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Dental Disease and Bisphosphonates Induce Osteonecrosis of the Jaws in Animal Models

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Oral Biology

by

Benjamin Kang

2012

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ABSTRACT OF THE DISSERTATION

Dental Disease and Bisphosphonates Induce Osteonecrosis of the Jaws in the Mouse

by

Benjamin Kang

Doctor of Philosophy in Oral Biology

University of California, Los Angeles, 2012

Professor Sotirios Tetradis, Chair

Bisphosphonates (BPs) have been used clinically for over 30 years to treat postmenopausal osteoporosis, hypercalcemia of malignancy and metabolic bone diseases. Although the use of BPs have been beneficial for these pathologies, several recent reports have linked BP usage to osteonecrosis of the jaw. A common factor amongst these case reports suggests a significant link of dental disease and bisphosphonate treatment in the development of bisphosphonate related osteonecrosis of the jaw (BRONJ). Since the pathophysiology of BRONJ is unknown, we developed two BRONJ animal models where aggressive periodontal and periapical disease was induced with zoledronic acid (ZA) treatment in the rat and mouse, respectively.

Ligature-induced periodontal disease caused significant alveolar bone loss while ZA treatment attenuated this effect in rats. Significant changes of alveolar bone morphology

including sequestration and extensive periosteal bone formation were observed by μCT in the ligated site of BP treated animals. These findings were confirmed histologically, demonstrating necrotic bone with diffuse loss of osteocytes and empty lacunae, rimming of the necrotic bone by squamous epithelium and inflammation, and exposure to the oral cavity. Importantly, the rat lesions were strikingly similar to those of BRONJ patients.

Mandibular tooth drillings causing pulpal exposure in the right first and second mandibular molars caused significant periapical lesion development in the mouse. Periapical lesion were quantified from radiographic images and showed a significant increase in lesion space in vehicle versus ZA treated mice. Significant qualitative differences were seen in the morphology of the alveolar bone in ZA treated mice through μCT analysis. Periosteal bone formation was significantly increased in periapical disease induced ZA treated mice versus control mice. Extensive necrotic bone areas were demonstrated the absence of lacunae in osteocytes. TRAP staining shows no significant difference in the number of osteoclasts between ZA versus vehicle treated animals. However, significant morphologic differences were observed by the detachment osteoclasts and flattening of the ruffled border in ZA treated mice. BRONJ developed in 100% of periapical disease induced ZA treated mice.

Our data suggest that dental disease and BP therapy is sufficient and necessary for BRONJ development. These animal models can serve as tools to understand the pathophysiology of BRONJ.

The dissertation of Benjamin Kang is approved.

Tara L. Aghaloo

Wenyuan Shi

Maie St. John

Sotirios Tetradis, Committee Chair

University of California, Los Angeles

2012

DEDICATION

I dedicate this work

to my parents

Jane

and my close friends

who have and continue to support and encourage me through life.

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LIST OF ACRONYMS

ANOVA	One-way analysis of variance
AOI	Area of interest
BPs	Bisphosphonates
BRONJ	Bisphosphonate related osteonecrosis of the jaw
BUN	Blood urea nitrogen
CEJ	Cementoenamel junction
D1	Distal of the first molar
D2	Distal of the second molar
EDTA	Ethylenediaminetetraacetic
LPS	Lipopolysaccharide
M1	Mesial of the first molar
M2	Mesial of the second molar
OM	Osteomyelitis
PMN	Polymorphonuclear
RANKL	Receptor of activator of NFκB ligand
TRAP	Tartrate resistant acid phosphatase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end
ZA	Zoledronate
μCT	MicroCT

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VITA

2004	Bachelor of Science, Mathematics University of California Los Angeles, California
2008	Masters of Science, Biology California Sate University Los Angeles, California

PUBLICATIONS AND PRESENTATIONS

Aghaloo TL, **Kang B**, Sung E, Schoff M, Ronconi M, Gotcher J, Bezouglaia O, Dry S, Tetradis S. *Periodontal Disease and Bisphosphonates Induce Osteonecrosis of the Jaws in the Rat*. J Bone Miner Res, 2011. 26(8): p. 1871-82. PMID: 21351151.

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Aghaloo TL, Bezouglaia O, Chaichanasakul N, **Kang B**, Franco R, Atti E, Tetradis S. Enhanced osteogenic potential of alveolar vs. long bone derived marrow stromal cells. American Society for Bone and Mineral Research, Montreal, Canada. Sept. 12-16. Poster presentation.

CHAPTER 1

INTRODUCTION

BISPHOSPHONATES

Bisphosphonates (BPs) are stable analogs of natural occurring pyrophosphates and are potent inhibitors of osteoclastic-mediated bone resorption that have been used for over 30 years. The discovery of BPs started in the 1960s when Neuman and Fleisch were studying mechanisms of calcification induced by collagen showing that inorganic pyrophosphate were present in serum and urine that prevented calcification by binding to newly forming crystals of hydroxyapatite [1]. This discovery led many researchers to study various pyrophosphate compounds that inhibited ectopic calcification in blood vessels, skin, and kidneys in animal models. However, one major drawback of translating this work to patients was the fact that pyrophosphates needed to be administered systemically because oral administration led to hydrolysis by mucosal brush border phosphatases [2]. Searching for a more stable analogue, BPs were discovered.

BPs shared common characteristics as pyrophosphates including their high affinity for bone mineral and their ability to prevent calcification both *in vitro* and *in vivo.* BPs were able to prevent pathological calcification when given orally to rats *in vivo.* However, the most important and novel property of BPs was its ability to inhibit the dissolution of hydroxyapatite crystals. This eventually led to a study showing BPs ability to inhibit osteoclast-mediated bone resorption both *in vitro* and *in vivo* [3,4].

Structurally, BPs are composed of a 'P-C-P' backbone which is responsible for the strong affinity of BPs for the skeleton based on the substitution in the R1 and R2 position on the carbon atom. BPs ability to bind to bone mineral is enhanced when the R1 side chain is a hydroxyl group allowing it to chelate calcium ions more effectively by tridentate over bidentate binding [5]. It has also been shown that BPs ability to inhibit bone resorption requires the P-C-P backbone and cannot be achieved with monophosphates [6]. Alterations to one or both phosphonate groups reduces the affinity for bone mineral [7]. Moreover, studies showed that different R2 side chains contributed to the potency of BPs. In particular, BPs containing a primary nitrogen atom in an alkyl chain (i.e. pamidronate and alendronate), were found to be 10-100 fold more potent than non-nitrogenous BPs (i.e. etidronate and clodronate). This discovery led to the synthesis of novel compounds to exploit new BPs by optimizing their antiresorptive effects containing a nitrogen atom within a heterocyclic ring (ie. risedronate and zoledronate. These BPs were shown to be 10,000-fold more potent than etidronate in some experimental studies.

Clinically, BPs have been used to treat postmenopausal osteoporosis, Paget's disease, hypercalcemia of malignancy and other metabolic bone disease [8-11]. In particular, etidronate and alendronate are approved for therapies in many countries due to their ability to increase bone mass and treat fractures of the spine, hip and other sites in postmenopausal women [12-16]. Zoledronate (ZA) and alendronate are examples of more potent nitrogen-containing BPs that are widely used in the management of cancer and skeletal disorders [17,18]. The clinical pharmacology of BPs is characterized by low intestinal absorption, but highly selective localization and retention in bone and significant side effects of BPs are minimal [19-21]. BPs are extremely similar in chemical structure and the capability in treating disorders of bone resorption reflect the history

of their clinical development, specific and functionality. Therefore, there are several BPs which include etidronate, clodronate, tiludronate, pamidronate, alendronate, risedronate and ibandronate that have been used for various clinical applications throughout the world in treating bone pathologies. The use of either intermittent dosing versus continuous, intravenous or oral administration, correlates to the severity of the pathology of the patient and highlights a constant need to improve their usage is constantly needed.

BISPHOSPHONATES AND OSTEOCLASTS

BPs affect osteoclast-mediated bone resorption in a variety of ways including effects on osteoclast recruitment, differentiation, and resorptive activity [22-26]. It is believed that BPs are directly internalized by osteoclasts rather than other cell types because of their accumulation in bone and the endocytic activity of osteoclasts. During bone resorption, the subcellular space beneath the osteoclast is acidified and BPs that have localized to hydroxyapatite are exposed at their highest concentrations. Following this exposure and release during the resportive process, BPs are ingested by osteoclasts, altering the resorptive function and survival of these cells [17,24,27]. This uptake of BPs by osteoclasts *in vivo* have been confirmed using radiolabeled alendronate, which was internalized into intracellular vacuoles and other subcellular compartments such as the cytoplasm, mitochondria and nuclei [24,28]. After cellular uptake, osteoclasts undergo a morphological change leading to the loss of its ruffled borders [29]. Several other studies showed that BPs also disrupt the cytoskeleton of osteoclasts where alendronate and tiludronate disrupt the formation of actin rings in resorbing osteoclasts [24,26]. Research has suggested that disruption

of the cytoskeleton could be through indirect inhibition of protein kinases or phosphatases that regulate cytoskeleton structure or through the loss of function of small GTPases such as Rho and Rac through the mevalonate pathway. This is accomplished by BPs inhibiting farnesyl diphosphate synthetase, an enzyme important in the synthesis of farnesyl pyrophosphate for cholesterol biosynthesis [30]. This prevents the prenylation of small GTPase-signaling proteins such as Rho and Ras, which are important for osteoclast regulation of the cytoskeleton, intracellular vesicular transport and cell survival [31].

In studies by Sato et al., and Murakami et al., alendronate and tiludronate were implicated to disrupt the formation of the cytoskeletal actin ring of osteoclasts [24,26]. In addition, Hughes et al., found that after treatment with clodronate, pamidronate and risedronate, morphological features of apoptosis could be identified in isolated murine osteoclasts both *in vitro* and histological sections of osteoclasts from BP-treated mice [22]. Fragmented and cleaved DNA could be seen in these apoptotic osteoclasts by TUNEL assay. In other studies, BPs were not only seen to induce apopotosis in osteoclasts, but also in macrophages (murine cell line J774) and human myeloma cells lines *in vitro* [32,33].

It still remains unclear that BP-induced apoptosis is the direct reason for the inhibition of bone resportion. It is perhaps more likely that BPs inhibit metabolic pathways that are important for osteoclast function and survival. Interference with such pathways prevents normal osteoclast functions that contribute to osteoclast cell death. In fact, studies have shown in BRONJ animal models that osteoclast numbers do not decrease in ONJ-developed animals, but significant morphological changes in attachment and flattening of the ruffled boards are notibly seen [34].

SITE SPECIFICITY

One of the most confounding questions is why BRONJ only occurs in the jaw. The oral cavity has several anatomical and embryological features that make it unique versus other skeletal sites. First, the oral cavity is composed of alveolar bone covered by a thin layer of periosteum and epithelium with an attenuated layer of connective tissue that is constantly being exposed to the outside environment [9]. This predisposes the oral bones to a wide variety of stresses that could lead to bone exposure from actions such as mastication, dental procedure or periodontal disease and caries. These factors combined with the thin layer of mucosa has proposed many studies suggesting the increased bone remodeling that occurs in the structures of the oral cavity versus other skeletal sites both in its osteogenic and osteoclastic activity [35]. In addition, it has been shown that the oral cavity and teeth are colonized by a complex microbial flora that contribute to oral disease such as caries and periodontitis unlike other bony sites [36]. The oral cavity consists of a diverse microflora of more than 700 bacterial species [36-38]. Changes in oral bacterial behaviors could lead to microbial infection and other inflammatory products to the underlying bone, a situation that is not found in any other part of the body. Investigators have documented many bone specimens of patients that developed BRONJ and shown bacterial morphotypes in biofilms ranged from 2 to 15 in quantity, including species from the genus *Fusobacterium,* bacillus, actinomyces, staphylococcus, streptococcus, *Selenomonas* and 3 different types of treponemes [39]. In another study by Kos et al., a chart review of 29 patients with BRONJ showed a significant increase in *Actinomyces* colonization in exposed bone of 61% versus non-BP treated group of 18% [40].

Actinomyces has been well documented as a primary component of dental plaque and calculus and periodontal disease and has been implicated as a major factor of osteomyelitis [41]. This has led other investigators to suggest the role of *Actinomyces* as a critical organism that contributes to the development of ONJ. Although it remains unclear if the colonization of this species is a primary or secondary event, there are numerous ONJ specimen examinations that have exhibited a large presence of the bacterial organism [42]

Embryologically, the development of the jaws is distinct from the axial and appendicular skeleton in that they arise from neural crest cells of the neuroectoderm and not from the mesoderm. The maxilla and mandible undergo intramembranous instead of endochondral ossification [43]. Differentiation in lineage of the precursor cells of the maxilla (mesenchymal) and mandible (hematopoietic) may utilize the key regulators as other skeletal sites, but it has been shown that several growth factors, receptors and associated signaling cascades play distinct roles in craniofacial versus axial and appendicular skeletal development [44]. Specifically, in the rat, mandibles lose significantly less trabecular bone and bone mineral density and at a lower rate compared to the primary spongiosa of the tibiae after ovariectomy and malnutrition, suggesting different homeostatic mechanisms of the two bones [45]. Investigators have also attributed the local occurrence of BRONJ to the inherent differences in response of inducible factors such as cytokine or hormonal influences on bone cells in the jaw versus other parts of the skeleton. Recent literature has shown differences in the osteogeneic potential of bone marrow stromal cells between long bones versus the mandible [35]. Stefanik *et al.,* [46] used pamidronate treatment of primary human mandibular and iliac crest bone marrow stem cells from the same donor and showed differences in cell survival, osteogenesis and osteoclast recruitment, suggesting possible

dysregulation of mandible bone homeostasis after BP exposure. Further studies are ongoing detailing site-specific cellular characteristics of the jaws and the potential differences BPs elicit to understand the pathophysiological mechanisms of BRONJ.

CLINICAL DIAGNOSIS AND MANAGEMENT

Historically, the risk of osteonecrosis was first described in the 19th century when workers in the matchmaking industry reported pain associated with bony jaw exposure and infection with sequestration coining the term 'phossy jaw' [47]. In 1999, ulceration of the oral mucosa associated with the complication of oral BP therapy was first described [48,49]. Finally, in 2003, osteonecrosis was identified to be associated with the use of BPs [48,50,51]. Interestingly, in the same year, Wang et al., reported patients who were concurrently on chemotherapy and pamidronate treatment that also manifested the complication [52]. Several reports followed with case reviews in clinics that were reported to the Food and Drug Administration along with a significant increase in scientific publications on BRONJ. In 2003, a group of investigators reported a possible association between patients treated with BPs and an atypical bone disorder termed osteonecrosis of the jaw (BRONJ) [53,54]. Several clinical reports followed associating BRONJ with the use of BPs [48,53,55]. In these reports, BRONJ was most frequently observed after dental interventions such as tooth extraction, periodontal disease and in patients receiving corticosteroid treatment [50,53,55].

