

CLODRONATE USE IN YEARLING, EXERCISING HORSES ON ENDOCRINE REGULATION
OF BONE GROWTH AND DEVELOPMENT

by

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DEDICATION

This work is dedicated to Dr. Amanda Bradbery. For showing me the joys of research and being a mentor beyond expectations.

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ABSTRACT

Extra-label bisphosphonate use in juvenile horses is widespread despite limited scientific understanding of biological and welfare impacts on skeletally immature, exercising horses. The objective of this study was to determine the effects of clodronate on endocrine regulation of bone growth, hypothesizing there would be no effect. To test this, 32 Quarter Horses were stratified by age (500 ± 13 d of age), BW (336 ± 26 kg), sex ($n = 16$ males, $n = 16$ females), and initial bone optical density by aluminum equivalence into four treatment groups for a 168-d trial. The experimental period was divided into two phases mimicking sales preparation (Phase I: d 0-83) and early performance training (Phase II: d 84-168). Investigators were blinded, and all horses received iso-volumetric intramuscular injections of either 1.8 mg/kg BW clodronate disodium (OSPHOS®) or saline (placebo) on d 0, 42, 84, and 126. Treatments consisted of control (CON; $n = 8$), single-dose (1X; $n = 8$; d 84), two-dose (2X; $n = 8$; d 0, 84), and four-dose groups (4X; $n = 8$; d 0, 42, 84, 126). Physical measurements including BW, wither height (WH), hip height (HH), body length (BL), and heart girth (HG) circumference were recorded every 42 d from d 0. Serum samples were collected every 42 d prior to treatment administration to quantify growth hormone (GH), calcitonin (CT), parathyroid hormone (PTH), and ionized calcium. Data were analyzed using PROC MIXED of SAS. All physical growth parameters increased ($P \leq 0.01$) and growth hormone tended to decrease over time ($P = 0.09$) following normal growth patterns. A treatment $\times$ time interaction was observed for PTH ($P = 0.05$) where concentrations increased on d 84 in 4X and on d 126 in 2x following the second administration of clodronate while 1X and CON remained unchanged. Ionized Ca decreased over time ($P < 0.01$) with no effect on CT concentrations ($P > 0.33$). These results suggest that while administration of bisphosphonate has no effect on growth morphometrics, its repetitive use alters PTH concentrations after two doses.

LITERATURE REVIEW

Introduction

Two bisphosphonates (BP), clodronate disodium and tiludronate disodium, were approved by the FDA in 2014 for treatment of navicular syndrome in horses 4 years of age and older. Previously, BPs have been used for the prevention and treatment of osteoporosis in humans for decades because of their mechanism of action to limit bone resorption by inhibiting osteoclastic activity (Recker et al., 2009), but the use in horses for navicular syndrome is well supported (Denoix et al., 2003; Whitfield et al., 2016; Mitchell et al., 2019). The benefits of BP in horses with bone abnormalities, however, have resulted in its extra-label use in horses with normal skeletal modeling/remodeling to enhance bone formation, even in the short-term, despite not being FDA approved.

In normal, healthy animals, bone strength and integrity are maintained by a careful balance between resorption and formation. In growing animals, anabolism is greater than catabolism; however, resorption by osteoclast regulation is important for removal of damaged bone, joint maturation, growth plate closure, and the maintenance of skeletal integrity (Drake et al., 2008; Gilday et al., 2020). Normal wear-and-tear from exercise training results in microdamage as bone adapts, and the healing of microdamage is dependent upon normal remodeling through a concerted effort by osteoclasts and osteoblasts. Bisphosphonates, however, inhibit osteoclast activity thereby inhibiting bone resorption and osteoblast-regulated bone formation (Sahni et al., 1993; Rodan and Fleisch, 1996a). Use of BPs in animals without bone degenerative disorders poses a significant risk to skeletal adaptive modeling.

Bone

Most research on equine bone is conducted in areas primarily related to the performance industries, such as racing, and include fracture resistance, structure, and density (Noble et al., 2016; Liley et al., 2018; Selim et al., 2018). This research, however, is primarily focused on mature horses that have already begun their performance careers. While there is some research in the use of exercise as a method of increasing bone and joint health in juvenile horses (Silvers et al., 2020; Logan et al., 2022), the effects of pharmaceuticals such as BPs on juvenile equine bone health and related pathologies are limited. Current understanding of bone tissue derived from research conducted in other species can be used to elucidate this relationship. Therefore, this review will focus on the mechanisms of BPs, bone tissue, and the primary endocrine regulators of bone metabolism: growth hormone (GH), parathyroid hormone (PTH), and calcitonin (CT).

Bone tissue is derived from multiple germ layers during embryonic development, with each layer developing into separate sections of the skeleton (Berendsen and Olsen, 2015). The paraxial mesoderm develops into the axial skeleton, the neural ectoderm develops into the craniofacial region of the skeleton, and the lateral plate mesoderm develops into the appendicular skeleton (Berendsen and Olsen, 2015). Mesenchymal cells from each of these regions migrate to the future sites of bone and differentiate into either osteoblasts or chondrocytes. Chondrocytes act as a blueprint for future bone development by forming cartilage matrices, while the osteoblasts form directly into bone (Berendsen and Olsen, 2015). Thus, formation of bone occurs in two distinct processes: endochondral ossification (chondrocyte lineages) and intramembranous ossification (osteoblast lineages).

Along with mesenchymal cells, bone tissue consists of three additional cell types: osteocytes, osteoblasts, and osteoclasts (Florencio-Silva et al., 2015). Osteoblasts are mononucleated cuboidal cells responsible for bone forming. Osteocytes are the most abundant cell type within bone and originate from differentiated osteoblasts during bone remodeling and bone formation. They are dendritic cells, but slightly differ in shape depending on their location, and serve a mechanosensitive function that allows bone to adapt to incurring forces (Florencio-Silva et al., 2015). Osteoclasts differ from osteoblasts and osteocytes in that they originate from haematopoietic stem cells and are multinucleated. Their function is to resorb bone tissue along the bone matrix (Florencio-Silva et al., 2015). These cells work in conjunction to facilitate multiple bone related processes such as endochondral ossification and bone remodeling.

Although endochondral ossification begins during the fetal stage of development, it continues to occur postnatally until skeletal maturation is achieved (Rolian, 2020). The process is triggered when the proliferated chondrocytes located in the anlage undergo hypertrophy, producing the extracellular matrix (ECM) and triggering vascularization. As blood vessels infiltrate the ECM, they carry osteoblasts that are deposited into the nearby matrix and form bone by mineralizing the chondrocytes within the ECM. This process establishes the primary center of ossification, as waves of bone tissue is developed through this continuous process. The vasculature near the ends of the bone develops one or more secondary centers of ossification using the same process. Convergence of the primary and secondary centers of ossification cause the formation of the physis with the primary center of ossification becoming the diaphysis and the secondary centers of ossification becoming the epiphyses. This process occurs in long bone

until the remaining chondrocytes in the physis calcify, fusing the epiphyses and diaphysis, and signaling skeletal maturation (Figure 1; Salhotra et al., 2020).

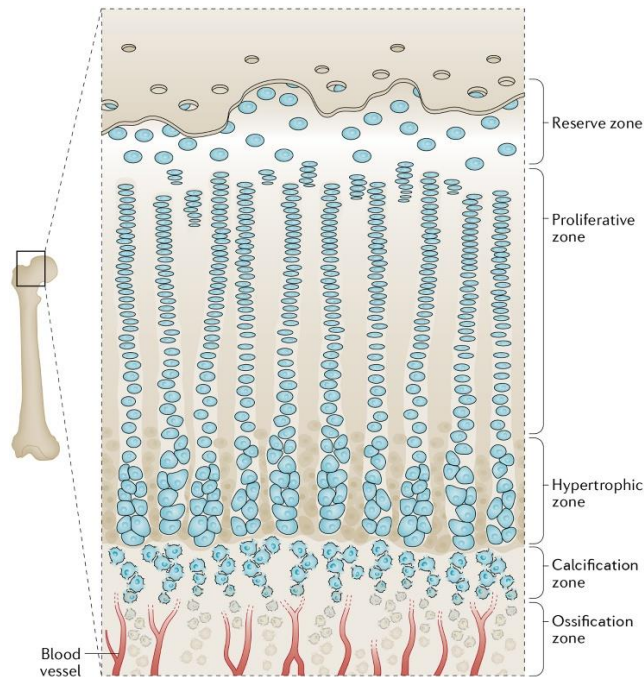


Figure 1. Endochondral ossification in the physis of a long bone with respective zones of activity (Chruścik et al., 2021).

