Short-Term Zoledronic Acid Reduces Trabecular Bone Remodeling In Aged Dogs

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ABSTRACT

Objective: Bisphosphonates are widely used to treat diseases of disproportionate bone resorption; however, possible side-effects include bisphosphonate-related osteonecrosis of the jaw (BRONJ). The susceptibility of various skeletal sites to BRONJ has been hypothesized to be related to the level of site-specific bone remodeling. Little is known about the physiologic trabecular bone remodeling (TBR) in the mandibular condyle of aged dogs or the effects of bisphosphonates on this TBR. The objectives of this study were to quantify and compare TBR in the mandibular condyle and vertebra of aged (2- to 3-year old) dogs and to evaluate the effects of short-term zoledronic acid on TBR. Methods: Seven (3 untreated, NT; 4 treated with 4 doses of 0.1 mg/kg/month zoledronic acid, ZOL) dogs were given a pair of calcein labels. Mineral apposition rate (MAR, µm/d), mineralizing surface (MS/BS, %), bone formation rate (BFR/BS, µm³/µm²/d), and micro-architectural histomorphometric parameters were quantified from undecalcified specimen (n=56; 4 condyle and 4 vertebra sections/dog) using histomorphometric methods and analyzed statistically (ANOVA, Tukey test, p<0.05). BFR/BS was the main variable of interest as it describes TBR. Results: TBR in NT group was significantly higher (>9-fold, p=0.0002) in the vertebra (BFR/BS 0.19±0.06) than the mandibular condyle (BFR/BS 0.020±0.012). In ZOL group, BFR/BS in both
skeletal sites was reduced to similar absolute levels (vertebra 0.008±0.010; mandibular condyle 0.007±0.012). BFR/BS in the vertebra was significantly reduced (-0.18; 96% reduction, p=0.0006), while it was not in the mandibular condyle (-0.014; 67% reduction, p=0.9). **Conclusions:** Physiologic TBR in aged dogs is vastly different in the mandibular condyle than in the vertebrae. This higher level of TBR in the vertebrae leads to greater reduction by short-term ZA treatment.
Dedicated to Abby and Kylie
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CHAPTER 1

INTRODUCTION

Bisphosphonate drugs (BPs) are widely used to treat diseases of disproportionate bone resorption, and their use is expected to increase because of the current lack of equally effective alternative treatments for these conditions. The Food and Drug Administration has recently approved once-yearly dosing of zoledronic acid, a potent intravenous BP, for treatment of osteoporosis (United States Food and Drug Administration website). One possible side-effect associated with the use of BPs is bisphosphonate-related osteonecrosis of the jaw, or BRONJ (Fujimura et al., 2009; Khosla et al., 2007; Zervas et al., 2006). As the number of adults seeking orthodontic care continues to increase, orthodontists are faced with many decisions regarding the treatment of patients with a history of BP therapy.

It is clear that BP’s principal mechanism of action is reducing bone remodeling; however, the pathogenesis of BRONJ is still not understood (Drake, Clarke, & Khosla, 2008; Russell, 2006). Various hypotheses have been proposed, the most popular of which relates BRONJ to inhibition of bone remodeling (Allen & Burr, 2009a). Since bone remodeling is necessary for homeostatic and healing mechanisms, alteration of bone remodeling could have severe consequences. If this hypothesis if true, it may suggest
that skeletal sites with greater physiologic bone remodeling may be more sensitive to BP-induced suppression and subsequently at a higher risk for developing BRONJ.

Not only do rates of bone remodeling differ among skeletal sites, they also differ among sites within the mandible and among different types of bone (Eriksen, Axelrod, & Melsen F., 1994; Huja et al., 2006b; Marotti & De Lena, 1966; Tricker, Dixon, & Garetto, 2002). There is, however, a lack of published data regarding physiologic trabecular bone remodeling in the mandibular condyle and any changes that occur to this remodeling activity with age. Several canine studies suggest that intracortical bone remodeling at various sites in the skeleton, including the jaws, is reduced by BP treatment; however, none report its effect on the trabecular bone remodeling of the mandibular condyle (Allen, Kubek, & Burr, 2009b; Li, Mashiba, & Burr, 2001; Mashiba et al., 2001b; Mashiba et al., 2005).
STATEMENT OF THE PROBLEM

Published information is lacking on: 1) the physiologic trabecular bone remodeling in the mandibular condyle and age-associated changes, 2) the effects of BP therapy on trabecular bone remodeling in the mandibular condyle. A better understanding of such events and effects may offer valuable information regarding etiologic factors associated with the development of BRONJ. The objectives of this study were to quantify and compare the physiologic trabecular bone remodeling of the mandible condyle and vertebra (axial skeleton) in aged (2- to 3-year old) canines and to evaluate the effects of short-term zolendronic acid on trabecular bone remodeling at each skeletal site. The hypotheses were that: 1) the rate of trabecular bone remodeling in aged canines remains significantly greater in the mandibular condyle than the axial skeleton, and 2) the trabecular bone remodeling in the mandibular condyle of aged canines is more reduced by short-term zolendronic acid than that in the axial skeleton.
REVIEW OF THE LITERATURE

Bone Remodeling

Bone is a dynamic tissue compared to other calcified tissues in the body—undergoing constant bone remodeling, or turnover. This continuous process of bone renewal occurs in response to physiologic calcium demands, wound healing, and mechanical factors associated with fatigue damage repair (Martin & Seeman, 2008; A. M. Parfitt, 1995). Remodeling prevents microdamage accumulation as a consequence of repetitive loading, and thus, increases the fatigue life of bone (Burr, 1993). Two types of remodeling have been proposed: 1) stochastic remodeling which is a normal, physiologic, non-site-specific remodeling that replaces damaged bone with new bone, and 2) targeted remodeling which is site-specific remodeling to remove microcracks (Li et al., 2001).

A bone remodeling cycle occurs at a focally discrete point on the bone surface (Martin & Seeman, 2008) and includes activation (A) of osteoclastic precursors, an osteoclast-mediated bone resorption (R) phase, and an osteoblast-mediated bone formation (F) and mineralization phase (Eriksen et al., 1994; Hattner, Epker, & Frost, 1965). Every remodeling cycle in humans with normal skeletal metabolism consists of this A-R-F sequence that lasts for approximately 100 days in cortical bone and 200 days in trabecular bone (Eriksen et al., 1994). Under normal skeletal physiologic conditions, the resorption and formation processes are coupled, and the volume of bone resorption is comparable to the volume of bone formation. Diseases of excessive bone resorption
result when these processes become unbalanced. These diseases include: multiple forms of osteoporosis, Paget disease of bone, osteogenensis imperfect, hypercalcemia, and malignancy metastatic to bone (Drake et al., 2008; Russell, 2006).

*Bisphosphonate Drugs*

BPs have become the primary therapy for treating diseases of disproportionate bone resorption (Drake et al., 2008). It has been suggested that over 190 million prescriptions for oral BPs have been dispensed worldwide (Advisory Task Force on Bisphosphonate-Related Osteonecrosis of the Jaws, American Association of Oral and Maxillofacial Surgeons, 2007), and an estimated 30 million BP prescriptions dispensed annually in the U.S. alone (Gutta & Louis, 2007). The molecular structure of BPs, similar to that of inorganic pyrophosphate, gives this class of drugs its very high affinity for binding to hydroxyapatite crystals in bone (Drake et al., 2008). Two generations of BPs (non-nitrogen-containing [clodronate, etidronate, tiludronate] and nitrogen-containing [pamidronate, alendronate, risedronate, ibandronate, and zoledronate]) have evolved as a result of molecular structural modifications, and their different molecular modes of action have been described in the literature (Drake et al., 2008; Russell, 2006). Once adsorbed to mineral surfaces in bone, both generations of BPs are internalized by bone-resorbing osteoclasts (Russell, 2006). Interference with specific biochemical processes leads to osteoclast apoptosis, which decreases bone resorption and subsequently the rate of coupled bone remodeling (Plotkin, Manolagas, & Bellido, 2006). In addition to this, BPs prevent osteoblast and osteocyte apoptosis, suggesting that the preserved osteocyte network and extended osteoblast life-cycle may play a role in increasing the fracture resistance of bone (Plotkin et al., 2006).
The potency of nitrogen-containing BPs is exponentially greater than that of non-nitrogen-containing BPs (Dunford et al., 2001; Russell, 2006). The most potent BP, zoledronic acid (Reclast®, Novartis Pharmaceuticals Corporation), is 10,000 times more potent than etidronate (Dunford et al., 2001; Russell, 2006). This potency has led to widespread use of nitrogen-containing BPs at low dosages (Reszka & Rodan, 2004) and is largely responsible for their extended half-life (Drake et al., 2008). The half-life of one potent BP, alendronate (Fosamax®, Merk & Co.), has been estimated to be greater than 10 years after a single IV administered dose (Khan et al., 1997). Intravenous administration allows a greater ability to deliver a defined dose compared to oral administration, which is poorly absorbed from the gastrointestinal tract. Intravenous administration also has less potential for gastrointestinal symptoms, greater patient convenience, and greater long-term adherence to treatment versus daily, weekly, or monthly oral doses (Cremers, Pillai, & Papapoulos, 2005; Drake et al., 2008; Russell, 2006).

Osteoporosis has been acknowledged as a serious, widespread disease in America (U.S. Department of Health and Human Services, 2004). The National Osteoporosis Foundation estimates that 10 million individuals in the U.S. have osteoporosis and almost 34 million more have an increased risk for the disease due to low bone mass (National Osteoporosis Foundation website). It is estimated that without dietary changes, half of all Americans over 50 will have weak bones and be at increased risk of osteoporosis by 2020 (U.S. Department of Health and Human Services, 2004). Oral BPs have been the first choice of treatment and prevention of osteoporotic fracture. In the HORIZON pivotal fracture trial, a large randomized controlled clinical trial, once-yearly intravenous
administration of zoledronic acid (5 mg) was shown to significantly reduce vertebral, hip, and other fractures in women with postmenopausal osteoporosis (Black et al., 2007). Since, the United States Food and Drug Administration has approved this regime for the treatment of postmenopausal osteoporosis, and more recently its approval has been extended to biennial (once every two years) administration to prevent osteoporosis in postmenopausal women with osteopenia (Biennial IV zoledronic acid (reclast) for prevention of osteoporosis, 2009).

With current and anticipated widespread use of this class of drugs, more attention has been given to the side effects that have been documented as associated with their use. Although the incidence of these side effects is relatively rare, the outcome can be devastating. One serious side effect is formation of painful, eroded, necrotic lesions in jaw bone referred to as BRONJ. Although a clear cause-and-effect relationship has not been established between BPs and BRONJ, hundreds of cases of BRONJ have been reported in the dental literature, and these cases have documented a higher incidence of BRONJ in: patients receiving intravenous versus oral BPs, the posterior mandible compared to the maxilla or other bones in the body, older patients (> 60 years), and patients with a history of invasive dental treatment or trauma (Khosla et al., 2007; Marx et al., 2007; Marx, Cillo, & Ulloa, 2007; Pazianas et al., 2007).