One problem that remained with these cases was that there was no universal agreement on a definition of the condition. In 2007, the task force on BRONJ by the American Society for

Bone and Mineral Research clinically defined the disease as "an area of exposed bone in the maxillofacial region that does not heal within 8 weeks after identification by a health care provider, in a patient who was receiving or had been exposed to a bisphosphonate and had not had radiation therapy to the craniofacial region" [56]. Also in 2007, the American Association of Oral and Maxillofacial Surgeons (AAOMS) defined BRONJ in patients if all of the following three characteristics were present: 1) current or previous treatment with a bisphosphonate; 2) exposed, necrotic bone in the maxillofacial region that has persisted for more than 8 weeks; 3) no history of radiation therapy to the jaws [57].

Clinically, BRONJ can be presented in a variety of different stages based on BP dosage regimens and treatment durations. Ruggiero et al., developed a clinical staging system to help categorize patients with BRONJ more accurately to systematically establish treatment guidelines and collect data to asses the prognosis in patients have either IV or oral BP use [58]. Generally, patients can be considered 'at risk' according to the AAOMS criteria or established with the disease. 'At risk' patients have no evidence of exposed or necrotic bone, but have been either exposed to an IV or an oral BP. Risk of developing BRONJ in these patients have been directly associated with the potency of the BP along with the duration of its use [59].

Staging of BRONJ development was initially proposed in 2007 by the AAOMS to assess severity and direct clinicians to probable treatment modalities [57]. It should be noted that in 2009, an update was made in the staging proposal as more information of BRONJ development and severity was observed and to fulfill the necessity to more accurately stratify patients [60]. Staging will continue to be modified as the pathophysiology of BRONJ is further elucidated and more research reveals the necessity to outline specifics in disease progression. The following includes

the most updated staging system used in clinics today.

Stage 0 is defined as patients with no clinical evidence of necrotic bone, but present with non-specific symptoms or clinical radiographic finds. Symptoms include dull, aching bone pain in the body of the mandible, sinus pain associated with inflammation and thickening of the maxillary sinus wall and altered neurosensory function. Clinical findings include periapical or periodontal fistula not associated with pulpal necrosis due to caries, thickening of the lamina dura and decreased size of the periodontal ligament space and changes in dense woven bone and persistence of unremodelled bone in extraction sockets. Treatment strategies at this stage include the use of medication for chronic pain and control of infection with antibiotics, when needed [59].

At stage 1, patients have exposed bone but are asymptomatic. No evidence of significant adjacent or regional soft-tissue inflammation, swelling or infection has occurred. It is, however, possible that patients have had symptoms of pain prior to radiological changes in the development of osteonecrosis of clinical evidence of exposed bone. Treatment and management strategies at stage 1 include the use of oral antimicrobial rinses, such as chlorhexidine 0.12%. Surgical treatment is not observed at this stage [59].

Stage 2 is characterized by exposed bone associated with pain, adjacent or regional soft-tissue inflammatory swelling, or secondary infection. Treatment strategies include the use of oral antimicrobial rinses in combination with antibiotic therapies such as metronidazole, clindamycin, doxycycline and erythromycin [59].

At stage 3, the disease has exposed bone with symptoms of pain, adjacent or regional soft-tissue inflammatory swelling, or secondary infection, in addition to pathologic fracture or an extra-oral fistula or radiographic evidence of osteolysis extending to the inferior border both in

the mandible and/or maxilla. Treatment modalities at stage 3 include debridement, including resection, in combination with antibiotic therapy, which may offer long-term palliation with resolution of acute infection and pain [59]. The progression of patients from Stage 1 or 2 to more advanced stages has not been determined, but may be dependent on variables such as duration of BP exposure and whether the patient is still receiving BP therapy [47,59].

Based on the stage of the disease, BRONJ management strategies have varied. The main goal in treating patients is always to eliminate clinical symptoms such as pain, treat any infection of soft tissue without exposing uninvolved bone and to minimize the progression of bone necrosis [61]. Although there is a small percentage of patients receiving BPs that develop osteonecrosis of the jaw spontaneously, the majority of affected patients experience this complication following simple dento-alveolar surgery such as extraction, dental implant placement or apical surgery. It is estimated that the incidence of BRONJ of patients receiving monthly intravenous infusions of ZA or pamidronate ranges from 0.8% to 12% where dento-alveolar trauma was performed. Some studies showed that with intravenous BP treatment in combination with a dento-alveolar surgery made it 7 times more likely to develop BRONJ than patients who had no dento-alveolar surgery [62]. BRONJ development with oral BPs is low and is estimated to be 0.7 cases per 100,000 in Australian populations in 2007 [57]. Osteoporosis patients receiving weekly alendronate, ranged from 0.01% to 0.04% in BRONJ development. For these reason, it is optimal to uphold the best dental health of patients who will receive or are receiving BP therapy.

BRONJ management can be performed either non-surgically or surgically. The use of antiseptic mouthwashes such as chlorhexidine gluconate or hydrogen peroxide and/or analgesia

is proposed for patients with at Stage 1 of the disease to prevent further progression of BRONJ and avoid infection of exposed bone. Those patients at Stage 2 are usually treated with antibiotics to treat a broad spectrum of microbial targets such as phenoxymethylpenicillin, amoxicillin or coamoxiclav, clindamycin with or without metronidazole [61,63]. In patients at Stage 3, surgical treatment to remove necrotic bone and create soft tissue coverage is the main goal. Since it is difficult to know how much bone removal is sufficient because of systemic BP distribution through the whole skeleton, there is effectively no unaffected bone. For this reason, it is recommended that the smallest amount of exposed bone removal and minimal soft tissue disturbance to debride affected areas be approached. Aside from all these treatment methodologies, adjunctive therapies for BRONJ management include hyperbaric oxygen, parathyroid hormone, platelet rich plasma and lasers [63].

PATHOPHYSIOLOGY OF BRONJ

Currently, the pathophysiology of BRONJ is unknown. However, several hypotheses have been proposed since numerous clinical reports of the disease occurred in 2003. The predominant hypothesis is the profound inhibition of osteoclast function in normal bone turnover. Since BRONJ is isolated in the jaw and no other skeletal site, speculative consideration of constant microdamge from constant mechanical loading or surgical injury has led researchers to attribute dysfunctional bone remodeling with bisphosphonate administration. In addition, BPs have been shown to have anti-angiogenic properties. ZA has been demonstrated to exert inhibitory effects on circulating levels of vascular endothelial growth factors *in vitro*

[64,65]. Limiting sufficient blood supply to the alveolar bone may contribute to ischemic changes to operate in concert with metabolic changes mediated by osteoclast suppression to produce local jaw bone necrosis. Since BRONJ occurs only to a minority of BP treated patients, researchers have looked at individual genetic variations that may contribute to the susceptibility disease development. Consideration to the epithelial wound healing process has also been hypothesized as a contributor to BRONJ development. Studies have shown inhibitory effects of BPs on epithelial cells and wound healing, suggesting apoptosis and/or senescence in these cell types. Finally, severe inflammation has been implicated with ONJ development. These hypotheses will be looked at in more detail.

BISPHOSPHONATES AND BONE REMODELING

The function of BPs in skeletal sites most at risk for fractures such as the vertebral spine, femoral neck of the femur and iliac crest are well known. Due to their ability to reduce osteoclastic activity, BPs have been used to treat pathologies involving bone loss. Chapurlat et al., examined transiliac bone biopsies from postmenopausal women and found no significant differences from controls in the number of microcracks within three years of BP treatment despite a marked reduction in bone turnover [66]. Stepan et al., reported on 38 postmenopausal women who showed low femoral neck density and increased microcrack accumulation following either an adjustment or cessation of alendronate treatment due to confounding factors [67]. Several other reports have shown that long-term use of BP in postmenopausal osteoporosis had significantly reduced associated fracture risks and returned and maintained patients from

experiencing significant bone loss [68].

BPs reduce the rate at which bone is remodeled by causing bone tissue to be more highly and uniformly mineralized and collagen to become more highly cross-linked [8]. Different BPs have differing binding affinities and potencies on osteoclastic enzymes. For this reason, variations of BPs are selectively used to suppress bone turnover based on overall risk factors. Other therapeutic drugs, such as Denosumab show similar effects as potent antiresorptive agents that utilize different mechanistic pathways [69].

Selective involvement of the maxilla and the mandible reflect the unique environment of the oral cavity in the development of BRONJ. BPs have been shown to preferentially deposit in bones with high turnover rate giving the maxilla and mandible elevated selectivity for BRONJ development [70]. Studies in dogs have shown intracortical remodeling in the alveolar bone of the mandible and maxilla to be higher than in the basal region [71]. In the alveolar region, the cortical bone is reported to have a turnover rate of approximately 25% per year, while the basal mandible is even lower at approximately 7% per year. Compared to intracortical remodeling of other skeletal sites, the rib has a rate of proximately 20% per year and the tibia or femur diaphysis has a rate of proximately 1 to 2% per year [72]. Within the maxilla alone, the remolding rate is heterogeneous. Rates of remodeling are much higher in the anterior portion (anterior of the second premolar) than posterior (posterior of the fourth premolar) [73]. In a mature skeleton, the alveolar mandible has a higher turnover rate than the alveolar maxilla.

A study by Allen et al. showed significant bone remodeling suppression of the mandible when compared to the rib and tibia of dogs treated with ZA in comparison to vehicle treated animals [72]. Mandibular remodeling was suppressed by 99% after 6 months of ZA treatment.

Similar findings were supported demonstrating suppression of intracortical bone formation of the mandible in beagle dogs [71]. Difference in mineral affinity and skeletal uptake could explain the differential remodeling suppression in these sites.

Finally, it has been shown that a linear relationship between BP dose and skeletal uptake exists which suggest that cumulative doses could have a significant impact on the amount of drug to which the skeleton is exposed [74]. Usage of intravenous versus oral BPs for different skeletal complications has been shown to have correlating bone suppression based on their potency.

BISPHOSPHONATES AND ANGIOGENESIS

Several authors have cited antiangiogenic effects of BPs in both *in vitro* and *in vivo* systems [54,65,75-81]. This compromise in blood supply to the alveolar bone has been compared to phenotypic results of ORN where necrotic bone develops as a consequence of decreased blood vascularity of radiation-induced fibrosis. However, distinctions between ORN and ONJ have been made clinically and radiographically. First, ORN rarely occurs in the maxilla versus the mandible. In ONJ, the ratio of occurrence between the two sites is less dramatic with many cases of necrotic bone development in the maxilla [9]. ORN development is prevalent due to anatomically disparities between the mandible and maxilla where one major vessel provides vascularity, oxygen supply and cellularity to the alveolar bone. Histologically, Hansen et al., showed differences in vessel hyalinization and decreased cellularity in ORN samples versus ONJ specimens [82].

It has been shown that ZA significantly suppresses new vessel sprouting in culture and angiogenesis when tissue chambers were implanted subcutaneously in mice [65]. This group also showed that endothelial cell adhesion, migration and vessel sprouting were significantly reduced by ZA administration. To investigate the effects of BPs *in vivo*, Fournier et al., examined revascularization in the prostate gland of rats induced by testosterone [76]. The researchers found that there was a 50% reduction in revascularization in rats that had been treated with ZA. Giraudo et al., showed the effects of ZA in a mouse model of human cervical carcinoma in inhibiting angiogenesis in tumor progression as well as occurrence of premalignant lesions of tumor growth [77]. These findings were supported by others showing reductions in tissue revascularization of rats and lower vessel densities in humans that had been treated with BPs.

In human studies, investigators showed significant reduction in serum VEGF levels when treated with ZA or pamidronate for patients with advanced solid cancers and associated bone metastases [64,80,81,83]. These studies were confirmed with supporting findings that reported further reductions in VEGF levels maintained through a period of 21 days. Vincenzi et al., also showed platelet-derived growth factor levels significantly dropped after only a single infusion of ZA in cancer patients [79]. Bezzi et al., showed the effects of ZA and clodronate on human umbilical vein endothelial cell (HUVEC) *in vitro* [75]. In these studies, ZA inhibited integrin-mediated adhesion and migration along with disrupting focal adhesions and actin stress fibers. ZA was also shown to cause tumor necrosis factor-induced cell death from nuclear fragmentation. Ferretti et al., conducted a study on 18 breast cancer patients with bone metastases that were treated with ZA and showed a transient significant decrease in serum levels of MMP-2, VEGF and bFGF after 2 days [83].

Incidences of ONJ occurrence in the maxilla may refute BP-induced inhibition of angiogenesis as its sole cause. It does not eliminate nor refute the antiangiogenic properties of BPs to contribute play a role in the multifactorial development and progression of BRONJ.

BISPHOSPHONATES AND WOUND HEALING

Clinically, BRONJ is characterized by exposure of necrotic bone by defective wound healing of the soft tissue. Researchers have attributed soft-tissue toxicity as a contributor of necrosis. The effects of BPs on epithelial, gastrointestinal, cervical, renal, prostate and oral mucosal cells have been well documented. Oral and IV BPs have been shown to cause ulcerations and gastric erosions, respectively. Twiss et al., used Caco-2 intestinal cells and showed that pamidronate had cytotoxic effects increasing cell permeability *in vitro*. Interestingly, when calcium was added to these cultures, both pamidronate and alendronate were cytotoxic to Caco-2 cells. However, when calcium was absent, pamidronate was the only BP to show toxicity to the cells. The nitrogenous-based BP was shown to create insoluble complexes with calcium that increased the intestinal cells to toxic accumulation of pamidronate. Supporting these findings, Suri et al., showed a dose-dependent increase of apoptotic Caco-2 cells, reduction in cell viability and inhibition of cell proliferation with pamidronate. These effects were prevented when geranylgeraniol was exogenously added into the cultures suggesting the direct effect BPs had on farnyldiphosphoate synthase of the mevalonate pathway and the insufficient production of cholesterol precursors for cell membrane biosynthesis. Other researchers have used a variety of different BPs to show similar increase in apoptosis in cervical epithelial cells,

renal cells and prostate epithelial cells.

In dose studies, Landesberg et al., showed the effects of pamidronate on mouse oral keratinocytes. Cellular proliferation of keratinocytes was inhibited by pamidronate at concentrations of 0.1 mM remaining consistent over a 7-day time point. Kim et al., showed that normal human oral keratinocytes underwent senescence with overexpressed senescence-associated beta-galactosidase, p16INK4A, IL-6 and IL-8. Moreover, the researchers showed that normal human oral fibroblast proliferation was inhibited mainly through apoptosis. Lastly, Scheller et al., showed BP-induced inhibition of P63 epithelial stratification of human oral keratinocytes.

BISPHOSPHONATES AND INFLAMMATION AND INFECTION

Many investigators have attribute bacterial infection and/or inflammation as key factors contributing to the pathogenesis of BRONJ.

Various studies that characterize the breath of bacterial colonization in ONJ patients have been made [36,39,84-87]. It is no secret that the oral cavity is comprised with a plethora of bacterial biofilms. These bacteria have been the source of a host of infections such as caries and periodontitis. In fact, it has been estimated that at least 35% of US adults between the ages of 30 and 90 have periodontitis. Specifically, 85% of affected patients have periodontitis during the diagnosis of BRONJ [55].