Unlike endochondral ossification, intramembranous ossification is not as well-understood (Abzhanov et al., 2007). It occurs when the mesenchymal cells differentiate into osteoblasts that directly turn into bone (Berendsen and Olsen, 2015) and does not utilize cartilage during the process. Fundamental information on the mechanisms of the developmental process is not fully understood and thus, contributes to the lack of understanding of endochondral bones overall (Percival and Richtsmeier, 2013). Due to the type of bones that are produced during intramembranous ossification, it does not play as large of a role in growth morphometrics, and while injuries to the craniofacial regions and similar flat bones do occur, they are less frequent

causing research to focus primarily on long bone injury in horses. This basic understanding of the process is, however, sufficient to understand the scopes of the study.

In addition to the above types of growth and development, bones also undergo two additional processes called modeling and remodeling (Clarke, 2008). Modeling is defined as the process in which mechanical forces or physiological influences trigger bones to change their overall shape as a response. It is under modeling where Wolff's law, which states that bones will adapt and change shape to accommodate for stresses placed on them can be applied. Modeling primarily occurs during the fetal and developmental stages, unlike remodeling which predominantly occurs during adulthood and lasts until death (Yamamoto et al., 2021). Furthermore, modeling operates with minimal involvement of osteoclasts and primarily utilizes osteoblast function for bone deposition.

Bone remodeling is the process of bone strength maintenance as well as mineral homeostasis through osteoclast mediated resorption of old or damaged bone followed by deposition of new, healthy bone by osteoblasts (Clarke, 2008). Mineral homeostasis is achieved by interfering with the balance of osteoblast and osteoclast function to bias bone deposition or bone resorption. Briefly, increased circulating mineral concentrations trigger CT to signal to osteoclasts to reduce their function and increase overall bone deposition, while decreased circulating mineral concentrations trigger PTH to signal for increased osteoclast function to resorb bone. Lastly, GH stimulates bone turnover by increasing both osteoblast and osteoclast activity (Olney, 2003). Unlike mineral homeostasis regulation, the balance between osteoblast and osteoclast function during remodeling is a key component in the repair of accumulated

microdamage within the bone and its overall health. The replacement of damaged bone with healthy bone allows the tissue to perform its functions throughout the life of the animal.

Bones have multiple key physiological functions: development of haematopoietic cells from the enclosed bone marrow, allow locomotion by acting as levers and attachment points for muscles, protect vital organs, and act as the primary storage of calcium and phosphorus within the body (Grabowski, 2009). The haematopoietic tissue within bone marrow contains adipocytes, macrophages, blood cells, and their precursors (Travlos, 2006). Haematopoiesis occurs within this tissue and is responsible for the formation of blood cells used throughout the body. Additionally, bone, due to its strength and rigid nature, acts as protection for internal organs against trauma and works in conjunction with muscle tissue to facilitate movement; with the musculoskeletal system sharing genetics and endocrine factors (Battafarano et al., 2020). As the largest store of calcium within the body, bone tissue is the primary mediator of ionic calcium concentrations (Bronner, 2001). Regulated by various hormonal signals, bone releases and uptakes calcium to maintain a balanced concentration within the body.

Serum Ionized Calcium

Calcium enters the body via dietary means and is absorbed in the form of calcium ions (Ca^{++}) following digestion through two methods: a paracellular route and transcellular route (Bronner, 2001). The paracellular route becomes significant in the lower half of the small intestine and depends on the differences in calcium concentration between body fluids and the intestinal lumen. The transcellular route is dominant in the duodenum and its activity inversely fluctuates with calcium intake (Bronner et al., 1986). Calcium leaves the body through urine and

feces, with excess calcium filtered out of blood through the kidneys or binding with unabsorbed calcium in digested material.

Extracellular concentrations of Ca^{++} are primarily regulated by bone. When Ca^{++} concentrations are high, bone favors deposition reducing overall resorption to deposit Ca^{++} into the bone matrix. When Ca^{++} concentrations are low, resorption increases to introduce stored Ca^{++} from the bone into circulation (Heaney, 2003). This balance of Ca^{++} concentration in the body is regulated by PTH and CT, the mechanisms of which will be discussed later in this review. Briefly, low Ca^{++} concentrations trigger PTH to signal osteoclasts to increase resorptive activity, increasing Ca^{++} concentrations in the bloodstream. High Ca^{++} concentrations trigger CT to signal osteoclasts to reduce or completely halt its resorptive activity, decreasing Ca^{++} released into the bloodstream.

This regulation of Ca^{++} concentrations is crucial to facilitate the integral functions of calcium within the body including muscle contractions, cardiac function, and bone health (Ebashi, 1972; Ikonomidis et al., 1990; Cashman, 2002). In skeletal muscle tissue, action potentials cause the release of Ca^{++} that binds to troponin exposing myosin binding sites ultimately resulting in muscle contraction (Figure 2; (Ebashi, 1972). Once the action potential subsides, Ca^{++} is recaptured in the sarcoplasmic reticulum and the muscle fiber relaxes. Like skeletal muscle, Ca^{++} plays a similar role in cardiac muscle tissue. It acts as a link between the mechanical shortening of cardiomyocytes and cardiac electrical activity (Ikonomidis et al., 1990). In bone tissue, proper Ca^{++} concentrations are required for normal growth and development as well as healthy bone mass (Cashman, 2002). Inadequate dietary Ca^{++} , especially during adolescence, can hinder development and bone fragility resulting in injury.

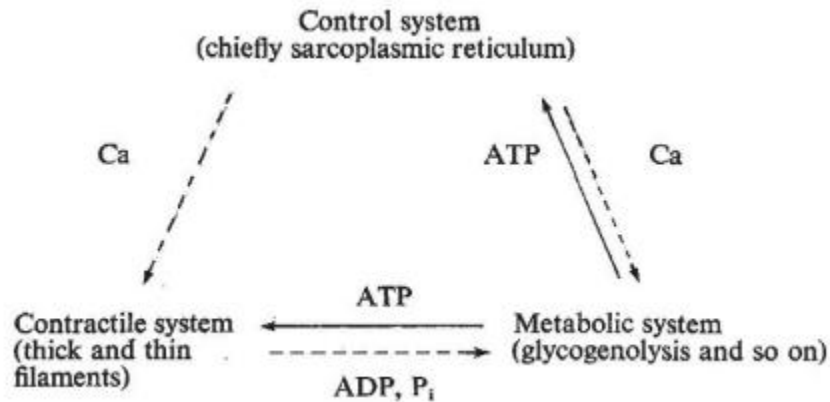


Figure 2. Influence of Calcium in the functional organization of muscle (Ebashi, 1972).

Bisphosphonate

Inorganic pyrophosphates (PPi) are naturally occurring compounds that consists of two phosphate groups linked via an ester bond. Within mammalian cells, PPi is generated as pyrophosphohydrolysis of the phosphodiesterase I bond in purine and pyrimidine nucleoside triphosphates or as a metabolic byproduct (Terkeltaub, 2001). Due to PPi's ability to influence various cell functions, there are many specialized mechanisms for multiple forms of interaction (production, transport, degradation) within cells. Most PPi appear to be primarily generated by the mitochondria and their energy metabolism process. Once generated, PPi has minor functions as a biochemical intracellular intermediate. It can act as a phosphate donor in protein phosphorylation and modify the activity of certain ATPases. However, most research focus has been towards PPi's relationship with bone cells. The compound directly inhibits osteoblast and epiphyseal chondrocyte capabilities to deposit minerals in the bone matrix. Simply, they bind to hydroxyapatite crystals and as a result, inhibit calcification (Rodan and Fleisch, 1996a). This function is the primary driver for the synthesis and subsequent improvements of BPs.

BPs are a form of bone modulating drug that are analogs of PPI, used to treat conditions such as osteoporosis and osteogenesis imperfecta in humans and navicular disease in horses (Drake et al., 2008; Antoniazzi et al., 2010). Like PPI, BPs also contain two phosphate groups, however they are joined by a nonhydrolyzed carbon atom with variations depending on side chains bonded to the central carbon atom (Figure 3; Drake et al., 2008). They bind to hydroxyapatite crystals, giving them a high affinity for bone mineral. Lastly, BPs also inhibit bone resorption by hindering calcification and hydroxyapatite breakdown.