The exact incidence of BRONJ is unknown, and it has been reported anywhere from 0.8%-18.6% (Ruggiero et al., 2009; Walter et al., 2008). A task force of the American Society for Bone and Mineral Research has reported that the risk of BRONJ associated with oral BP therapy for osteoporosis seems to be low; however, the risk in patients treated with high doses of intravenous BPs is in the range of 1-10 per 100
patients depending on the duration of therapy (Khosla et al., 2007). Zoledronic acid has been commonly associated with BRONJ throughout the literature, and one study concluded that its administration produced 9.5-fold greater risk for developing BRONJ than pamidronate (Zervas et al., 2006).

The exact pathogenesis of BRONJ is not clear, and there has been a general lack of success in developing animal models for study of BRONJ (Allen & Burr, 2009a; Huja et al., 2009; Khosla et al., 2007). Various hypotheses have been proposed, but the most popular of these is the “remodeling suppression hypothesis” which identifies BP’s reduction of bone remodeling as a key player in the pathogenesis of BRONJ (Allen & Burr, 2009a). Support for this hypothesis comes from animal studies showing that remodeling rates are high in the jaw compared to long bones (Huja et al., 2006b; Tricker et al., 2002). Since remodeling is responsible for homeostatic and healing mechanisms in osseous tissue, alteration of bone remodeling by BPs could have severe consequences, leading to the development of BRONJ.

This hypothesis suggests that a skeletal site with a higher rate of physiologic bone remodeling may be at a greater risk of developing BRONJ. Skeletal uptake and retention after intravenous or oral administration varies widely due to variations in bone turnover and subsequent BP binding site availability (Cremers et al., 2005). Factors, such as dosing, length of treatment, and route of administration, may determine the amount of BP available at the bone surface for skeletal uptake and retention. High levels of bone surface remodeling activity increase the potential for osteoclast uptake of BP and subsequent suppression of bone remodeling. Information regarding the level of physiologic bone remodeling at individual skeletal sites may be valuable in determining
the effects of this drug class at individual skeletal sites and possibly the site-specific predisposition to development of BRONJ.

**Bone remodeling variables**

The rate of physiologic bone remodeling is important baseline information in studying modalities that alter bone metabolism and rates of remodeling, such as mechanical loading, hormones, disease, or drug therapy. Variables that may influence physiologic bone remodeling and the effect of BP therapy are: bone type, skeletal site, and age.

**Bone Type**

Bone can be classified into cortical and trabecular types based on its macroscopic architecture. Cortical bone, predominating in the appendicular skeleton, is compact, dense bone that is arranged in concentrically-layered lamellae that form osteons (Eriksen et al., 1994). Trabecular bone, or cancellous bone, predominates in the axial skeleton and consists of a honeycomb pattern of bony struts, plates, or spicules. Trabecular bone remodeling begins on bone surfaces, and it has a greater effect on the skeleton due to the fact that the surface-to-volume ratio of trabecular bone is much higher than that of cortical bone (Eriksen et al., 1994; Foldes et al., 1991). The rate or remodeling of cortical bone is estimated to be 2-10%/year, whereas the rate of remodeling of trabecular bone is estimated at 30-35%/year (A. M. Parfitt, 1983a). This different behavior of cortical and trabecular bone may helps to explain their different responses to physiologic changes and pharmacologic treatment (Eriksen et al., 1994).

In the canine model, trabecular bone remodeling is less understood than cortical bone remodeling, and data that does exist focuses on axial and appendicular skeletal
remodeling in an effort to better understand osteoporosis and its various treatment
modalities. For instance, multiple studies have reported mineral apposition rates of
trabecular bone in the appendicular (humerus and ulna) and axial (pelvis and lumbar
vertebrae) skeleton (Mashiba et al., 2001a; Mashiba et al., 2001b). The predominate
source of trabecular bone available in the canine mandible is located in the head of the
mandibular condyle. A previous study has established limited baseline
histomorphometric data for trabecular bone in the mandibular condyles of 5-month- and
13-month-old canines (Huja, Rummel, & Beck, 2008b); however, complete static and
dynamic histomorphometric data does not exist for trabecular bone at this site.

At a tissue level, BPs reduce cortical bone remodeling in the appendicular
skeleton (Allen et al., 2009b; Li et al., 2001), axial skeleton (Allen et al., 2008a; Mashiba et al.,
2001a; Mashiba et al., 2001b), and the mandible (Allen & Burr, 2008b; Allen et al.,
2009b). It is likely that BPs would have a similar effect on remodeling in the trabecular
bone of the mandible; however, this needs to be examined.

A better understanding of how physiologic trabecular bone remodeling in the
mandibular condyle relates to that of the axial skeleton and how it is affected by BP
therapy may contribute to our understanding of the role that trabecular bone plays in
development of BRONJ.

*Skeletal site*

The rates of bone remodeling differ among skeletal sites. Various sites have been
studied and documented: lumbar vertebra, ilium, femur (Mashiba et al., 2001a), thoracic
vertebra, rib (Mashiba et al., 2001b), iliac crest (Mashiba et al., 2005), maxilla, mandible
(Huja et al., 2006b; Huja & Beck, 2008a), and mandibular condyle (Cottingham K.L., 1998; Cottingham K.L., 1998; Huja et al., 2008b).

There is considerable interest in understanding the tissue level properties of bone in the mandibular condyle (Herring & Ochareon, 2005; Huja et al., 2008b; Teng & Herring, 1996; van Eijden, van Ruijven, & Giesen, 2004). It is formed by endochondral ossification, a mechanism of bone formation also found in the development of long bones and ribs comprising the appendicular skeleton and vertebral bodies comprising the axial skeleton (Nanci A., Whitson S.W., Bianco P., 2003). Despite this similarity, the mandibular condyle is a unique bone as it is part of one of the most complex joints in the body, and it undergoes a unique pattern of loading that is generated by masticatory function rather than weight (Okeson, 2003). The stress created in the trabecular bone of the mandibular condyle by normal function is not well understood. One study attempted to characterize stress distribution in the mandible as a result of excursive movements using a 3D finite element model and concluded that the concentrated areas of stress in three different human loading patterns were in the condylar neck, posterior surface of the coronoid process, and the mandibular angle (Sun et al., 2004). In light of its unique functional demands and blood supply, data from studies of other joints and skeletal sites may not accurately convey the characteristics, behavior, and age-related changes of mandibular condyle trabecular bone.

Extensive data exists regarding the physiologic and architectural bone properties of the vertebrae as result of osteoporosis research. Different functional demands placed on various bones and joints may contribute to differing physiologic bone activities. For this reason, it has been hypothesized that the trabecular bone of the mandible is not likely
comparable to weight bearing sites, including the vertebrae (Huja et al., 2008b).

Differences in the architecture of the trabeculae in the mandibular condyle and vertebrae exist. The thick, but separated, struts of bone comprising the trabeculae of the canine mandibular condyle have been contrasted to the more numerous, thinner, and less separated trabeculae of canine vertebrae. Trabecular bone at both sites is surrounded by a shell of cortical bone, thicker in the condyle than the vertebrae (Huja et al., 2008b).

BP therapy can suppress trabecular bone remodeling in both the appendicular and axial skeletons of humans. In the appendicular skeleton, considerable suppression of bone formation and increase in bone mineral density in iliac crest bone has been associated with the oral BP alendronate (Boivin, et al., 2000; Chavassieux et al., 1997; Odvina et al., 2005) and intravenous zoledronic acid (McClung et al., 2007). Likewise in the axial skeleton, daily oral dosing of various BPs, including risedronate (Harris et al., 1999; Reginster et al., 2000), ibandronate (Chesnut III et al., 2004), etidronate (Harris et al., 1993; Harris et al., 1999; Storm, et al., 1990), and alendronate (Bone et al., 2004), in women with postmenopausal osteoporosis or low bone mineral density demonstrate reduced risk or prevented vertebral fractures. More currently, 5 mg/year of zoledronic acid has shown a 70% reduction in morphometric vertebral fractures and 77% reduction in clinical vertebral fractures (Black et al., 2007).

In the animal model, the effects of BP therapy on bone turnover, microdamage accumulation, mechanical properties, and collagen cross-linking in canine vertebrae have been documented (Allen et al., 2006b; Allen & Burr, 2007; Allen et al., 2008a; Mashiba et al., 2001a; Mashiba et al., 2001b). In addition to this, zoledronic acid has been shown
to diminish the loss of vertebral bone and mechanical strength associated with estrogen deficiency in rats (Glatt et al., 2004).

Though this class of drug has been used for over 4 decades, the literature lacks data on the effects of BPs on the jaw bone (Allen & Burr, 2008b). With the extensive investigations of the effects of a variety of BPs on the trabecular bone of the vertebrae, it is of interest to know how similar bone type in the jaws responds to this class of drugs.

**Age**

The effects of aging on the physiologic activity and architecture of bone have been studied at length. The mineral content of human bone increases with age (Currey, 1979); however, both cortical and trabecular bone mass are lost with age, as indicated by reduction in thickness of the cortex and trabecular bone volume, respectively (A. M. Parfitt, 1983a). This bone loss has been, in part, attributed to a decline in bone formation due to decreased osteoblastic function (Kelly et al., 1989) and an increase in osteoclastic bone resorption (Jowsey J., 1960). Age-related factors are also probably responsible for the degradation of the mechanical properties of human cortical bone (Currey, Brear, & Zioupos, 1996). For instance, cortical bone in the femur of old humans (63 years-old) shows a tendency to develop local hypermineralized areas that crack easily and may reduce toughness (Boyce & Bloebaum, 1993). Reported age-related changes in trabecular bone, predominately micro-architectural changes, include: decreased trabecular thickness, decreased apparent density, decreased volume fraction, increased surface-to-volume ratio, increased trabecular separation, decrease connectivity of trabeculae, and decreased trabecular number (Aaron, Makins, & Sagreiya, 1987;
Compston, Mellish, & Garrahan, 1987; McCalden, McGeough, & Court-Brown, 1997; Mosekilde, 1988).

Trabecular microarchitecture plays an important role, along with bone mass, in determining bone strength (Delling & Amling, 1995; Silva & Gibson, 1997). In human lumbar vertebrae specimens, vertical trabeculae, predominantly involved in support, appear to have increased trabecular thickness (Atkinson, 1967; Pugh, Rose, & Radin, 1973). Broad trabeculae offer support along major lines of force and stress, while fine trabeculae add supplemental strength and allow absorption of complex stresses, such as dynamic pressures produced in joints and stresses produced by weight bearing and muscle pull. Changes in trabeculae are seen before gross changes in bone density are apparent (Liu et al., 2006). Treatment to increase trabecular bone volume can only occur by thickening of existing trabeculae, not by increasing the number of trabeculae (Silva & Gibson, 1997).

Bone turnover rate in young individuals is high, indicated by both high formation and resorption rates and is believed to decrease with age (Jowsey J., 1960). For example, bone turnover in the alveolar process of younger canines is higher than that in 10- to-12-year old canines (Dixon R.B., Tricker N.D., Garetto L.P., 1997). Furthermore, it was 21% higher in the appendicular skeleton of young (5-month old) canines than in the mandibular alveolar process; however, this mandibular BFR remains relatively elevated with age and skeletal maturation while it decreases in the appendicular skeleton (Huja et al., 2006b; Huja & Beck, 2008a; Tricker et al., 2002).