P. gingivalis is strongly associated as the causative agent of adult periodontitis. C57BL/6J mice deficient in the vascular adhesion receptors ICAM-1 showed increased bone loss

when infected with *P. gingivalis* [88]. Bone loss in these studies were attributed to alterations in the immune defense of the bacteria [89]. Other studies using antibody-mediated depletion of CD-4 lymphocytes resulted in decreased alveolar bone resorption in the presence of local injections of E. *coli* lipopolysaccaride [90]. These studies demonstrate a bacterial-induced immunological response that could promote osteoclastic activity. BP administration could alter alveolar bone remodeling in bacteria-induced infections of dental disease contribute to BRONJ development.

Using culture-independent molecular methods, researchers have detected over 500 species or phylotypes in subgingival plaque of healthy subjects. Localization of the structures in the oral cavity has also been analyzed to determine site specificity of bacterial colonization. In a study by Sedghizadeh et al., sequestered necrotic bone was analyzed in BRONJ patients. Results of the study by SEM evaluation showed large areas occluded with biofilms comprised of bacteria, yeast and extracellular polymeric substances. *Fusobacterium*, bacillus, actinomyces, staphylococcus, streptococcus, *selenomonas* and three different types of treponemes were observed. Yeast was identified consistent with *Candida* species. Although biofilms were present in all ONJ samples, results generally identified a breath of bacterial players that may contribute to BRONJ development. With such a wide variety of bacterial influence in the oral cavity, the teeth and alveolar bone are subjected to disease development such as periodontal infection. These infections can lead to the start of chronic osteomyelitis in an asymptomatic and subclinical necrotic bone development with BP administration eliciting further host inflammatory responses [91-93].

By far, inflammation has been attributed as a major factor in BRONJ development. Clinical resected necrotic bone of patients revealed extensive inflammatory infiltrates in previous

studies. Lesclous et al., observed bone marrow inflammation that positively correlated with the clinical extent of ONJ [94]. In addition, the investigators were able to link osteocyte apoptosis and empty osteocyte lacunae to the degree of inflammation. Osteocytes were the only cell type that was recognize undergoing apoptosis suggesting BP-induced osteocyte apopotosis in inflammatory recruitment [94]. It has also been shown that ZA is a novel inhibitor of suppressor of cytokine signaling-3 (SOCS3) in primary macrophages in human ONJ biopsy specimens [95]. Inhibition of SOCS3 by ZA resulted in increase production of IL-6. These studies have alluded to the influence macrophage-released cytokines have also on other cell types including fibroblasts and endothelial cells [95]. Ultimately, the dysregulation of macrophage functions by BPs could contribute not only to bone remodeling, but altered would healing and vascularity needed to preserve viable alveolar bone. On going studies are revealing the complications BPs have in the enhancement and destruction of inflammation.

RATIONALE AND HYPOTHESIS

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a complex side effect attributed to bisphosphonate (BP) therapy used in patients suffering from skeletal disorders or cancer. BRONJ is clinically defined as an area of exposed bone in the maxillofacial region that does not heal within 8 weeks after identification by a health care provider, in a patient who was receiving or had been exposed to a bisphosphonates without a history of radiation therapy to the craniofacial region.

BRONJ is a unique disease that locally affects only the jaw bones. This may be attributed to a multitude of anatomical, functional and microbiological features unique to bones in the craniofacial region. A thin layer of periosteum, epithelium and connective tissue covers the alveolar bone in the jaws, which predisposes these structures to a wide variety of stresses and trauma resulting from mastication, dental procedure or periodontal disease and caries. In addition, the oral cavity consists of a diverse microflora of more than 700 bacterial species that could lead to microbial infection and other inflammatory products to the underlying bone. These conditions are not found in any other part of the body.

Investigators have hypothesized many factors that contribute to BRONJ. These include BP induced inhibition of osteoclastic function, suppression of angiogenesis, apoptosis of epithelial cells and dysregulation of immune cells in an inflammatory response. Although much progress has been made both in *in vitro* and *in vivo* systems, a complete understanding in the mechanistic pathophysiology of BRONJ remains unclear.

To test these hypotheses, several BRONJ animal models have been developed to study the disease. These models mainly follow clinical observations of BP treated patients who develop osteonecrosis after dental intervention, predominantly tooth extraction. Although these extraction-induced BRONJ animal models are helpful, various added factors such as the use of dexamethasone or vitamin D deficient diets, have been necessary to recreate the disease. These factors have limited understanding the direct effect of BPs to the development of BRONJ.

Considering the multifactorial contributes to BRONJ pathophysiology, we *hypothesize* that the combination of BP therapy and aggressive dental disease will create BRONJ in both the rat and mouse. To test this *hypothesis* and further elucidate contributing factors of pathogenesis we propose the following specific aims:

SPECIFIC AIMS

SPECIFIC AIM 1: To establish a periodontal disease-induced BRONJ rat model.

- Develop BRONJ in the rat with bisphosphonate administration and wire ligature induced periodontal infection
- Qualitatively and quantitatively evaluate the effect of bisphosphonates and periodontal disease for BRONJ development in the rat by radiographic, μCT, histological, and immunological analysis

SPECIFIC AIM 2: To establish a periapical disease-induced BRONJ mouse model.

- Develop BRONJ in the mouse with bisphosphonate administration and pulp exposure to induce periapical disease
- Qualitatively and quantitatively evaluate the effect of bisphosphonates and periapical disease for BRONJ development in the mouse by radiographic, μCT, histological, and TRAP analysis

SPECIFIC AIM 3: To evaluate contributing factors in the development of BRONJ pathophysiology.

- Qualitatively and quantitatively evaluate the effects of BP on bone remolding in dental disease
- Qualitatively and quantitatively evaluate the effects of BP on osteoclasts
- Evaluate changes in blood vessel number, epithelium, bacteria and inflammation

CHAPTER 2

MATERIALS AND METHODS

Animal Care, Ligature Placement and Periapical Lesion Induction

All animals and surgical procedures were handled in accordance with guidelines of the Chancellor's Animal Research Committee of the Office for Protection of Research Subjects at the University of California, Los Angeles. Three-month old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in light and temperature controlled facilities and given food and water ad libitum. Animals received intraperitoneal (IP) injections of vehicle (sterile saline) or 66 μg/kg ZA three times per week for 3 weeks prior to intraoral procedures. This dose corresponds to the 4 mg/60 kg ZA dose administered monthly to cancer patients for bone disease control. Rats were anesthetized with isoflurane and a sterile 28-gauge wire ligature was placed around the cervical portion of the right first molar. Animals were monitored weekly to ensure presence of the ligature, and were given a regular diet throughout the duration of the experiment. Ligatures remained in place for 12 weeks, and then animals were sacrificed. At the time of sacrifice, blood was drawn from all animals via cardiac puncture to evaluate serum chemistry. Whole maxillas were removed, placed in 10% formalin for 48 hours and stored in 70% ethanol.

Weights of the rats were measured three times per week. At the end of the experiment, animals were euthanized, blood was collected via cardiac puncture, and serum biomarkers

including Na, K, Cl, CO_2, blood urea nitrogen (BUN), creatinine, Ca, Mg, phosphorus, albumin and alkaline phosphatase were measured. No significant differences in weight or any of the serum markers between vehicle- and ZA- treated animals were detected (data not shown).

Four-month old C57BL/6J male mice (Jackson Laboratories, Bar Harbor, ME, USA) were housed in light and temperature controlled facilities and given food and water ad libitum. Animals received intraperitoneal (IP) injections of vehicle (endotoxin free water) or 200 μg/kg ZA three times per week for 1 week prior to periapical lesion induction. Mice were anesthetized with isoflurane and mounted on a jaw retraction board. Pulpal exposures were performed with a size 1/4 round bur to the depth of the diameter of the bur, so that furcal perforation would be avoided. Exposed teeth were left open to the oral environment. The operative procedure was carried out on the right mandibular first and second molars in all animals. Animals were monitored weekly and continuously injected with ZA three times a week for 7 weeks. Thereafter, mandibles were removed, placed in 4% paraformaldehyde for 48 hours and stored in 70% ethanol.

MicroCT Scanning and Radiographs

Rat maxillas were imaged by μCT scanning (μCT 40; Scanco Medical AG, Basserdorf, Switzerland) at 16 μm resolution, volumetric data were converted to DICOM format and imported in the Dolphin Imaging software (Chatsworth, CA) to generate 3D and multiplanar reconstructed images. An oral and maxillofacial radiologist (ST), blinded to the specific animal treatment, evaluated the μCT images of all animals to identify and score bony changes, and

performed all linear measurements utilizing Dolphin software tools.

Radiographic evaluation of patient records through the approval by the Office for Protection of Research Subjects at the University of California, Los Angeles was obtained to review the radiographic records of seventeen patients with diagnosis of BRONJ. The patients were imaged at the Oral and Maxillofacial Radiology Clinic at the UCLA School of Dentistry utilizing the high-resolution cone beam computed tomography (CBCT) 3D Accuitomo scanner (J Morita, Japan). BRONJ and unaffected sites were reviewed and the cortical thickness of the buccal or lingual cortex of the affected alveolar ridge and of the corresponding site of the healthy alveolar ridge were measured at axial, coronal or sagittal reconstructed sections. Then, the cortical thickness of the alveolar ridge at the BRONJ site was expressed as % thickness of the healthy site.

Mouse mandibles were imaged by μCT scanning (μCT SkyScan 1172; SkyScan, Kontich, Belgium) at 12 μm resolution. Volumetric data were converted to DICOM format and imported in the Dolphin Imaging software (Chatsworth, CA) to generate 3D and multiplanar reconstructed images. To quantify periapical lesion between vehicle versus ZA treated animals, the imaged volume was oriented with body of the mandible parallel to the horizontal plane and the midline of the mandible perpendicular to the horizontal plane and parallel to the mid-sagittal plane. The shortest midline vertical distance from the apex of the root to the inferior alveolar bone was measured for the distal and mesial root of the first and second molar, respectively, and compared to the contralateral healthy side.

Histology and TRAP Histochemistry

Rat maxillas were decalcified (Fisher Scientific) for 4 days, and then samples were paraffin embedded and five-micron thick sections were stained with Hematoxylin and Eosin (H&E). All histology was performed at the Translational Pathology Core Laboratory (TPCL) at UCLA.

Mouse bones were decalcified in 14.5% ethylenediaminetetraacetic acid (EDTA) solution for three weeks. Samples were then paraffin embedded and 5μm-thick sections were cut coronally, perpendicular to the long axis of the alveolar ridge. Each section included a complete cross section through the mandible to allow a side-by-side comparison of bone, teeth and soft tissues from the periapical induced side to the control side. Area of osteonecrosis and periosteal thickness was quantified as previously described [96].

Osteoclasts were identified by tartrate-resistant acidic phosphatase (TRAP) histochemistry using a leukocyte acid phosphatase kit (Sigma, St. Louis, MO), following the procedures recommended by the manufacturer. Multinucleated TRAP positive cells were quantified for the defined region of interest.

TUNEL Immunohistochemistry

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling **(**TUNEL) staining was performed using the DeadEnd Colorimetric TUNEL System Kit (Promega, San Luis Obispo, CA, USA) on adjacent sections to the H&E stain. TUNEL-positive osteocytes and

total osteocytes were counted manually within 1mm adjacent to osteonecrotic foci or (if osteonecrosis was not present) within a 1 mm along the buccal alveolar bone.

For immunohistochemistry, slides were peroxidase quenched in 0.3% H_2O_2 in methanol, washed 3 times for 5 minutes in PBST (0.05% Tween-20), and then blocked for 30 minutes in 5% rat serum. Primary antibodies were applied overnight at 4°C in PBS; anti-CD31 (1:100), followed by washes in PBST, and then biotinylated secondary antibodies (anti–rat 1:100), streptavidin-HRP (1:250 dilution), and diaminobenzidine (DAB) peroxidase substrate were added.

Inflammation of the diseased side was identified using a standard brightfield microscope, in areas of mucosa overlying the alveolar crest. The high-power (40X) field with the greatest numbers of inflammatory cells was selected, and the numbers of polymorphonuclear cells and lymphocytes were counted manually.

Statistics

Data among groups were analyzed using one-way analysis of variance (ANOVA) and the Student-Newman-Keuls post hoc test. Data between groups were analyzed using the Student Ttest. Data among groups represent at least three independent experiments. Statistical analysis was performed using Student's *t*-test where *p* values less than 0.05 were considered significant.

CHAPTER 3

PERIODONTAL DISEASE AND BISPHOSPHONATES INDUCE OSTEONECROSIS OF THE JAWS IN THE RAT

ABSTRACT

Bisphosphonates (BPs) are commonly used medications to treat primary and metastatic bone cancer, as well as osteoporosis. Though BPs improve bone mineral density, reduce fracture risk, and reduce hypercalcemia of malignancy, some patients may develop osteonecrosis of the jaws. BP related osteonecrosis of the jaws (BRONJ) is a devastating complication, presenting as clinically exposed bone in the maxillofacial region for more than eight weeks. Despite an increasing number of BRONJ cases since first reported, the disease pathophysiology remains largely unknown. Since published studies suggest a significant role for dental disease in the pathophysiology of BRONJ, we developed a BRONJ animal model where aggressive periodontal disease is induced by ligature placement around the crown of the right maxillary first molar in the presence of vehicle or zoledronic acid (ZA), a potent BP. Ligature placement induced significant alveolar bone loss while ZA treatment attenuated this effect. Significant changes of alveolar bone morphology including sequestration and extensive periosteal bone formation were observed by μCT in the ligated site of BP treated animals. These findings were confirmed histologically, demonstrating necrotic bone with diffuse loss of osteocytes and empty lacunae, rimming of the necrotic bone by squamous epithelium and inflammation, and exposure to the

oral cavity. Importantly, the rat lesions were strikingly similar to those of BRONJ patients. Our data suggest that dental disease and potent BP therapy are necessary and sufficient for BRONJ development in the rat.

