only one animal was observed to be lame twice during the study. The prevalence of lameness on different study days did not change throughout the study (Table 8).

Table 8. The number of animals with and without lameness at each time point based on a subjective grading scale [150].

Day	n	Frequency	
		Animals without lameness	Animals with lameness
0	20	20	0
16	20	19	1
30	20	18	2
44	20	18	2
58	20	20	0
80[1]	20	19	1
Total observations	120	114	6

[1]Lameness evaluation was assessed on the last day of exercise.

Serum bone biomarkers

ELISA kits were validated; all kits showed percentage recoveries between 80-120% as previously described [155]. PINP data was not included in the analysis for day 56 due to laboratory error and the lack of additional serum samples. No differences were detected in bone formation and mineralization markers (BALP or PINP) throughout the study. Bone resorption markers, RANKL, TRAP5b, and CTX-1 varied throughout the study. TRAP5b was increased on

day 28 (30.7 ± 28.5 ng/ml) compared to day 0 (22.4 ± 14.1 ng/ml, P = 0.009). In contrast, CTX-I was higher (P ≤ 0.05) on days 0 (9.8 ± 2.9 ng/ml) and 56 (9.5 ± 2.5 ng/ml) compared to days 28 (7.7 ± 2.0 ng/ml) and 84 (7.3 ± 1.6 ng/ml). Further, RANKL was increased on day 56 (32.6 ± 7.0 ng/ml) compared to days 28 (25.9 ± 9.4 ng/ml; P = 0.04) and 84 (24.8 ± 11.9 ng/ml; P = 0.007, Table 9).

Table 9. Blood serum bone biomarkers in 20 juvenile sheep evaluated every 4 weeks for 84 days.

Day	Bone formation markers		Bone resorption markers		
	BALP (ng/ml)	PINP (ng/ml)	RANKL (ng/ml)	CTX-I (ng/ml)	TRAP5b (ng/ml)
0	7.7 ± 4.3	1.7 ± 1.3	31.8 ± 11.9^{ab}	9.8 ± 2.9^{a}	22.4 ± 14.1^{b}
28	9.1 ± 6.4	1.9 ± 1.2	25.9 ± 9.4^{b}	7.7 ± 2.0^{b}	30.7 ± 28.5^{a}
56[1]	10.7 ± 9.6	-	32.6 ± 7.0^{a}	9.5 ± 2.5^{a}	30.4 ± 31.7^{ab}
84	8.0 ± 5.2	1.6 ± 1.0	24.8 ± 11.9^{b}	7.3 ± 1.6^{b}	27.3 ± 31.8^{ab}

Abbreviations: BALP = bone-specific alkaline phosphatase. PINP = procollagen type I amino-terminal propeptide. RANKL = receptor activator of nuclear factor NF-κB ligand. CTX-I = cross-linked C-terminal telopeptides of type I collagen. TRAP5b = tartrate-resistant acid phosphatase isoenzyme 5b. Means followed by a common letter are not significantly different by the Tukey-Kramer test at the 5% level of significance. Values are expressed as mean ± SD.
[1]Day 56 PINP data were excluded from the analysis due to laboratory error and lack of sufficient serum samples.

DISCUSSION

Multiple large animal models are available for the investigation of musculoskeletal disease and therapeutic interventions [101,138–140,156–159]. Previous ovine models have incorporated treadmill exercise [143–145], which is labor intensive, may not reflect normal movement, and can only be performed in a straight line on a specific surface. This study aimed to evaluate the feasibility of an ovine group exercise model utilizing a commercially-available walker system. Specifically, the study aimed to evaluate the animals' ability to be trained as a group using a walker and incremental distance and speed increases. Outcome measures included physical parameters, lameness, and serum bone.

After a 2-week adaptation period, the juvenile sheep easily learned their routine, facilitating ease of handling during the study period. Subjectively, the animals displayed a

positive interest in using the walker, waiting for their pen's gate to be open and moving quickly to the walker. Sheep showed clear readiness to exercise, especially at the beginning of each week, after 96 h of rest. Each exercise session required approximately a total of 5 minutes for guiding the animals to the walker and back to their pen, and only one person was needed to handle all animals. This contrasts with previous exercise studies involving sheep, where the animals were individually trained to use a treadmill for several weeks [143–145], requiring a significant amount of time, and the potential that animals may refuse to cooperate with the exercise protocol [143–145], No sheep refused the exercise protocol in the present study. Group exercise has many advantages over individual treadmill exercise. Sheep can be highly stressed by isolation [160]. Some researchers have attempted to mitigate this stress by exercising the animals in pairs [161] or in the company of other sheep next to the treadmill [162]. In contrast, our method of exercise utilizes the herd dynamic to facilitate exercise and minimize handling stress.

There are multiple advantages to using sheep for musculoskeletal research; sheep tend to be a more uniform population than other large research animals like horses. In our study, the 20 sheep were of relatively similar size according to their sex (Table 6) and close in age (254 ± 5 days of age). This is especially important when studying younger populations as juveniles undergo both bone modeling and remodeling. Bone modeling involves the shaping and growth of bone during development and in response to mechanical forces, while bone remodeling is the coordinated process of resorption and formation by osteoclasts and osteoblasts, respectively, allowing them to adapt to changing mechanical loads and repair damage throughout life [163]. Understanding bone modeling and remodeling is essential for the investigation of pharmacological and/or surgical interventions in juvenile animals, as these interventions may have different outcomes in juveniles than in adults. Additionally, the availability of both male

and female sheep is important in preclinical and translational research because it allows for the study of sex-specific differences and responses to interventions. This is particularly relevant to current public funding entity requirements such as the U.S. National Institutes of Health, which encourage the inclusion of both sexes in preclinical research [164]. In contrast, when using other large animal models such as dairy calves, male animals may be easier to acquire [165]. In summary, the use of sheep has multiple noteworthy advantages including the relative genetic and physical uniformity, the availability of both sexes, and the willingness to exercise in large groups.

The use of a walker as a mechanism for exercising sheep offers multiple advantages. Circular exercise is a common training method for species like horses, which may be ridden or worked on the ground in round pens or by lunging [163]. The walker allows researchers to assess the effects of circular exercise at different speeds and different diameters. Previous studies have used calves as an animal model for horses to investigate the effects of circular exercise [165]. However, calves are resistant to exercising for greater than 30 minutes, requiring constant verbal encouragement to move, and sustain lower speeds (1.1-1.5 m/s) [165]. Sheep in this study were able to easily sustain 30 min of exercise and reach faster speeds (2.0 m/s) with minimal morbidity. Further increases in speed and duration of exercise are possible in this model, making it potentially suitable to investigate more challenging exercise protocols.

The walker may result in a more natural gait than treadmill exercise. Prior research in horses and humans suggests treadmill exercise significantly alters gait [166,167], including stride length and stance duration [147]. Further, in cases where animals are required to be tethered with a halter to the treadmill may result in significant alterations in gait, leading to differences in bone characteristics between left and right limbs, including cortical area and fracture force [165]. In

contrast, using a walker enables natural gait and bidirectional exercise, which may prevent significant confounding factors when studying bone characteristics in musculoskeletal research. Additionally, the walker exposes the animals to a footing experience that can be similar to that of common overground surfaces and provides the possibility of testing different overground surface materials. No substantial lameness was observed during the study period, despite exercising 4 days per week. The maximum lameness score observed was “2” on a scale from “0” to “6” [150]. In total, five animals were identified as having mild lameness during the 84-day period, suggesting that the exercise protocol did not cause substantial lameness in the sheep [168]. All lamenesses were transient. Only one individual showed lameness in two consecutive lameness examinations.

Although significant lameness was not observed, sheep lost approximately 4% of their BW over the course of the study, and this may have been due to a combination of factors, including exercise and heat stress. According to a previous study using Merino sheep, the exercise group (1 hour of exercise per day at speeds up to 2.5 m/s) had a lower carcass weight (0.5 to 0.9 kg) than the sedentary group, suggesting that the lower weight gain was attributed to their exercise protocol [169]. Weather may have also been a contributing factor in weight loss [154]. Average maximum temperatures ranged from 25.6 °C (19.6–32.7 °C) in June to 28.8 °C (22.7–32.1 °C) in August [170]. Feed consumption and weight gain are negatively impacted by heat stress [154]. The animals were exercised in the morning and their pen was located indoors to mitigate heat stress. However, the increase in average maximum temperatures, as well as exercise, may have played a role in the sheep’s weight loss during the study. Despite a 4% loss in BW, sheep were able to complete the exercise protocol during the warmer days, without the presence of other comorbidities detected during the physical examinations and daily monitoring.

Physical examination parameters may also reflect environmental factors and exercise acclimation throughout the study. Resting HR decreased from baseline (day 0), which may indicate a modest improvement in fitness. However, HR and RR remained above the normal resting range for sheep [152,153]. This can likely be attributed to the stress of handling [171] coupled with environmental factors [154]. Future research could use telemetric monitoring methods [172], to accurately measure resting HR without stressing animals and provide a better real-time measurement of HR response to handling and exercise [173], allowing researchers to adjust the exercise protocol intensity.

In addition to physical measurements, serum markers of bone metabolism can provide a minimally invasive and specific way to assess the dynamic changes in bone metabolism (bone formation and resorption) [151]. Decreases in bone resorption markers (CTX-I and RANKL) were observed on days 28 and 84 in our study. CTX-I is indicative of osteoclast activity [174], while RANKL is the ligand that induces maturation of osteoclasts [175]. The reduction of CTX-I would indicate an overall reduction in bone resorption. However, a contradictory increase in TRAP5b was observed on day 28. TRAP5b is a marker for osteoclast numbers [174] and has been associated with increased bone resorption in some conditions [176,177]. TRAP5b is released from both mature and immature/pre-fusion osteoclasts [174] and may reflect overall osteoclast numbers instead of an active resorption process underway. To further understand the seemingly contradictory findings, future studies could include cathepsin K, which is only produced in mature resorbing osteoclasts [174]. In contrast to our findings, CTX-1 has been found to increase in foals [90] or remain the same in yearlings [178] under exercise; however, our animals were considerably more mature entering the study period at 80% of their adult body weight. Bone formation markers (BALP and PINP) did not change during the present study. This

is consistent with previous studies where no changes in bone formation markers were found when juvenile horses were exercised 3 days per week [178,179]. Bone serum markers may also reflect growth-related changes [180]. Sheep were approximately 254 ± 5 days of age (8.5 months), and animals grew taller. Future studies should include an aged-matched, non-exercising control group to determine the effect of exercise versus growth.

Whenever utilizing an animal model, differences between species must be recognized and results interpreted within the limitations of the model. Sheep have been widely used in orthopedic research [138–140], despite their clear physiological and anatomical differences from humans and other species. When comparing sheep and horses, there are noticeable differences in their anatomy and physiology. Horses are perissodactyls with one toe per hoof, while sheep are artiodactyls with two cloven toes per hoof [181]. These anatomical differences can make it challenging to study diseases involving structures distal to the metacarpo/metatarsophalangeal (fetlock) joints. However, sheep may be useful for addressing pathologies originating proximal to the fetlock joint or systemic responses to intravenous or intramuscular drugs like bisphosphonates. Bone formation is consistently favored in response to mechanical strain across various animal species [163] including horses [182], calves [183], gilts [184], and even roosters [185]. Furthermore, sheep and horses are quadrupeds with comparable kinetics and kinematics when adjusted for body size [142]. That stated, all results must be interpreted whilst recognizing the limitations associated with using sheep to model horses. An additional limitation of the study was infrequent sampling for serum bone biomarker analysis. Serum bone biomarkers were measured at 4 different time points during the study. It is well-established that serum bone biomarkers can respond differently to acute and chronic exercise [88], thus, more frequent sampling, particularly following physical activity may have provided a more accurate

representation of the acute effects of physical activity [88,186]. Further, the study lacks complementary measurements of bone structural changes, such as bone microarchitecture imaging analysis, which could have provided further insight into the relationship between serum bone biomarker responses and bone structural changes during the study period.