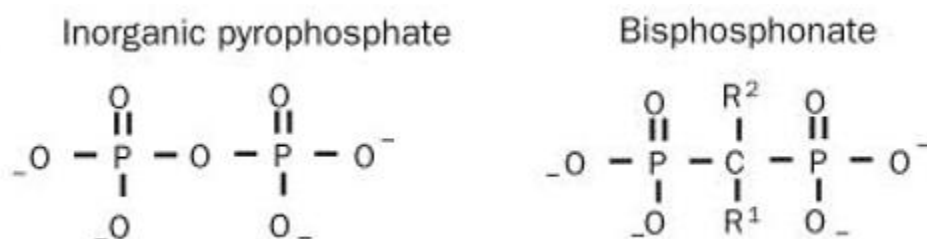


Figure 3. Comparison of the skeletal structures of Inorganic Pyrophosphate and Bisphosphonate (Drake et al., 2008).

Most variants of BP are alike in that they contain a hydroxyl group in the R^1 position of the compound, which further increases its affinity for hydroxyapatite crystals. The R^2 group, however, differs from each variant and is the primary differentiator in a BP's potency (Drake et al., 2008). The antiresorptive potency of a BP exponentially increases with the presence of an amino group or a nitrogen in the R^2 position, therefore, BPs can be divided into two groups: non-nitrogen-containing and nitrogen-containing BPs. Broad application of the mechanism of action of both groups of BPs are very similar. They deposit where bone is undergoing remodeling as the bone mineral in these locations are exposed to surrounding fluids (Rodan and Fleisch, 1996). When the location of BP deposit is resorbed in subsequent iterations of bone remodeling the compound is released.

However, the specific mechanisms greatly differ between the two groups of BPs. Earlier iterations of BPs are considered the first-generation and consist of non-nitrogen-containing BPs including etidronate, clodronate, and tiludronate. They have the lowest potency of BPs and have a simpler mechanism of action compared to their counterparts. Once resorbed by osteoclasts, class II aminoacyl-transfer RNA synthases incorporate non-nitrogen-containing BPs into molecules of adenosine triphosphate (ATP) (Drake et al., 2008). These nonhydrolyzable ATP analogues accumulate within the osteoclast and inhibit multiple ATP-dependent processes causing apoptosis.

Nitrogen-containing BPs make up the second- and third-generation BPs and include alendronate, risedronate, ibandronate, pamidronate, and zoledronic acid. The presence of a nitrogen atom allows these BPs to bind to and inhibit farnesyl pyrophosphate synthase activity (Dunford et al., 2001). Inhibition of farnesyl pyrophosphate synthase interferes with the mevalonic acid pathway and prevents the isoprenylation of key proteins, such as Rab and Rho, in regulation of osteoclast activity causing osteoclast apoptosis (Figure 4; Luckman et al., 1998; Drake et al., 2008).

Two non-nitrogen containing BPs (clodronate and tiludronate) have been approved by the FDA for use in horses aged 4 years or older for treatment of navicular disease (Mitchell et al., 2019). Any usage outside of this specified situation is considered “extra-label” and is not recommended by the manufacturer as such uses have not been studied by the FDA nor the manufacturer. The industry has gone as far to ban extra-label use of bisphosphonates in equine athletes, with regulatory bodies such as the Association of Racing Commissioners International prohibiting the use of BPs in horses younger than 4 and specifying its allowed use (ARCI, 2019).

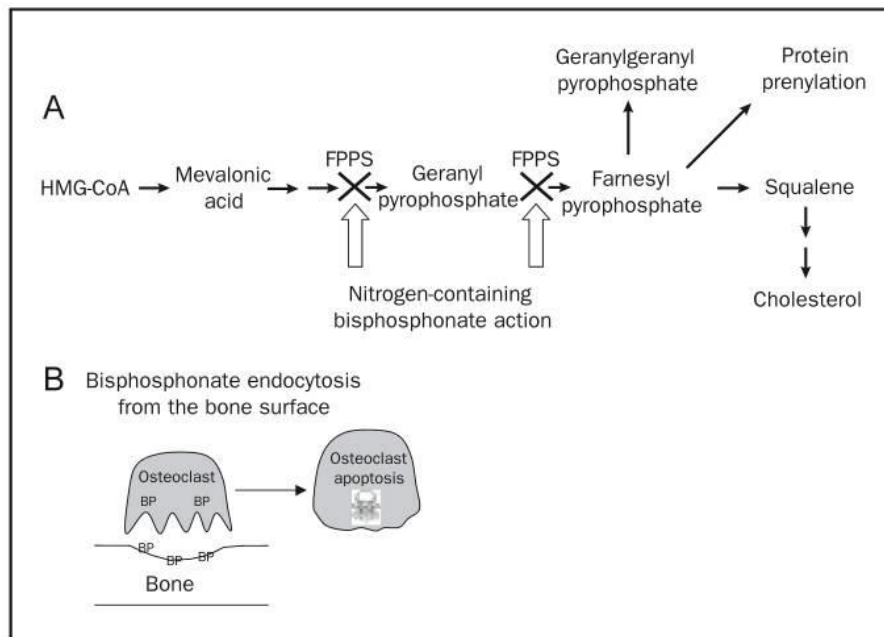


Figure 4. Mechanism of action of nitrogen-containing bisphosphonates on the mevalonic acid pathway. HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A; FPPS = farnesyl pyrophosphate synthase; BP = nitrogen-containing bisphosphonate. (Drake et al., 2008; Luckman et al., 1998).

Although its efficacy in treating navicular disease is well accepted by veterinarians and owners, causing widespread use throughout the industry, the few studies testing BP efficacy (Denoix et al., 2003; Whitfield et al., 2016) report negative complications with bone tissue. Despite BP studies conducted on humans that report both beneficial and adverse effects (Khosla et al., 2007; Abrahamsen, 2010; Adler, 2016), such effects on the juvenile horse have yet to be studied. Although this lack of data is cause for concern, the extra-label use continues with sesamoiditis being a primary cause for extra-label use, especially in juvenile horses (Suva et al., 2021). BPs are used in juvenile cases of sesamoiditis to improve visual discrepancies on radiographs for prepurchase exams. This form of BP use is not backed by scientific evidence, and when considering the mechanisms of BP may cause more harm to the juvenile horse through accumulation of microdamage in bone and eventual catastrophic failure. Until the potential

adverse effects of extra-label use on skeletal health and bone regulatory factors are elucidated, such use should be approached with caution (Suva et al., 2021; Mitchell et al., 2019). To better understand the effects of BP use on endocrine osteoregulatory factors within the scope of this study, their mechanisms must first be discussed.

Parathyroid Hormone

Parathyroid hormone is a peptide hormone secreted by chief cells in the parathyroid gland and plays a large role in the regulation of Ca^{++} homeostasis within the body (Toribio et al., 2003; Gaffney-Stomberg et al., 2017). The synthesis and secretion of PTH are regulated by the calcium-sensing receptor (CaSR) that is G-protein linked and megalin. CaSR and megalin respond to minute decreases in serum Ca^{++} concentrations (hypocalcemia) because its response curve has an inverse sigmoidal shape and thus, signal a rapid response from PTH. PTH increases serum Ca^{++} concentrations through three organ systems: kidneys, small intestine, and bone (Figure 5; Gaffney-Stomberg et al., 2017). However, if hypocalcemia persists for an extended period, the rapid degradation of PTH is inhibited, PTH gene transcription is increased, and parathyroid cell proliferation is increased to accommodate.