INTRODUCTION

Bisphosphonate related osteonecrosis of the jaws (BRONJ) is defined clinically as an area of exposed, necrotic bone in the maxilla or mandible of patients receiving bisphosphonates (BPs) that is present for at least eight weeks with or without the presence of pain, infection, or previous trauma [56,59]. The clinical presentation is similar to osteomyelitis (OM) or osteoradionecrosis (ORN) of the jaws, the former caused by an infection in the bone and the latter by high doses of localized radiation therapy [55,97,98]. However, BRONJ patients have no history of radiation therapy, may have a secondary bacterial infection, and classic therapies for OM and ORN are usually ineffective [48,55,99]. Though studies do not identify a direct causal relationship between BP use and osteonecrosis of the jaws (ONJ); [100,101], the only consistent variable for many ONJ cases is BP therapy, most commonly through intravenous (IV) administration. From these data, the primary involvement of BPs in the pathophysiology of ONJ is strongly suggested [48,55,99,102,103]. Overall, the risk of BRONJ development for patients on intravenous BPs is estimated between 1 and 28% [102-108], which increases after longer treatment time and in patients on zoledronic acid (ZA); [99]. BRONJ may even be underreported due to mostly retrospective data collection from chart reviews and lack of routine dental evaluation [106,108]. BRONJ in patients taking oral BPs for osteoporosis is rare, estimated at 0.001-0.1% [56,109,110]. The recent Kaiser Permanente study demonstrates an increased number of BRONJ

cases, possibly due to chronic oral BP use and thorough screening [110]. In the great majority of cases, BRONJ occurs after extraction of teeth deemed unrestorable due to the severity of dental disease, or around teeth with active periodontal or periapical disease [55,59,106,111,112]. Indeed, Marx RE in a series of 152 BRONJ cases reported that in 85 patients (56%) the initiating event for ONJ was either extraction due to periodontitis, extraction due to failing root canal, uncontrolled periodontitis, periodontal surgery or apicoectomy. An additional 25 patients (16.4%) received tooth extraction due to caries. It would be safe to assume that in most of these 25 patients deep caries requiring tooth extraction extended to the pulp causing pulpal necrosis and periapical inflammation [113]. Thus, although not always reported as the initiating factor, inflammation of the periodontal tissues is present in the majority of BRONJ cases. Interestingly, oral preventive measures decrease BRONJ incidence [114,115], further emphasizing the importance of dental disease in BRONJ pathophysiology. To investigate the significance of dental disease in the development of BRONJ, we have developed a clinically relevant animal model. We induced aggressive periodontal inflammation in rats treated with vehicle or ZA, a potent BP. We observed alveolar bone necrosis with bone sequestration and/or periosteal bone reaction. Importantly, the appearance of the osteonecrotic bone in the rat closely resembles BRONJ in patients.

RESULTS

Common methods to induce experimental loss of periodontal bone include lipopolysaccharide (LPS) injection in the interproximal gingiva of the maxillary molar teeth or ligature placement around the maxillary first molar crown [30,116]. LPS injections create chronic

inflammation with inflammatory cell infiltrate, increased inflammatory cytokines, and eventually loss of alveolar bone [116]. Alternatively, ligature placement leads to plaque accumulation and periodontal inflammation [116,117]. The experimental periodontal ligature model creates more aggressive bone loss compared to LPS injections [116]. For all our studies described herein, we adapted the well characterized ligature model by placing a sterile wire ligature around the crown of the right first maxillary molar as shown in Fig. 1A.

Animals were treated for three weeks with IP injections of vehicle or ZA, then the sterile ligature was placed and animals continued to receive vehicle or ZA injections for 12 more weeks. Weight of animals was measured three times per week. At the end of the experiment, animals were euthanized, blood was collected and serum biomarkers including Na, K, Cl, CO2, blood urea nitrogen (BUN), creatinine, Ca, Mg, Phosphorus, Albumin and Alkaline Phosphatase were measured. No significant differences in weight or any of the serum markers between vehicle and ZA treated animals was detected (data not shown).

To ensure that ligature placement was effective in inducing experimental periodontal, dissected maxillae were imaged by μCT as described earlier. The distance from the cementoenamel junction (CEJ) to the alveolar crest (AC) was measured at the distal-buccal root of the first maxillary molar (D1) and the mesial-buccal root of the second maxillary molar (M2) in both the ligature and non-ligature sites (right and left respectively; Fig. 1B). Increase in the CEJ-AC distance represents bone loss. The distance in the ligature site (experimental) was expressed as % of the same distance in the non-ligature site (control) and the % increase in the ligature site was measured. Ligature placement caused a significant increase in the CEJ-AC distance in both vehicle and ZA treated animals (Fig. 1C). Interestingly though, vehicle treated

animals experienced augmented periodontal bone loss, as reflected by the significantly increased CEJ-AC distance in these compared to the ZA treated animals (Fig. 1C).

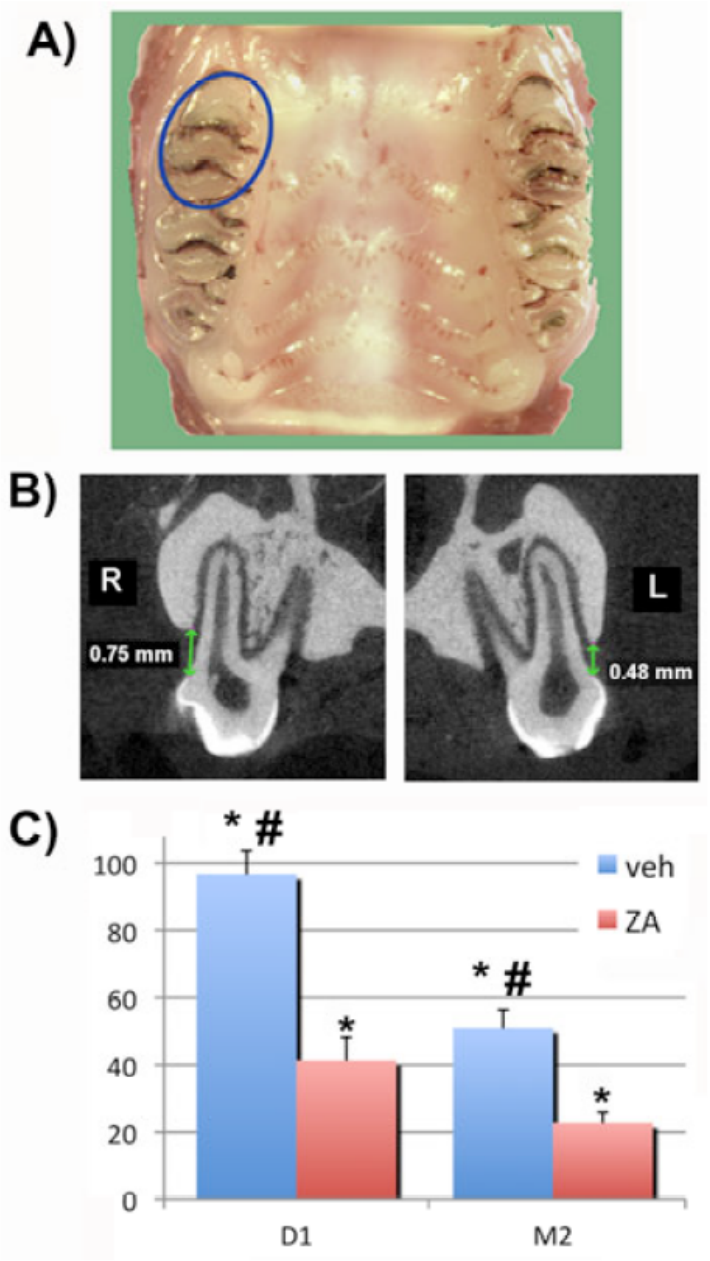


Figure 1. *In Vivo* Periodontal disease model.
A) Diagram of in vivo periodontal disease model. A sterile ligature was placed subgingivally around the maxillary first molar unilaterally (blue circle) to induce periodontal disease in vehicle

or ZA treated animals. B) To quantify ligature-induced alveolar bone loss, the distance between the cementoenamel junction (CEJ) and alveolar crest (AC) in ligated (R) and non-ligated (L) site was measured. Longer distance denotes bone loss. The CEJ-AC distance in the ligated site was expressed as % of the same distance in the non-ligated site. C) The % increase in CEJ-AC distance at the distal-buccal root of the first molar (D1) and the mesial-buccal root of the second molar (M2) were determined. * statistically significantly different from the non-ligated site, # statistically significantly greater than ZA treated animals, $p<0.01$.

Then multiplanar and 3D reformatted μCT images from vehicle and ZA treated animals were reviewed for evaluation of radiographic findings indicative of osteonecrosis. Representative 3D reformatted images of non-ligated and ligated maxillae from vehicle or ZA treated animals are depicted in Fig. 2. Maxillae in the non-ligated site from both vehicle (Fig. 2A) or ZA treated animals (not shown) demonstrate a normal alveolar crest that extends just inferior of the CEJ, covering most of the root, as well as the root furcation of the first and second molars (purple arrow; Fig. 2A). In the ligated site of vehicle treated animals, loss of alveolar crest height with root exposure and visualization of the furcation areas characteristic of periodontal bone destruction was seen (yellow arrow; Fig. 2B). The ligated site of nine ZA treated animals presented a different radiographic appearance of the alveolar bone. Some animals demonstrated bony fragments with irregular borders, separated from the remaining alveolar ridge (orange arrow; Fig. 2C). These fragments had the characteristic appearance of a sequestrum (a necrotic piece of bone), very common in BRONJ patients [109,118]. In other ZA treated animals, substantial expansion of the buccal thickness of the alveolar ridge, probably due to extensive periosteal new bone formation was observed (green arrow; Fig. 2D). Periosteal bone formation and ridge expansion are common findings in BRONJ patients [109,118].

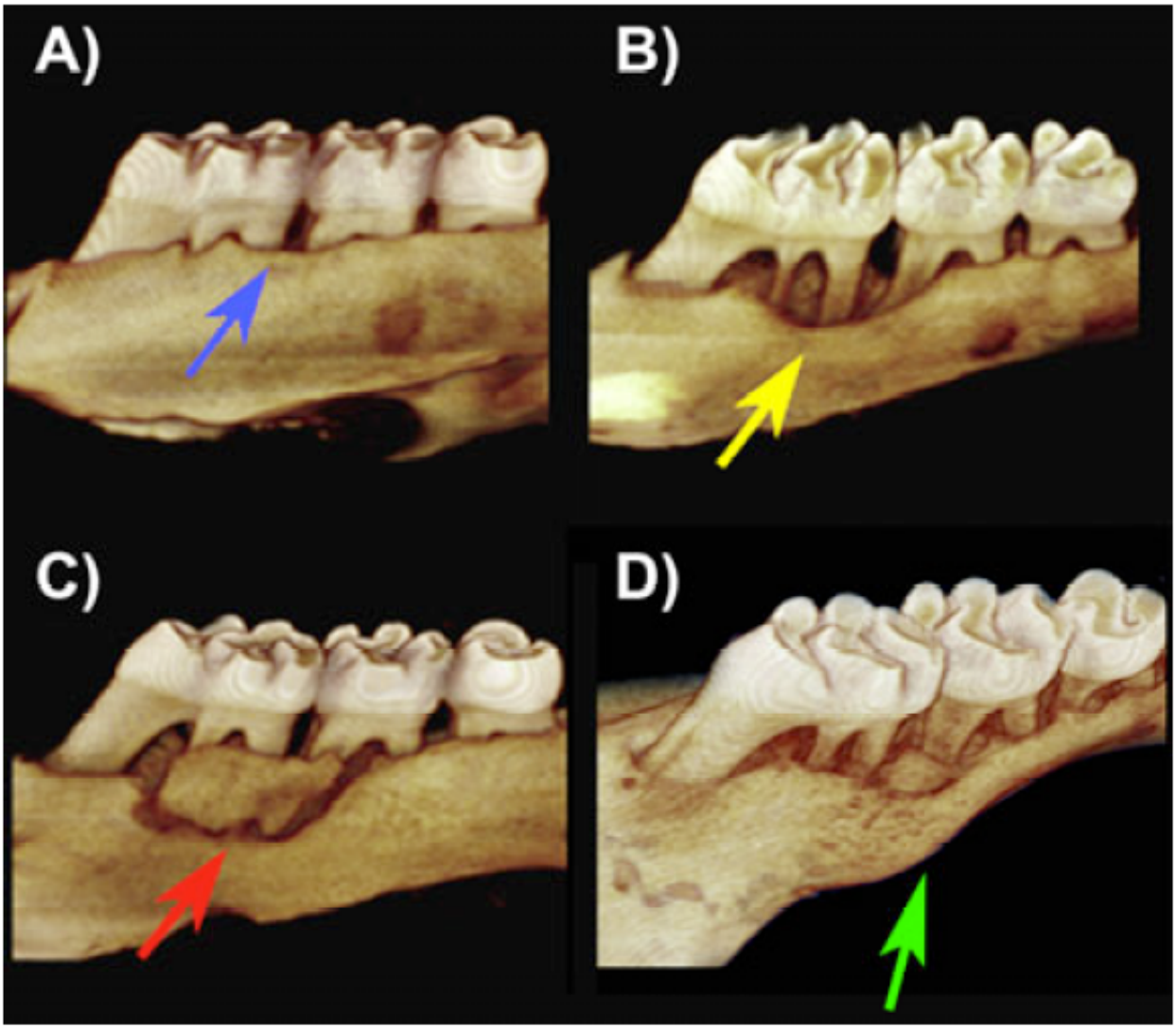


Figure 2. 3D μCT reconstructed images of the rat maxilla.
A) Unligated site in vehicle treated rat. B) Ligature induced periodontal disease in vehicle treated rat. C, D) Ligature induced periodontal disease in ZA treated rat. Blue arrow in A) points to normal alveolar bone in the interproximal area of the distal of the first molar and mesial of the second molar. The yellow arrow (B) points to periodontal bone loss in vehicle treated animals at the area of the ligature. In ZA treated animals, red arrow points to a sequestrum formation (C) and green arrow points to extensive periosteal new bone formation (D) at the area of the ligature.

Since the alveolar bone in the periodontal disease site of ZA treated animals exhibited BRONJ radiographic features, we contrasted the radiographic appearance of the alveolar ridge

from the animals to that of BRONJ lesions from patients that have been treated at the UCLA School of Dentistry clinic. Fig. 3 depicts μCT multiplanar views of rat from Fig. 2C and corresponding multiplanar CT views from a patient. Both rat and patient images demonstrate the appearance of an irregular radiopacity surrounded by an erosive radiolucent zone, consistent with a bony sequestrum that is discontinuous from the remaining alveolar bone as seen in sagittal, coronal, and axial slices (Fig. 3, arrows).

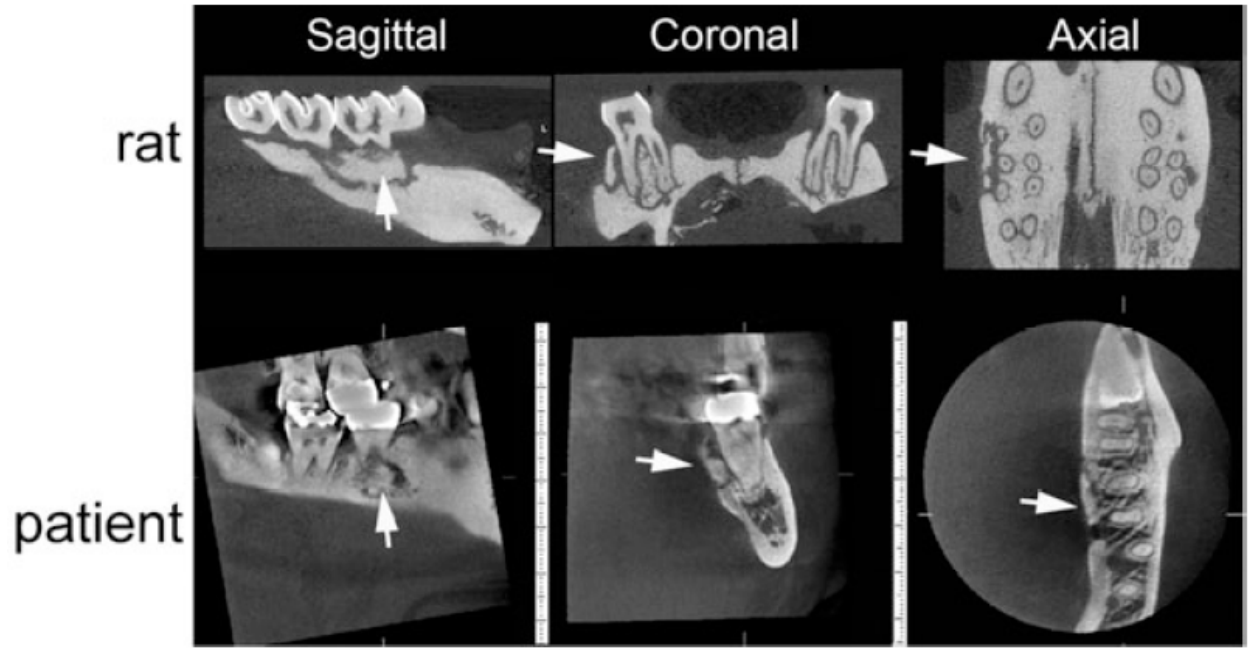


Figure 3. Similarity of rat and human radiographic findings in BRONJ.
Sagittal, coronal, and axial CT slices from a rat and a patient with BRONJ demonstrate a bony sequestrum in the alveolar bone (arrows).