Data from histomorphometric analysis of trabecular bone in the mandibular condyles of canines has shown higher bone forming activity and greater bone mass in
younger (~5-months old) condyles than adult (13-month-old) condyles (Huja & Beck, 2008a). It can be hypothesized that this trend of diminishing bone mass and physiologic bone activity continues beyond 13 months of age; however, histomorphometric analyses is needed in aged canines to further define this aging effect. If true, aging could negatively impact the effectiveness of BP therapy since their uptake and integration into bone are dependent upon physiologic bone activity.

Studies in kids with osteogenesis imperfect who are being treated with high doses of BPs bring uncertainty to the remodeling suppression hypothesis. To date, no cases of BRONJ have been reported in these young patients (Chahine et al., 2008; Malmgren, Astrom, & Soderhall, 2008; S. Schwartz, et al., 2008). If the remodeling suppression hypothesis is indeed true, it is not yet clear why BPs have such a vastly different affect on the remodeling in the jaws of young and old patients. This offers further support for a better understanding of the bone remodeling activity in the mandibular condyle of aged canines.

Histomorphometric Analysis

Past and current cellular activity is represented in a sample of bone. The ability to qualitatively analyze these cellular events is necessary in order to understand the kinetics of bone turnover and the changes in bone structure associated with the effects of pathology, pharmacology, or aging (Eriksen et al., 1994). Accurate analysis of bone on the cellular level is challenging because three-dimensional projections of a section of bone are seen as a two-dimensional image of profiles under microscopic viewing (A. M. Parfitt, 1983b). Stereology is a method that utilizes random, systematic sampling of two-dimensional planar sections to provide unbiased and quantitative data about a three-
dimensional material. Bone histomorphometry applies stereologic principles and formulas to obtain three-dimensional data about bone structure using two-dimensional sections of undecalcified bone in series and at different orientations (Briarty, 1975; Elias & Hyde, 1980; A. M. Parfitt, 1983b). Bone histomorphometry uses brightfield, polarized light, and fluorescence microscopy and an optical grid system that ensures unbiased measurements through random positioning of the grid (Eriksen et al., 1994).

The morphologic characteristics, or static parameters, of bone architecture as well as the kinetic characteristics, or dynamic parameters, of bone physiologic processes can be measured using histomorphometry (Eriksen et al., 1994). Primary histomorphometric indices are measured directly on the bone section and include such variables as area estimates derived from quantifying number of grid points on bone, surface estimates derived from quantifying number of grid lines intercepting bone surfaces, and interlabel width measured as the distance between two fluorescent bone labels (Eriksen et al., 1994). Secondary, or derived, histomorphometric indices are calculated from primary indices to extrapolate three-dimensional data from two-dimensional data (Eriksen et al., 1994; A. M. Parfitt, 1983b). Direct measurement of dynamic parameters, such as remodeling activity and the rate of bone formation, requires the administration of fluorescent labels which are incorporated into forming bone serving as time markers for measurement (Frost, 1969).

Schwartz and Recker studied the validity of measuring trabecular bone volume using histomorphometry versus direct measurement (Schwartz, M.P. & Recker, 1981). They concluded that stereologic measures of bone volume are highly accurate. Although inter- and intra-patient variation in bone volume measurement did exist, they concluded
that it is largely due to variation within different biopsy sites, not variation in the actual measurement. They also concluded that quantification of 20 fields is sufficient for accurate and precise quantification of volume or surface density (M. P. Schwartz & Recker, 1981). Little data exists regarding validity of measuring other parameters using histomorphometric methods.

**Rational for drug potency, experimental period, and animal model**

While most patients are probably taking oral forms of BPs, such as alendronate, the main difference when compared to zoledronic acid is its potency. By using a more potent BP, the duration of the animal experiment can be decreased. The rate of intracortical bone remodeling in canines receiving 0.067 mg/kg/month zoledronic acid was significantly suppressed after only 3 months (Allen et al., 2009b), and physical and microstructural changes were seen in the trabecular bone of humeral head and lumbar vertebrae in canines after 12-week treatment with 0.5 mg/kg/day of alendronate (Hu et al., 2002). Furthermore, while patients who receive no other drug than oral BPs can develop BRONJ (Marx et al., 2007), humans on high dose BPs can develop BRONJ with an exposure period as short as 6-18 months (Durie, Katz, & Crowley, 2005; Magopoulos et al., 2007). An experiment of short-duration using the canine model may also in part be justified by the fact that A-R-F remodeling periods in canine and human rib have been estimated to last three months and four to five months, respectively (Storm et al., 1990; Takahashi H et al., 1980). This suggests that canines may have up to a 40% shorter remodeling cycle.

Animal models to study bone remodeling include rabbits, pigs, canines, and non-human primates. Remodeling in the canine model is well-established and described in
the literature providing baseline information for multiple skeletal sites (Mashiba et al., 2001a; Mashiba et al., 2001b; Mashiba et al., 2005) including the jaws (Huja et al., 2006b; Huja & Beck, 2008a; Huja et al., 2008b; Marotti & De Lena, 1966). Information regarding trabecular bone remodeling in the mandible of the canine model, however, is limited compared to that for femur, ribs, and vertebral bodies. The canine model has also been used to study alteration in remodeling, tissue quality, and mechanical properties in response to BP therapy (Allen et al., 2006b; Allen et al., 2006c; Burr et al., 2003; Day et al., 2004; Hu et al., 2002; Mashiba et al., 2005). Characterization of bone remodeling in this older age and greater skeletal maturity is important because of the vast changes that occur with age (Huja & Beck, 2008a; Tricker et al., 2002).
SPECIFIC AIMS AND HYPOTHESES

Aims

This study investigated the trabecular bone characteristics of the mandible and vertebrae of untreated and BP-treated, aged (2- to 3-years old) canines to help answer the following questions:

1. What physiologic level of trabecular bone remodeling exists in the mandible of an aged canine model and how does this relate to that in vertebra?
2. Does physiologic trabecular bone remodeling in the mandible change with age?
3. Does short-duration, high dose intravenous BP therapy affect trabecular bone remodeling in aged canines? If so, to what degree?
4. Do the effects of BPs on trabecular bone remodeling differ between the mandible and axial skeleton?

Hypotheses to be tested

1. The physiologic trabecular bone remodeling in the mandible remains relatively elevated compared to that in the axial skeleton of aged canines.
2. Short-duration, high dose intravenous BP therapy suppresses trabecular bone remodeling in both the mandible and axial skeleton of aged canines.
3. The degree of BP-induced suppression of trabecular bone remodeling is greater in the mandible than the axial skeleton of aged canines.

Null hypotheses

1. The physiologic trabecular bone remodeling in the mandible is no different than that in the axial skeleton of aged canines.

2. Short-duration, high dose intravenous BP therapy does not suppress trabecular bone remodeling in the mandible or the axial skeleton of aged canines.

3. The degree of BP-induced suppression of trabecular bone remodeling is the same in the mandible as it is in the axial skeleton of aged canines.
CHAPTER 2

MATERIALS AND METHODS

Experimental design

All protocols had IACUC (Institutional Animal Review) approval. Seven male, beagle canines were purchased from Marshall Farms USA (North Rose, NY). The canines were assigned to age-matched treatment groups [n=4 treatment group (ZOL); n=3 no treatment group (NT)]. After one week acclimatization, intravenous infusions of zoledronic acid (0.1 mg/kg/month) were administered monthly for four months. Urine was collected by cystocentesis immediately prior to- and one week after each zoledronic acid administration. Each canine was administered a pair (14 days apart) of calcein bone labels (5mg/kg, Sigma, St. Louis, Missouri)—the second label administered approximately 22 weeks after the first zoledronic acid treatment (Figure 1). These labels marked bone formation at anabolically active bone surfaces. Zoledronic acid and calcein labels were diluted in sodium chloride and administered into the cephalic vein over 20 minutes. Acepromazine was administered subcutaneously prior to all urine collections, zoledronic acid administrations, and bone label administrations. Three to 14 days after the second calcein label was administered, all the canines were euthanized (IV pentobarbital) in accordance with the recommendation of the American Veterinary Medical Association Panel on Euthanasia, and the maxilla, mandible, ninth rib, femur,
and lumbar vertebrae were harvested. The mean age of the canines at sacrifice was 33 months (ZOL 33 months ± 0.4 months; NT 33 months ± 4.8 months).

**Bone sample preparation**

The mandibular condyles and L3, L4, or L5 vertebrae were prepared following the previously described protocol for histologic slide preparation for bone histomorphometric analysis (Huja et al., 2008b). The undecalcified, unstained bone specimen were placed in 70% ethanol, further dehydrated in graded alcohols, and embedded in methyl methacrylate. Standard histologic techniques were followed to cut sections of bone (~80-100 µm thick) along the sagittal plane of each specimen using a diamond wire saw (Delaware Diamond Knives, Wilmington, DE) under water lubrication (Donath & Breuner, 1982). Four bone sections from each condyle and vertebrae from each animal (n=56 bone sections) were mounted on glass slides for complete histomorphometric analyses of bone labels (Table 1).

**Histomorphometric analysis**

The histologic slides were blinded to conceal treatment group during analyses, and all analyses was completed by a single observer. All raw data and measurements were obtained at 100X under epifluorescent light (Olympus, BX 51, Tokyo, Japan), except interlabel thickness (Ir.L.Th) which was measured at 400X. Primary histomorphometric measurements (area, length, and distance) were quantified using standard hit/intercept methods with the aid of a Merz grid (Anderson, 1982; Merz & Schenk, 1970; A. M. Parfitt, 1983b). The square Merz grid was mounted in the eye-piece of the microscope and consists of 36 lattice points delineating 36 square areas and 6 horizontally oriented, hemispherical lines (Anderson, 1982). Primary parameters were
used to calculate derived (kinetic and structural) histomorphometric indices (A. M. Parfitt et al., 1987). All parameters measured (Table 2) were reported in accordance with ASBMR nomenclature and had been previously published (A. M. Parfitt et al., 1987).

BFR can be measured using a surface (BS) or volume (BV) referent. Parfitt suggested that expressing BFR per unit of bone surface (BFR/BS) may be most applicable when considering hormonal effects on remodeling, and bone formation rate per unit of bone volume (BFR/BV) when determining bone age and various age-dependent properties of bone (A. M. Parfitt, 1983b). This concept may have limited applicability to the present study due to its short-term nature. Trabecular bone remodeling is frequently reported using a surface referent. Bone formation rate was calculated as BFR/BS (µm³/µm²/d, µm³/µm²/yr, and mm³/mm²/yr) as well as BFR/BV (%/yr) to allow for comparisons with other studies.