CONCLUSIONS

In summary, this study shows that using a walker to exercise juvenile sheep in groups can be an effective and practical way of studying different orthopedic interventions without causing significant morbidity. The sheep readily acclimated to the walker, displaying a positive interest in exercise. The animals were easily trained and each exercise session required only one person to handle all animals. No animals in this study refused to exercise. Additionally, exercising the sheep in groups mitigated the stress and welfare concerns caused by individual training on a treadmill, providing a more natural footing surface than treadmills. Despite the observed mild weight loss, the sheep were able to exercise without significant comorbidities. Further investigation using this translational model should include a sedentary group and focus on evaluating additional exercise protocols. Overall, this ovine, exercising model has multiple advantages including ease of handling, availability of serum biomarkers, and the ability to test multiple exercise characteristics including duration and intensity in an efficient and practical manner.

CHAPTER 5: Clodronate disodium does not produce measurable effects on bone metabolism in an exercising, juvenile, large animal model

ABSTRACT

Bisphosphonates are commonly used to treat and prevent bone loss, but their effects in active, juvenile populations are unknown. This study examined the effects of intramuscular clodronate disodium (CLO) on bone turnover, serum bone biomarkers (SBB), bone mineral density (BMD), bone microstructure, biomechanical testing (BT), and cartilage glycosaminoglycan content (GAG) over 165 days. Forty juvenile sheep (253 ± 6 days of age) were divided into four groups: Control (saline), T_0 (0.6 mg/kg CLO on day 0), T_{84} (0.6 mg/kg CLO on day 84), and T_{0+84} (0.6 mg/kg CLO on days 0 and 84). Sheep were exercised 4 days/week and underwent physical and lameness examinations every 14 days. Blood samples were collected for SBB every 28 days. Microstructure and BMD were calculated from tuber coxae (TC) biopsies (days 84 and 165) and bone healing was assessed by examining the prior biopsy site. BT and GAG were evaluated postmortem. Data, except lameness data, were analyzed using a mixed-effects model; lameness data were analyzed as ordinal data using a cumulative logistic model. CLO did not have any measurable effects on the skeleton of sheep. SBB showed changes over time ($P \leq 0.03$), with increases in bone formation and decreases in some bone resorption markers. TC biopsies showed increasing bone volume fraction, trabecular spacing and thickness, and reduced trabecular number on day 165 versus day 84 ($P \leq 0.04$). These changes may be attributed to exercise or growth. The absence of a treatment effect may be explained by the lower CLO dose used in large animals compared to humans. Further research is needed to examine whether low doses of bisphosphonates may be used in active juvenile populations for analgesia without evidence of bone changes.

INTRODUCTION

Bisphosphonates are a class of drugs that have been used for over 40 years in human medicine for their antiresorptive biological effects [3,105]. Bisphosphonates work by impairing osteoclast-mediated bone resorption [24,28]. As a result, these drugs have been shown to increase bone mineral density (BMD) [187,188], reduce serum bone biomarkers (SBB) of bone resorption [188,189], and change the mechanical properties of bones [190,191]. Bisphosphonates treat and prevent bone loss in various diseases such as postmenopausal osteoporosis [21,192], osteogenesis imperfecta [44,46,59], and Paget's disease [91,193]. They are also used in veterinary medicine for different pathologies, such as canine osteosarcoma [194], feline idiopathic hypercalcemia [195], and equine navicular syndrome [75,196].

The effect of bisphosphonates in young, active humans is poorly understood [197–199], and concern exists over the use of bisphosphonates in juvenile, high-performance animals such as racehorses [4,5,79,102,106]. Growth and adaptation to exercise depend on a normal bone modeling and remodeling processes in which osteoclasts play a central role [19]. Osteoclasts are active and abundant in the subchondral bone of juvenile animals [80] and humans [2]; thus, the potential impairment of this group of cells may be detrimental in juvenile individuals [10,200]. While serious side effects resulting from bisphosphonate use, such as osteonecrosis of the jaw [60,62] and atypical femur fractures [61], have been reported in adults and replicated in several animal models [64,67,68,70,109], but have not been investigated in a young, active population.

Bisphosphonate effects have been assessed in different animal models [64,65,67,68,70,109] including sheep [106]. Sheep can be conditioned to forced exercise [101,143,144] making them a particularly applicable model species for the effects of therapeutics on active individuals. Further, significant volumes of blood and tissues may be acquired. When

used in a terminal study, bone mechanical testing and skeletal advanced imaging may be combined with gross dissection and sampling, creating a robust data set.

This study aimed to investigate the skeletal effects of the intramuscular (i.m.) administration of clodronate disodium (CLO), an FDA-approved bisphosphonate in horses, using a juvenile ovine model under forced exercise. CLO was selected as it is an FDA-approved bisphosphonate for use in a large animal species [38], has shown clinical efficacy for musculoskeletal disease [38,201], and the pharmacokinetics have been described in sheep [202]. Outcome measures included physical examinations, lameness evaluations, SBB, advanced imaging methods, biomechanical testing (BT), and sulfated glycosaminoglycan content (GAG) in cartilage. We hypothesized that CLO administration would result in (1) a reduction in bone resorption markers indicating a decrease in bone turnover, (2) an increase in BMD and an increase in fracture force, (3) a decrease in bone healing, and (4) an increase in cartilage GAG content.

MATERIALS AND METHODS

Animals and management

All animal protocols were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (2020000264) and the experimental procedures and results are described according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines [203]. Forty juvenile, cross bred Dorset-Polypay sheep (253 ± 6 days of age), consisting of 20 castrated males (wethers, 87 ± 5 kg), and 20 females (ewes, 72 ± 8 kg) were acquired from the MSU Sheep Teaching and Research Center. Castrated males were used as the farm orchiectomizes the rams (intact males) shortly after birth. Juvenile sheep were defined as animals that had not attained 80% of their mature size (body mass) [148,204]. Sheep

were sheared nine weeks prior to the beginning of the study. Two weeks prior to the beginning of the study, sheep were transported to the MSU Bennett Road Farm and then housed in two indoor pens (21.6 m^2 each, Figure 1S). The study was comprised of a 2-week acclimation period followed by a 24-week study period. Animals were randomly assigned to each indoor pen, with 20 animals housed in each pen. Sheep had access to a total mixed ration that contained 90% dry matter of chopped hay (82%), a corn/soybean blend (16.5%), and a 1.5% of a mineral blend (Marvo Mineral Company, Osseo, MI). The sheep were fed this total mixed ration once per day at a mass equivalent to an average of 2.0–2.3% of their body weight in dry matter over the 24-week experimental period and were provided *ad libitum* access to water.

During the 2-week habituation period, sheep were acclimated to their housing, a 3-way manual weigh crate (Prattley, Temuka, New Zealand), and the 20-m diameter exerciser (Q-Line Horse Exerciser, Aromas, CA, USA). Twice per week, animals were moved through an indoor chute system connected to the weigh crate to acclimate the sheep to sample collections and physical examinations. A 32-meter fence connecting the outside walker to the inside pens allowed sheep to easily move from the barn to the walker. During the habituation period, sheep were placed in the exerciser 4x/week and encouraged to walk for 5 min/day (Figure 1S).

Physical examinations and exercise protocol

Physical examinations (PE) including body weight (BW), height measured at the withers (height), heart rate (HR), respiratory rate (RR), and rectal temperature (RT) were performed every 14 days throughout the study period, beginning on day 0. All physical examinations were performed using the previously mentioned indoor chute system and weigh crate. Sheep were confirmed to be without lameness prior to the start of the study, and lameness evaluations were performed every 14 days starting on week 0 by two veterinarians (A.C.C, F.B.V) using a

standardized sheep subjective lameness evaluation scoring system that ranges from "0" (no lameness) to "6" (animal incapable of movement) [150]. The sheep were exercised in groups of 20 to ensure their safety, as the exerciser's bay could not accommodate all 40 sheep at once, and to maintain the original distribution of animals from each pen. No exercise was completed during week 12 as sheep were sedated and bone biopsies were collected from the tuber coxae (TC); sheep resumed exercise during week 13 (Table 10).

Table 10. Juvenile sheep exercise protocol and estimated distance traveled using a circular high-speed exerciser for 24 weeks.

Week	Exercise duration	Estimated distance traveled
1	10 min (WS)	780 m
2	15 min (WS)	1,170 m
3	20 min (WS)	1,560 m
4	25 min (WS)	1,950 m
5	30 min (WS)	2,340 m
6–8	13 ¾ min (WS) → 2 ½ min (TS) → 13 ¾ min (WS)	2,445 m
9–11	12 ½ min (WS) → 5 min (TS) → 12 ½ min (WS)	2,550 m
12[1]	–	–
13	30 min (WS)	2,340 m
14	12 ½ min (WS) → 5 min (TS) → 12 ½ min (WS)	2,550 m
15–17	8 min (WS) → 3 ¾ min (TS) → 6 ½ min (WS) → 3 ¾ min (TS) → 8 min (WS)	2,655 m
18–24	7 min (WS) → 5 min (TS) → 6 min (WS) → 5 min (TS) → 7 min (WS)	2,760 m

Abbreviations: WS, walking speed at 1.3 m/s; TS, trotting speed at 2.0 m/s. Sheep were exercised 4x/week alternating between clockwise and counterclockwise directions each day. The TS bout was incorporated in the middle of the 30 min exercise protocol, starting on week 6.

[1]No exercise due to bone biopsies.

Treatment distribution

Sheep were stratified by sex and weight, randomly assigned a number from #1 to #40, and allocated to one of three CLO treatment groups (T_0, T_{84}, T_{0+84}) or a saline control group (Con). Treatment groups received CLO (0.6 mg/kg i.m., OSPHOS®, Dechra Veterinary Products, Overland Park, KS, USA) administered on day 0 (T_0), CLO (0.6 mg/kg i.m.) administered on day 84 (T_{84}), or CLO (0.6 mg/kg i.m.) administered on day 0 and day 84 (T_{0+84}).

To maintain proper controls, animals not receiving CLO on days 0 or 84 was administered saline solution i.m., and control animals received saline solution i.m. on days 0 and 84. The dose of CLO was selected based on a pilot study which compared plasma concentrations of CLO following 3 different doses of CLO (0.6, 1.8, 3.0 mg/kg i.m.) over 48 hours in 12 adult sheep (n = 4/treatment group) [202]. CLO administered at 0.6 mg/kg i.m. resulted in similar pharmacokinetic parameters to the FDA-approved dose of CLO (1.8 mg/kg i.m.) for horses [202].

Serum harvest

Twenty milliliters of blood were harvested by jugular venipuncture between 07:00 and 09:00 h every 28 days, starting on day 0. Blood was placed in serum-separator vacutainer tubes, facilitating coagulation on ice for 1 h prior to centrifugation at 2,000 × *g* for 15 min. Serum was aliquoted into 2-mL microcentrifuge tubes and stored at -80 ºC for later evaluation.

Tuber coxae biopsy

At week 12, TC bone biopsies were performed to assess bone healing and microstructure; the left or right TC was randomly chosen regardless of the treatment group, using a random sequence generator (https://www.random.org/sequences). Sheep were fasted for 24 h and deprived of water for 12 h prior to surgery. Sheep received a single dose of penicillin G procaine (22,000 UI/kg i.m.; VetOne, Boise, ID, USA) prior to administration of midazolam (0.26 mg/kg i.m.; Hikma Pharmaceuticals USA Inc., Berkeley Heights, NJ, USA), ketamine (3.25 mg/kg i.m.; Akorn Operating Company LLC, Gurnee, IL, USA), and xylazine (0.01-0.02 mg/kg i.m. as needed; Patterson Veterinary Supply Inc., Loveland, CO, USA) to achieve recumbent sedation. The biopsy site was clipped and aseptically prepared, and the skin was infiltrated with 5 mL of mepivacaine hydrochloride. An approximately 4-cm incision was made through the skin and

subcutaneous tissues over the TC. An 8-mm Michele trephine was used to remove a sample which included a cartilage cap, cortical bone, and trabecular bone. Biopsy samples were stored in 4% paraformaldehyde solution for 96 h and then transferred to 70% ethanol for later micro-computed tomography (micro-CT) analysis. The subcutaneous tissue was closed using a 0-monocryl suture in a continuous pattern and the skin incision was closed using a 2-0-monocryl suture in a simple, interrupted pattern. All animals were administered a single dose of meloxicam (1 mg/kg PO; Zydus Pharmaceuticals Inc., Pennington, NJ, USA) after the skin incision was sutured.