Fig. 4 depicts μCT multiplanar views of rat from Fig. 2D and corresponding multiplanar CT views from another BRONJ patient. Both rat and patient images demonstrate the appearance of substantial new bone formation at the periphery seen in sagittal, coronal, and axial slices (Fig.

4, arrows) that causes significant expansion of the alveolar ridge (compare affected with unaffected sides Fig. 4 coronal and axial slices for the rat).

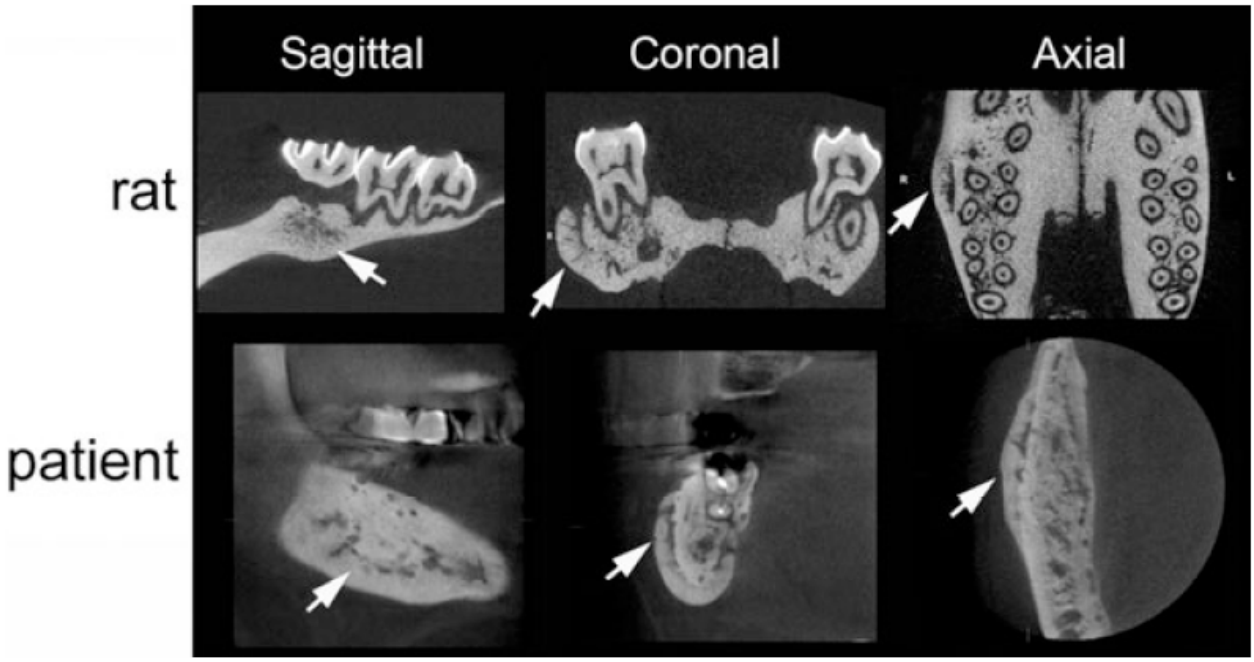


Figure 4. Similarity of rat and human radiographic findings in BRONJ.
Sagittal, coronal, and axial CT slices from a rat and a patient with BRONJ demonstrate significant periosteal bone reaction and expansion of the alveolar ridge dimensions (arrows).

Because extensive periosteal bone formation was observed in the ligated site of several ZA treated animals, we quantified the buccal bone thickness in the ligated versus non-ligated site in vehicle versus ZA treated animals (Fig. 5). The buccal width of the alveolar bone was measured at the mesial and distal buccal roots of the first (M1 and D1) and second (M2 and D2) maxillary molars (Fig. 5A). An increase in buccal width of the ligated site was observed in vehicle treated animals and involved D1, M2 and D2 (Fig. 5B). Importantly, ZA treated animals demonstrated a significantly higher increase of the buccal width in ligated versus non-ligated site

compared to vehicle treated animals that extended to a larger area involving M1, D1, M2 and D2 (Fig. 5B).

To investigate whether the increased alveolar bone width in ZA treated animals is relevant for BRONJ patients, we measured the cortical thickness of the alveolar bone at BRONJ lesions and the same alveolar bone area of the non-involved site on multiplanar CBCT images of patients treated at the UCLA School of Dentistry clinic (Fig. 5C). A two fold statistically significantly increased alveolar cortical thickness was observed at the BRONJ site compared to the non- BRONJ site (Fig. 5D).

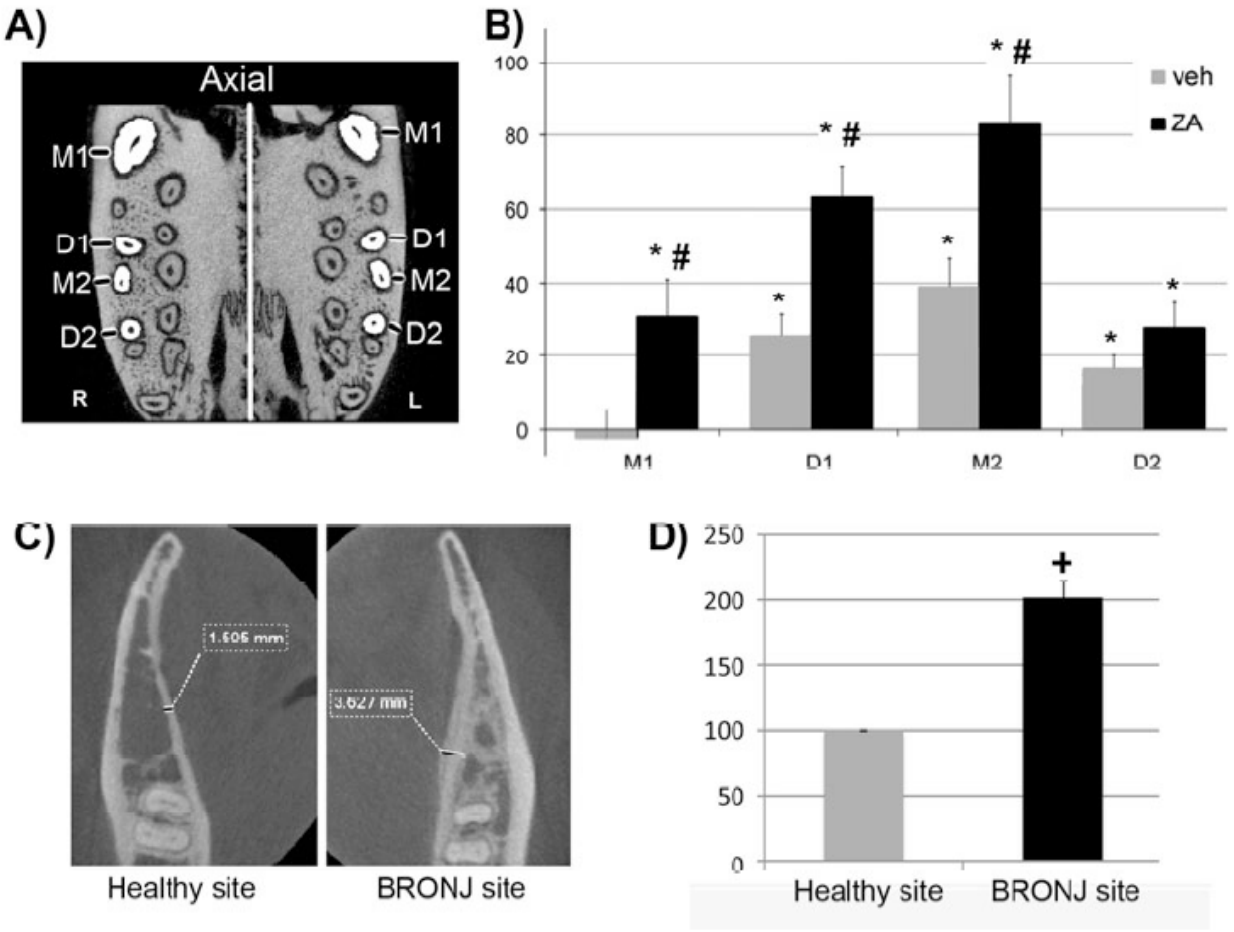


Figure 5. Comparison of periosteal bone formation in BRONJ and healthy sites in the rat and patient.
To quantify buccal bone thickness in vehicle versus ZA treated rats, the buccal width of the alveolar bone was measured at the mesial-buccal and distal-buccal roots of the 1st (M1 and D1) and second (M2 and D2) molars in the ligated and non-ligated sites on axial μCT slices (A). The measurements on the periodontal site were calculated as % of the same measurements on the healthy site and the % increase was determined. (B). To evaluate bone width in BRONJ involved versus noninvolved sites, cortical bone thickness was measured at the BRONJ site and the same alveolar bone area of the non-involved site on CBCT images of patients (C). Cortical thickness was expressed as % thickness of the non-involved site (D). (* statistically significantly different from the non-periodontal site, $p<0.01$, # statistically significantly different from the vehicle treated group, $p<0.01$, + statistically significantly different from the non-involved site, $p<0.01$).

To investigate the histologic appearance of the alveolar bone and periodontal ligament at the non-ligated and ligated sites of vehicle versus. ZA treated animals, sections from the D1 to the M2 roots were examined following decalcification and H&E staining (Fig. 6). Sections from non-ligated site of vehicle treated animals (Fig. 6A, A1) or ZA treated animals (not shown) showed viable lamellar bone surrounding nearly the entire tooth root length (black arrow). Sections from ligated site of vehicle treated maxillae (Fig. 6B, B1) demonstrated inflammation (green arrow) and marked bone loss/resorption; viable lamellar bone surrounded the lower 25% of the root (black arrow). In sections from the ligated site of ZA treated animals (Fig. 6C, C1) necrotic lamellar bone (yellow arrows), characterized by diffuse loss of osteocytes with confluent areas of empty lacunae (inset C1, D1) was observed. In Fig. 6C and C1, the mucosal surface of this necrotic bone is not covered by epithelium and is exposed to the oral cavity (red arrow); deeper portions of the sequestrum are rimmed by squamous epithelium (blue arrow). In Fig. 6D and D1, substantial periosteal bone formation (double white arrow) is present adjacent to necrotic bone; unlike Fig. 6C, the necrotic bone is not sequestered but is continuous with the remaining viable bone. Inflammation continues to be seen in periodontal with BP treatment

(green arrows Fig. 6C, C1, D, D1). This histologic appearance of necrotic bone with exposure to the oral cavity confirms osteonecrosis of the maxilla in ZA treated rats with experimental periodontal.

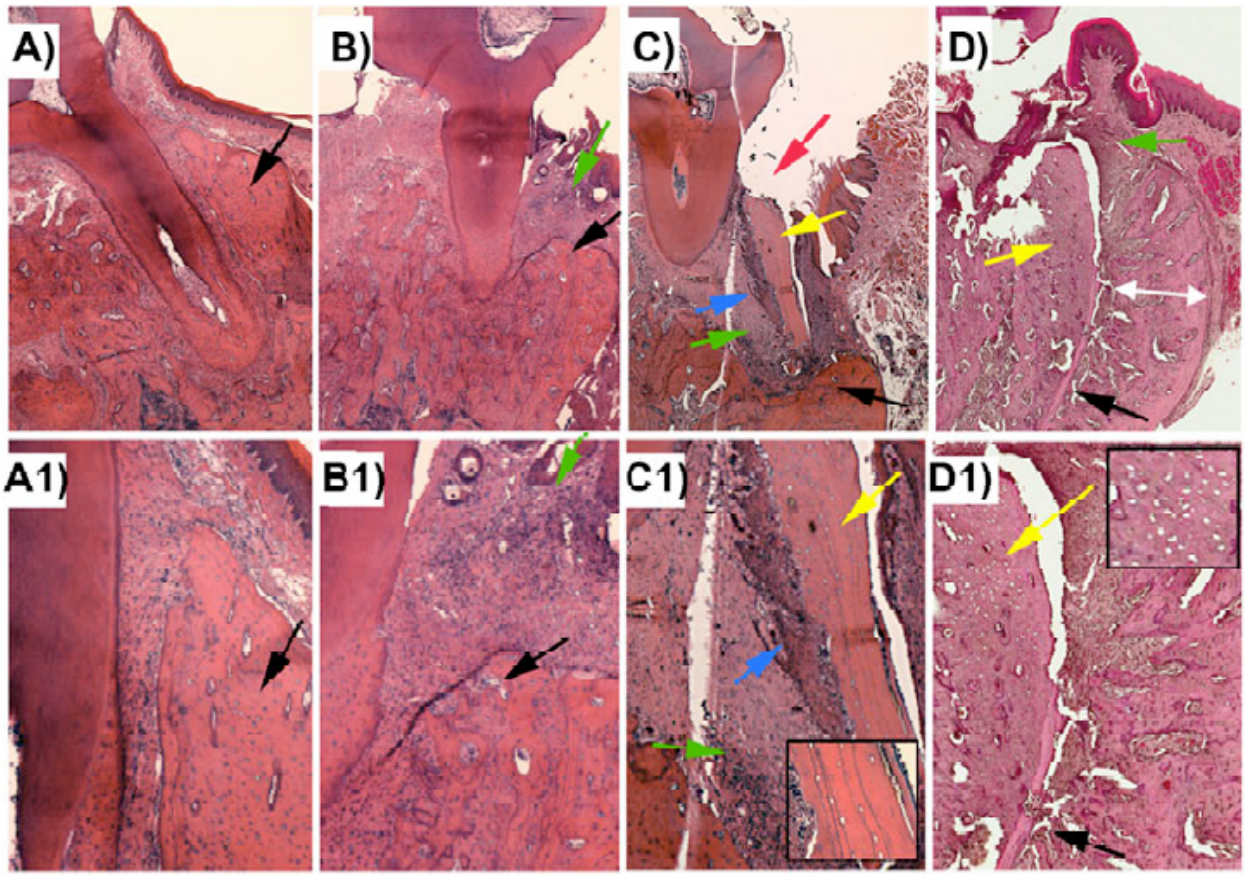


Figure 6. Histologic examination of experimentally induced BRONJ.
A, A1) Unligated site in vehicle treated animal. B, B1) Ligature induced periodontal disease in untreated rat. C, C1, D, D1) Ligature induced periodontal disease in ZA treated rat. A, B, C, D are at 10X magnification, while A1, B1, C1, and D1 demonstrate a magnified area of A, B, C, and D. Necrotic bone (yellow arrows) is seen in the ZA treated periodontal site. 40X (box) of C1 and D1 show extensive osteocyte loss with confluent empty lacunae.