All trabecular bone in each mandibular condyle section was analyzed since the focus of this study was trabecular bone in the mandibular condyle. In the vertebra, a 4x4 mm area (16 fields of view) in each section was randomly selected for analysis. Ir.L.Th of random double labels outside of the selected area of analysis in each vertebra section to ensure that an accurate average Ir.L.Th for the bone section was calculated. Specific methods have been suggested to account for missing data, such as double labels, in low-turnover subjects to avoid introducing bias into collected data (Hauge, Mosekilde, & Melsen, 1999). If no double label is present, then the MAR and other derived indices using MAR cannot be calculated. Leaving missing data with the value of 0 can lead to erroneous or skewed derived parameters. If only single labels are present, MAR used in calculating derived indices should come from either a minimum biologic value (0.3 µm/d
or 0.1 µm/d) or the mean measured value from other surfaces (Foldes, Shih, & Parfitt, 1990; Hauge et al., 1999). In this study, the latter of the two suggested methods was used, and an average MAR value from the same slide or corresponding slide was used in calculating derived indices for fields with missing double labels in the presence of single labels. When no bone existed in a field of view BV/TV and Tb. N were 0, and other derived indices using bone hits was not available.

Statistical analyses

After all data was collected, each slide was decoded, an average for each histomorphometric variable in each skeletal sites for each canine was calculated by averaging the values from the four bone sections taken for that site in each canine, and statistical comparisons were made. Descriptive statistics were calculated for micro-architectural (BV/TV, Tb.N, Tb.Th, and Tb.Sp) and bone remodeling (MAR, MS/BS, BFR/BS, and BFR/BV) histomorphometric parameters. A factorial repeated measures analysis of variance (ANOVA) was used to test the difference in each bone remodeling variable between group (ZOL vs. NT) and skeletal site (mandibular condyle vs. vertebra) while controlling for the between canine variance. Group and skeletal site were included as main effects in the statistical models, and the two-way interaction was also tested. Post hoc tests were conducted using the Tukey Kramer method. Differences were considered statistically significant when \( p < 0.05 \). A power analysis was not carried out in this study since the number of canines available was predetermined from a previous study. All statistical tests were performed using SAS software (SAS Institute, Inc.).
Title: Short-term zoledronic acid reduces trabecular bone remodeling in aged dogs

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Running title: Mandibular condyle, trabecular bone, and zoledronic acid
Abstract

**Objective:** Bisphosphonates (BP) are widely used to treat diseases of disproportionate bone resorption; however, possible side-effects include bisphosphonate-related osteonecrosis of the jaw (BRONJ). The susceptibility of various skeletal sites to BRONJ has been hypothesized to be related to the level of site-specific bone remodeling. Little is known about the physiologic trabecular bone remodeling (TBR) in the mandibular condyle of aged dogs or the effects of bisphosphonates on this TBR. The objectives of this study were to quantify and compare TBR in the mandibular condyle and vertebra of aged (2- to 3-year old) dogs and to evaluate the effects of short-term zoledronic acid on TBR. **Methods:** Fourteen (7 untreated, NT; 7 treated with 4 doses of 0.1 mg/kg/month zoledronic acid, ZOL) dogs were given a pair of calcein labels. Mineral apposition rate (MAR, µm/d), mineralizing surface (MS/BS, %), bone formation rate (BFR/BS, µm³/µm²/yr; and BFR/BV,%/yr), and micro-architectural histomorphometric parameters were quantified from undecalcified specimen (n=112; 4 condyle and 4 vertebra sections/dog) using histomorphometric methods and analyzed statistically (ANOVA, Tukey test, p<0.05). BFR/BS was the main variable of interest as it describes TBR. **Results:** TBR in NT group was significantly higher (>6-fold, p=<0.0001) in the vertebra (BFR/BS 60.4±16.7) than the mandibular condyle (BFR/BS 9.9±6.1). In ZOL group, BFR/BS in both skeletal sites was reduced to similar absolute levels (vertebra 2.6±3.0; mandibular condyle 1.4±3.3). BFR/BS in the vertebra was significantly reduced (-57.8; 96% reduction, p=<0.0001), while it was not in the mandibular condyle (-8.5; 86% reduction, p=0.29). **Conclusions:** Physiologic TBR in aged dogs is vastly different in the
mandibular condyle than in the vertebrae. This higher level of TBR in the vertebrae leads to greater reduction by short-term ZA treatment.

Keywords: Bisphosphonate, mandibular condyle, TBR histomorphometry
1. Introduction

Bone remodeling, or turnover, is a continuous renewal process that occurs in response to mechanical factors associated with fatigue damage repair, physiologic calcium demands, and wound healing (Martin & Seeman, 2008; A. M. Parfitt, 1995). This remodeling process is important as it increases the life of bone by preventing microdamage accumulation as a consequence of repetitive loading (Burr, 1993). Each bone remodeling cycle includes osteoclast-mediated bone resorption and osteoblast-mediated bone formation phases (Eriksen, Axelrod, & Melsen F., 1994; Hattner, Epker, & Frost, 1965). Under normal skeletal conditions these resorption and formation processes are coupled, and the volume of bone formation is comparable to the volume of bone resorption.

Levels of physiologic bone remodeling differ among skeletal sites, regions within skeletal sites, different types of bone, and with age (Eriksen et al., 1994; Huja, Fernandez, Hill, & Li, 2006b; Marotti & De Lena, 1966; A. M. Parfitt, 1983a; Tricker, Dixon, & Garetto, 2002). The rate of physiologic bone remodeling is important baseline information in studying modalities that alter bone metabolism and rates of remodeling, such as mechanical loading, hormones, disease, or drug therapy. Published information is lacking on the physiologic TBR in the mandibular condyle and age-associated changes, as well as the affects of BP therapy on TBR at this skeletal site.

BPs have become the primary therapy for treating diseases of unbalanced bone resorption (Drake, Clarke, & Khosla, 2008), and their use is expected to increase because of the current lack of equally effective alternative treatments for these conditions. With such widespread use of this class of drugs, attention has been given to the possible side
effects that have been associated with their use, such as BRONJ (Fujimura et al., 2009; Khosla et al., 2007; Zervas et al., 2006). The pathogenesis of BRONJ is still not understood (Drake et al., 2008; Russell, 2006). Various hypotheses have been proposed, the most popular of which relates BRONJ to inhibition of bone remodeling (Allen & Burr, 2009a). Since bone remodeling is necessary for homeostatic and healing mechanisms, alteration of bone remodeling could have severe consequences.

Trabecular bone, or cancellous bone, predominates in the axial skeleton and consists of a honeycomb pattern of bony struts, plates, or spicules. Extensive data exists regarding the bone properties of the vertebrae as result of osteoporosis research. The greatest source of trabecular bone available in the canine mandible is located in the head of the mandibular condyle. A previous study has established limited baseline histomorphometric data for trabecular bone in the mandibular condyles of 5-month- and 13-month-old canines (Huja, Rummel, & Beck, 2008b); however, complete histomorphometric data regarding remodeling activity and microarchitecture does not exist for trabecular bone at this site. (Diab, Allen, & Burr, 2009)

On a tissue level, BPs reduce trabecular and cortical bone remodeling in the appendicular skeleton (Allen, Kubek, & Burr, 2009b; Li, Mashiba, & Burr, 2001) and axial skeleton (Allen, Gineyts, Leeming, Burr, & Delmas, 2008a; Mashiba et al., 2001a; Mashiba et al., 2001b), as well as cortical bone in the mandible (Allen & Burr, 2008b; Allen et al., 2009b; Allen et al., 2009b) of 1- to 2-year old canines. It is likely that BPs would have a similar effect on remodeling in the trabecular bone of the mandible and in canines older than 1- to 2-years old; however, investigation is warranted to confirm such hypotheses.
It is of interest to know how physiologic TBR in the mandible relates to that of the axial skeleton, as well as the affects that BP therapy has on TBR in the mandible. Such information may ultimately contribute to our understanding of BP-related side effects. The objectives of this study were to quantify and compare the physiologic TBR of the mandibular condyle and vertebra in 2- to 3-year old (aged) canines and to evaluate the affects of short-term zolendronic acid on this TBR. Based on previous cortical bone studies, the hypotheses were that: (1) the rate of TBR in aged canines remains significantly greater in the mandibular condyle than the axial skeleton, (2) short-term zolendronic acid reduces TBR in both the mandibular condyle and axial skeleton of aged canines, and (3) this reduction is greater in the mandibular condyle than the axial skeleton.

2. Methods and Materials

2.1 Materials

All protocols had IACUC (Institutional Animal Review) approval. Fourteen male, beagle canines were purchased from Marshall Farms USA (North Rose, NY). The canines were assigned to age-matched treatment groups [n=7 treatment group (ZOL); n=7 no treatment group (NT)]. After one week acclimatization, intravenous infusions of zoledronic acid (0.1 mg/kg/month) were administered monthly for four months. Each canine was administered a pair (14 days apart) of calcein bone labels (5mg/kg, Sigma, St. Louis, Missouri)—the second label administered approximately 22 weeks after the first dose of zoledronic acid. These labels marked bone formation at anabolically active bone surfaces. Zoledronic acid and calcein labels were diluted in sodium chloride and
administered into the cephalic vein over 20 minutes. Urine was collected by
cystocentesis immediately prior to- and one week after each zoledronic acid
administration; and Acepromazine was administered subcutaneously prior to all urine
collections, zoledronic acid administrations, and bone label administrations. Three to 14
days after the second calcein label was administered, all the canines were euthanized (IV
pentobarbital) in accordance with the recommendation of the American Veterinary
Medical Association Panel on Euthanasia, and the mandible and lumbar vertebrae were
harvested. The mean age of the canines at sacrifice was 32 months (ZOL 32 months ±
1.2 months; NT 32 months ± 4.3 months).

2.2 Bone sample preparation

The mandibular condyles and L3, L4, or L5 vertebrae were prepared following
the previously described protocol for histologic slide preparation for bone
histomorphometric analysis (Huja et al., 2008b). The undecalcified, unstained bone
specimen were placed in 70% ethanol, further dehydrated in graded alcohols, and
embedded in methyl methacrylate. Standard histologic techniques were followed to cut
sections of bone (~80-100 µm thick) from each specimen using a diamond wire saw
(Delaware Diamond Knives, Wilmington, DE) under water lubrication (Donath &
Breuner, 1982). Four bone sections from each condyle and vertebrae from each animal
(n=112 bone sections) were mounted on glass slides for complete histomorphometric
analyses of bone labels.

2.3 Histomorphometric analysis

The histologic slides were blinded to conceal treatment group during analyses,
and all analyses was completed by two calibrated observers using standard hit/intercept
methods with the aid of a Merz grid (Anderson, 1982; Anderson, 1982; Anderson, 1982; Merz & Schenk, 1970; A. M. Parfitt, 1983b). All raw data and measurements were obtained at 100X under epifluorescent light (Olympus, BX 51, Tokyo, Japan), except interlabel thickness (Ir.L.Th) which was measured at 400X. All trabecular bone in each mandibular condyle section was analyzed, while an area of approximately 16 mm² (16 fields of view) in each vertebra section was randomly selected and analyzed.

Primary and secondary histomorphometric parameters of interest are presented (Table 1). Primary parameters were used to calculate secondary parameters, which describe bone remodeling and microarchitecture. If a bone section did not have a double label to calculate MAR, mean MAR from corresponding bone sections were used to calculate secondary indices for that bone section (Foldes, Shih, & Parfitt, 1990; Hauge, Mosekilde, & Melsen, 1999). All parameters measured were reported in accordance with ASBMR nomenclature (A. M. Parfitt et al., 1987).