Euthanasia and specimen collection

At the conclusion of the 24-week study, all sheep were humanely euthanized using a captive bolt pistol at the MSU Meat Laboratory. Mandibles, right fused metacarpi (MC_{3+4}), and fourth lumbar vertebrae (L4) were collected from each animal, wrapped in saline-soaked paper towels, stored in plastic bags, and immediately placed on ice. The medial condyle of the left MC_{3+4} (LMC) and the third lumbar vertebra (L3) were placed in 4% paraformaldehyde. The TC which had been previously biopsied (during week 12) was removed en bloc and an 8-mm biopsy was obtained from the contralateral TC for preservation in 4% paraformaldehyde. All samples in 4% paraformaldehyde were transferred to 70% ethanol after 96 h. Articular cartilage was harvested from the proximal surface of the right radius using a scalpel. The harvested cartilage was immediately placed in microcentrifuge tubes and stored on ice, then transferred to storage at -20 ºC until GAG analysis.

Computed tomography

The mandible, right MC_{3+4}, and L4 from each sheep were CT scanned at 120 kV and 320 mAmp, with a slice thickness of 0.625 mm (GE Revolution Evo Scanner; GE Healthcare,

Princeton, NJ, USA). All CT scans were analyzed using Mimics 23.0 (Materialise NV, Leuven, Belgium). The whole-slice BMD of the mandible was calculated with a mask threshold of 250 Hounsfield Units (HU) at the midpoint of the diastema between the fourth incisor and premolar; the BMD of the left and right hemi-mandibles was averaged. The midpoint of each right MC_{3+4} was used to measure BMD and the following dimensions with a mask threshold value of 400 HU: cross-sectional area (CSA), external dorsopalmar (DP) and lateromedial (LM) diameters (cortex), internal DP and LM diameters (medullary cavity), and cortical widths (anterior, posterior, lateral, and medial). The BMD of L4 was measured at the first full cranial slice, midpoint, and last full caudal slice and averaged. Additionally, the vertebral body length and CSA dimensions of the L4 were measured. To calculate the CSA, the average cranial and caudal full slice areas were used. Vertebral scans were identified and separated by masking at 226 HU. A calcium hydroxyapatite phantom (Image Analysis, Inc., Columbia, KY, USA) with rows representing 0, 75, and 150 milligrams of calcium hydroxyapatite per cubic centimeter (mg HA/cm^3) was included in each scan. All density measurements in HU were converted to mg HA/cm^3 using linear equations calculated from the phantom on each scan, as previously described [205]. After CT scans were completed, all samples were wrapped in saline-soaked gauze and stored at -20 ºC until later analysis by BT.

Biomechanical testing

The right MC_{3+4} were removed from the freezer, wrapped in saline-soaked gauze, and allowed to slow thaw at 4.8 ºC over a 5-day period. Soft tissue was removed after thawing and immediately prior to biomechanical testing. The right MC_{3+4} were subjected to 4-point bending using an electromechanical testing system equipped with a 60 kN load cell (MTSCriterion, Model 43, Eden Prairie, MN, USA). Right MC_{3+4} for each sheep were positioned individually,

with the palmar aspect of the metacarpus facing upward toward the force applicators, as previously described [165] (Figure 2S). All samples were loaded to failure at a rate of 10 mm/min [206]. Flexural stress (maximal force to failure/CSA) and modulus of elasticity were calculated. Modulus of elasticity (*E*) was calculated based on the formula: $E = EI/I$. The *EI* (flexural rigidity) was calculated by the following equation: $EI = (F/V)(a^2/12)(3 \times L - 4a)$, where *F/V* (stiffness) was obtained from the linear portion of the curve between 0.7 to 1.2 mm of compression with an R^2 of 0.99. The distance between the bottom support stands minus the width of the support stands was *L* (51.9 mm); *a* (3.8 mm) corresponds to *L* minus the distance between the force applicators divided by two (Figure 2S). Moment of inertia (*I*) was determined by calculating a hollow ellipse as previously described [89,165]: $I = 0.049[(B \times D^3) - (b \times d^3)]$ (*B* = exterior lateromedial diameter, *D* = exterior dorsopalmar diameter, *b* = interior lateromedial diameter, *d* = interior dorsopalmar diameter).

L4 were allowed to thaw at 4.8 ºC for 3 days prior to the removal of soft tissues. Once thawed, the soft tissues, vertebral arch, and transverse processes were removed (Figure 3S). The superior and inferior vertebral endplates were embedded in polyurethane resin (TC-808, BJB Enterprises, Tustin, CA, USA) (Figure 4S) and the specimens were wrapped in saline-soaked gauze. Compression tests were performed using an electromechanical testing system equipped with a 100 kN load cell (Instron Model 5982, Norwood, MA, USA). A flat 3-mm thick metal plate was included between the specimen and the compression piston's end to guarantee a uniformly distributed axial load applied over the vertebral body (Figure 5S). All specimens were loaded to failure at a rate of 1 mm/min [206]. Compressive stress (maximal compressive force divided by the average CSA calculated from the CT data for each vertebral body) was calculated. Compressive modulus of elasticity was calculated as the slope of the stress-strain curve between

60 to 80% of the maximum compressive stress using the Bluehill® Universal software (Norwood, MA, USA).

Micro-computed tomographic analysis

Fixed whole TC, TC biopsies, LMC, and L3 vertebral bodies were analyzed using micro-computed tomography (micro-CT). Micro-CT images were obtained using a PerkinElmer Quantum GX system (Waltham, MA, USA) with a voltage of 90 kV, current of 88 µA, and reconstruction resolution of 50 µm. Image analysis was performed using Dragonfly Software (v.2022.1.0.1259, Object Research Systems, Quebec, Canada) to differentiate bone (mineral) from bone marrow (non-mineral) and segment TC biopsy slices using the Otsu threshold algorithm [207]. A cylindrical region of interest (ROI) was established to calculate bone volume fraction (BV/TV), trabecular separation (TbSp), trabecular thickness (TbTh), trabecular number (TbN), trabecular connectivity density (ConnD), and BMD of the TC biopsies. To assess bone healing, a 6-mm diameter cylinder was established to calculate BV/TV, TbSp, TbTh, TbN, and ConnD at the TC biopsy site. Cortical thickness (CtTh) was determined by measuring and averaging the cortical bone thickness in 5 different regions of a representative slice of the TC. Separation of cortical from the trabecular bone in L3 and LMC was done using the Buie bone segmentation tool in the Bone Analysis tool [208], resulting in the calculation of BV/TV, TbSp, TbTh, TbN, and CtTh. Growth plates were present in the LMC and L3 samples; therefore, those regions were excluded to standardize image analysis. For the micro-CT images, the BMD was calculated from the intensity of the bone and converted to mg HA/cm^3, as previously described [205], using a micro-CT calcium hydroxyapatite phantom (QRM, Möhrendorf, Germany).

Serum bone biomarker analysis

Serum samples were thawed immediately before testing. Serum concentrations of ovine-

specific bone markers [151] were assessed using enzyme-linked immunoassays according to the manufacturer's instructions (Kendall Scientific, Lincolnshire, IL, USA). Bone markers analyzed included bone-specific alkaline phosphatase (BALP), procollagen type I amino-terminal propeptide (PINP), receptor activator of nuclear factor-κB ligand (RANKL), cross-linked C-terminal telopeptides of type I collagen (CTX-I), and tartrate-resistant acid phosphatase isoenzyme 5b (TRAP5b). The assays were analyzed using a SpectraMax ABS Microplate Reader (Molecular Devices, LLC, San Jose, CA, USA) at 450 nm. The remodeling index BALP/CTX-I [209,210] and resorption index CTX-I/TRAP5b [174,211] were also determined.

Cartilage glycosaminoglycan content

Cartilage samples were thawed, weighed, digested in papain buffer (0.1M sodium acetate, 0.05 EDTA, pH 5.53), and activated with 0.005M L-cysteine HCl hydrate overnight in a 60 ºC water bath. One µg of papain (26 mg/mL) was added per milligram of cartilage, as previously described [212]. The digested GAG content was determined using a 1,9-dimethylmethylene blue colorimetric assay as previously described [213]. The colorimetric reaction was measured at 520 nm using a SpectraMax 384 Microplate Reader (Molecular Devices) and compared to a chondroitin sulfate standard (bovine trachea).

Statistical analysis

An a priori power analysis, with a significance criterion of alpha = 0.05, was conducted based on mean differences and pooled standard deviations (SD) between treatment groups for BALP (bone formation marker) and CTX-I (bone resorption marker). The analysis revealed that a sample size of 10 animals per treatment group would provide sufficient power to detect a significant difference. For BALP, the estimated power was 99.9% with a mean difference of 7 ng/mL and a SD of 3 ng/mL [214]. For CTX-I, the estimated power was 98.1% with a mean

difference of 135 ng/mL and a SD of 75 ng/mL using an ELISA method [64]. Sample size and power were calculated using OpenEpi (Version 3.01). The authors recognized the limited information available on bisphosphonate administration in juvenile sheep and, therefore, maximized the power of the study by using 10 animals per treatment group.

Physical parameters and SBB of sheep were analyzed using a mixed-effects model that considered the fixed effects of treatment, time, sex, and all possible 2- and 3-way interactions, with repeated measures of time and subject effect of sheep. The results are presented as mean values ± SD and were analyzed using the MIXED procedure of SAS 9.4 (SAS Inc., Cary, NC, USA). CT (mandibles, right MC_{3+4}, and L4), micro-CT (TC, TC biopsies, L3, and LMC), BT data (right MC_{3+4} and L4), and cartilage GAG content were evaluated with the fixed effects of treatment, sex, as well as the interaction between treatment and sex. Normality was assessed using diagnostic plots of residuals for each independent variable. All data, except for SBB and lameness evaluations, were deemed to be normally distributed. SBB data was log-transformed and subsequently followed a normal distribution after transformation. Height and BW were assessed as potential covariates in this model, because the size of the animals could have influenced the results of the independent variables. No significant correlations were detected between height or BW and the independent variables; therefore, they were removed from the final model. An effect of sex was observed in sheep BW, height, right MC_{3+4} dimensions, L4 dimensions and BT, and LMC BV/TV and BMD. For the rest of the analyses, the effect of sex was not included in the results, as it was not significant. Post-hoc comparisons using least-squares means separated by the Tukey-Kramer test were used when the effects were significant ($P \leq 0.05$).

Lameness data were analyzed as ordinal data. Lameness scores from two veterinarians were averaged and rounded to the nearest integer for each sheep. A cumulative logistic model was

applied using RStudio v.2022.07.2 (RStudio Inc., Boston, MA, USA), calculating the estimated likelihood of lameness score, measured as predicted probability for each treatment and day and standard error. Post-hoc multiple comparisons were performed using the Tukey method and the R package 'emmeans'. Statistical significance was set at $P \leq 0.05$.