To evaluate the histologic fidelity of our rat model, we compared the appearance of the BP treated rat with periodontal to a histologic specimen from a BRONJ patient (Fig. 7). Similar

to our rat model (Fig 7A, C), sections from a human BRONJ patient (Fig. 7B, D) demonstrated osteonecrosis of lamellar bone with empty lacunae (yellow arrows), rimming of necrotic bone by squamous epithelium (blue arrows) and inflammation (green arrows).

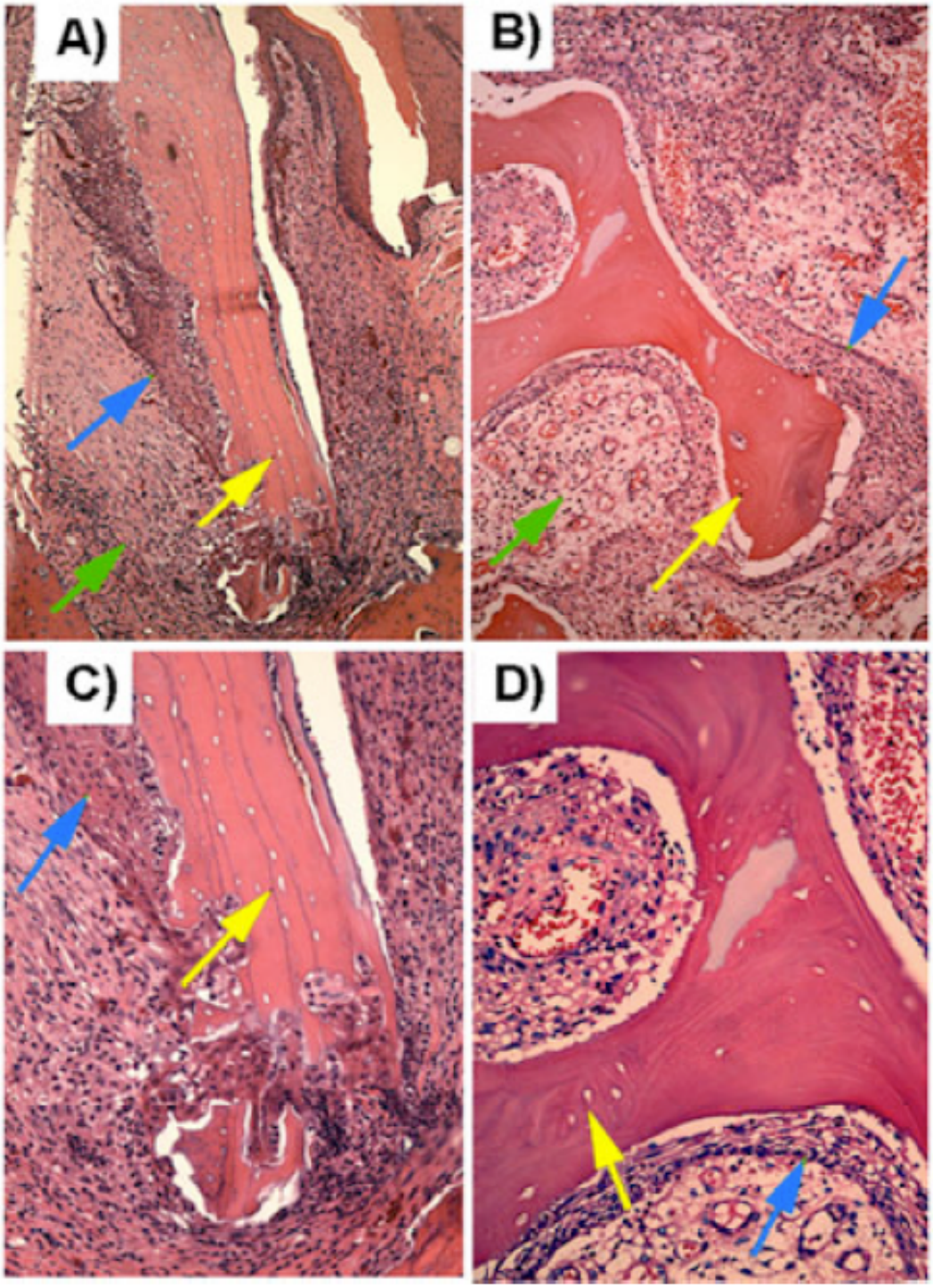
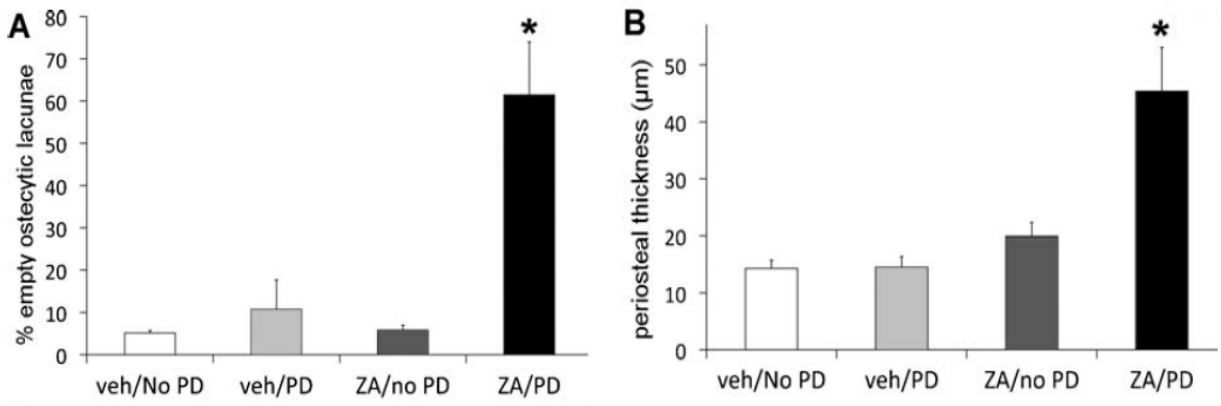


Figure 7. Rat and human histologic findings in BRONJ.
Sections through the jaw of rat (A-20X, C-40X) and patient (B-20X, D-40X) show osteonecrosis (yellow arrows) with squamous epithelium rimming necrotic bone (blue arrows) and

inflammation (green arrows).

Because of the μCT-observed increased periosteal bone formation (Fig. 5B), we measured the thickness of the periosteum at the buccal aspect of the alveolar ridge (Fig. 8B). The ligated site of ZA-treated animals showed a significantly increased periosteal thickness, whereas no differences were observed in the other group. Gingival inflammation induced by the ligature was similar in vehicle- and ZA-treated animals, with increased numbers of polymorphonuclear neutrophils (PMNs) that were statistically significantly different compared with the non-ligated site but not different between vehicle- and ZA-treated animals (Fig. 8C). Ligature also increased the number of lymphocytes in both vehicle- and ZA-treated animals (Fig. 8D). Although this did not reach statistical significance ($p \leq 0.0557$), lymphocyte increase was higher in ZA-treated animals.



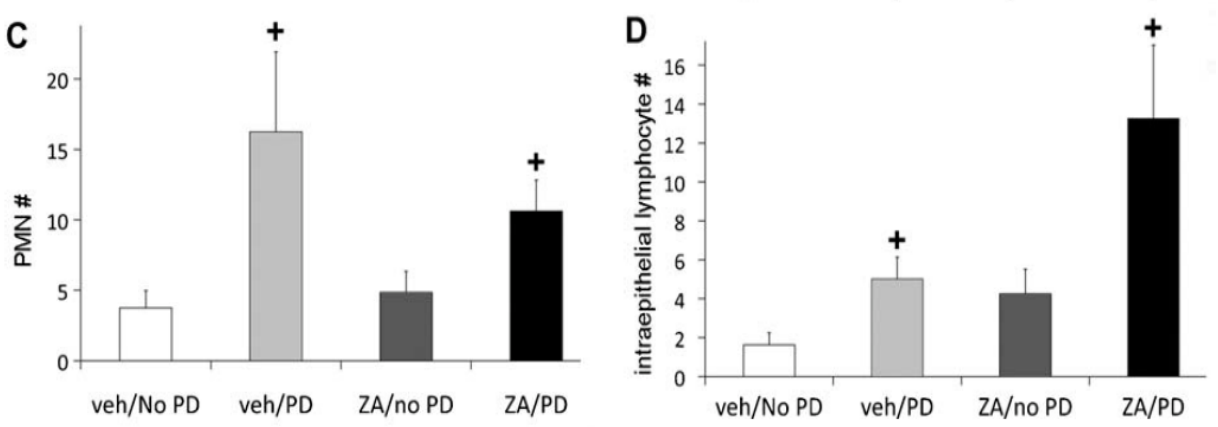


Figure 8. Quantification and characterization of osteonecrosis and inflammation.
(A) Number of osteocytic lacunae at the buccal alveolus was measured, and empty lacunae were expressed as a percent of total. (B) Thickness of periosteum at the buccal alveolus was determined. (C) Number of polymorphonuclear neutrophils and (D) intraepithelial lymphocytes was quantified. Statistically significantly greater than the non-ligated site of ZA-treated animals and the non-ligated or ligated site of vehicle-treated animals, $p<.01$. Statistically significantly greater than the non-ligated site, $p<.05$.

Finally, TUNEL assays were performed to detect evidence of apoptotic cell death (Fig. 9). No TUNEL positive cells were observed in sections from non-ligated or ligated site of vehicle treated animals (Fig. 9A, B) or from non-ligated site of ZA treated animals (Fig. 9C). However, several TUNEL positive osteocytes were seen in lamellar bone adjacent to areas of histological obvious typical osteonecrosis in the ligated site of ZA treated animals (Fig. 9D). Table 1 summarizes the radiographic and histologic findings of the 19 vehicle and 19 ZA treated animals that were included in our studies. Radio graphically, six (32%) ZA and one (5%) vehicle treated animals presented irregular bone fragments detached from the surrounding alveolus, characteristic of sequestrum formation. Additionally, six (32%) ZA treated animals displayed a greater than 2 fold compared to the non-ligated site, increase in buccal cortical width, suggestive of substantial periosteal bone formation. No vehicle treated animal demonstrated

such dramatic increase in buccal alveolar bone thickness. Histologically, four (21%) ZA treated animals showed bone not covered by epithelium indicating clinical bone exposure. Finally, nine (47%) ZA and one (5%) vehicle treated animal revealed clear histologic presentation of bone necrosis with extensive empty osteocytic lacunae.

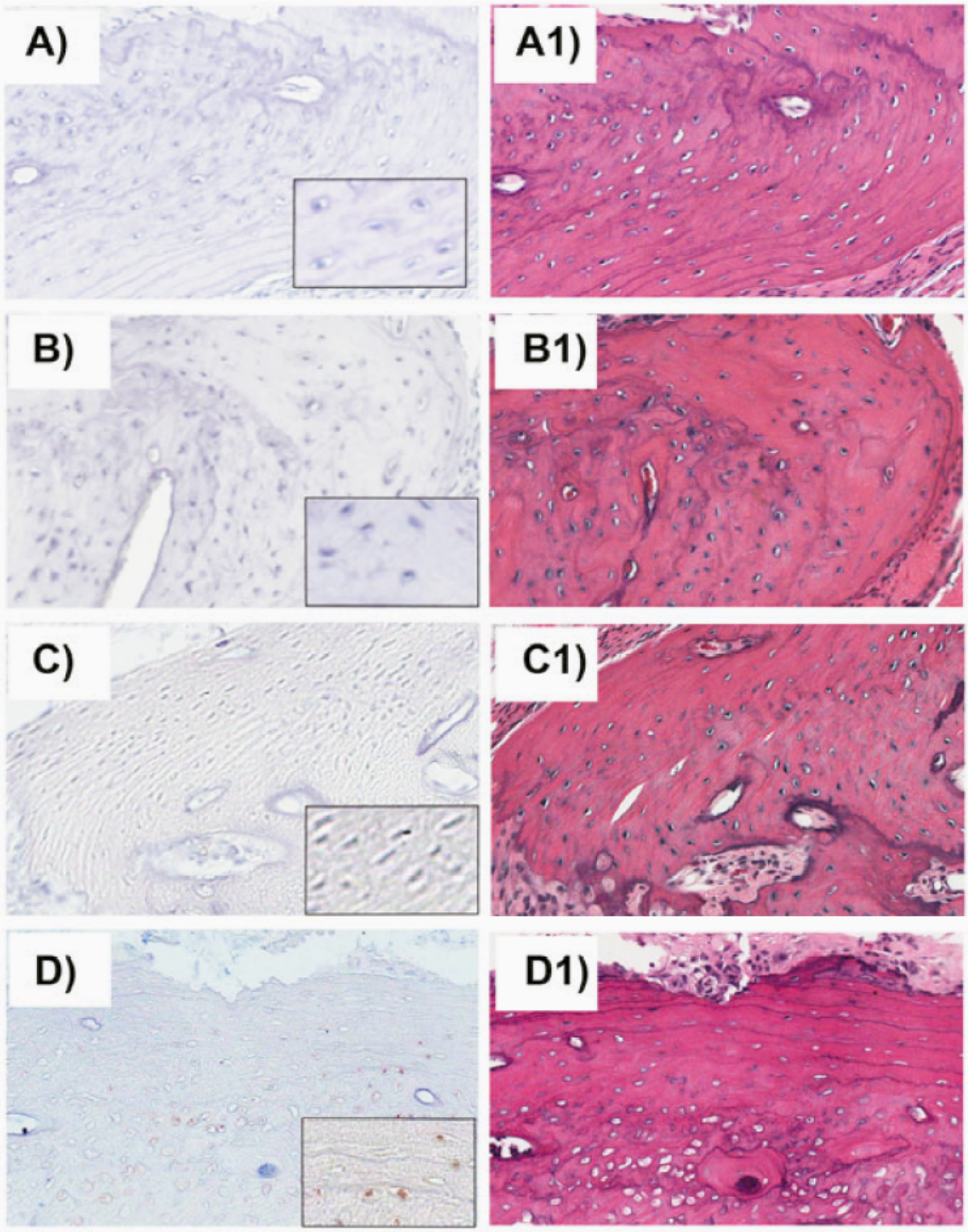

A)
A1)
B)
B1)
C)
C1)
D)
D1)

Figure 9. TUNEL staining on osteocytes in necrotic area.
20X TUNEL (A, B, C, D) and H&E (A1, B1, C1, D1) stained adjacent sections from non-ligated (A, A1) and ligated (B, B1) site of a vehicle treated animal, or a non-ligated (C, C1) and ligated (D, D1) site of a ZA treated animal. Several TUNEL positive osteocytes are present in D close to osteonecrotic area (D1). No apoptotic figures are seen in (A, B, C). 40X insets show TUNEL – and + cells in detail.