2.4 Statistical analyses

An average for each histomorphometric variable for each skeletal sites in each canine was calculated by averaging the values from the four bone sections taken for that site in each canine. Means and standard deviations were calculated for all secondary histomorphometric parameters. Statistical comparison of each secondary histomorphometric parameter using a factorial repeated measures analysis of variance (ANOVA) was made to detect differences between group (ZOL and NT), skeletal site (mandibular condyle and lumbar vertebra), and interaction between group and skeletal site (Table 2). Post hoc tests were conducted using the Tukey Kramer method.
Differences were considered statistically significant when \( p < 0.05 \). All statistical tests were performed using SAS software (SAS Institute, Inc.).

### 3.0 Results

No adverse effects due to the drugs or procedures were noticed, and all animals exhibited normal behavior and weight maintenance during the experimental period.

Means (SD) for secondary parameters are reported (Table 1). Secondary histomorphometric parameters from NT group indicate bone remodeling activity and microarchitecture under physiologic conditions in 2- to 3-year old canines. Changes in these parameters induced by zoledronic acid administration are indicated by the histomorphometric parameters from ZOL group. BFR/BS is the main histomorphometric parameter of interest as it represents bone remodeling.

*Microarchitecture*

Physiologic bone volume was 10% greater in the condyle than the vertebra \( (p=0.004, \text{ Table 2}) \) due to nearly 2-fold greater Tr.Th in the condyle than the vertebra \( (p=<0.0001, \text{ Table 2}) \).

*Bone Formation Rate*

Bone formation rate is the product of the mineral apposition rate (MAR) and the number of bone formation sites (MS/BS) within a specified surface area. There was a 6-fold difference \( (p<0.0001, \text{ Table 2 and Figure 1}) \) between the BFR/BS of the vertebra and the mandibular condyle in the NT group due to both lower MAR \( (p=0.04, \text{ Table 2}) \) and MS/BS \( (p<0.0001, \text{ Table 2}) \) in the mandibular condyle. In the ZOL group, absolute levels of BFR/BS were similar between skeletal sites \( (1.8\text{-fold}, p=0.99, \text{ Table 2}) \) due to a significant suppression of BFR/BS in the vertebra of ZOL group \( (96\%, p=<0.0001, \text{ Table} \)
2 and Figure 1), while BFR/BS in the mandibular condyle was not significantly suppressed (86%, \( p=0.29 \), Table 1 and Figures 1 and 2). This suppression in the BFR/BS of the vertebrae was due to lower MS/BS (94%, \( p<0.0001 \), Table 2) compared to that in the NT group.

4.0 Discussion

The selected duration of zoledronic acid administration was over 4 months; however, the actual time for the pharmacologic effects to be expressed was from the first administration of the drug to the second calcein label administration, which totaled 5.5 months. The selection of such short-duration is supported by previous studies which showed significant suppression of intra-cortical bone remodeling rate in canines receiving 0.067 mg/kg/month of zoledronic acid after only 3 months (Allen et al., 2009b) and physical and microstructural have been reported in the trabecular bone of canine humeral head and lumbar vertebrae after the same duration of treatment with 0.5 mg/kg/day of alendronate (Hu et al., 2002). In addition to this, the remodeling cycles in canine and human rib have been estimated to last three months and four to five months, respectively (Storm, Thamsborg, Steiniche, Genant, & Sorensen, 1990; Takahashi H, Norimatsu H, Watanabe G, Konno T, Inoue J, Fukuda M., 1980). This suggests that canines may have up to a 40% shorter remodeling cycle.

Microarchitecture

Comparison of mean microarchitectural parameters between skeletal sites shows a tendency for trabecular struts in the condyle to be fewer in number, thicker, and more separated compared to those in the vertebra. These data agree with previous qualitative studies using the canine model, the thick, but separated, struts of bone comprising the
The trabeculae of the canine mandibular condyle have been contrasted to the more numerous, thin, and less separated trabeculae of canine vertebrae (Cottingham K.L., 1998; Huja et al., 2008b). Trabecular bone in the mandibular condyle may be described as an architectural intermediate between cortical bone and trabecular bone of the vertebrae. These microarchitectural differences may be the result of different functional demands between these two skeletal sites.

No statistically significant differences were found for any of the trabecular bone microarchitectural parameters between groups (Table 2) in this age group of canines using the selected dose and duration of BP therapy.

Physiologic trabecular bone remodeling

The relative surface area undergoing mineralization was nearly 5-times greater in the vertebra than the condyle. This highlights that the trabecular bone forming activity in the mandibular condyles is vastly different than that in the vertebrae of 2- to 3-year old canines. This difference in MS/BS was reflected as a 6-fold greater BFR in the vertebra than the mandibular condyle. This relative elevation of bone remodeling in the axial skeleton compared to the mandible contrasts previous studies reporting a relative elevation in the cortical mandibular alveolar bone compared to the appendicular skeleton in 1- to 2-year old and 10- to 12-year old canines (Dixon R.B., Tricker N.D., Garetto L.P., 1997; Huja & Beck, 2008a). Based on this relative elevation of cortical bone remodeling in the mandible alveolar bone, it was hypothesized that the trabecular bone of the mandibular condyle would also be elevated relative to the axial skeleton; however, it was not. Similar to the intermediate nature of the trabecular bone microarchitecture in the mandibular condyle compared to cortical bone and vertebral trabecular bone, the bone
remodeling activity in the mandibular condyle is also suggestive of an intermediate nature since it appears to behave more similar to the low activity level of cortical bone in the long bones than to the higher activity level of trabecular bone in the vertebrae.

This different remodeling behavior of the trabecular bone in the mandibular condyle and vertebrae, like microarchitecture, could be the result of different functional demands and mechanical strains that may exist on the trabecular bone of these two skeletal sites. One possible explanation cited for the relative BFR elevation found in the cortical mandibular alveolar bone with age was that bone turnover may be important to maintain bone surrounding teeth (Garetto & Tricker, 2002). Likewise, different loading patterns that are transferred to the trabecular bone of the mandibular condyle and vertebrae may exist as a result of functional or anatomical differences. A better understanding of these loading patterns may aid in better understanding the relationship between function, structure, and TBR.

Zoledronic acid on trabecular bone remodeling

Two groups of canines were studied—one receiving no treatment and the other receiving high doses of a potent intravenous BP, zoledronic acid. Age-related changes are accounted for since the groups were age-matched. The selected dose (0.1 mg/kg/month) of zoledronic acid was on a mg/kg basis for each canine and is equivalent to a 7 mg dose for a 70 kg human. Although this dose was higher than the intravenous doses of ibandronate (3 mg every 3 months) and zoledronic acid (5 mg once-yearly) that have been most commonly reported in human trials (Cosman, 2009), it was within a range of pharmacologic dosing. Cancer dosing regimens of single intravenous doses of zoledronic acid have been reported as high as 16 mg (Berenson et al., 2001a) and
monthly doses as high as 8 mg (Berenson et al., 2001b). Furthermore, dosing of 0.25 mg/kg of intravenous zoledronic acid has been reported to appear safe in canines after multiple administrations (Fan et al., 2008).

No published studies were found regarding the effects of BP therapy on the TBR in the mandibular condyle. There was statistically significant difference in the physiologic MAR found in the NT group between skeletal sites; however, the biologic significance of such difference may be questionable since a minimum biological value has been estimated as low as 0.1 µm/d and normal values for MAR can range from 0.5-2 µm/d (Hauge et al., 1999).

Physiologic MS/BS and BFR were both statistically different between skeletal sites in the NT group; however, these values were reduced to similar, near-zero absolute levels of TBR (Figure 2). This suggests that treatment with this dose and duration of zoledronic acid greatly suppresses TBR without completely eliminating it in both skeletal sites with high levels of physiologic TBR as well as those with low levels of physiologic TBR. It also suggests that sites with low levels of physiologic remodeling are not inherently at a greater risk of over suppression. Regardless of the site-specific level of physiologic remodeling, a low level of remodeling appears to be maintained, indicating a limit to the suppression that occurs with this dose of short-term zoledronic acid. Similar results have been reported in a 3-year study comparing the suppressive effects of 0.2 and 1.0 mg/kg doses of alendronate on the TBR of the femoral neck and lumbar vertebrae in ~1-year old canines. These sites were chosen for their different physiologic remodeling rate (femoral neck 33% lower); however, both sites were suppressed to similar absolute levels under both dosing regimens. This study, along with the current study, offers
support for the existence of a lower limit of BP-induced suppression of TBR (Diab et al., 2009).

Although the reductions in the MS/BS and BFR/BS of the condyle were not statistically significant, 85% suppression is likely to be biologically significant. The absolute amount of reduction (Figure 2) was almost 7-fold greater in the vertebrae (57.9 \( \mu m^3/\mu m^2/yr \)) than the condyle (8.5 \( \mu m^3/\mu m^2/yr \)). This greater absolute reduction in the vertebra may be the result of greater physiologic bone remodeling resulting in greater BP binding site availability and greater uptake and retention of zoledronic acid (Cremers, Pillai, & Papapoulos, 2005).

One popular hypothesis regarding development of BRONJ is that inhibition of osteoclast function and subsequent bone remodeling may decrease remodeling rates to levels below those needed to maintain homeostatic and healing mechanisms. If this is true, the critical level of remodeling at which homeostatic and healing mechanism are lost is not yet known. Skeletal sites with higher physiologic bone remodeling may be more susceptible to BP-induced suppression, but it is unclear whether these sites are subsequently at a higher risk for developing BRONJ. If the critical level of remodeling is the same in all skeletal sites, it would appear that sites of high physiologic remodeling would not be at any greater risk than low-level remodeling sites because BP therapy suppress both sites to similar levels. This may be supported by the fact that there have been few, if any, reports in the literature of BRONJ affecting the trabecular bone of either the vertebra or the condyle. If, on the other hand, the critical level of remodeling is greater in the sites of high physiologic remodeling, then these sites may be in jeopardy of
suppression beyond their critical level of remodeling, possibly resulting in devastating side effects.