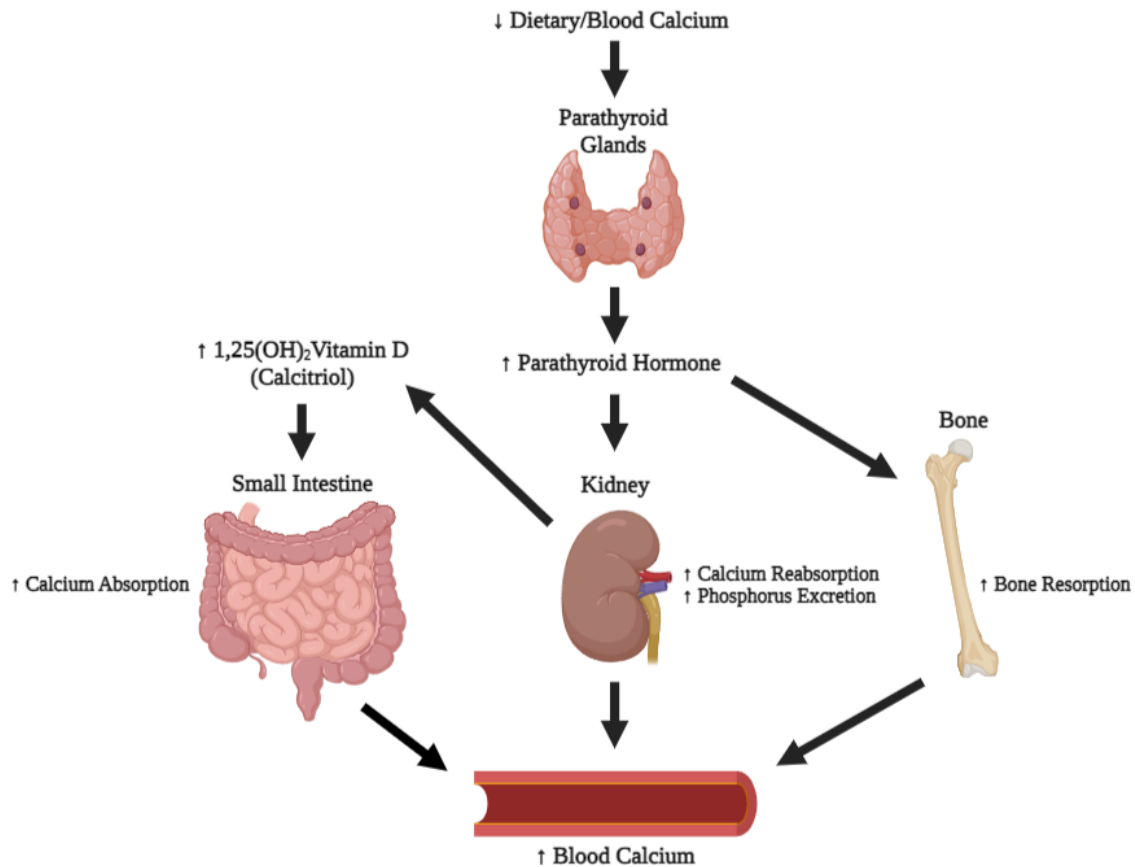


Figure 5. Pathways of Parathyroid Hormone secretion in response to decreased Serum Calcium Ion concentrations (Modified from Gaffney-Stomberg et al., 2017).

When PTH reaches the kidneys, it triggers direct and indirect pathways to increase serum Ca^{++} concentrations (Gaffney-Stomberg et al., 2017). In the direct pathway, PTH signals the renal distal tubule to resorb Ca^{++} using active transport via NCX1, a $\text{Na}^{+}\text{-Ca}^{+}$ exchanger, and plasma membrane ATPase that allows Ca^{++} to be resorbed into the renal cell using potential vanilloid 5, a transient receptor, bound to calbindin-D28k. From within the cell, Ca^{++} is ultimately passed into the lumen through PMCA1b, a plasma membrane calcium-ATPase (Blaine et al., 2015). Additionally, PTH reduces expression of the brush-border membrane in NaPi2a and NaPi2c, that are sodium-phosphate cotransporters (Gaffney-Stomberg et al., 2017).

This causes the kidneys to decrease urine calcium excretion while increasing phosphate excretion. In the indirect pathway, PTH activates 25-hydroxyvitamin D-1- α -hydroxylase into its active form 1,25(OH)₂D₃ (calcitriol) (Gaffney-Stomberg et al., 2017). Calcitriol performs two functions acting as negative feedback against PTH production and signaling the intestines to express calcium transport proteins (Fleet and Schoch, 2010). Once the small intestine is signaled by calcitriol, it increases the expression of calcium transport proteins that absorb Ca⁺⁺ from the digesta. The small intestine also increases absorption directly when a high calcium diet is ingested, and potentially through a PTH receptor found on small intestine absorptive cells (Gentili et al., 2003).

PTH's regulation of serum Ca⁺⁺ concentrations via bone is most pertinent within the scope of this study. Regarding increasing Ca⁺⁺, PTH plays a catabolic role by increasing osteoclast function (Figure 6; Gaffney-Stomberg et al., 2017). It does so by communicating via the RANKL-OPG-RANK pathway. PTH signals osteoblasts to increase expression of receptor activator of nuclear factor kappa- β ligand (RANKL) which, in conjunction with macrophage colony-stimulating factor, increases mature osteoclast function and differentiation of precursor cells into new osteoclasts. Additionally, PTH inhibits osteoprotegerin (OPG), a receptor activator of nuclear factor kappa- β (RANK) antagonist, expression increasing the ratio between RANK:OPG and allowing more binding between RANKL and RANK. This binding results in increased osteoclastogenesis and ultimately bone resorption.

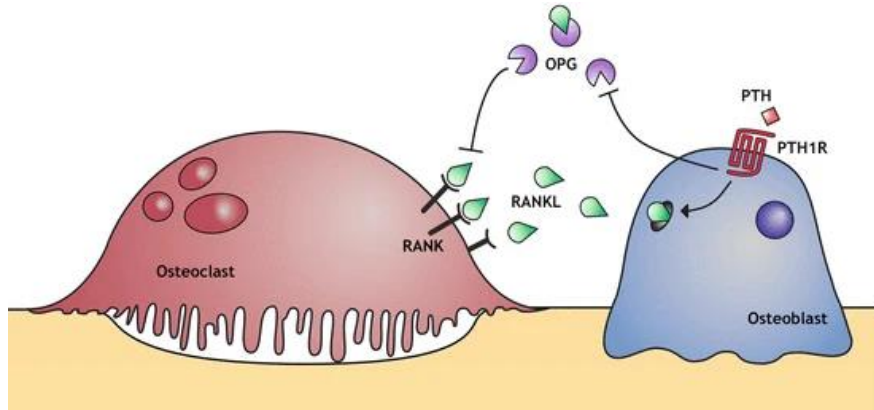


Figure 6. Regulation of osteoclast function through Parathyroid Hormone signaling of the RANKL-OPG-RANK pathway (Gaffney-Stomberg et al., 2017).

Interestingly, the above function of PTH only occurs when PTH is released intermittently (Reeve and Zanelli, 1986; Gaffney-Stomberg et al., 2017). On the other hand, when PTH is released in a continuous fashion it begins to display anabolic effects. When PTH is injected intermittently to mimic its cyclic manner, it exhibits anabolic properties in elderly men and postmenopausal women increasing bone mass and reducing incidence of fracture (Hodsman et al., 2005). This mechanism can be explained by the relationship between PTH and sclerostin, an inhibitory regulator of osteoblast differentiation, proliferation, and activity (Figure 7: Gaffney-Stomberg et al., 2017). Secreted by osteocytes, sclerostin interferes with the Wnt/ β -catenin pathway by competing to bind to Fzd, the receptor for the Wnt/ β -catenin pathway on the osteoblast. When PTH is intermittently released, it downregulates the expression of sclerostin in the osteocyte, allowing for Wnt to bind to Fzd in the osteoblast resulting in bone anabolism (Silva and Bilezikian, 2015).

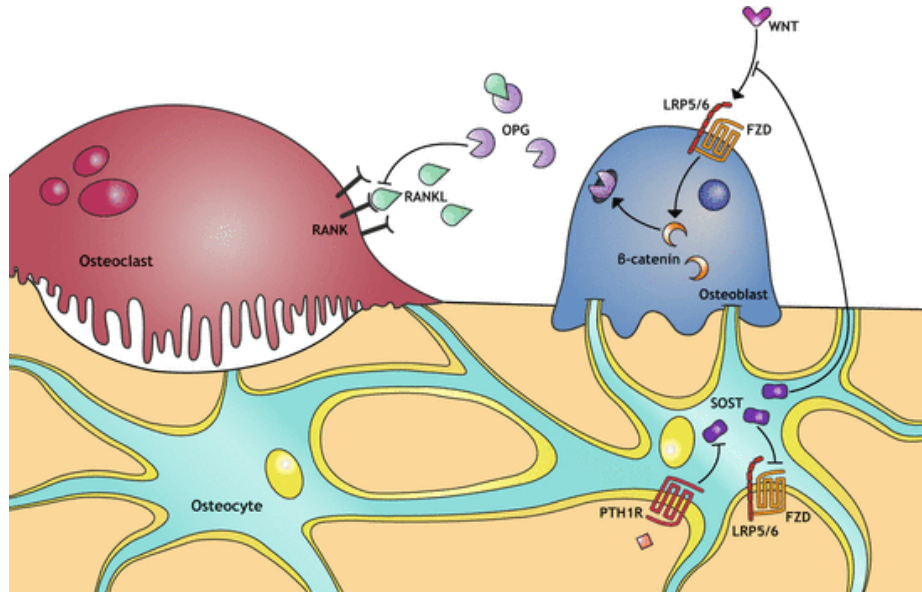


Figure 7. Inhibition of Sclerostin function by Parathyroid Hormone, allowing for bone anabolism (Gaffney-Stomberg et al., 2017).