RESULTS

Physical examinations and lameness evaluations results

During the study, two sheep experienced moderate morbidity leading to intervention. During week 8, sheep #7 (female, T_0 group) developed a fever and lethargy; she was treated with penicillin G procaine (22,000 UI/kg i.m.) for 10 days every 12 h (7 days of exercise missed). She fully recovered until week 12, when she experienced persistent lameness following TC biopsy. Due to persistent lameness, exercise was discontinued, and sheep #7 was excluded from the study. One additional sheep developed a mild fever and lethargy following TC biopsy (#37, female, T_{84} group) and received oxytetracycline (20 mg/kg) every 48 h, receiving 2 doses in total (no days of exercise missed).

As anticipated with growth, BW and height increased over time ($P < 0.001$) but did not differ with treatment administration over time ($P \geq 0.24$, Table 11).

Table 11. Initial and final body weights (BW) and heights of 40 juvenile sheep.

Sex	BW (d 0)	BW (d 163)	P-value
Males (kg)	79 ± 7^{b}	86 ± 6^{a}	0.01
Females (kg)	70 ± 7^{c}	74 ± 9^{bc}	
Average BW (kg)	75 ± 8	80 ± 10	<0.001
Sex	Height (d 0)	Height (d 163)	P-value
Males (cm)	69 ± 3^{x}	80 ± 2^{z}	0.01
Females (cm)	67 ± 3^{x}	75 ± 4^{y}	
Average height (cm)	68 ± 3	77 ± 4	<0.001

^{abc}BW means followed by a common letter are not significantly different ($P \leq 0.01$)
^{xyz}Height means followed by a common letter are not significantly different ($P \leq 0.01$)
Values are expressed as mean ± SD.

Lameness evaluations

There were no differences in lameness between treatment groups at any time point. However, on day 94, sheep in the T_0 group were less likely to be sound (score "0", 0.53 ± 0.10, $P \leq 0.05$) compared to days 0, 30, 44, 58, 72, 114, 142 and 163. Additionally, they were more likely to receive a score of "1" (0.21 ± 0.04, $P \leq 0.05$) compared to days 30, 72, 114, and 142. A single sheep from the T_0 group (sheep #7) had a lameness score of "4" (reluctance to bear weight during movement) at day 94 following TC biopsy. This lameness persisted despite treatment with meloxicam (1 mg/kg PO) every 24 h for five days. Because the animal could not complete the exercise protocol, it was removed from further analyses, as previously indicated. No other sheep received a score greater than "2" at any point during the study period. Table 12 shows the frequency for each lameness score according to their treatment groups and days.

Table 12. Frequency of sheep lameness scores by day and treatment group.

	Day	Frequency of lameness score						Day	Frequency of lameness score				
		0	1	2	3	4			0	1	2	3	4
Con	0	10	0	0	0	0	T_0	0	8	2	0	0	0
	16	9	0	1	0	0		16	9	0	1	0	0
	30	8	1	1	0	0		30	9	1	0	0	0
	44	8	2	0	0	0		44	10	0	0	0	0
	58	10	0	0	0	0		58	10	0	0	0	0
	72	10	0	0	0	0		72	10	0	0	0	0
	94[1]	5	3	2	0	0		94[1]	6	3	0	0	1
	100	8	2	0	0	0		100	9	0	0	1	0
	114	10	0	0	0	0		114	9	0	1	0	0
	128	9	1	0	0	0		128	7	0	3	0	0
	142	10	0	0	0	0		142	9	0	1	0	0
	156	9	0	1	0	0		156	9	0	1	0	0
	163	10	0	0	0	0		163	9	0	1	0	0
	Total observations	116	9	5	0	0		Total observations	114	6	8	1	1

Table 12 (cont'd)

	Day	Frequency of lameness score						Day	Frequency of lameness score				
		0	1	2	3	4			0	1	2	3	4
T_{84}	0	10	0	0	0	0	T_{0+84}	0	10	0	0	0	0
	16	10	0	0	0	0		16	9	0	1	0	0
	30	10	0	0	0	0		30	10	0	0	0	0
	44	10	0	0	0	0		44	9	1	0	0	0
	58	10	0	0	0	0		58	8	1	1	0	0
	72	9	0	1	0	0		72	10	0	0	0	0
	94[1]	7	2	1	0	0		94[1]	8	2	0	0	0
	100	9	0	1	0	0		100	10	0	0	0	0
	114	9	1	0	0	0		114	10	0	0	0	0
	128	9	0	1	0	0		128	10	0	0	0	0
	142	10	0	0	0	0		142	10	0	0	0	0
	156	10	0	0	0	0		156	9	1	0	0	0
	163	10	0	0	0	0		163	9	1	0	0	0
	Total observations	123	3	4	0	0		Total observations	122	6	2	0	0

Abbreviations: Con, control group with no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO.
No lameness evaluations were recorded between 5–6.
[1]Lameness evaluations were recorded on day 94 instead of day 86 due to bone biopsies and lack of exercise during week 12.

Computed tomography

No treatment differences or sex differences were found in the BMD of the mandibular, MC_{3+4} cortices and whole slice at the midpoint, or averaged L4 (Table 13).

Table 13. Treatment means and SD of bone mineral density (BMD) measured using computed tomography at various locations: midpoint of the mandibular diastema, dorsal, palmar, lateral, medial cortices, whole slice at the midpoint of the right fused metacarpus (MC_{3+4}), and average of the fourth lumbar vertebral body (L4).

	BMD (mg HA/cm^3)						
	Mandible	MC_{3+4}					L4
Treatments	Midpoint diastema	Dorsal cortex	Palmar cortex	Lateral cortex	Medial cortex	Midpoint whole slice	Vertebral body
Con	805 ± 74	1,180 ± 85	767 ± 145	1,201 ± 67	1,179 ± 60	901 ± 140	390 ± 35
T_0	813 ± 65	1,209 ± 69	765 ± 143	1,213 ± 35	1,240 ± 55	898 ± 143	426 ± 29
T_{84}	809 ± 51	1,203 ± 61	717 ± 93	1,207 ± 35	1,233 ± 52	834 ± 87	421 ± 50
T_{0+84}	808 ± 53	1,202 ± 85	792 ± 153	1,193 ± 64	1,225 ± 90	963 ± 161	403 ± 45
P-value	0.99	0.82	0.68	0.88	0.21	0.24	0.22

Abbreviations: Con, control treatment no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO.

No treatment differences were found for any of the right MC_{3+4} dimensions. However, sex differences were found in all right MC_{3+4} dimensions. Males had greater external and internal DP and LM diameters ($P \leq 0.03$), as well as dorsal, palmar, lateral, medial CW, CSA, and bone length ($P \leq 0.02$). The L4 vertebral body dimensions (length and CSA) did not differ among treatments, but males had greater vertebral body length and CSA measurements than females ($P<0.003$, Table 14).

Table 14. Means and SD for the right fused metacarpus (MC_{3+4}) including the internal and external diameters of at the dorsopalmar (DP) and lateromedial (LM) locations, cortical widths (CW) at the dorsal, palmar, lateral, and medial cortex, bone length, cross-sectional area (CSA) and means and SD for the fourth lumbar vertebral body (L4) CSA.

Measurements	MC_{3+4} dimensions				
	Con	T_0	T_{84}	T_{0+84}	P-value
DP external diameter (mm)	13.6 ± 0.9	13.9 ± 0.7	13.5 ± 1.0	13.6 ± 0.8	0.31
DP internal diameter (mm)	7.4 ± 0.8	7.7 ± 0.8	7.0 ± 0.6	7.3 ± 0.9	0.19
LM external diameter (mm)	20.0 ± 1.0	20.9 ± 1.5	20.2 ± 1.3	20.6 ± 1.4	0.26
LM internal diameter (mm)	12.0 ± 1.0	12.6 ± 1.7	11.6 ± 1.1	12.2 ± 1.5	0.34
Dorsal CW (mm)	3.8 ± 0.5	4.0 ± 0.5	4.0 ± 0.5	3.9 ± 0.5	0.79
Palmar CW (mm)	2.4 ± 0.3	2.4 ± 0.2	2.5 ± 0.3	2.4 ± 0.2	0.81
Lateral CW (mm)	4.1 ± 0.4	4.1 ± 0.4	4.2 ± 0.3	4.2 ± 0.3	0.67
Medial CW (mm)	4.0 ± 0.4	4.2 ± 0.4	4.4 ± 0.4	4.2 ± 0.4	0.11
Bone length (mm)	150.8 ± 9.7	154.0 ± 9.1	150.6 ± 9.9	150.6 ± 8.2	0.69
CSA (mm^2)	214.5 ± 25.3	235.9 ± 30.0	216.3 ± 31.4	221.8 ± 30.9	0.25
	Males		Females		P-value
DP external diameter (mm)	14.2 ± 0.6		13.1 ± 0.7		<0.001
DP internal diameter (mm)	7.7 ± 0.8		7.0 ± 0.8		0.02
LM external diameter (mm)	21.3 ± 1.0		19.6 ± 1.1		<0.001
LM internal diameter (mm)	12.6 ± 1.4		11.6 ± 1.2		0.03
Dorsal CW (mm)	4.1 ± 0.4		3.7 ± 0.4		0.003
Palmar CW (mm)	2.5 ± 0.3		2.3 ± 0.2		0.03
Lateral CW (mm)	4.3 ± 0.2		4.0 ± 0.3		0.003
Medial CW (mm)	4.4 ± 0.4		4.1 ± 0.4		0.02
Bone length (mm)	158.8 ± 4.7		143.5 ± 4.6		<0.001
CSA (mm^2)	240.2 ± 21.8		202.3 ± 23.6		<0.001

Table 14 (cont'd)

Measurements	L4 dimensions				
	Con	T_0	T_{84}	T_{0+84}	P-value
Vertebral body length (mm)	39.1 ± 1.9	39.2 ± 1.9	39.1 ± 1.6	39.0 ± 2.2	0.69
CSA (mm^2)	561 ± 48	593 ± 80	563 ± 48	582 ± 73	0.25
	Males		Females		P-value
Vertebral body length (mm)	40.1 ± 1.8		38.3 ± 1.5		0.003
CSA (mm^2)	619 ± 50		540 ± 44		<0.001

Abbreviations: Con, control treatment no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO.

Biomechanical testing

No treatment, sex, or treatment-by-sex interaction differences were found in any BT values for the right MC_{3+4}. No treatment differences were found for L4 BT variables. However, sex differences were observed. Females had greater compressive stress (P<0.001) and males had a greater modulus of elasticity (P = 0.04, Table 15).

Table 15. Means and SD for flexural stress (N/mm^2) and modulus of elasticity (GPa) reported for the right fused metacarpus (MC_{3+4}). Compressive stress and modulus of elasticity are reported for the fourth lumbar vertebra (L4).

Treatments	MC_{3+4}		L4	
	Flexural stress (N/mm^2)	Modulus of elasticity (GPa)	Compressive stress (N/mm^2)	Modulus of elasticity (GPa)
Con	17.9 ± 4.0	3.0 ± 0.7	22.4 ± 3.5	25.2 ± 5.5
T_0	17.2 ± 5.9	3.1 ± 0.7	21.6 ± 5.1	30.7 ± 4.3
T_{84}	19.3 ± 4.9	3.3 ± 0.7	23.8 ± 3.4	29.5 ± 5.1
T_{0+84}	18.2 ± 4.7	3.2 ± 0.7	21.1 ± 3.8	26.9 ± 6.7
P-value	0.84	0.77	0.30	0.13
Sex	Flexural stress (N/mm^2)	Modulus of elasticity (GPa)	Compressive stress (N/mm^2)	Modulus of elasticity (GPa)
Males	18.7 ± 5.5	2.9 ± 0.5	19.9 ± 3.3	29.8 ± 6.4
Females	17.6 ± 4.0	3.4 ± 0.8	24.8 ± 3.0	26.1 ± 4.3
P-value	0.49	0.09	<0.001	0.04

Abbreviations: Con, control treatment no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO; GPa, Gigapascals.