The aims of this study were to quantify and compare physiologic TBR in the mandibular condyle and vertebra of 2- to 3-year old dogs and to evaluate the affects of short-term zoledronic acid on this TBR. It was concluded that: (1) physiologic TBR in the mandibular condyle and lumbar vertebrae are vastly different in aged dogs; (2) short-term intravenous BP therapy does suppress TBR in aged canines; (3) the higher level of physiologic TBR in the vertebrae compared to the mandibular condyle leads to greater suppression by short-term BP therapy.
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References


Figure 1. Bone formation rate (BFR/BS) by group and skeletal site measured in \(\mu m^3/\mu m^2/d\). There was a non-significant reduction of 86% between the NT and ZOL groups in the mandibular condyle, and a statistically significant reduction of 96% in the vertebra. † denotes a 6-fold difference (\(p<=0.0001\)) between the condyle and vertebra in the NT group. No significant difference between skeletal sites was found within the ZOL group. Means, SD, and percent differences, and \(p\) values <0.05 are depicted.
**Figure 2.** Comparison of the effect of zoledronic acid on TBR suppression for each skeletal site. a) Absolute reduction was calculated as amount of BFR in ZOL group relative to NT group. Almost a 7-fold difference existed between skeletal sites. b) Percent reduction was also calculated relative to BFR values for NT group. Means, SD, and percent differences, and p values <0.05 are depicted.
Figure 3. Epifluorescent photomicrographs of undecalcified sections (~80 µm) of trabecular bone (magnification 12.5x) in the mandibular condyle from A) ZOL group and B) NT group. Thick, widely separated trabeculae are apparent. Minimal green calcein label is present in ZOL group compared to that seen in NT group specimen.
Figure 4. Epifluorescent photomicrographs of undecalcified sections (~80 µm) of trabecular bone (magnification 12.5x) in the lumbar vertebra from A) ZOL group and B) NT group. Numerous, thin trabeculae are apparent compared to the mandibular condyle. Minimal green calcein label is present in ZOL group compared to NT group.
Table 1. Histomorphometric parameters of interest and their abbreviation, unit of measurement, and formula (A. M. Parfitt et al., 1987). Primary parameters are measured from bone hits, bone intercepts, and direct measurement of bone label. Secondary parameters are calculated using primary parameters.
<table>
<thead>
<tr>
<th>Index</th>
<th>Vertebra</th>
<th>Condyle</th>
<th>Fold-Diff.</th>
<th>p</th>
<th>Vertebra</th>
<th>Condyle</th>
<th>Fold-Diff.</th>
<th>p</th>
<th>Vertebra</th>
<th>P</th>
<th>Condyle</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td><strong>MAR (µm/day)</strong></td>
<td>1.10 (0.08)</td>
<td>0.85 (0.20)</td>
<td>-1.3</td>
<td>0.038</td>
<td>0.84 (0.23)</td>
<td>0.75 (0.23)</td>
<td>1.1</td>
<td>0.96</td>
<td>-23%</td>
<td>0.10</td>
<td>-11%</td>
<td>0.97</td>
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<tr>
<td><strong>MS/BS (%)</strong></td>
<td>15.6 (4.1)</td>
<td>3.3 (1.9)</td>
<td>-4.7</td>
<td>&lt;0.0001</td>
<td>0.87 (1.04)</td>
<td>0.52 (1.22)</td>
<td>-1.7</td>
<td>0.99</td>
<td>-94%</td>
<td>&lt;0.0001</td>
<td>-84%</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>BFR/BS (µm³/µm²/d)</strong></td>
<td>0.17 (0.05)</td>
<td>0.03 (0.02)</td>
<td>-6.1</td>
<td>&lt;0.0001</td>
<td>0.007 (0.008)</td>
<td>0.004 (0.009)</td>
<td>-1.8</td>
<td>0.99</td>
<td>-96%</td>
<td>&lt;0.0001</td>
<td>-86%</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>BFR/BS (µm³/µm²/y)</strong></td>
<td>60.4 (16.7)</td>
<td>9.9 (6.1)</td>
<td>-6.1</td>
<td>&lt;0.0001</td>
<td>2.6 (3.0)</td>
<td>1.4 (3.3)</td>
<td>-1.8</td>
<td>0.99</td>
<td>-96%</td>
<td>&lt;0.0001</td>
<td>-86%</td>
<td>0.29</td>
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<tr>
<td><strong>BFR/BV (%)</strong></td>
<td>93.5 (21.1)</td>
<td>10.0 (7.1)</td>
<td>-9.3</td>
<td>&lt;0.0001</td>
<td>4.0 (4.3)</td>
<td>1.5 (3.4)</td>
<td>-2.7</td>
<td>0.97</td>
<td>-96%</td>
<td>&lt;0.0001</td>
<td>-85%</td>
<td>0.47</td>
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<td><strong>BV/TV (%)</strong></td>
<td>27.9 (1.7)</td>
<td>38.2 (5.4)</td>
<td>1.4</td>
<td>0.004</td>
<td>30.6 (5.5)</td>
<td>35.9 (6.0)</td>
<td>1.2</td>
<td>0.15</td>
<td>10%</td>
<td>0.70</td>
<td>-6%</td>
<td>0.80</td>
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<td><strong>Th.Th (µm)</strong></td>
<td>130 (18)</td>
<td>254 (46)</td>
<td>1.9</td>
<td>&lt;0.0001</td>
<td>127 (30)</td>
<td>220 (47)</td>
<td>1.7</td>
<td>&lt;0.0001</td>
<td>-2%</td>
<td>1.0</td>
<td>-13%</td>
<td>0.30</td>
</tr>
<tr>
<td><strong>Th.N (#/mm)</strong></td>
<td>2.20 (0.18)</td>
<td>1.79 (0.36)</td>
<td>-1.2</td>
<td>0.05</td>
<td>2.56 (0.48)</td>
<td>1.75 (0.22)</td>
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<td>0.0004</td>
<td>16%</td>
<td>0.18</td>
<td>-2%</td>
<td>0.99</td>
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<tr>
<td><strong>Th.Sp (µm)</strong></td>
<td>368 (26)</td>
<td>468 (180)</td>
<td>1.3</td>
<td>0.28</td>
<td>317 (101)</td>
<td>430 (65)</td>
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<td>0.78</td>
<td>-8%</td>
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**Table 2.** Descriptive statistics, between site comparisons, and between group comparisons of secondary histomorphometric parameters from NT and ZOL groups. Descriptive statistics expressed as mean (SD); ZOL, zoledronic acid 0.1 mg/kg/month; fold-difference: (-) condyle less than vertebra, (+) condyle greater than vertebra.
CHAPTER 4

RESULTS AND COMPREHENSIVE DISCUSSION

RESULTS

No adverse effects due to the drugs or procedures were noticed, and all animals exhibited normal behavior and weight maintenance during the experimental period.

Descriptive statistics and statistical analysis of the histomorphometric variables of interest for each group (NT and ZOL) and skeletal site (vertebra and mandibular condyle) are presented in Table 3. Corresponding graphical representation of this data can be found in Figures 2 - 10. Histomorphometric parameters from NT group indicate bone activity under physiologic conditions in 2- to 3-year old canines. The suppression of trabecular bone remodeling induced by zoledronic acid administration is indicated by the histomorphometric parameters from ZOL group. This suppression is compared between groups at both sites (Table 3).

Microarchitectural Parameters

*Bone volume/total volume*

This parameter indicates the density, or lack of porosity, of the trabecular bone. The mean (SD) BV/TV in the NT group was 27.48% (2.29%) in the vertebra and 32.97% (1.90%) in the mandibular condyle. In the ZOL group, this parameter increased to
32.04% (6.90%) and 39.36% (1.94%) in the vertebra and condyle, respectively (Figure 2).

**Trabecular thickness**

This parameter indicates the average strut thickness of the trabecular bone. The Tb.Th in the NT group was 130.7 µm (20.6 µm) in the vertebra and 252.7 µm (73.4 µm) in the condyle. In the ZOL group, Tb.Th was increased 9% to 142.5 µm (30.3 µm) and decreased 1% to 250.1 µm (26.9 µm) in the vertebra and mandibular condyle, respectively (Figure 3).

**Trabecular number**

This parameter indicates the average number of struts/mm in the trabecular bone. The Tb.N in the NT group was 2.13 struts/mm (0.16 struts/mm) in the vertebra and 1.54 struts/mm (0.45 struts/mm) in the condyle. In the ZOL group, the Tb.N increased 12% to 2.39 struts/mm (0.39 struts/mm) and 11% to 1.71 struts/mm (0.30 struts/mm) in the vertebra and mandibular condyle, respectively (Figure 4).

**Trabecular separation**

This parameter indicates the average amount of space between each strut of trabecular bone. The Tb.Sp in the NT group was 381 µm (SD 17 µm) in the vertebra and 603 µm (SD 187 µm) in the mandibular condyle. In the ZOL group, the Tb.Sp decreased 13% to 330 µm (108 µm) and 28% to 434 µm (70 µm) in the vertebra and condyle, respectively (Figure 5).

**Dynamic Parameters**

*Mineral apposition rate*
This index indicates the tissue level activity of the osteoblasts since these cells are responsible for new matrix production, which is normally followed by new mineralization (Frost, 1966). The mean MAR in the NT group was 1.04 µm/d (0.07 µm/d) in the vertebrae and 0.87 µm/d (0.16 µm/d) in the condyle. These values showed a non-significant decrease in the ZOL group to 0.71 µm/d (0.07 µm/d) and 0.75 µm/d (0.23 µm/d) in the vertebra and condyle, respectively (Figure 6A). Because no statistically significant interaction between group and skeletal site was found, main-effects were analyzed for the two factors involved (Figure 6B). Skeletal site yielded no statistical significance; however, the mean MAR in the NT group [0.95 µm/d (0.15 µm/d)] was significantly greater (23%, p=0.03) than that in the ZOL group [0.73 µm/d (0.16 µm/d)].

Mineralizing bone surface

This parameter indicates the percentage of bone surface that is mineralizing, or labeled, compared to the total bone surface available. There was a 7.5-fold difference (p= 0.0006) between the MS/BS of the vertebra [18.39% (4.01%)] and condyle [2.44% (1.41%)] in the NT group, while this difference was reduced in the ZOL group to a non-significant 1.2-fold (Figure 7). In the ZOL group, MS/BS was significantly suppressed 94% (p=0.0003) in the vertebra to 1.11% (1.28%) and non-significantly reduced by 63% (p=0.72) in the condyle to 0.91% (1.58%).

Bone formation rate

This parameter is the product of the mineral apposition rate (MAR) and the number of bone formation sites (MS/BS) within a specified surface area (BFR/BS) or volume (BFR/BV). There was a 9.4-fold difference between the BFR of the vertebra
(BFR/BS 0.19 \mu m^3/\mu m^2/d (0.06 \mu m^3/\mu m^2/d); BFR/BV 104.7%/y (24.8%/y)) and the mandibular condyle (BFR/BS 0.020 \mu m^3/\mu m^2/d (0.012 \mu m^3/\mu m^2/d); BFR/BV 11.15%/y (9.35%/y)) in the NT group (Figure 8). This difference between skeletal site was reduced to 1.2-fold in the ZOL group as BFR/BS significantly decreased 96% (p=0.0006) in the vertebra to 0.008 \mu m^3/\mu m^2/d (0.010 \mu m^3/\mu m^2/d) and decreased non-significantly 67% (p=0.87) in the condyle to 0.007 \mu m^3/\mu m^2/d (0.012 \mu m^3/\mu m^2/d). Similarly, BFR/BV in the ZOL group significantly decreased 96% (p=0.0002) to 4.28%/y (4.71%/y) and non-significantly decreased 77% (p=0.72) to 2.55%/y (4.44%/y) in the vertebra and mandibular condyle, respectively (Figure 10)
DISCUSSION

An animal model of known age was used in this study to describe the bone formation activity and micro-architecture of trabecular bone within the vertebrae and mandibular condyle of untreated and BP-treated 2- to 3-year old canines. Intravenous doses of 3 mg of ibandronate given every 3 months and 5 mg of zoledronic acid given once-yearly have been reported most commonly in human trials (Cosman, 2009). The selected dose (0.1 mg/kg/month) of zoledronic acid was on a mg/kg basis for each canine, and although this dose is equivalent to a 6 mg dose for a 60 kg human, it was selected in order to shorten the duration of the animal experiment and was within a range of pharmacologic dosing. Studies involving cancer patients have reported single intravenous doses of zoledronic acid as high as 16 mg (Berenson et al., 2001a) and monthly intravenous doses as high as 8 mg (Berenson et al., 2001b). Furthermore, one study using 0.25 mg/kg of intravenous zoledronic acid reported this dose to appear safe after multiple administrations in canines (Fan, de Lorimier, Garrett, & Lacoste, 2008).