Calcitonin

Calcitonin is a single-chain polypeptide hormone secreted by para-follicular cells of the thyroid gland in response to increased serum Ca^{++} concentration (Srinivasan et al., 2020). Its primary target tissue is bone where it hinders bone resorption by inhibiting osteoclast function. Osteoclasts use their ruffled borders to create a seal around a portion of bone matrix, where they release acids and acid hydrolases to degrade bone (Horne et al., 1994). Once bound to an osteoclast, CT promotes the ruffled border proteins to be internalized within intracellular vesicles, inhibiting the osteoclast's ability to secrete acid and break down bone. Reduced osteoclast function allows osteoblasts to continue to deposit bone, gradually decreasing serum Ca^{++} concentrations (Srinivasan et al., 2020). The mechanism of CT in conjunction with PTH facilitate serum Ca^{++} homeostasis.

CT also has secondary target tissues including the kidneys and the central nervous system (Horne et al., 1994). CT signals the kidneys to reduce Ca^{++} absorption from the blood along with other minerals like potassium, chloride, and sodium. In the central nervous system, CT induces analgesia, anorexia, and stomach acid secretion. The mechanisms behind the analgesic effects of CT have yet to be elucidated, however there are some proposed hypotheses. A study in rats found that the number of serotonin receptors are reduced, while expression of thalamic serotonin receptors increased due to CT function (Yeh et al., 2016).

An industry precursor to BP to treat metabolic bone diseases such as osteoporosis and hypercalcemia, CT was the treatment of choice since its discovery as a serum Ca^{++} lowering hormone (Srinivasan et al., 2020). CT from numerous species were studied due to the large variance in their affinity for the CT receptors. Salmon CT has the highest affinity for CT receptors in all studied species with a longer half-life, and thus salmon CT was the standard form used in human treatments (Andreotti et al., 2006).

Upon discovery and acceptance of BP use however, CT use began to fade away (Srinivasan et al., 2020). One of the primary reasons for this transition is that the literature surrounding BP is more promising and seems to show more anti-fracture efficacy compared to CT. The lack of widely available oral forms of CT also contributed to its replacement by BP (Maricic, 2012). Additionally, most common route of administration (nasal) increased the risk of cancer by 8.9% in the treatment group in a study by Chesnut and colleagues in 2000, further dissuading its use.

Growth Hormone

Although growth hormone plays a role in bone remodeling and Ca^{++} homeostasis, its involvement is limited when compared to the interplay between PTH and CT. GH is a polypeptide hormone that is secreted in a pulsatile fashion by somatotrope cells in the anterior pituitary gland (Figure 8; KATO et al., 2002). Its release is regulated by the hypothalamus via growth hormone releasing hormone (GHRH), ghrelin, and somatostatin. Ghrelin is also released from the pituitary gland and the stomach, while somatostatin is produced by the pancreas and hypothalamus. GH's main function is to promote post-natal longitudinal growth and does so through various tissue types including lipid, bone, and cartilage with its primary target being the liver (Kopchick and Andry, 2000). Most of GH's actions are mediated by insulin-like growth factor-1 (IGF-1), a hormone that is synthesized primarily by the liver, but also other tissue such as bone, in response to a stimulus from GH. Thus, GH can act on target cells directly or indirectly through IGF-1.

IGF-1 production in the liver occurs when GH binds to growth hormone receptors (GHR) causing phosphorylation of the GHR and subsequently Janus kinase 2 (JAK2) (Kopchick and Andry, 2000). Then, signal transducer and activator of transcription 5 (STAT 5), a cytoplasmic protein, binds the GHR where it is phosphorylated by JAK2 and in turn, activated. This activation of the JAK/STAT pathway results in the production of insulin-like growth factor 1 (IGF-1), which is then release from the liver into circulation. From circulation, IGF-1 travels to its target tissues to carry out its functions along with GH. This review will discuss the effects of GH and IGF-1 on muscle tissue, cartilage tissue, and bone tissue ultimately resulting in growth.

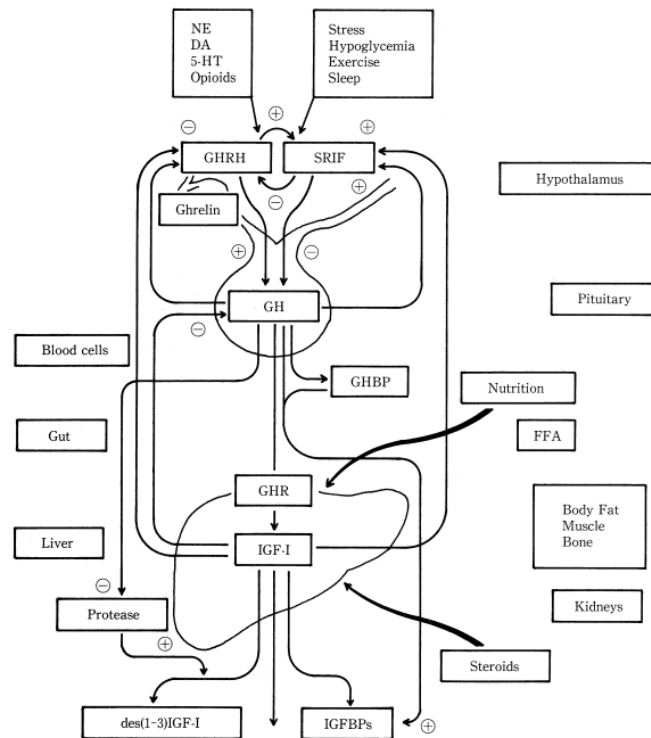


Figure 8. Schematic regulation of Growth Hormone secretion (Kato et al., 2002).

Growth hormone targets muscle tissue through the mediation of IGF-1, stimulating protein synthesis that leads to skeletal and somatic growth (Yarasheski, 1994). The effect GH has on muscle growth and development becomes apparent when GH-deficient adults are observed. These adults have significantly reduced lean body mass with literature consensus stating a reduction of 7-8% compared to healthy adults (Lissett and Shalet, 2000). Trials where GH-deficient adults are given GH replacement therapy show an increase in lean body mass of approximately 2-5.5 kg (Bengtsson et al., 1993; Johannsson et al., 1997). Furthermore, net protein synthesis, including muscle protein synthesis, increased in elderly individuals with low GH concentrations following 1 month of GH or IGF-1 treatment (Butterfield et al., 1997).

Growth hormone interaction with cartilage and bone tissue is integral in the facilitation of longitudinal growth as both play a role in endochondral ossification (Giustina et al., 2008).

During endochondral ossification chondrocytes within the epiphyseal plate undergo multiple phases or zones as they mature and progress from the epiphysis to the diaphysis (Long and Ornitz, 2013). The first phase is the reserve zone where small chondrocytes reside within the matrix. The chondrocytes do not actively participate in growth; however, this zone is responsible for attaching the epiphysis to the rest of the long bone. The proliferative zone is where the chondrocytes begin to undergo mitosis to replace dying chondrocytes in further phases. The most predominant zone is the zone of maturation and hypertrophy where chondrocytes undergo hypertrophy, traveling closer to the diaphysis as they increase in size.

The final two zones facilitate the transition between chondrocytes and osteoblasts. Chondrocytes that reach the zone of calcified matrix die as the surrounding matrix begins to calcify and nutrients are restricted. This phase connects the epiphyseal plate to the diaphysis. The final zone is incorporated into the diaphysis and is called the ossification zone. Osteoblasts and capillaries from this zone start to penetrate the zone of calcified matrix and the osteoblasts secrete bone tissue onto the calcified matrix, converting it to bone. Throughout this process the bone physically grows longer and contributes to longitudinal growth. The stimulus of chondrocytes and osteoblasts, primarily by IGF-1, ultimately results in increased growth.

GH and IGF-1 also work in conjunction to stimulate bone remodeling. While GH directly impacts osteoblastogenesis, the osteoblasts express their own IGF-1 under stimulus of PTH, further increasing bone formation (Tritos and Klibanski, 2016). Additionally, IGF-1 causes the synthesis of RANKL on osteoblasts, increasing osteoclast synthesis and function. The increased function of both osteoclasts and osteoblasts results in bone turnover and thus bone health. Furthermore, the stimulated bone remodeling process indirectly assists in serum Ca^{++} regulation

as more bone deposition and resorption occurs. Understanding how BPs interact with GH, PTH, and CT may yield results that can support future research on extra-label BP use and its implications.