Micro-computed tomography

No treatment differences were found for the BV/TV, TbSp, TbTh, TbN, ConnD, and BMD of the biopsy site and CtTh of the whole TC (Table 7). Similarly, no treatment-by-time differences were found for BV/TV, TbSp, TbTh, TbN, ConnD, and BMD of TC biopsies. However, time differences were observed for some of the TC biopsy parameters. On day 165, BV/TV, TbSp, and TbTh were increased compared to day 84 ($P \leq 0.04$), and TbN was decreased compared to day 84 ($P<0.001$). No time differences were found for ConnD or BMD (Table 17).

Table 16. Treatment means and SD of the bone healing measured as bone volume fraction (BV/TV), trabecular separation (TbSp), trabecular thickness (TbTh), trabecular number (TbN), trabecular connectivity density (ConnD), and bone mineral density (BMD) of the tuber coxae (TC) biopsy site and whole TC cortical thickness (CtTh).

Measurement	TC Bone healing				
	Con	T_0	T_{84}	T_{0+84}	P-Value
BV/TV (%)	33.6 ± 6.3	32.6 ± 7.2	38.3 ± 11.0	33.7 ± 9.4	0.47
TbSp (mm)	1.87 ± 0.64	1.76 ± 0.85	1.64 ± 0.82	1.56 ± 0.73	0.81
TbTh (mm)	0.58 ± 0.10	0.53 ± 0.10	0.59 ± 0.08	0.50 ± 0.12	0.18
TbN (mm^{-1})	0.44 ± 0.11	0.52 ± 0.26	0.50 ± 0.15	0.55 ± 0.21	0.59
ConnD (mm^{-3})	6.7 ± 4.6	7.0 ± 3.3	6.0 ± 3.3	6.9 ± 3.9	0.95
BMD (mg HA/cm^3)	238 ± 60	277 ± 54	292 ± 63	281 ± 80	0.29
TC CtTh (mm)	0.86 ± 0.10	0.86 ± 0.11	0.85 ± 0.13	0.84 ± 0.05	0.98

Abbreviations: Con, control treatment no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO.

Table 17. Treatment means and SD of the bone volume fraction (BV/TV), trabecular separation (TbSp), trabecular thickness (TbTh), trabecular number (TbN), trabecular connectivity density (ConnD), and bone mineral density (BMD) of the tuber coxae (TC) bone biopsies.

Days	TC bone biopsies				
	BV/TV (%)				
	Con	T_0	T_{84}	T_{0+84}	Average
84	32.9 ± 4.7	33.9 ± 4.6	36.0 ± 9.4	35.1 ± 3.0	34.5 ± 5.8
165	33.8 ± 3.0	37.0 ± 4.4	39.1 ± 6.9	36.5 ± 2.7	36.6 ± 4.8
P-Value			0.80		0.04
Days	TbSp (mm)				
	Con	T_0	T_{84}	T_{0+84}	Average
84	0.49 ± 0.08	0.48 ± 0.07	0.49 ± 0.11	0.47 ± 0.05	0.48 ± 0.07
165	0.54 ± 0.04	0.50 ± 0.05	0.49 ± 0.06	0.50 ± 0.04	0.51 ± 0.05
P-Value			0.44		0.02
Days	TbTh (mm)				
	Con	T_0	T_{84}	T_{0+84}	Average
84	0.25 ± 0.08	0.25 ± 0.01	0.27 ± 0.02	0.25 ± 0.02	0.26 ± 0.02
165	0.27 ± 0.02	0.27 ± 0.01	0.29 ± 0.03	0.27 ± 0.02	0.28 ± 0.02
P-Value			0.99		<0.001
Days	TbN (mm^{-1})				
	Con	T_0	T_{84}	T_{0+84}	Average
84	1.37 ± 0.10	1.38 ± 0.14	1.34 ± 0.16	1.40 ± 0.13	1.37 ± 0.13
165	1.24 ± 0.07	1.29 ± 0.08	1.29 ± 0.09	1.29 ± 0.07	1.28 ± 0.08
P-Value			0.53		<0.001
Days	ConnD (mm^{-3})				
	Con	T_0	T_{84}	T_{0+84}	Average
84	10.0 ± 2.9	10.8 ± 3.9	10.7 ± 4.4	8.0 ± 3.8	9.8 ± 3.8
165	8.4 ± 3.2	9.8 ± 2.5	10.2 ± 3.5	9.6 ± 2.7	9.5 ± 3.0
P-Value			0.38		0.64
Days	BMD (mg HA/cm^3)				
	Con	T_0	T_{84}	T_{0+84}	Average
84	364 ± 77	390 ± 71	406 ± 68	365 ± 29	381 ± 64
165	363 ± 45	392 ± 76	396 ± 60	391 ± 43	386 ± 56
P-Value			0.78		0.67

Abbreviations: Con, control treatment no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO.

No treatment differences were found for BV/TV, CtTh, TbSp, TbTh, TbN, and BMD of the LMC. Sex differences were found only for the BV/TV and BMD values, with greater values for females than males ($P \leq 0.006$). No treatment, sex, or treatment-by-sex interaction differences were found for BV/TV, CtTh, TbSp, TbTh, TbN, and BMD of the L3 (Table 18).

Table 18. Treatment means and SD of the bone volume fraction (BV/TV), cortical thickness (CtTh), trabecular separation (TbSp), trabecular thickness (TbTh), trabecular number (TbN), and bone mineral density (BMD) of the left metacarpal medial condyle (LMC) and third lumbar vertebra (L3).

Treatments	LMC					
	BV/TV (%)	CtTh (mm)	TbSp (mm)	TbTh (mm)	TbN (mm^{-1})	BMD (mg HA/cm^3)
Con	74.1 ± 4.6	1.34 ± 0.32	0.65 ± 0.17	0.47 ± 0.04	0.92 ± 0.16	3,770 ± 151
T_0	74.9 ± 5.3	1.57 ± 0.46	0.64 ± 0.24	0.46 ± 0.05	0.94 ± 0.16	3,601 ± 227
T_{84}	75.9 ± 3.1	1.55 ± 0.15	0.81 ± 0.30	0.47 ± 0.03	0.81 ± 0.30	3,786 ± 172
T_{0+84}	76.6 ± 5.0	1.58 ± 0.24	0.60 ± 0.12	0.46 ± 0.05	0.95 ± 0.10	3,646 ± 265
P-value	0.55	0.33	0.17	0.95	0.18	0.16
Sex	BV/TV (%)	CtTh (mm)	TbSp (mm)	TbTh (mm)	TbN (mm^{-1})	BMD (mg HA/cm^3)
Males	73.4 ± 4.2	1.47 ± 0.31	0.74 ± 0.24	0.46 ± 0.04	0.87 ± 0.16	3,596 ± 209
Females	77.4 ± 3.9	1.56 ± 0.32	0.61 ± 0.20	0.47 ± 0.04	0.94 ± 0.14	3,807 ± 170
P-value	0.006	0.33	0.09	0.25	0.13	0.003
Treatments	L3					
	BV/TV (%)	CtTh (mm)	TbSp (mm)	TbTh (mm)	TbN (mm^{-1})	BMD (mg HA/cm^3)
Con	47.6 ± 3.7	0.94 ± 0.11	0.60 ± 0.04	0.32 ± 0.02	1.10 ± 0.05	1,718 ± 140
T_0	49.2 ± 2.4	1.02 ± 0.16	0.56 ± 0.04	0.31 ± 0.01	1.15 ± 0.06	1,830± 148
T_{84}	50.7 ± 4.0	1.06 ± 0.17	0.57 ± 0.05	0.32 ± 0.01	1.13 ± 0.06	1,844 ± 191
T_{0+84}	47.8 ± 4.1	1.00 ± 0.13	0.58 ± 0.04	0.31 ± 0.02	1.12 ± 0.05	1,835 ± 139
P-value	0.21	0.35	0.26	0.64	0.22	0.21
Sex	BV/TV (%)	CtTh (mm)	TbSp (mm)	TbTh (mm)	TbN (mm^{-1})	BMD (mg HA/cm^3)
Males	47.7 ± 4.1	0.97 ± 0.15	0.58 ± 0.04	0.31 ± 0.01	1.12 ± 0.05	1,789 ± 164
Females	50.0 ± 3.0	1.03 ± 0.14	0.58 ± 0.04	0.32 ± 0.02	1.12 ± 0.06	1,824 ± 156
P-value	0.06	0.20	0.65	0.30	0.93	0.57

Abbreviations: Con, control treatment no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO.

Serum bone biomarkers

Day 56 of PINP was excluded from the analysis due to laboratory error and lack of additional samples. No treatment or treatment-by-time interactions were found in SBB. However, time differences were observed for bone formation (BALP and PINP) and resorption

markers (CTX-I and TRAP5b). Bone formation markers were higher on day 28 compared to day 0 ($P \leq 0.03$). CTX-I was higher on days 0, 56, and 140 ($P \leq 0.03$) compared to other days, while TRAP5b was higher on days 28 and 112 ($P \leq 0.02$) compared to other days. RANKL had no changes over time (Table 19).

Table 19. Treatment means and SD of serum bone marker bone-specific alkaline phosphatase (BALP), procollagen type I amino-terminal propeptide (PINP), receptor activator of nuclear factor-κB ligand (RANKL), tartrate-resistant acid phosphatase isoenzyme 5b (TRAP5b), and carboxy-telopeptide of type I collagen cross-links (CTX-I).

			BALP (ng/ml)		
Day	Con	T_0	T_{84}	T_{0+84}	Average
0	7.7 ± 5.0	6.3 ± 3.5	7.7 ± 3.8	6.0 ± 2.2	6.9 ± 3.7[c]
28	9.9 ± 8.1	9.4 ± 6.0	8.2 ± 4.5	9.3 ± 5.2	9.2 ± 5.9[ab]
56	12.0 ± 12.5	9.5 ± 6.6	9.3 ± 5.8	10.8 ± 6.8	10.4 ± 8.1[a]
84	9.0 ± 5.8	7.1 ± 4.0	7.1 ± 4.6	8.4 ± 3.9	7.9 ± 4.5[bc]
112	9.6 ± 9.9	8.6 ± 4.8	7.3 ± 3.7	8.0 ± 3.9	8.4 ± 6.0[abc]
140	10.3 ± 11.6	7.4 ± 3.7	6.9 ± 4.7	7.3 ± 3.1	8.0 ± 6.7[abc]
163	9.5 ± 10.0	6.2 ± 2.4	6.6 ± 4.5	6.1 ± 3.0	7.1 ± 5.8[bc]
P-Value			0.92		<0.001
			PINP (ng/ml)		
Day	Con	T_0	T_{84}	T_{0+84}	Average
0	1.7 ± 1.5	1.2 ± 0.9	1.8 ± 1.3	1.3 ± 0.7	1.5 ± 1.1[b]
28	2.1 ± 1.5	1.6 ± 1.3	1.8 ± 0.9	1.9 ± 1.4	1.8 ± 1.3[a]
56[1]	-	-	-	-	-
84	1.8 ± 1.2	1.4 ± 0.8	1.5 ± 0.9	1.8 ± 1.1	1.6 ± 1.0[ab]
112	1.5 ± 1.1	1.5 ± 1.0	1.4 ± 0.9	1.5 ± 0.8	1.5 ± 0.9[ab]
140	1.7 ± 1.3	1.4 ± 0.8	1.4 ± 0.9	1.7 ± 1.0	1.5 ± 1.0[ab]
163	1.4 ± 0.9	1.1 ± 0.5	1.2 ± 0.8	1.4 ± 0.8	1.3 ± 0.7[b]
P-Value			0.85		<0.001
			RANKL (ng/ml)		
Day	Con	T_0	T_{84}	T_{0+84}	Average
0	33.7 ± 14.3	37.6 ± 14.3	29.9 ± 9.3	37.8 ± 14.6	34.7 ± 13.2
28	23.6 ± 8.3	34.0 ± 18.8	29.4 ± 10.5	26.6 ± 7.0	28.0 ± 11.8
56	33.3 ± 7.4	28.0 ± 8.8	31.9 ± 6.9	31.5 ± 9.9	31.2 ± 8.2
84	27.1 ± 15.2	33.6 ± 14.7	22.2 ± 6.8	39.1 ± 14.7	31.0 ± 14.5
112	31.7 ± 16.7	24.5 ± 8.1	25.4 ± 13.5	35.6 ± 13.5	29.4 ± 13.7
140	24.0 ± 9.2	34.3 ± 17.3	30.4 ± 16.9	31.7 ± 13.7	30.0 ± 14.5
163	31.5 ± 17.5	36.2 ± 14.6	25.9 ± 15.8	24.5 ± 9.1	29.2 ± 14.5
P-Value			0.62		0.28