DISCUSSION

Clinically, BPs have advanced therapy for malignant diseases such as multiple myeloma, and breast and prostate cancer by decreasing life-threatening skeletal-related complications, including hypercalcemia of malignancy, pathologic fractures, spinal cord compression, and severe bone pain [119,120]. Long-term studies suggest that potent BPs like ZA decrease tumor burden in animals [121], and improve quality of life and survival in certain patient groups [122]. BPs are also very effective in treating osteoporosis and Paget's disease, by increasing bone mineral density and decreasing fracture risk, and inhibiting bone resorption, respectively [101,123,124]. Despite the great clinical benefits in cancer and osteoporotic patient management, BP use has been recently associated with BRONJ [59,106].

To date, a major unanswered question is the occurrence of BP related osteonecrosis only in the jaws, sparing the long bones and axial skeleton. Indeed, the orofacial complex is a complicated system composed of teeth, oral mucosa, periodontal tissues, muscles, tongue, salivary glands, and alveolar bone. These tissues interact to perform unique functions that range from mastication and speech to swallowing and taste. The maxilla and mandible are the only bones covered by mucosa in close proximity to the external environment [125], where bacterial infections, such as caries and periodontal disease, commonly occur [126,127]. Hypotheses that

attempt to explain the specificity of BRONJ include altered bone remodeling, angiogenesis inhibition, constant microtrauma, and bacterial infection [55,128]. However, thus far the specific parameters that contribute to the increased jaw sensitivity to BPs remain unidentified. In vitro studies have provided conflicting findings on the direct BP effects on osteoblast function, differentiation, and apoptosis. In various osteoblastic cell types, BPs decrease or increase alkaline phosphatase (ALP) activity [129,130], decrease or increase proliferation and differentiation [130-133], and decrease or increase apoptosis [131,133]. Additionally, BP type [132] or concentration differentially regulate in vitro osteoblast function [130]. Overall, the BP effect on osteoblasts is difficult to characterize. Furthermore, most in vitro studies do not address any specific BP effects on alveolar bone cells.

A recent in vivo study in beagle dogs that received three years of alendronate demonstrated matrix necrosis of alveolar bone, where the necrotic areas were void of patent canaliculi. Additionally, dynamic histomorphometry revealed significant decrease of intracortical bone turnover [71]. Similar results in the same animal model were observed with other BPs at different time points [134]. Interestingly, these BP effects occurred in the absence of jaw trauma or inflammation. Thus, in vitro and in vivo studies of BP regulated osteoblast, and specifically craniofacial bone cell function, begin to elucidate effects on alveolar bone homeostasis. The absence of animal models to evaluate parameters that affect BRONJ occurrence, progression and severity has been a major obstacle in understanding BRONJ pathophysiology. Recently, Sonis et al. demonstrated delayed wound healing with mucosal ulceration and bony sequestration after maxillary tooth extraction in rats that received ZA and dexamethasone combination therapy [135].

Most BRONJ lesions appear after extraction of teeth that cannot be restored due to advanced dental disease, or around teeth with periodontal or periapical infection [55,59,106,111,112]. Furthermore, aggressive dental hygiene reduces the incidence of BRONJ in multiple myeloma [114] and metastatic cancer [115] patients. This apparent close association of active dental disease with BRONJ in patients prompted us to explore the necessary and sufficient requirement of aggressive periodontal and BP treatment in BRONJ development in a rat animal model. In this model, aggressive periodontal was induced with a sterile ligature around the maxillary first molar. Consistent with previous studies [117], significant alveolar bone loss occurred in the vehicle treated animals, where the ligature was utilized. Also as expected, ZA treatment attenuated ligature-induced alveolar bone loss [136].

Importantly, μCT analysis revealed significant changes of alveolar bone morphology in ZA treated animals in the presence of periodontal. Sequestration or extensive periosteal bone formation, classic features of BRONJ in patients [109,118], were present in 47% of the periodontal affected ZA treated maxillas. Specifically, cortical bone thickness at the periodontal site of ZA treated animals was significantly increased over the periodontal site of vehicle treated rats. This increase paralleled BRONJ patient findings that demonstrated significant increase in periosteal bone formation in the area of BRONJ as compared to the contralateral, unaffected site.

Histologic examination confirmed the presence of necrotic bone with diffuse loss of osteocytes and empty lacunae, diagnostic of osteonecrosis and the hallmark of BRONJ histopathology in humans. Apoptotic osteocytes were observed in the proximity of empty lacunae indicating expansion of the osteonecrotic areas. Histologically, the similarities between rat and patient were striking, both demonstrating necrotic bone with empty lacunae, rimmed by

squamous epithelium and inflammation. These findings were observed in 9 out of 19 BP-treated animals with the induction of periodontal (47%). Interestingly, one of 19 vehicle treated animals (5.2%) developed an osteonecrotic lesion in the area of experimental periodontal. Although this finding was unexpected, necrotic bone exposure has been reported in the jaws of patients not receiving BPs [137-139]. BRONJ is a clinically defined disease described as exposed bone for duration of more than eight weeks. However, we observed bone exposure only in 4 (21%) of the ZA treated animals, while we observed radiographic and histologic osteonecrosis in 47% of the same animals. This would suggest that bone necrosis precedes mucosal retraction and bone exposure. Indeed, sequestrum formation in patients receiving BPs without signs of clinically exposed bone have been reported [140].

Based on our findings and other published data, we propose a model of BRONJ pathophysiology (Fig. 10), where the combination of robust alveolar bone resorption by BPs and the presence of aggressive dental disease provide a unique environment for BRONJ development. Fig. 10A portrays a healthy periodontium. The alveolar bone demonstrates a uniform, well-defined cortical outline and normal trabecular pattern. Dental disease such as periodontal (Fig. 10B) creates an inflammatory environment (gray shadowed area) inducing alveolar bone resorption (note the crestal bone loss between A and B). Bone loss in the presence of inflammation could be essential to remove bone from the area and to allow mounting of an effective immune response. In the absence of dental disease, BPs (Fig. 10C) attenuate osteoclastic activity and increase alveolar bone density. Periodontal in the presence of BPs (Fig. 10D) similarly creates periodontal tissue inflammation (depicted by the gray shadowed area). However, osteoclastic activity is greatly diminished. As a result, alveolar bone cannot be

resorbed away from the inflammatory nidus but is exposed to an environment rich in bacterial toxins, inflammatory cytokines or oxidative stress. Such an environment is highly toxic to bone cells and results in osteonecrosis. Thus, BRONJ develops.

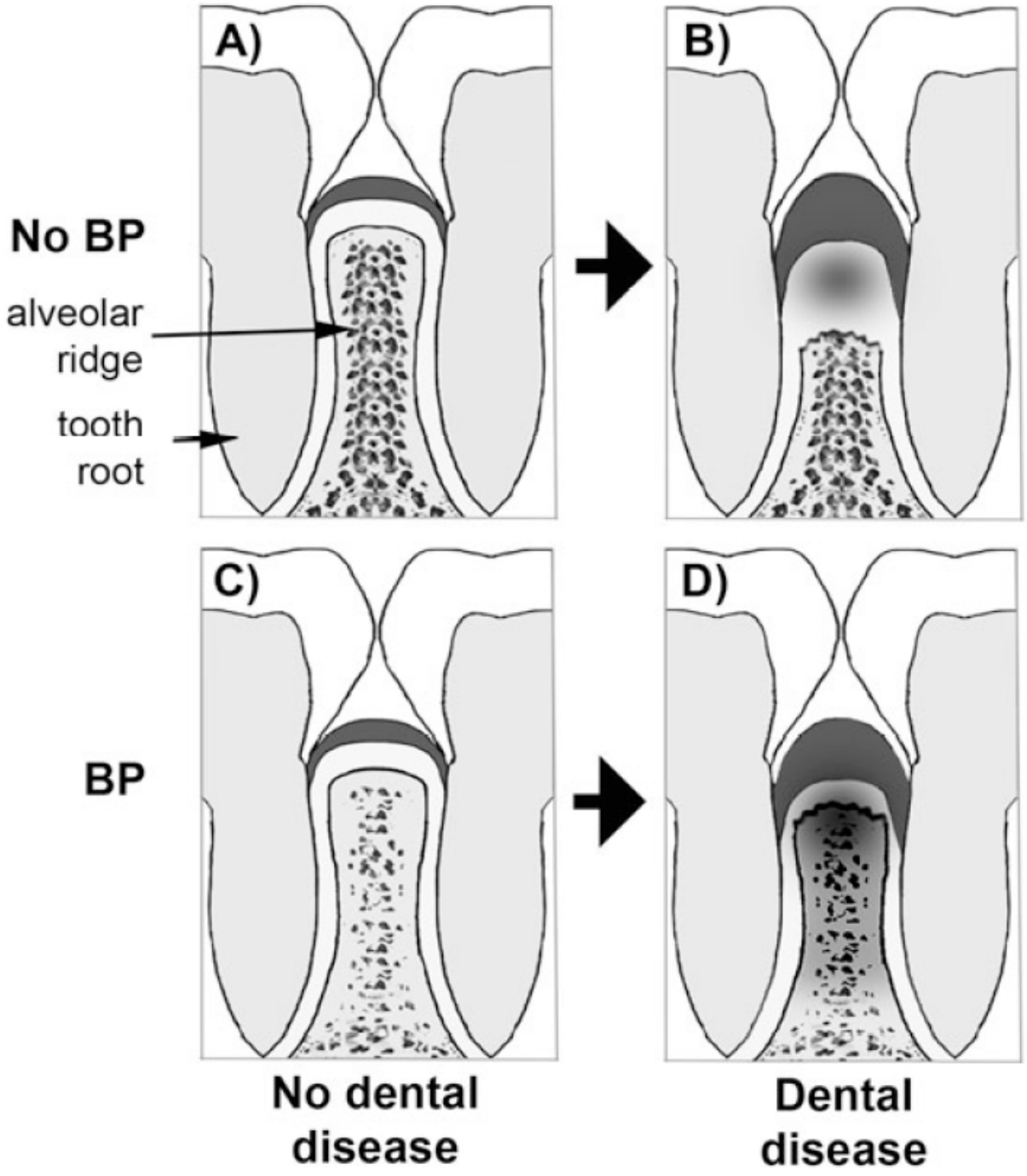


Figure 10. Model of BRONJ pathophysiology.
Response of alveolar bone to health (A and C) or dental disease (B and D) in the absence (A and B) or presence (C and D) of BP treatment. BRONJ, depicted by the darker color in bone D, requires the presence of both BP and dental disease.

Two conclusions can be drawn from this model. First, periodontal tissue inflammation would increase local bone turnover and thus is expected to increase incorporation of BPs in the inflammatory area. In fact, increased uptake of technetium-99m labeled diphosphonate is observed in the presence of dental disease [119]. Increase BP uptake in areas of inflammation might reinforce the observed high correlation of dental disease with BRONJ lesions [55,59,106,111,112]. Second, this model emphasizes bone resorption as crucial in BRONJ development. This would predict that other strong osteoclast inhibitors could, similar to BPs, predispose patients to ONJ. Indeed, we and others have reported ONJ in patients receiving Denosumab, an anti-RANKL monoclonal antibody [141]. The incidence of ONJ development between ZA and Denosumab appears to be comparable [69].

In conclusion, we have created in the rat a condition that closely mimics BRONJ in patients. Although BRONJ pathophysiology is complex and many factors could influence its prevalence, duration and severity, our data suggest that dental disease and inhibition of bone resorption by a potent BP are necessary and sufficient for BRONJ development.

TABLE 1. Radiographic and histologic findings in vehicle vs. ZA treated rats.

Treatment	Number of animals	Sequestrum formation	Exuberant (>200%) periosteal bone formation	Presence of exposed bone	Histologic Osteonecrosis
Veh	19	1 (5%)	0 (0%)	0 (0%)	1 (5%)
ZA	19	6 (32%)	6 (32%)	4 (21%)	9 (47%)

CHAPTER 4

PERIAPICAL DISEASE AND BISPHOSPHONATES INDUCE OSTEONECROSIS OF THE JAW IN THE MOUSE

ABSTRACT

Bisphosphonates (BPs) have been used clinically for over 30 years to treat postmenopausal osteoporosis, hypercalcemia of malignancy and metabolic bone diseases. Although they have been beneficial for managing these pathologies, several recent reports have linked BPs to osteonecrosis of the jaw (ONJ). A common factor amongst these case reports suggests a significant link of dental disease and BP treatment in the development of bisphosphonate related ONJ (BRONJ). Since the pathophysiology of BRONJ is unknown, we have developed a mouse model that replicates features of the disease. C56BL/6 male mice were injected with 200 μg/kg zoledronic acid (ZA) three times per week for 8 weeks. Following one-week of ZA pretreatment, pulpal exposure in the right first and second mandibular molars was performed to induce periapical disease. After 8 weeks, mouse mandibles were analyzed qualitatively and quantitatively using μCT, histology and tartrate-resistant acidic phosphatase (TRAP) staining. Periapical lesion were significant larger in vehicle- versus ZA-treated mice. Significant qualitative differences were seen in the morphology of the alveolar bone in ZA-treated mice with Periapical disease, including periosteal bone formation, eroded cortical outlines and irregular trabeculation. Periosteal bone formation was significantly increased in

ZA- versus vehicle-treated animals. Extensive necrotic bone areas, characterized by empty osteocytic lacunae were observed histologically in 100% of ZA- and in none of the vehicle-treated mice. TRAP staining revealed no significant difference in the number of osteoclasts between ZA- versus vehicle-treated animals. However, significant morphologic differences were observed in the ZA-treated group including detached, hyperchromatic osteoclasts with flattened ruffled border. Our data suggest that periapical disease and BP therapy cause BRONJ like lesions in the mouse. This animal model should prove useful in studying BRONJ pathophysiological mechanisms in future studies.

INTRODUCTION

Bisphosphonates (BPs) are stable analogs of natural occurring pyrophosphates and are potent inhibitors of osteoclastic-mediated bone resorption that have been used for over 30 years. BPs have been used to treat postmenopausal osteoporosis, Paget's disease, hypercalcemia of malignancy and other metabolic bone disease [8-11]. Due to their structural 'P-C-P' backbone, BPs rapidly localize to hydroxyapatite *in vivo* and are ingested by osteoclasts, altering the resorptive function and survival of these cells [17,24,27]. Zoledronate (ZA) and alendronate are examples of more potent nitrogen-containing BPs that are widely used in the management of cancer and skeletal disorders [17,18]. Mechanistically, BPs affect the mevalonate pathway by inhibiting farnesyl diphosphate synthetase, an enzyme important in the synthesis of farnesyl pyrophosphate for cholesterol biosynthesis [30]. This prevents the prenylation of small GTPase-signaling proteins such as Rho and Ras, which are important for osteoclast regulation of the cytoskeleton,

intracellular vesicular transport and cell survival [31].