Implications in the Horse

Increasing popularity of extra-label bisphosphonate use may pose significant risks, especially to juvenile horses. Because young horses are still undergoing growth and development, it is imperative that healthy development of their skeletal system is facilitated. Although specific adverse effects have not yet been elucidated in the horse, evidence collected in research on humans and canines show an increase in fragility fractures due to oversuppression of bone turnover and an accumulation of microdamage, respectively (Mashiba et al., 2000; Schmidt et al., 2010). A better understanding of the effects of extra-label bisphosphonate use on bone health and endocrine regulators of bone in juvenile horses will allow for better advocacy for the horse's wellbeing.

MATERIALS AND METHODS

All procedures and handling of horses were approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP #2020-0254).

Horse Treatments and Groups

Thirty-two yearling Quarter horses (335 ± 4 kg BW, 500 ± 13 d of age, 16 geldings and 16 fillies) were stratified by age, BW, sex, and initial radiographic bone aluminum equivalence (RBAE) into four treatment groups for a 168-d trial. Bisphosphonate treatments consisted of control (CON; $n = 8$), single-dose (1X; $n = 8$), two-dose (2X; $n = 8$), and four-dose groups (4X; $n = 8$). All horses received iso-volumetric intramuscular injections of either 1.8 mg/kg BW clodronate disodium (OSPHOS[®]) or saline (placebo) on d 0, 42, 84, and 126. Horses were housed in individual stalls (3.6 m $\times$ 7.2 m), fed 0.5% BW commercial concentrate feed (ProElite Growth, Cargill, Wayzata, MN) every 12 hours, and offered *ad libitum* access to coastal bermudagrass (*Cynodon dactylon*) hay and water. Diets were formulated to meet or slightly exceed nutrient requirements for yearling horses undergoing exercise (NRC, 2007).

Exercise Protocol

The experimental period was divided into two phases to mimic industry standards in management practices. Phase I began on d 0 and continued for 84 d to represent sales preparation. Phase II began on d 85 and continued until d 168 to represent early exercise training. Horses were adapted to and exercised using an 8-panel free-stall exerciser (Priefert, Mount Pleasant, TX) from d 0 to 83. The exercise protocol began at the walk, 25 min/d for 5 d/wk

working clockwise and counterclockwise, equally. Exercise duration increased 5 min weekly until total walking exercise duration was achieved on d 35. Beginning on d 36, 5 min of trot at 4.0 m/s was included in both directions totaling 5 min of trot and 40 min of walk for 45 min/d for 5 d/wk of total exercise until the end of Phase I. On d 84 horses were transitioned into Phase II, in which the intensity of exercise increased over time using the same 8-panel free-stall exerciser (Table 1). Maximum exercise intensity was maintained for the duration of the study once achieved on d 105 until d 168.

Table 1. Exercise protocol for transition to Phase II exercise (m/s).

Day ¹	Counterclockwise²					Clockwise				
	2 min	5 min	2 min	5 min	1 min	1 min	5 min	2 min	5 min	2 min
84-90	1.75	4.0	1.75	4.0	1.75	1.75	4.0	1.75	4.0	1.75
91-97	1.75	4.0	1.75	6.0	1.75	1.75	4.0	1.75	6.0	1.75
98-104	1.75	6.0	1.75	6.0	1.75	1.75	6.0	1.75	6.0	1.75
105-168	1.75	6.0	1.75	6.5	1.75	1.75	6.0	1.75	6.5	1.75

¹Day of Phase II exercise transition.

²Every two days, starting direction will alternate so horses will equally exercise going clockwise and counterclockwise.

Sample Collection

Physical growth parameters were measured by the same researcher every 42 d prior to morning feeding and included BW, wither height (WH), hip height (HH), body length (BL), and heart girth (HG). BW was measured with a platform scale. An altitude stick (Sullivan Supply, Inc., Hillsboro, TX) was used to record WH and HH. BL and HG measurements were recorded using a single soft tape measure for each timepoint. Blood samples were collected d 0, 42, 84, 126, and 168 via jugular venipuncture into 10 mL additive-free, evacuated blood collection tubes (BD Vacutainer, Franklin Lakes, NJ). Samples were kept at room temperature to allow for

adequate clotting until centrifugation at $2,000 \times g$ at 10°C for 20 minutes (ALC, PM140R, Thermo Fisher Sci., Waltham, MA), serum was harvested, and stored at -80°C until further analysis.

Sample Analysis

Serum concentrations of PTH and Ca^{2+} were determined by the Michigan State University Veterinary Diagnostic Laboratory (East Lansing, MI) using an immunoradiometric assay (IRMA; Code 20033). GH (LifeSpan BioSciences, Inc., Seattle, WA) and CT (MyBioSource, Inc., San Diego, CA) concentrations were measured using commercial ELISA kits previously validated for use in the horse. Final concentrations of both markers were read using a microplate reader with optical density set at 450 nm (Gen5™, BioTek Instruments, Inc., Winooski, VT). Intra-assay coefficients of variability (CVs) were established as acceptable at $\leq 15\%$ for both GH and CT ELISAs. Inter-assay precision was $\leq 12\%$ for GH and $\leq 15\%$ for CT.

Statistical Analysis

All data were analyzed using the PROC MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). The model contained main effects of treatment, day, and treatment $\times$ day interactions to determine effects of bisphosphonate administration on physical growth parameters and serum concentrations of GH, CT, PTH, and Ca^{2+} . Means are reported as LSMeans $\pm$ SEM. Significance was defined for all variables at $P \leq 0.05$, and a trend towards significance was defined at $0.05 < P \leq 0.10$.

RESULTS

Physical Growth Parameters

There were no treatment differences ($P \geq 0.62$) or treatment $\times$ time interactions ($P \geq 0.25$) for BW, WH, HH, BL, and HG measurements. All parameters, however, increased over time ($P \leq 0.01$) (Table 2).

Table 2. Physical growth parameters of juvenile, exercising horses given placebo (saline) (CON), single dose (1X), two doses (2X) or four doses (4X) of clodronate disodium (OSPHOS[®]) at 1.8 mg/kg BW.

Item	Day	Treatment				SEM	P - Value		
		CON	1X	2X	4X		TRT ^a	Time	TRT $\times$ Time
Body Weight (kg)	0	740.30	740.30	746.50	726.80	19.2	0.91	< 0.01	0.82
	168	907.70	935.30	918.30	918.70				
Wither Height (cm)	0	137.16	137.16	137.72	136.68	0.84	0.95	< 0.01	0.82
	168	143.58	143.67	144.30	144.21				
Hip Height (cm)	0	142.64	142.48	143.43	142.95	0.83	0.85	< 0.01	0.90
	168	148.04	146.76	147.95	147.30				
Body Length (cm)	0	142.40	142.88	142.32	140.18	1.36	0.62	< 0.01	0.25
	168	150.29	151.05	150.02	149.72				
Heartgirth	0	159.07	159.31	159.46	158.27	1.60	0.99	< 0.01	0.62
Circumference (cm)	168	174.50	174.07	173.12	173.75				

^aTRT = Main effect of treatment

Endocrine Factors

A treatment $\times$ time interaction was revealed ($P = 0.05$) for PTH concentrations driven by treatment groups that received two or four doses of clodronate disodium over the course of the experimental period. Specifically, concentrations increased on d 84 in 4X and on d 126 in 2X following their second administration of clodronate on d 42 and 84, respectively. Comparatively, 1X and CON remained unchanged (Figure 9). Although there was no main effect of treatment for

PTH ($P = 0.50$), concentrations increased over time ($P = 0.03$). Despite an increase in PTH concentrations in 2X and 4X, there was no effect of treatment ($P = 0.98$), time ($P = 0.33$), nor treatment $\times$ time interaction ($P = 0.61$) for CT concentrations (Figure 10). There was no effect of treatment ($P = 0.26$) nor treatment $\times$ time interaction ($P = 0.32$) for concentrations of serum ionized calcium in spite of increased PTH in 2X and 4X. However, concentrations decreased over time ($P < 0.01$) (Figure 11). GH concentrations tended to decrease over time ($P = 0.09$) with no effect of treatment ($P = 0.44$) or treatment $\times$ time interactions ($P = 0.81$) for all treatment groups (Figure 12).