Table 19 (cont'd)

CTX-I (ng/ml)					
Day	Con	T_0	T_{84}	T_{0+84}	Average
0	10.3 ± 2.9	9.3 ± 3.3	9.4 ± 3.1	10.6 ± 3.7	9.9 ± 3.2^{a}
28	8.0 ± 2.3	8.4 ± 2.5	7.5 ± 1.8	9.5 ± 2.8	8.3 ± 2.4^{bcd}
56	9.7 ± 3.0	8.6 ± 1.6	9.2 ± 2.1	10.3 ± 3.0	9.5 ± 2.5^{ab}
84	7.2 ± 1.5	8.2 ± 2.2	7.3 ± 1.7	9.4 ± 2.9	8.0 ± 2.2^{cd}
112	7.1 ± 1.0	7.7 ± 2.2	7.2 ± 2.4	7.7 ± 1.1	7.4 ± 1.7^{d}
140	9.0 ± 2.7	9.2 ± 1.9	8.2 ± 1.6	10.7 ± 3.0	9.3 ± 2.5^{abc}
163	7.0 ± 2.2	8.5 ± 2.1	6.3 ± 1.7	9.1 ± 3.3	7.7 ± 2.6^{d}
P-Value			0.63		<0.001
TRAP5b (ng/ml)					
Day	Con	T_0	T_{84}	T_{0+84}	Average
0	22.7 ± 16.6	18.3 ± 9.3	22.2 ± 12.1	18.3 ± 8.1	20.4 ± 11.7^{c}
28	34.9 ± 38.0	24.3 ± 15.9	26.6 ± 15.2	29.5 ± 15.7	28.8 ± 22.7^{ab}
56	33.5 ± 39.8	21.2 ± 13.3	27.2 ± 22.8	28.4 ± 22.7	27.6 ± 25.8^{bc}
84	33.2 ± 44.1	20.2 ± 10.4	21.5 ± 10.5	25.1 ± 11.3	25.0 ± 23.6^{bc}
112	33.5 ± 29.1	29.7 ± 19.1	26.4 ± 16.7	32.0 ± 18.2	30.4 ± 20.7^{a}
140	28.2 ± 29.0	20.9 ± 10.2	19.8 ± 10.1	22.3 ± 7.7	22.8 ± 16.4^{bc}
163	26.7 ± 33.4	18.6 ± 9.2	18.0 ± 9.3	18.8 ± 5.9	20.6 ± 18.0^{c}
P-Value			0.98		<0.001

Abbreviations: Con, control treatment no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO. Means followed by a common letter are not significantly different by the Tukey-Kramer test at the 5% level of significance.
[1]Day 56 PINP data excluded from the analysis due to laboratory error and lack of sufficient samples.

No treatment differences were found for BALP/CTX-I and CTX-I/TRAP5b indexes. However, time differences were observed. BALP/CTX-I on days 28 and 56 were higher ($P \leq 0.009$) than day 0. In contrast, CTX-I/TRAP5b on days 28, 84, and 112 were lower ($P \leq 0.02$) than on days 0 and 56 (Table 20).

Table 20. Treatment means and SD of the remodeling ratio (BALP/CTX-I index) and resorption ratio (CTX-I/TRAP5b index).

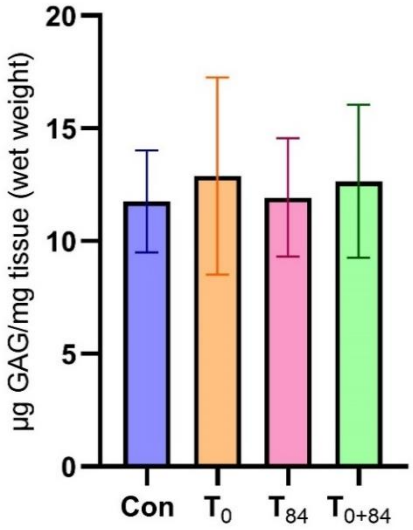
BALP/CTX-I index					
Day	Con	T_0	T_{84}	T_{0+84}	Average
0	0.81 ± 0.56	0.80 ± 0.56	0.93 ± 0.60	0.62 ± 0.29	0.79 ± 0.51[b]
28	1.43 ± 1.28	1.31 ± 0.90	1.19 ± 0.53	1.17 ± 0.73	1.28 ± 0.87[a]
56	1.41 ± 1.64	1.07 ± 0.69	1.16 ± 1.02	1.13 ± 0.69	1.19 ± 1.05[a]
84	1.25 ± 0.77	0.94 ± 0.63	0.99 ± 0.61	1.00 ± 0.59	1.05 ± 0.64[ab]
112	1.35 ± 1.26	1.12 ± 0.57	1.16 ± 0.79	1.09 ± 0.62	1.18 ± 0.83[ab]
140	1.25 ± 1.49	0.83 ± 0.44	0.88 ± 0.61	0.76 ± 0.41	0.93 ± 0.85[ab]
163	1.35 ± 1.27	0.75 ± 0.26	1.12 ± 0.77	0.77 ± 0.46	1.00 ± 0.81[ab]
P-Value			0.99		<0.001
CTX-I/TRAP5b index					
Day	Con	T_0	T_{84}	T_{0+84}	Average
0	0.67 ± 0.47	0.67 ± 0.46	0.52 ± 0.27	0.67 ± 0.37	0.64 ± 0.39[a]
28	0.36 ± 0.19	0.49 ± 0.40	0.32 ± 0.15	0.38 ± 0.22	0.39 ± 0.25[bc]
56	0.63 ± 0.53	0.54 ± 0.26	0.52 ± 0.29	0.61 ± 0.57	0.58 ± 0.42[a]
84	0.42 ± 0.25	0.51 ± 0.28	0.39 ± 0.15	0.46 ± 0.28	0.45 ± 0.24[bc]
112	0.33 ± 0.21	0.34 ± 0.18	0.36 ± 0.22	0.33 ± 0.19	0.34 ± 0.19[c]
140	0.56 ± 0.40	0.54 ± 0.28	0.50 ± 0.22	0.57 ± 0.33	0.54 ± 0.30[ab]
163	0.46 ± 0.27	0.53 ± 0.21	0.43 ± 0.20	0.55 ± 0.34	0.49 ± 0.26[ab]
P-Value			0.84		<0.001

Abbreviations: Con, control treatment no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO. Means followed by a common letter are not significantly different by the Tukey-Kramer test at the 5% level of significance.

Sulfated glycosaminoglycans (GAG)

Cartilage GAG content did not differ between treatments (Figure 4).

Figure 4. Treatment means and SD of the sulfated glycosaminoglycan (GAG) content after tissue papain digestion (µg GAG/mg tissue) of cartilage samples from the right proximal articular surface of the radius subjected to the following treatments: Con, control treatment no drugs administered; T_0, group treated once on day 0 with clodronate disodium (CLO); T_{84}, group treated once on day 84 with CLO; T_{0+84} group treated on day 0 and 84 with CLO. No differences between treatments were found (P = 0.82).



DISCUSSION

are one of the most common drugs used to treat and prevent bone resorption via osteoclast impairment [3,24,28,42]. However, the effects of bisphosphonates on immature skeletal development while undergoing exercise are largely unknown [5,10,80,106,200]. Therefore, we sought to determine the effects of a single or repeated i.m. administration of CLO in a juvenile sheep model subjected to exercise. We failed to reject our null hypotheses, as no differences were detected among treatments. However, sex differences were realized for multiple outcome measures including BW, height, right MC_{3+4} (dimensions), L4 (dimensions and BT), and LMC (BV/TV and BMD). Also, BW, height, TC biopsies (BV/TV, TbSp, and TbTh), and SBB (BALP, PINP, CTX-I, and TRAP5b) outcome measures were noted to change over the study period.

Treatments with bisphosphonates impair osteoclast function [3,24]; these biological

changes can be detected through SBB [188,189], changes in BMD [188,189], and/or BT [190,191]. Our results suggested that CLO did not produce any measurable effects on bone metabolism in juvenile sheep subjected to exercise. Our findings are similar to previous reports that showed bisphosphonates administered to large animals (i.e., horses) were clinically effective [56,75,201,215], although did not result in changes in SBB, BMD, micro-CT measurements, and/or BT [38,56,75,196,201,215] using FDA-approved doses [38,74]. This may be due to the lower dose used in large animals compared to humans. We used a dose in sheep that resembled the pharmacokinetic values of therapeutic CLO administered in horses [202]. The dose used in horses (1.8 mg/kg) has shown clinical efficacy in reducing musculoskeletal pain in multiple studies [38,56,75,201]. However, humans treated for osteoporosis receive 200 mg i.m. every 2 weeks (approximately 2.7 mg/kg based on a 75 kg person) which results in detectable BMD increases [216]. A linear relationship is described between the dose and frequency of bisphosphonates and their effects [190,216,217], where a greater dose correlates with a greater inhibition of osteoclasts and subsequent effects on bone turnover. Therefore, lower CLO doses may lead to clinical effects without significant antiresorptive outcomes in the skeleton [218].

Clodronate has a lower anti-resorptive potency compared to other bisphosphonates which may explain the lack of measurable bone effects. Bisphosphonates are categorized into nitrogen-containing and non-nitrogen-containing bisphosphonates [22,126], and it is well established that the non-nitrogen-containing bisphosphonates (e.g., CLO) have a lower binding affinity and antiresorptive potency than nitrogen-containing bisphosphonates (e.g., ibandronate or zoledronate) [22,126]. Consequently, the class of bisphosphonate, coupled with the lower dose employed in this study, may have fallen below the threshold needed to produce detectable osteoclast inhibition. Notably, exercise and age influence the bisphosphonate distribution in the

body. Exercise decreases the glomerular filtration rate [219], which in turn reduces the excretion of CLO and enhances blood supply to the bones [220], potentially increasing CLO availability within the skeletal system. Additionally, age influences bisphosphonate absorption by the bones, with higher absorption expected in young populations due to greater bone turnover compared to adults [33,221]. Even though this study used juvenile animals subjected to exercise, which could have increased their exposure to CLO, no measurable skeletal effects were observed. Administering a higher dose and/or evaluating a higher-intensity training may result in detectable changes in bone parameters.

Although SBB and TC biopsies did not show any time-by-treatment differences, there was an effect of time. In this study, bone formation markers increased on days 28 (BALP and PINP) and 56 (BALP), while CTX-I, which is a marker of osteoclast activity [211], decreased on several days. Similarly, the BALP/CTX-I remodeling index increased on days 28 and 56, suggesting further increase in bone formation on those days. This idea is supported by an increase observed on BV/TV driven by an increase of TbTh in the TC biopsies. However, TRAP5b, a marker of osteoclast numbers [174,211], increased on days 28 and 112, while RANKL did not increase during the study, indicating that there was not a significant osteoclast activation [11]. TRAP5b can be released from both mature and immature osteoclasts [174,222]; hence, an increase in this marker may not necessarily indicate increases in bone resorption. Moreover, when CTX-I is evaluated in relation to TRAP5b (CTX-I/TRAP5b), it may provide a more comprehensive understanding of the biological activity of bone resorption. The CTX-I/TRAP5b resorption index showed a consistent decrease on days 28, 84, and 112, in similar fashion to the response in BALP, PINP, and CTX-I. Future studies should include other markers, such as cathepsin K, which is released by mature osteoclasts only [174].