Micro-architectural parameters

Detecting changes in the micro-architectural parameters was not a primary focus of this study. Based on a 3-month trabecular remodeling cycle in the canine (Takahashi et al., 1980), these parameters were not expected to significantly change in this short-duration study, and statistical analysis to compare micro-architectural parameters
between skeletal sites and treatment groups was not conducted. Because of this, no conclusions can be drawn regarding the micro-architectural parameters; however descriptive statistics were reported (Table 3) and some trends were observed.

Qualitative comparison of micro-architectural parameters between skeletal sites suggests that the struts in the condyle, although fewer in number, are thicker and more separated than those of the vertebrae (Figures 11A and 12A). Trabecular bone in the mandibular condyle could be described as an architectural intermediate between cortical bone and trabecular bone of the vertebrae. The struts of the vertebrae can be described as more fine, greater in number, and less separated, which is possibly a reflection of the functional demands that are placed on the vertebrae to bear compressive forces. This description of the trabecular struts in the mandibular condyle and vertebrae is supported by previous qualitative descriptions of trabecular bone architecture in similar skeletal sites (Cottingham K.L., 1998; Huja et al., 2008b).

The mean Tb.Sp was markedly higher in the mandibular condyle than in the vertebra of the NT group (Table 3). This was in part due to substantial variation found in the trabecular architecture of the condylar specimen, particularly in two of the three control canines (665 µm, 753 µm, and 393 µm).

The BV/TV in the vertebrae (27.48%) is similar to that in previous studies of 1- to 2-year old canines ranging from 21.9 – 25.4% (Mashiba et al., 2001a; Mashiba et al., 2001b). In contrast, condylar BV/TV (32.97%) appears to be lower than that which has been previously reported in younger canine samples ranging from 60.2% in 5-month old canines to 41.8% in 1- to 2-year-old canines (Huja et al., 2008b). From these
comparisons, it is possible to hypothesize that there may be more of an aging effect on the trabecular bone mass in the condyle compared to that in the vertebrae; however, further investigation is needed in order to make any definitive conclusions. Qualitative analysis provides support for an aging-effect in the mandibular condyle as trabecular porosity increases with age and skeletal maturation (Figure 13).

*Age-related trends in trabecular bone remodeling*

The MAR value for vertebra (1.04 µm/d) in this sample of 2- to 3-year old canines tended to be lower than most previously reported values for younger canines. Lee reported 2.5 (1.0) µm/d in a 3-month old canine and 1.1 (0.04) µm/d in a 1- to 2-year-old canine (LEE, 1964). More contemporary studies with more appropriately sized samples involving 1- to 2-year-old canines report MAR values (ranging from 1.04 – 1.56 µm/d) that are similar to slightly higher than those in the current study (Mashiba et al., 2001a; Mashiba et al., 2001b). The trabecular MAR (0.87 µm/d) in this sample was slightly higher compared to the 0.7 µm/d reported in the mandibular condyle of 1- to 2-year old canines (Cottingham K.L., 1998). No data has been published regarding trabecular MAR in young canines. Weak trends, if any, may exist in this data regarding the aging effect on MAR in either skeletal site location. Further investigation is needed regarding the possibility of an age effect, particularly in the early years of life in the vertebrae, on MAR in the trabecular bone before definitive conclusions can be made.

The MS/BS for the vertebra (18.39%) was similar to other studies (ranging from 18.02 – 21.5%) reporting MS/BS in 1- to 2-year-old canines (Mashiba et al., 2001a; Mashiba et al., 2001b). On the contrary, MS/BS in the mandibular condyle of 1- to 2-
year old canines has been reported at 12.6%, which is 5-fold higher than the 2.4% found in this study (Cottingham K.L., 1998). A similar trend of decreasing bone forming activity with age has been described in the trabecular bone of the mandibular condyles in ~5-month and 13-month old canines (Huja & Beck, 2008a). The possibility exists for there to be a more dramatic age-related effect on the trabecular bone forming activity of the mandibular condyle than the vertebrae, but further investigation is warranted to better characterize this effect.

The BFR/BS in the vertebra (70.24 µm³/µm²/y) was only slightly lower than the range (81.5 - 122 µm³/µm²/y) reported for 1- to 2- year old canines (Mashiba et al., 2001a; Mashiba et al., 2001b), while BFR/BV in the mandibular condyle (11.5%/yr) was 3.5-fold lower (39.7%/yr) than that reported in 1- to 2-year old canines (Cottingham K.L., 1998). This comparison of BFR between 2- to 3- and 1- to 2-year old canines suggests trends that are similar to those found for MS/BS. This is no surprise since BFR is the product of MAR and MS/BS.

It may be possible that if age-related changes exist in the trabecular bone remodeling between the mandibular condyle and vertebra, they may be an adaptation to mechanical stress and strain, which may also change over time with age.

Comparison of trabecular bone remodeling between skeletal sites

There was a statistically significant difference between the MS/BS of the vertebra (18.39%) and mandibular condyle (2.44%), indicating that the relative surface area undergoing mineralization was 7.5-times greater in the vertebra than the condyle (Figure 7). This highlights that the trabecular bone forming activity in the mandibular condyle is
vastly different than that in the vertebra of 2- to 3-year old canines. This vast difference in MS/BS was, again, reflected in the BFR as a 9.5-fold greater (p=0.0015) BFR in the vertebra than the mandibular condyle.

The relative elevation of trabecular bone forming activity in the vertebra (axial skeleton) compared to the mandibular condyle contrasts the relative elevation that has been previously reported in the cortical mandibular alveolar bone compared to the appendicular skeleton in 1- to 2-year old and 10- to 12-year old canines (Dixon R.B., Tricker N.D., Garetto L.P., 1997; Huja & Beck, 2008a). It was hypothesized this relative elevation of BFR found in the cortical mandibular alveolar bone would also be found in the trabecular bone of the mandibular condyle; however, this was not the case. Similar to the micro-architecture of the trabecular bone in the mandibular condyle being described as an intermediate between trabecular and cortical bone earlier, the bone forming activity is also suggestive of an intermediate nature as it appears to behave more similar to the low activity level of cortical bone in the long bones than to the higher activity level of trabecular bone in the vertebrae.

This different behavior of the trabecular bone in the mandibular condyle and vertebrae could also be the result of vastly different functional demands and mechanical strains that may exist in the trabecular bone of these two skeletal sites. One possible explanation cited for the relative BFR elevation found in the cortical mandibular alveolar bone with age was that bone turnover may be important to maintain bone surrounding teeth (Garetto & Tricker, 2002). Likewise, different loading patterns on the trabecular bone of the mandibular condyle and vertebrae may exist as a result of functional
differences or even anatomical differences that may partially insulate functional loads, such as the shock-absorbing cartilaginous disc and the thick shell of cortical bone surrounding the trabecular bone within the mandibular condyle. A better understanding of the loading patterns that are transferred to the trabecular bone at these two skeletal sites may aid in better understanding of the relationship between function, structure, and trabecular bone remodeling with age.

The effects of zoledronic acid on trabecular bone remodeling parameters

Two groups of canines were studied—one receiving no treatment and the other receiving high doses of a potent intravenous BP. Age-related changes are accounted for since the groups were age-matched (mean 32.75-months old at second calcein label). Zoledronic acid was administered once monthly for 4 months; however, the actual time for the pharmacologic effects to be expressed was from the first administration of the drug to the second calcein label administration, which totaled 5.5 months.

No data has been published regarding the effects of BP drug therapy on the trabecular bone remodeling in the mandibular condyle. No interaction between group (ZOL and NT) and skeletal site (vertebra and mandibular condyle) was detected for MAR; however, MAR did significantly differ for the group effect alone (NT group 0.95 µm/d; ZA group 0.73 µm/d; p=0.03; Figure 6). The biologic significance of such difference may be questionable since a minimal biological value has been estimated as low as 0.1 µm/d and normal values for MAR can range from 0.5-2 µm/d (Hauge et al., 1999). Multiple studies that treated younger canines with oral and intravenous BPs at widely differing doses have all reported statistically significant reductions in MAR in
trabecular bone of the lumbar vertebrae (Allen et al., 2009b; Diab, Allen, & Burr, 2009; Mashiba et al., 2001a). Another study treated canines with low and high doses of etidronate over 7 months and showed no change in vertebral trabecular bone MAR in the low dose group but significant suppression in the high dose group (Mashiba et al., 2001b). These results along with the current study may suggest that BPs can affect osteoblast activity and suppress MAR if given at a high enough potency, high enough dose, and/or long enough duration. The current study suggests that potent BP therapy at high dosing may alter MAR, even in short duration. Reduction in MAR may be an indirect result of overall suppressed bone remodeling.

There was substantial reduction of MS/BS and BFR in trabecular bone of the vertebra and mandibular condyle after four months of treatment with intravenous BP therapy; however, the reductions in the condyle were not statistically significant (Figures 7, 8, 9). This may have been the result of insufficient statistical power due to large biologic variability and/or a small sample size. MS/BS and BFR were both statistically different between skeletal sites in the NT group; however, no differences existed between the skeletal sites in the ZOL group. Regardless of the vastly different physiologic trabecular bone remodeling that existed between these sites, both skeletal sites were reduced to similar absolute levels of bone turnover slightly greater than zero. The absolute amount of reduction (Figure 9) was almost 13-fold greater in the vertebrae (0.18 µm³/µm²/d reduction) than the condyle (0.013 µm³/µm²/d reduction). This greater absolute reduction in the vertebra is likely the result of greater physiologic bone formation activity that leads to greater BP binding site availability and greater uptake and
retention of zoledronic acid at this skeletal site than in the significantly-less-active mandibular condyle.

Another factor that may contribute to different suppression potentials at different skeletal sites may be the amount of bone surface available at that site. For example, a skeletal site with finer, more numerous and closely arranged trabeculae, such as the vertebra, has relatively greater surface area available for remodeling to take place and BPs to exert their effect.