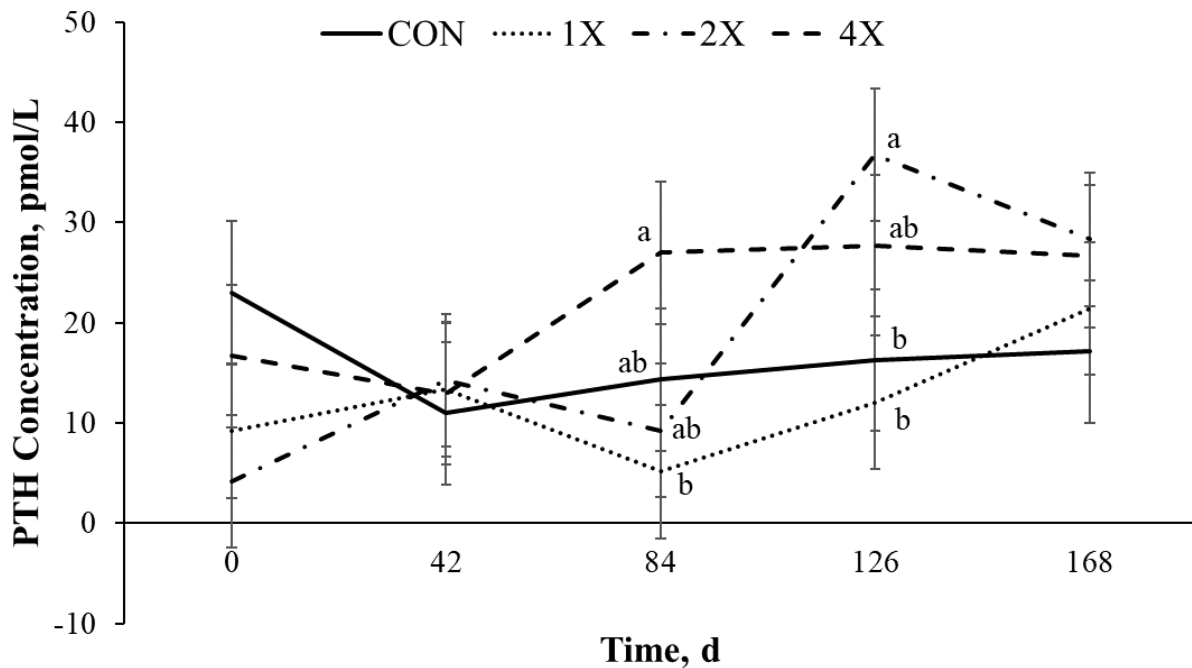


Figure 9. Mean parathyroid hormone (PTH) concentrations (pmol/L; least squares means $\pm$ SEM) over time (d) in juvenile, exercising horses given placebo (saline) (CON), single dose (1X), two doses (2X) or four doses (4X) of clodronate disodium (OSPHOS[®]) at 1.8 mg/kg BW. ^{a,b} Different superscripts denote differences ($P < 0.05$) in PTH concentrations among treatment groups within a timepoint.

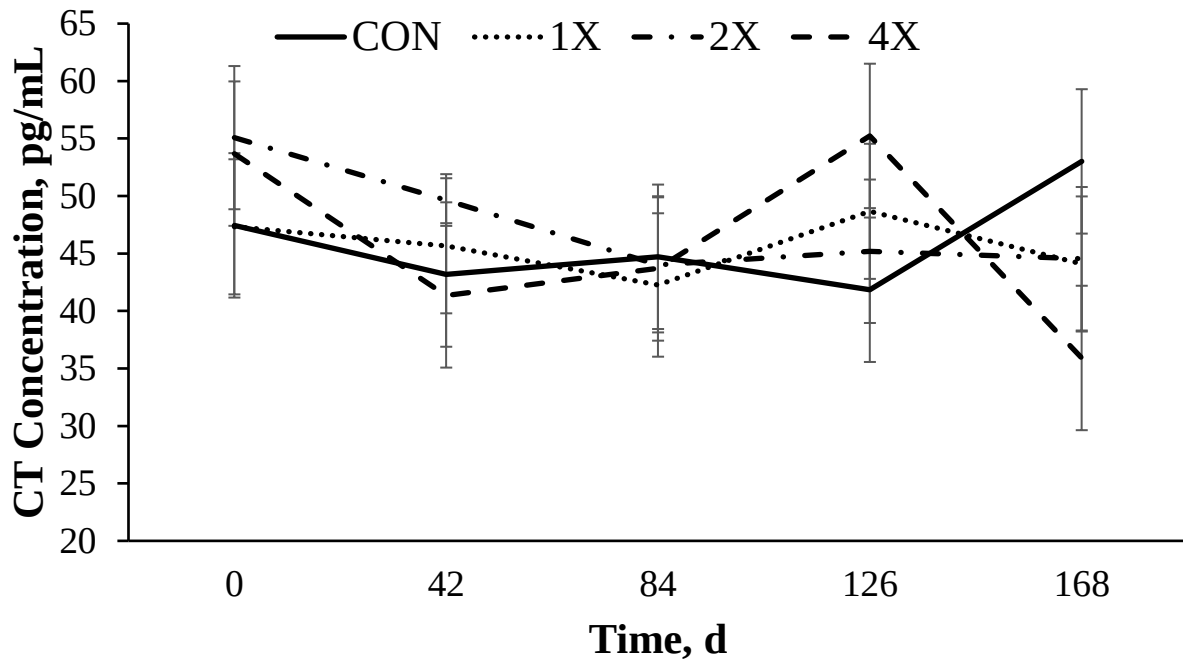


Figure 10. Mean calcitonin (CT) concentrations (pg/mL; least squares means $\pm$ SEM) over time (d) in juvenile, exercising horses given placebo (saline) (CON), single dose (1X), two doses (2X) or four doses (4X) of clodronate disodium (OSPHOS[®]) at 1.8 mg/kg BW.

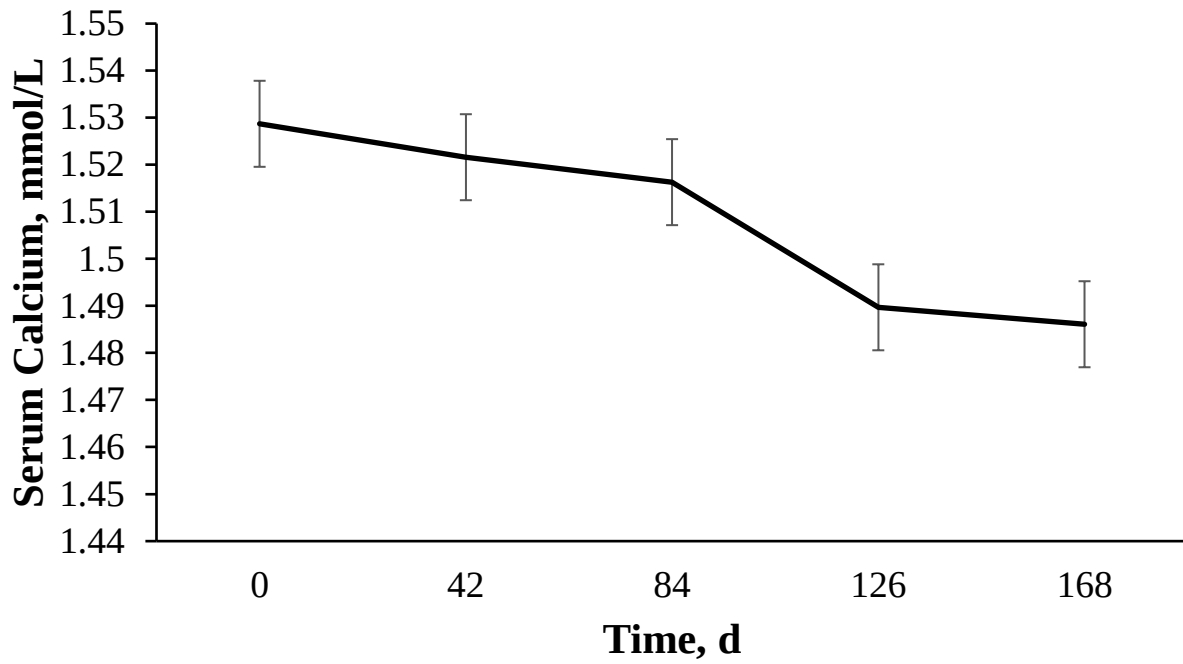


Figure 11. Mean serum ionized calcium (Ca^{++}) concentrations (mmol/L; least squares means $\pm$ SEM) over time (d) in juvenile, exercising horses given placebo (saline) or clodronate disodium (OSPPOS[®]) at 1.8 mg/kg BW. Values indicate mean concentrations for all horses in each time point.