The observed changes in TC biopsies could be explained by the exercise protocol, as physical activity favors increased bone formation and decreased bone resorption, as observed in the SBB [223]. However, TC is a non-weight bearing bone and may not respond to exercise [224]. Therefore, increases in BALP and PINP (bone formation markers), and no changes in RANKL with decreases in CTX-I (bone resorption markers), may be more related to animal growth than exercise. Sheep were approximately 80% developed at the beginning of the study and showed significant growth during the study period. Increases in BV/TV and TbTh are commonly found in the iliac bone of growing children [225], which is similar to the TC changes observed in the juvenile sheep. For this reason, future studies should include a sedentary control group in order to differentiate between the effects of exercise and/or growth when using juvenile animals.

Sex differences were observed in several parameters, including BW, height, right MC_{3+4} and L4 dimensions, L4 BT, and LMC BV/TV and BMD. Sexual dimorphism is a natural characteristic of this species, particularly regarding body size [226,227]. This study utilized castrated males. Orchiectomy leads to a decrease in sex hormones, resulting in reduced bone mass due to decreased bone accrual during growth and increased bone resorption [228–230]. Therefore, the reductions in LMC BV/TV and BMD of males compared to females can be explained by the reduction of sex hormones. Furthermore, the castrated male L4 specimens exhibited decreased compressive stress at failure and an increased modulus of elasticity compared to the intact female L4 specimens. This apparent contradiction between decreased compressive stress at failure and increased modulus of elasticity can be explained by the lack of circulating sex hormones as well. Studies show that vertebrae compensate for bone loss from reduced sex hormones by cranio-caudally rearranging their collagen and HAP [231,232]. This

rearrangement results in an increased modulus of elasticity, despite a decrease in compressive stress at failure [231,232]. Although L4 specimens did not undergo micro-CT analysis, L3 microstructure was analyzed and no differences between sexes were found. However, there was a trend ($P = 0.06$) towards increased BV/TV in females compared to males in L3 specimens, suggesting that even small changes in BV/TV may significantly affect the mechanical structure of the lumbar vertebrae. Thus, castrated male L4 specimens may have experienced cranio-caudal collagen and HAP rearrangement as a compensatory response, although this was not addressed in the present study. Notably, no significant BMT sex differences in weight-bearing bones were found (e.g., right MC_{3+4}), indicating that the lack of sex hormones' effect on weight-bearing bones can be compensated for by physical activity [230,233–235]. Based on our findings, future studies should consider the effects of castration influencing sex hormone levels and bone microstructure. This will aid in translating results from the sheep model to other species, including humans.

In addition to the PE, lameness evaluations showed differences in the T_0 group only, with an increase in the probability of lameness observed on day 94. This increase is likely attributed to a temporary increase in lameness following the bone biopsy procedure performed on day 84. In this group, one individual remained consistently lame following TC biopsy; this individual was eventually removed from the future analysis. No other increases in probability of lameness were detected, suggesting that the sheep tolerated the exercise protocol well and CLO did not result in significant lameness.

Detectable levels of CLO are achieved in synovial fluid following i.m. administration in horses [39]. To assess the potential effect of CLO on cartilage health in juvenile, exercising animals, GAG content was measured from cartilage samples. GAG analysis of cartilage samples

from the proximal surface of the radius did not reveal treatment differences. This finding is consistent with a previous *in vitro* investigation that evaluated the impact of various concentrations of CLO on cartilage explants, chondrocytes, and synoviocytes and found no evidence of either cytoprotective or cytotoxic effects [236]. High doses of bisphosphonates have shown *in vitro* cytotoxicity in joint tissues, such as bovine chondrocytes [237] and equine cartilage [238]. Bisphosphonate content in the synovial fluid of joints was not measured in the current study. Future experimental studies should explore different doses of CLO *in vivo*, as low doses of CLO may provide a clinical effect without cartilage toxicity.

Another known effect of bisphosphonates is their analgesic properties. CLO has also been used to treat chronic pain in humans [239,240]. Analgesic effects of CLO may be attributed to blockade of the vesicular nucleotide transporter [57]; CLO is the strongest bisphosphonate inhibitor of this transporter [241]. These analgesic effects are desirable in painful bone-related conditions, such as osteogenesis imperfecta or bone cancer [239]. Bisphosphonates' analgesia can have long-lasting effects [239], and can be independent of antiresorptive effects [218], as demonstrated in multiple studies where analgesic effects are found in the absence of skeletal changes [38,56,74,75,196]. Therefore, low doses of CLO may be used as treatment for refractory pain without skeletal changes [218]. The analgesic effects of CLO were not evaluated in this juvenile sheep model. However, future studies using this model are warranted to assess the analgesic effects of low doses of CLO considering the lack of negative skeletal effects in growing, active individuals. This could be particularly promising for children experiencing musculoskeletal pain for which long-term pain management with non-steroidal anti-inflammatories, opioids or steroids could result in significant morbidity.

While we were able to compare treated animals to an untreated control group in the

exercising study population, without a sedentary group we are not able to identify the effect of exercise, exercise intensity, or growth. In addition, SBB can show changes within hours after exercise. Having more sample points may have allowed for a better characterization of the SBB' response over time [223]. Further, bisphosphonates may produce glomerulosclerosis [58], and additional blood and urine samples may have helped to characterize the risk of CLO use by exercising individuals. Finally, the sex should be considered in future studies as castration may have influenced some skeletal outcomes.

CONCLUSIONS

In conclusion, our study found no measurable skeletal effects on juvenile, exercising sheep following the administration of a single or repeated dose of 0.6 mg/kg of CLO. The lack of effects could be attributed to the lower dose used in animals. Further investigation is required to explore bisphosphonates' analgesic effects, as low doses of CLO may provide analgesic benefits with minimal negative skeletal effects. Long-lasting analgesia without negative skeletal effects may be particularly advantageous considering the morbidity associated with long term use of other commonly used analgesics such as non-steroidal anti-inflammatories, steroids, and opioids. Future studies should explore effects of sex, include a sedentary group, and investigate additional doses in conjunction with exercise. Moreover, additional studies should investigate the effects of more potent bisphosphonates, such as zoledronic acid, or newer bisphosphonates, such as lidadronate (IG9402) [105], on the skeleton of juvenile, active individuals.

CHAPTER 6: Conclusions

The skeleton is influenced by various factors, including activity, nutrition, hormones, and pharmacological interventions. Bisphosphonates (BPs) are a group of drugs that inhibit bone resorption by impairing osteoclast function, thus preventing bone loss. Although two BPs have been approved since 2014 for use in horses over four years old, concerns have been raised following their FDA approval. Currently, there is no known association between equine catastrophic injuries and bisphosphonate use. However, evidence from animal models and human studies suggests that these drugs may have significant negative consequences. Therefore, the primary objective of this dissertation was to investigate how BPs can affect the skeleton and joints, particularly in juvenile exercising animals. The focus was on juvenile animals, where bone modeling and remodeling are highly active and osteoclast impairment could be detrimental to growth and adaptation to exercise.

In this dissertation, we examined the effects of clodronate disodium (CLO) on equine joint tissues *in vitro*, assessed the pharmacokinetics of CLO in sheep, and investigated the effects of CLO on juvenile exercising sheep as a model for horses. Our initial study investigated the potential effects (beneficial or detrimental) of CLO on joint tissues, including cartilage explants, chondrocytes, and synoviocytes, using three different CLO doses (5, 50, and 100 ng/mL). These doses were chosen based on previous literature indicating that CLO reaches the synovial fluid after intramuscular administration of the drug. Our findings indicated that CLO did not induce cytotoxicity in joint tissues; however, we did not observe any anti-inflammatory effects using an equine recombinant IL-1β inflammatory model. These results suggest that administering CLO to horses may not adversely affect joint tissues. Although an anti-inflammatory effect was not observed, it is possible that CLO acts on other cells, such as macrophages.

We used a sheep model to further evaluate the effects of CLO *in vivo*. Sheep have been used previously as models for bisphosphonate administration and exercising horses. To evaluate the suitability of juvenile sheep as an animal model for horses, we conducted a pharmacokinetic (PK) study using 11 juvenile sheep and a CLO dosage of 0.6 mg/kg. Our study concluded that a CLO dose of 0.6 mg/kg IM in sheep closely resembles the PK parameters reported in previous studies on horses. In addition, the plasma protein binding (PPB) of clodronate is similar between horses and sheep. Therefore, based on our evaluation of PK and PPB, sheep appear to be a viable animal model for studying the effects of CLO in juvenile horses.

A novel exercise protocol was developed in order to investigate the effect of CLO on juvenile, exercising sheep as a model for horses. We successfully trained a group of 40 juvenile sheep using an increasing exercise protocol on a high-speed exerciser. The sheep demonstrated excellent adaptability to training, and serum bone biomarkers showed potential for assessing bone metabolism.

Administration of CLO at 0.6 mg/kg IM in juvenile, exercising sheep over a 165-day period did not result in measurable skeletal effects or changes in bone serum biomarkers. In previous human and animal studies, BPs are noted to result in increased bone density and reduction of serum bone resorption markers. Though, the dose used in humans is higher than those utilized in horses and newer generation BPs used in human medicine are more potent (e.g., zoledronate) in comparison to CLO. The lack of significant skeletal effects with CLO at relatively low dose in our study may suggest that the positive outcomes observed in previous horse studies, such as improved lameness, could be attributed to the analgesic properties of BPs, as no changes in bone were detected. However, this is merely hypothetical as this dissertation work did not assess the analgesic effects of BPs. Additionally, assay sensitivities or differences

in drug metabolism between species (sheep vs. horses) could have also played a role in the lack of effects detected in the skeleton.

The results of this dissertation work suggest that CLO use could be safe for the skeleton of exercising, juvenile animals up to two doses, possibly due to the relative low potency of CLO and low dose used in this study. However, extra-label use reported by a retrospective study in horses may involve up to nine doses. More frequent doses may induce negative effects in the skeleton, such as increased bone brittleness and/or increased risk of fractures, as BPs accumulate in bone due to very extended half-lives. Therefore, future studies should investigate the PK of BPs for longer periods of time (months or years) and in repeated doses. Also, further research should evaluate how exercise may alter the PK of BPs as renal and skeletal blood flow decreases and increases, respectively. Additionally, future research should assess the CLO analgesic effects by optimizing dose regimens, using younger animal models, and investigating alternative administration routes (e.g., i.v. and/or s.c. routes). Such investigations into the analgesic properties of CLO have the potential to significantly impact palliative care for both humans and animals, particularly if analgesic effects are detected at low doses, with minimal or without skeletal changes, as those found in this dissertation work.

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APPENDIX

Figure S1. Diagram and pictures of facilities used for the exercise protocol. A: satellite image is used to show the location of indoor pens (dark blue, 21.6 m2 each), the distance between the walker and indoor pens (light blue, 32 m of fencing), the 18 m diameter high-speed walker (Q-Line Horse Exerciser, Aromas, CA). B: Panoramic view of the high-speed walker. C: Inside view of sheep walking at 1.3 m/s. Sheep walked clockwise and counterclockwise on alternate days.