One popular hypothesis regarding development of BRONJ is that inhibition of osteoclast function and subsequent bone remodeling may decrease remodeling rates to levels below those needed to maintain homeostatic and healing mechanisms. If this is true, the critical level of remodeling at which homeostatic and healing mechanism are lost is not known. Skeletal sites with higher physiologic bone remodeling may be more susceptible to BP-induced suppression, but it is unclear whether these sites are subsequently at a higher risk for developing BRONJ. If the critical level of remodeling is the same in all skeletal sites, it would appear that sites of high physiologic remodeling would not be at any greater risk than low-level remodeling sites because both sites are suppressed to virtually identical absolute levels. This may be supported by the fact that there have been few, if any, reports in the literature of BRONJ affecting the trabecular bone of either the vertebra or the condyle. If, on the other hand, the critical level of remodeling is higher in the high-remodeling sites, then these sites may be in jeopardy of suppression beyond their critical level possibly resulting in devastating side effects.
The current data suggests that sites with low levels of physiologic remodeling are not inherently at a greater risk of over suppression due to their very nature of low-level remodeling sites. Regardless of the site-specific physiologic remodeling level, some extremely low level of remodeling appears to be maintained, indicating a limit to the suppression that occurs with this dose of short-term zoledronic acid in the trabecular bone of the mandibular condyle and vertebrae. This limited suppression in trabecular bone has been previously reported in a 3-year study comparing the suppressive effects of 0.2 and 1.0 mg/kg doses of alendronate on the trabecular bone remodeling of the femoral neck and lumbar vertebrae in ~1-year old canines. These sites were chosen for their different physiologic remodeling rate (femoral neck 33% lower), similar to the mandibular condyle and vertebrae in the current study. Both femoral neck and vertebra sites were suppressed to similar absolute levels under both dosing regimens. This study, along with the current study, offers support for the existence of a lower limit of BP-induced suppression of trabecular bone remodeling (Diab et al., 2009).

The results of this study should be interpreted with caution due to the limited sample size. Caution must also be taken in comparing the results of this study to previous studies using BP drugs. This comparison does not offer any definitive conclusions because of substantial differences among published literature regarding type of BP, dosing, length of treatment, route of administration, and age of sample.
GENERAL CONCLUSIONS

This study investigated the trabecular bone characteristics in the mandible and lumbar vertebrae of untreated and BP-treated, 2- to 3-year old canines. Complete descriptive statistics for the histomorphometric parameters of interest were provided. The primary findings of this study were that physiologic trabecular bone remodeling in the mandibular condyle and vertebra of aged dogs were both relatively low but significantly different; and short-term, high dose intravenous BP therapy significantly reduced trabecular bone remodeling in the vertebra but not in the mandibular condyle.

Null hypotheses

$H_{o1}$: The physiologic trabecular bone remodeling in the mandible is no different than the trabecular bone remodeling in the axial skeleton of aged canines.

Hypothesis rejected.

$H_{o2}$: Short-duration, high dose intravenous BP therapy does not suppress trabecular bone remodeling in the mandible or the axial skeleton of aged canines.

Hypothesis rejected.

$H_{o3}$: The degree of BP-induced suppression is the same in the mandible as it is in the axial skeleton of aged canines.

Hypothesis rejected.
**Conclusion:** Physiologic trabecular bone remodeling in the mandibular condyle and lumbar vertebrae are vastly different in aged dogs. Regardless of this difference, trabecular bone remodeling following short-term, monthly intravenous zolendronic acid treatment was reduced to similar absolute levels at both skeletal sites. This indicates: 1) that BPs can suppressive trabecular bone remodeling in aged animals; 2) that BPs have a more profound suppressive effect in the trabecular bone of the vertebrae than the mandibular condyle; and 3) that a lower limit of BP-induced remodeling suppression in the trabecular bone of both skeletal sites may exist.
FIGURES AND TABLES

<table>
<thead>
<tr>
<th>Event Description</th>
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<tr>
<td>2-3-year-old canines (total n = 7; ZOL group = 4, NT group = 3)</td>
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<td>*Pair of alizarin bone labels (n = 1 ZOL group, 3 NT group)</td>
<td>Week 1 and 3</td>
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<td>0.1 mg/kg/month intravenous zoledronic acid (ZOL group) (4 months)</td>
<td>Week 4, 8, 12, 16</td>
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<td>*Extractions and mini-implant surgery (maxilla and mandible)</td>
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<tr>
<td>Pair of calcein bone labels (n = 7)</td>
<td>Week 24 and 26</td>
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<td>Sacrifice (mean age: 33 months)</td>
<td>Week 27</td>
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<td>Harvest maxilla, mandible, rib, femur, vertebrae</td>
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<td>Labeled bone sections mounted on slides</td>
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<td>Static and dynamic histomorphometry</td>
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</table>

**Figure 1.** Overall experimental design and timeline. Calcein labels were given 14 days apart. *Denotes events that actually occurred but were not applicable to the current study design. Total time for pharmacologic effects of zoledronic acid to take place was 5.5 months (first administration of zoledronic acid to second calcein label).
Figure 2. Bone volume/total volume (BV/TV) by group and skeletal site measured as the % of total volume that comprised of bone. Means and SD are depicted. No statistical analysis was carried out to detect differences. There is a trend toward greater BV/TV in the condyle than the vertebra.
Figure 3. Trabecular thickness (Tr. Th.) by group and skeletal site measured in µm. Means and SD are depicted. No statistical analysis was carried out to detect differences. There is a trend toward greater Tr. Th. in the condyle than the vertebra.
Figure 4. Trabecular number (Tr. N.) by group and skeletal site measured in #/mm. Means and SD are depicted. No statistical analysis was carried out to detect differences. There is a trend toward greater Tr. N. in the vertebra than the condyle.
Figure 5. Trabecular separation (Tr. Sp.) by group and skeletal site measured in µm. Means and SD are depicted. No statistical analysis was carried out to detect differences. There is a trend toward greater Tr. Sp. in the condyle than the vertebra however, significant variability existed in the condyles of the untreated group.
Figure 6. Mineral apposition rate (MAR) by A) group and skeletal site, B) group only.

A) No significant group and skeletal site interaction was present despite a non-significant trend toward decreased MAR in the vertebra. B) MAR for ZOL group was significantly less than that for the NT group. Means, SD, and percent differences, and $p$ values <0.05 are depicted.
**Figure 7.** Mineralizing surface/bone surface (MS/BS) by group and skeletal site measured as % total bone surface undergoing mineralization. There was a non-significant reduction of 63% between the NT and ZOL groups in the condyle, and a statistically significant reduction of 94% in the vertebra. † denotes a 7.5-fold difference (p=0.0006) between the condyle and vertebra in the NT group. No significant difference between skeletal sites was found within the ZOL group. Means, SD, and percent differences, and p values <0.05 are depicted.
Figure 8. Bone formation rate (BFR/BS) by group and skeletal site measured in \( \mu m^3/\mu m^2/d \). There was a non-significant reduction of 67% between the NT and ZOL groups in the mandibular condyle, and a statistically significant reduction of 96% in the vertebra. † denotes a 9.4-fold difference (p=0.0015) between the condyle and vertebra in the NT group. No significant difference between skeletal sites was found within the ZOL group. Means, SD, and percent differences, and p values <0.05 are depicted.
Figure 9. Zoledronic acid affects on trabecular bone remodeling for each skeletal site. a) Absolute reduction was calculated as amount of BFR in ZOL group relative to NT group. Almost a 13-fold difference existed between skeletal sites. b) Percent reduction was also calculated relative to BFR values for NT group. Means, SD, and percent differences, and $p$ values $<0.05$ are depicted.
Figure 10. Bone formation rate (BFR/BV) by group and skeletal site measured as %/yr.

There was a non-significant reduction of 77% between the NT and ZOL groups in the mandibular condyle, and a statistically significant reduction of 96% in the vertebra. † denotes a 9.4-fold difference (p=0.0009) between the condyle and vertebra in the NT group. No significant difference between skeletal sites was found within the ZOL group. Means, SD, and percent differences, and p values <0.05 are depicted.
Figure 11. Epifluorescent photomicrographs of undecalcified sections (~80 µm) of trabecular bone (magnification 12.5x) in the mandibular condyle from A) ZOL group and B) NT group. Thick, widely separated trabeculae are apparent. Minimal green calcein label is present in ZOL group compared to that seen in NT group specimen.
Figure 12. Epifluorescent photomicrographs of undecalcified sections (~80 µm) of trabecular bone (magnification 12.5x) in the lumbar vertebra from A) ZOL group and B) NT group. Numerous, thin trabeculae are apparent compared to the mandibular condyle. Minimal green calcein label is present in ZOL group compared to NT group.
Figure 13. Epifluorescent photomicrographs of undecalcified sections (~80 µm) of mandibular condyle from canines: a) 5-months old (Huja et al., 2008b), b) 13-months old (Huja et al., 2008b), and c) 33-months old (same specimen as in 10b). Green calcein bone label can be seen in each image allowing qualitative comparison of age-affect on physiologic trabecular bone forming activity. Also, a decrease in trabecular number and an increase in trabecular separation, or porosity, is apparent with age and skeletal maturation.
<table>
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<tr>
<th>Canine</th>
<th>Group</th>
<th>Right condyle</th>
<th>Left condyle</th>
<th>L3 vertebra</th>
<th>L4 vertebra</th>
<th>L5 vertebra</th>
<th>Total sections</th>
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<td>8</td>
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**Total bone sections analyzed** 56

**Table 1.** Bone sections analyzed from each canine, group, and skeletal site.
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<th>Type</th>
<th>Name</th>
<th>Abbr.</th>
<th>Unit</th>
<th>Formula</th>
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<td>Primary (static)</td>
<td>Bone volume</td>
<td>BV</td>
<td>µm²</td>
<td># bone hits</td>
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<td></td>
<td>Void volume</td>
<td>Vd.V</td>
<td>µm²</td>
<td># void hits</td>
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<td></td>
<td>Bone surface</td>
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<td>µm</td>
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<td></td>
<td>Double labeled surface</td>
<td>dLS</td>
<td>µm</td>
<td># double label intercepts</td>
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<td></td>
<td>Interlabel thickness</td>
<td>Ir.L.Th</td>
<td>µm</td>
<td>Average*(mag. factor)</td>
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<td>Secondary (dynamic)</td>
<td>Bone volume</td>
<td>BV/TV</td>
<td>% of total volume</td>
<td>(BV/(BV + Vd.V)*100</td>
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<td>Trabecular thickness</td>
<td>Tb.Th</td>
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<td>% of total volume</td>
<td>(BV/(BV + Vd.V)*100</td>
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<td>Mineralizing surface</td>
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<td>% of bone surface</td>
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<td>Mineral apposition rate</td>
<td>MAR</td>
<td>µm/day</td>
<td>Ir.L.Th/interlabel time in days</td>
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<td>BFR/BS</td>
<td>µm³/µm²/d, µm³/µm²/y, mm³/mm²/y</td>
<td>MAR*[(dLs + sLS/2)/BS]</td>
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<tr>
<td></td>
<td>BFR/BV</td>
<td></td>
<td>%/y</td>
<td>MAR*[(dLs + sLS/2)/BV]<em>100</em>365</td>
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</table>

**Table 2.** Histomorphometric parameters of interest and their abbreviation, unit of measurement, and formula (A. M. Parfitt et al., 1987). Primary parameters are measured from bone hits, bone intercepts, and direct measurement of bone label. Derived parameters are calculated using primary parameters.
Table 3. Descriptive statistics and statistical comparison of histomorphometric parameters between-sites in both NT and ZOL groups and between-groups. Descriptive statistics expressed as mean (SD). ZOL, zoledronic acid 0.1 mg/kg/month. Fold-difference: (-) condyle less than vertebra, (+) condyle greater than vertebra. † no statistical analysis performed.