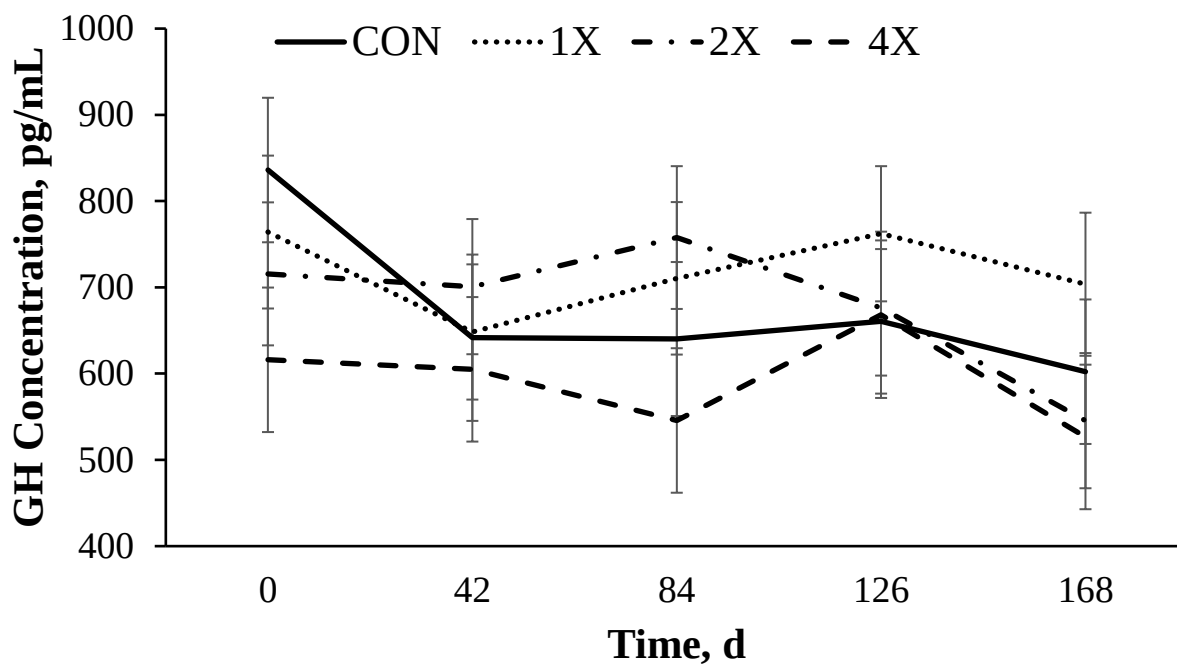


Figure 12. Mean growth hormone (GH) concentrations (pg/mL; least squares means $\pm$ SEM) over time (d) in juvenile, exercising horses given placebo (saline) (CON), single dose (1X), two doses (2X) or four doses (4X) of clodronate disodium (OSPHOS[®]) at 1.8 mg/kg BW.

DISCUSSION

The present study delved into the effects of clodronate on endocrine regulation of bone growth in the juvenile horse. Interactions between BPs and endocrine regulators have been primarily studied in the field of human medicine, particularly the use against bone related ailments. However, these interactions are understudied in horses (Antoniazzi et al., 2010; Khan et al., 2021).

Participating yearlings exhibited growth in all physical parameters over time regardless of treatment effects and treatment $\times$ time interactions corresponding to expectations of bisphosphonate use and aligning with results found in applications with growing humans and mice (Brumsen et al., 1997; Zhu et al., 2014). Higher activity of osteoblasts compared to activity of osteoclasts during endochondral ossification and overall anabolic effects of growth overshadow any effect resulting from reduced osteoclast function via bisphosphonates (Rolian, 2020). Additionally, a diet formulated to meet or exceed requirements of growing horses undergoing light exercise support these results (NRC, 2007).

The tendency for GH concentrations to decrease over time may be explained by the gradual decline in concentrations that accompany normal aging (Rudman et al., 1981). Lack of treatment effects and treatment $\times$ time interactions can be supported through the differences in mechanisms between GH and BPs. Although GH influences both osteoblasto- and osteoclastogenesis, its bias towards osteoblast function and synthesis during anabolic processes like bone modeling and endochondral ossification outweigh any influence BP may have on osteoclasts (Giustina et al., 2008). Furthermore, the mechanistic action of BPs has no interaction with that of GH allowing expression of both without interference (Drake et al., 2008).

Serum Ca^{++} concentrations decreased over time across all treatment groups, and values were below the reference range of 1.58 – 1.90 mmol/L used for the IRMA. The decrease is explained by the nature of BP's function; as the decrease in osteoclast population inevitably causes hypocalcemia (Drake et al., 2008). Additionally, serum ionized Ca^{++} levels can be associated with fluctuations of GH levels, with Ca^{++} levels increasing as GH levels increase (Ahmad et al., 2003). This aligns with the decrease in Ca^{++} concentration overtime and the tendency of GH concentration decrease as seen in this study. The effect of BP on calcium levels was a large motivator in investigating how the regulators of calcium homeostasis, PTH and CT, were also impacted by BP use. Interestingly, there was no treatment effect nor treatment $\times$ time interactions. It is possible that PTH is compensating in treatment groups that received multiple doses resulting in no effect on serum ionized Ca^{++} .

Treatment $\times$ time interactions for PTH whereby concentrations were higher in treatment groups that received two or more doses of BP indicate that repetitive use of BPs alters PTH concentrations. This behavior of PTH is supported by its ability to react rapidly to deviations in Ca^{++} homeostasis by stimulating osteoclastogenesis (Gaffney-Stomberg et al., 2017). All concentrations, however, were lower than the validated values of 55.7 ± 8.1 pg/mL in mature horses (Estepa et al., 2003). The disparity of PTH concentrations in the juvenile may be due to the lack of osteoclast function in the endochondral ossification process that is prioritized over bone remodeling in juvenile horses (Salhotra et al., 2020). Furthermore, the introduction of a second dose may cause an extended hypocalcemic environment leading to additional PTH secretion. Increases in concentrations over time are likely also explained by this mechanism of PTH and its relation to the BP induced reduction in osteoclast activity causing a decline in serum

Ca^{++} concentrations. To compensate, PTH may rely on the kidneys and small intestine to decrease calcium excretion via urine and increase calcium uptake from the diet, respectively (Gaffney-Stomberg et al., 2017). Increased phosphorus excretion from this compensatory mechanism may cause unintended phosphorus deficiencies. It is unlikely, however that serum Ca^{++} concentrations can be fully managed by the kidneys and small intestine alone, as 99% of the body's total calcium is stored in bone (Toribio, 2011).

No effects of time, treatment, nor treatment $\times$ time interactions were observed in CT concentrations despite the increases in PTH. Concentration values for each treatment were above a baseline of 40 pg/mL determined from normal, mature equine CT values (Rourke et al., 2009). Although normal CT ranges have not been established in various equine age groups, the disparity between the concentrations of this study and the baseline established by Rourke and colleagues may be due to CT expression in horses decreasing with age as seen in humans (Shamonki et al., 1980). CT responds to increases in serum Ca^{++} by inhibiting osteoclast function to reduce calcium uptake (Srinivasan et al., 2020). The failure of PTH to increase serum Ca^{++} concentrations to a hypercalcemic, let alone homeostatic state within this study can explain the lack of significance observed. Furthermore, the mechanism of BP may hinder the ability of CT to properly signal its target cells. As BP causes more osteoclasts to undergo apoptosis, CT is unable to directly signal osteoclasts to reduce hydroxyapatite breakdown due to decreased osteoclast numbers (Drake et al., 2008; Srinivasan et al., 2020). It is possible, however, that in an environment that is hypercalcemic when introduced to BPs, the concentration of CT may increase in attempt to combat the apoptotic effects of BP. The prevention of demineralization of bone in osteoclasts caused by CT may hinder the BP bound to hydroxyapatite crystals from

being released (Srinivasan et al., 2020). If BP is not absorbed by osteoclasts, apoptosis cannot occur (Rodan and Fleisch, 1996). Further studies must be done to determine the precise relationship between endogenous CT and BP administration.

In conclusion, extra-label use of BP in juvenile horses increased concentrations of PTH in treatment groups that received two or more doses, indicating a physiological shift in endocrine regulation associated with osteoclast inhibition. As expected, physical growth parameters and GH concentrations were not affected regardless of BP administration. Unexpectedly, however, no changes were observed in CT concentrations while concentrations of serum Ca^{++} decreased over time. With little to no research on the effects of BP use on endocrine regulation of growth, let alone extra-label use within the industry, more work must be done to maintain standards of equine wellbeing.

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