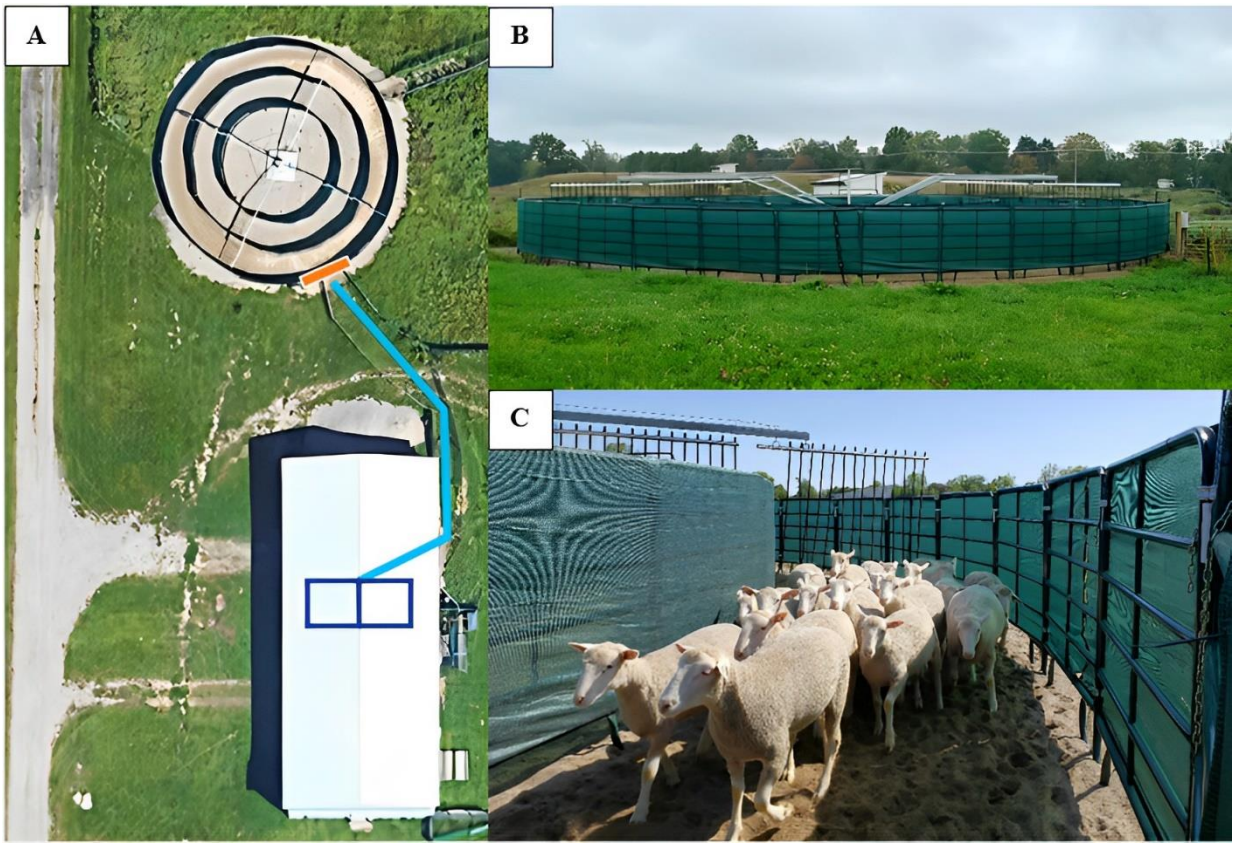

Figure S2. A: Placement of the right fused third and fourth metacarpal (MC_{3+4}) for four-point bending test using electromechanical testing system (60 kN load cell, MTSCriterion, Model 43, Eden Prairie, MN, USA). Specimens were kept with wrapping paper to avoid dehydration and loss of fragments after fracture failure. B: Diagram of a right MC_{3+4} subjected to 4-point bending on an Instron. The load exerted (*F*) on the bone is depicted as the Instron measures the bone's displacement (*V*). *L* is the span length, which was 51.9 mm; a is the distance between *F* and the supports on either end of the bone with a value of 3.8 mm. Each support was 24.5 mm wide.

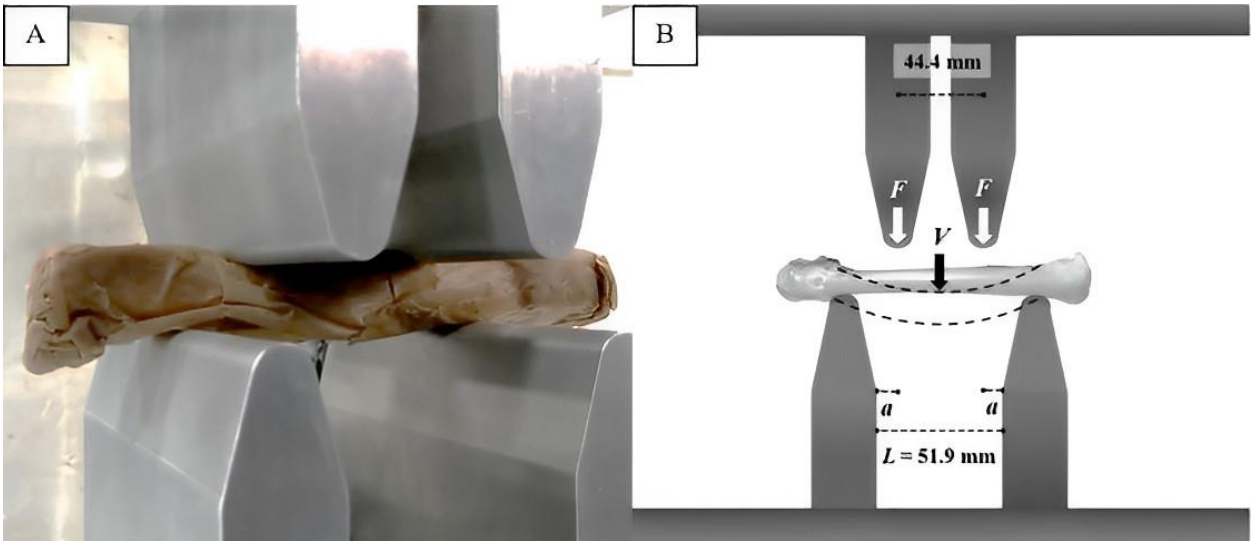

Figure S3. Preparation of vertebral bodies prior to embedding with polyurethane resin. Dissection from soft tissues, transverse processes, and dorsal arch.

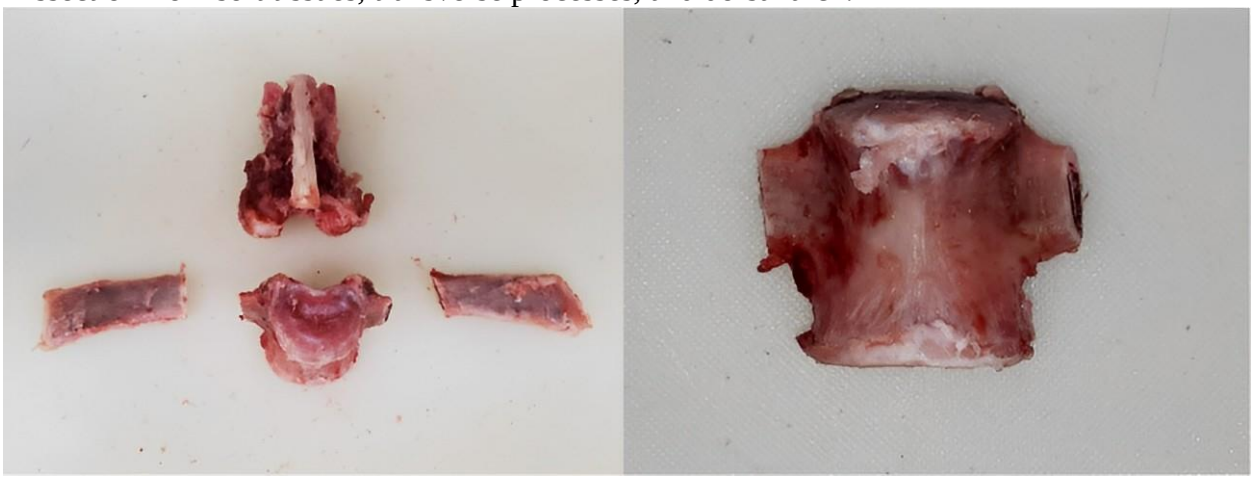

Figure S4. A: 3D print holder to keep cranial and caudal-leveled surfaces. B: Silicone molds and 3D print used holder for polyurethane resin embedding (TC-808, BJB Enterprises, Tustin, CA, USA) of vertebral bodies prior to compression tests.

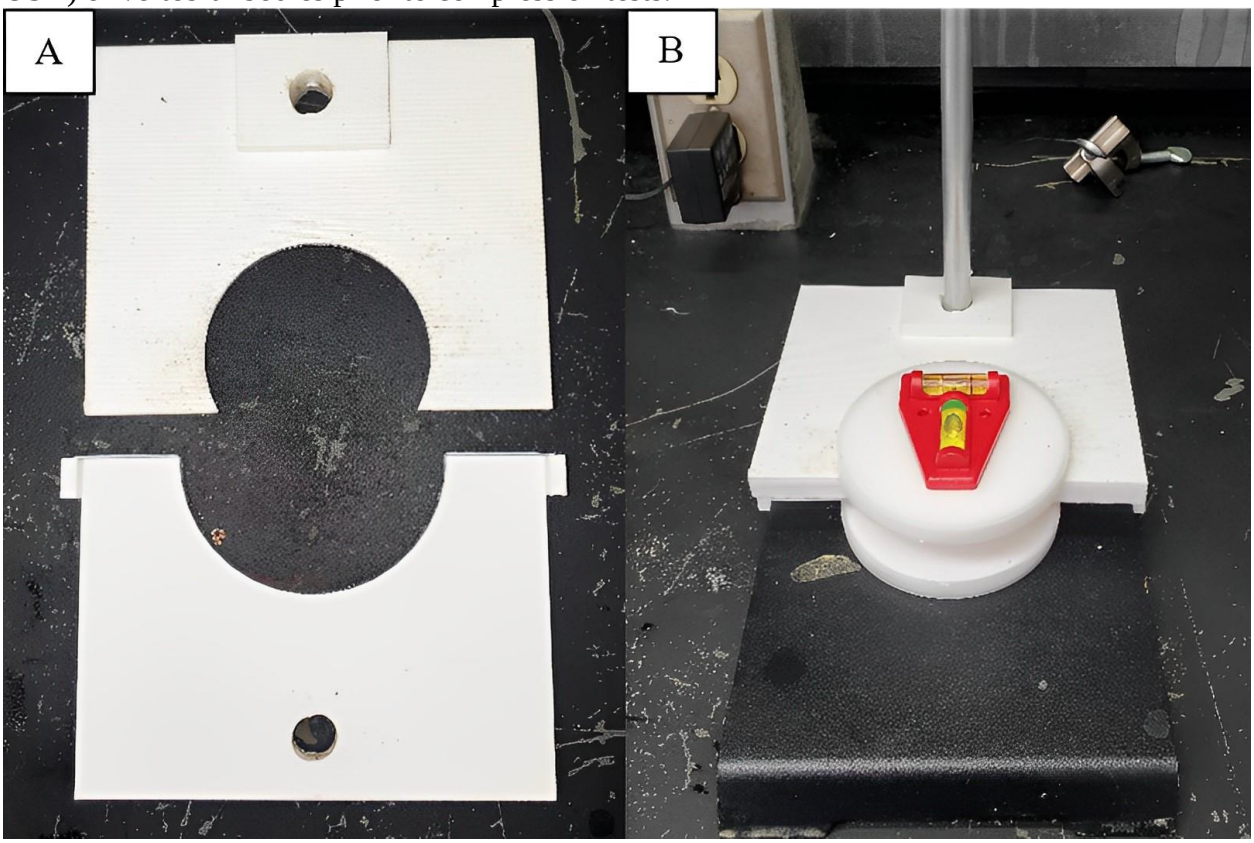

Figure S5. Lateral view of fourth lumbar vertebra for electromechanical testing compression test equipped with a 100kN load cell (Instron model 5982 Norwood, MA, USA) and a flat 3 mm thick metal plate.