Although the use of BPs is beneficial for cancer and skeletal diseases treatments, several investigators have reported significant side effects from their long-term usage. In 2003, a group of investigators reported a possible association between patients treated with BPs and an atypical bone disorder termed osteonecrosis of the jaw (BRONJ) [53,54]. Several clinical reports followed associating BRONJ with the use of BPs [48,53,55]. In these reports, BRONJ was most frequently observed after dental interventions such as tooth extraction, periodontal disease and in patients receiving corticosteroid treatment [50,53,55]. In 2007, the task force on BRONJ by the American Society for Bone and Mineral Research clinically defined the disease as "an area of exposed bone in the maxillofacial region that does not heal within 8 weeks after identification by a health care provider, in a patient who was receiving or had been exposed to a bisphosphonate and had not had radiation therapy to the craniofacial region" [56].

Periapical disease is one of the most common inflammatory dental diseases in humans. Periapical lesions of the mandible have been shown to cause bacterial infections of the dental pulp that result in soft-tissue destruction and periapical bone resorption [142-147]. In 2005, Sarathy *et al.,* reported a case study of two individuals that developed osteonecrosis of the jaws both in the maxilla and mandible [148]. Based on other clinical reports and previous animal models, aggressive dental disease or surgical trauma in combination with BP treatment has been shown to be factors that contribute to BRONJ development. It should be also noted that these animal models showed BRONJ development either in the maxilla or mandible. However, clinically, it has been reported that osteonecrosis has been observed more frequently in the mandible than the maxilla [50].

Therefore, to establish a clinical relevant BRONJ model, we induced periapical disease in mice treated with ZA and observed alveolar bone necrosis with bone sequestration and periosteal bone reaction.

RESULTS

To evaluate the qualitative differences in the mandibular alveolar bone caused by periapical lesion induction in vehicle-versus ZA-treated mice, representative μCT and radiographic images were evaluated. Multiplanar radiographic views were shown in the coronal, sagittal and axial orientations to see changes in the alveolar bone (Fig. 1). Both vehicle- and ZA-treated mice showed normal height from the CEJ to the lingual alveolar crest on the non-drilled side (Fig. 1A and D). However, significant changes were seen in vehicle-treated animals on the contralateral drilled side where irregular alveolar crest borders along with widening of the periodontal ligament space around the molars were observed in comparison to ZA-treated animals (Fig. 1A, *arrow* and D). A significant widening of the periapical space around the all the roots associated with the first and second molars were seen in vehicle- versus ZA-treated animals (Fig. B and E). ZA-treated animals, in contrast, showed increase bone thickness in the alveolar bone throughout the drilled sites. In addition, significant thickening of the lamina dura was observed between the furcation of the first and second molars in ZA- versus vehicle-treated animals (Fig. 1E and B, respectively). Exuberant periosteal bone thickening was observed in ZA-treated animals in drilled sites (Fig 1F). Vehicle-treated animals showed qualitatively significant regions of bone loss in the axial view in comparison to ZA-treated animals (Fig 1C).

Throughout all of the images, stark contract in bone loss versus bone thickening was seen as significant differences between vehicle- versus ZA-treated animals.

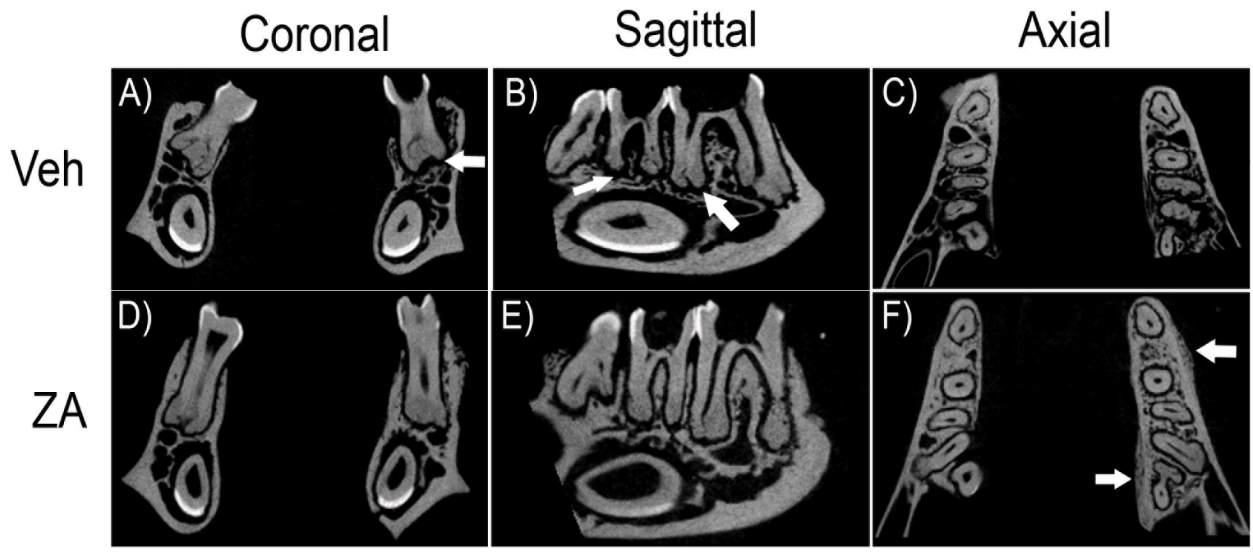


Figure 11. Radiographic analysis of pulp exposure in the mandible.
Multiplanar radiographic views of vehicle-treated animals show increased bone loss on drilled sites around the periapical region and widening of the periodontal space (A, B, C). ZA-treated mice show increase bone density (D, E, F) along with periosteal bone formation on the drilled site shown in the axial view (F). Significant amount of bone thickening is seen in the lamina dura between the furcation of the roots in ZA versus vehicle-treated animals (E).

To ensure periapical lesion development, disarticulated mandibles were imaged by μCT using previous published protocols [96]. Mandibles were oriented and linear measurement were made in the coronal view of the distance from the apex of the root to the lower adjacent alveolar bone for the distal root of the first molar (D1) and the mesial root of the second molar (M2) on the corresponding healthy and drilled side (Fig 2A). This region was chosen because the most significant qualitative bone changes in these drilled and non-drilled sites of the mandible. Significant increase in the distance from the apex of the root to the alveolar bone was seen on the

drilled side of vehicle-treated mice in comparison to the contralateral healthy side in both D1 and M2 (Fig 2B) of the same animal. ZA-treated animals showed a similar statistically significant increase in linear periapical distance in the D1 region, but not for the M2 region. However, ZA-treated animals showed a significant decrease in the linear periapical space distance on drilled sites in comparison to corresponding drilled sites of vehicle-treated animals in both D1 and M2 (Fig 2B). No significant differences were observed when comparing the non-drilled sites between vehicle- versus ZA-treated animals.

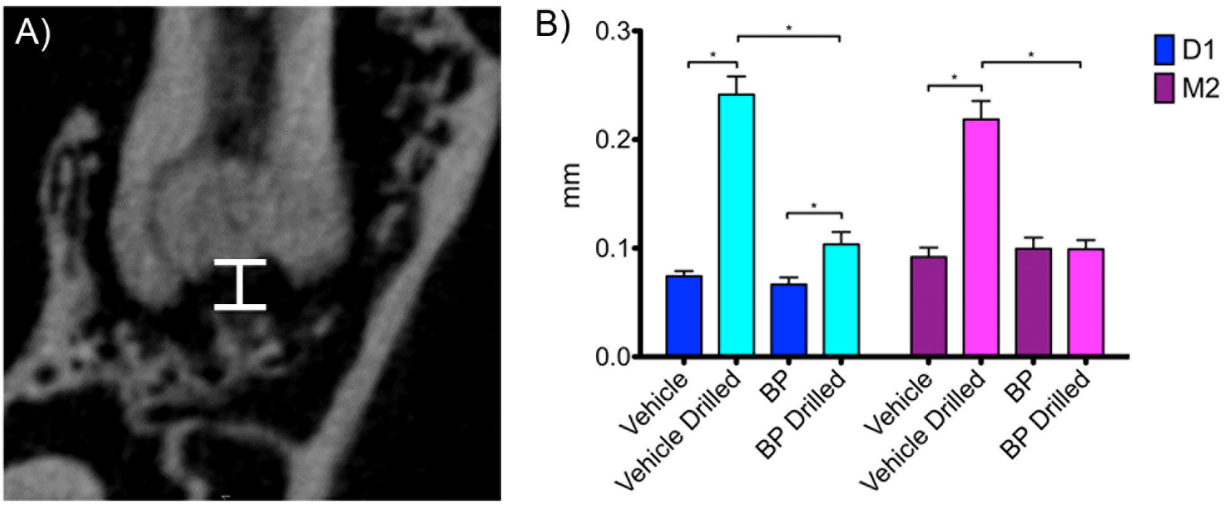


Figure 12. Quantification of periapical lesion development.
Linear distance measurements were made of the periodontal ligament space, from the apex of the root to the alveolar bone for the distal root of the first molar (D1) and the mesial root of the second molar (M2) for both the healthy and drilled sites of vehicle- versus ZA-treated mice (A). Vehicle-treated drilled sites showed a significant increase in linear distance (apical space) in comparison to vehicle treated non-drilled sites and ZA-treated mice (B).

Since exuberant periosteal bone formation was observed in the drilled sites of all ZA-treated animals, we quantified lingual bone thickness in the drilled versus non drilled sites in vehicle- versus ZA-treated animals as previously described [96]. Linear measurements were made

from the lingual side of the distal root of the first molar (D1L) and the lingual side of the mesial root of the second molar (M2L) (Fig. 3A). Vehicle-treated animals showed a significant decrease in lingual bone thickness in comparison to the contralateral healthy side of the corresponding molars for both D1L and M2L in the same animal (Fig 3B). In striking contrast, ZA-treated animals showed a significant increase in lingual bone thickness not only in respect to their contralateral healthy side, but also in comparison to the drilled sites of vehicle-treated animals for both D1L and M2L.

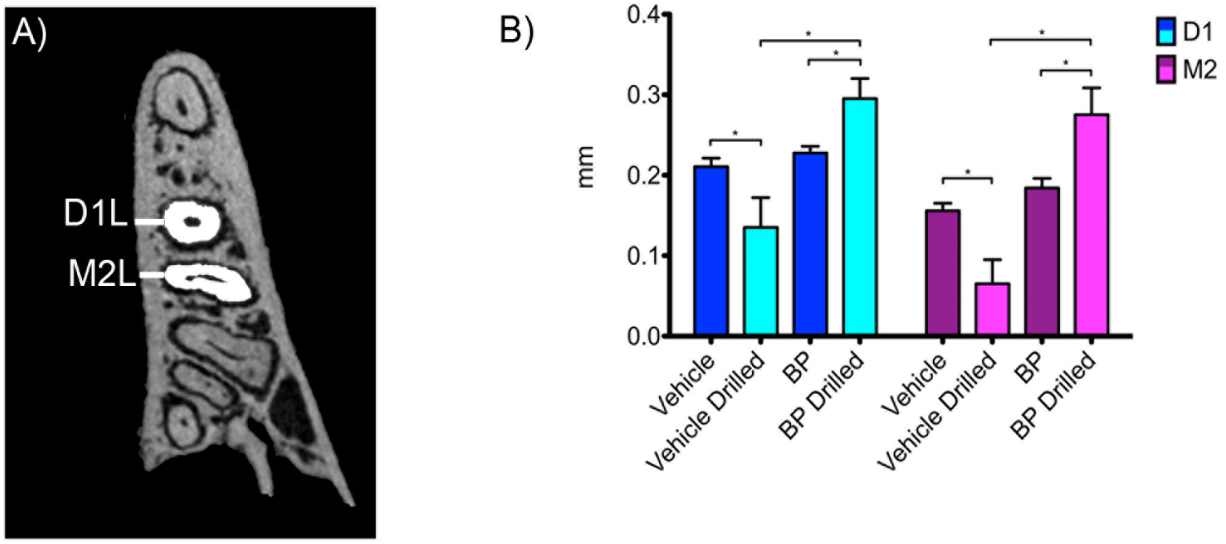


Figure 13. Quantification of lingual bone thickness.
Lingual bone thickness was measured at both the distal (D1) and the mesial (M2) root of the first and second molars, respectively (A). Vehicle-treated animals showed a significant decrease in lingual bone thickness at both D1 and M1, while ZA-treated animals showed a significant increase in bone thickness.

Histological analysis showed significant qualitative differences in the appearance of the alveolar bone between drilled and non-drilled sites of vehicle- versus ZA-treated animals. Mandibles were decalcified and an initial cut between the D1 to the M2 roots were made

perpendicular to the alveolar bone was made. Serial sections were then performed to capture the area of interest (AOI) followed by H&E staining (Fig 4). Images in figure 4 are focused on the periapical region (Fig. 4A, B, E and F) as well as the periodontal region (Fig. 4C, D, G and H) of the first molar due to extensive differences seen in both areas. In the periapical region of vehicle-treated animals, a marked increase in inflammation was seen in the drilled sites with increase bone loss/resorption around the periapical space in comparison to healthy sites (Fig 4B, *blue arrow* and A, respectively). There were no qualitative differences seen in the presence of red blood vessels and viable alveolar bone with confluent areas of filled osteocytes in vehicle-treated animals (Fig 4A and B, *yellow and green arrows, respectively*). In the periodontal region, vehicle-treated animals showed increased bone remodeling characterized by irregularity of buccal alveolar bone (Fig. 4D, *green arrow*). The lingual alveolar ridge height was significantly reduced when compared to the healthy contralateral side in vehicle-treated animals. Marked increase in inflammation was seen in drilled sites along with the presence of blood vessels in remodeling area, respectively (Fig. 4D, *blue and yellow arrows, respectively*). Similarly, both healthy and drilled sites of vehicle-treated animals showed viable alveolar bone (Fig. 4C and D, *green arrows*).

Significant histological changes different from vehicle-treated animals were seen in ZA-treated mice (Fig. 4E-H). In the periapical region, drilled sites of ZA-treated animals showed increased inflammation in comparison to the contralateral healthy sites (Fig. 4F, *blue arrows*). Moreover, periapical spaces in drilled sites were attenuated in ZA- versus vehicle-treated animals. In the periodontal region, drilled sites in ZA-treated animals showed significant areas of necrotic bone characterized by diffuse loss of osteocytes with confluent areas of empty

lacunae (Fig. 4H, *red arrows*). These necrotic areas were abutted with a large presence of inflammation and in some cases bacterial colonization (Fig 4F, H and Fig. 5A, respectively). Adjacent to these areas were extensive periosteal bone formation was present both on buccal and lingual surfaces (Fig. 4H, *double white arrows*). Inflammatory infiltrates surrounded the periodontal region in all drilled sites of ZA-treated animals in comparison to the contralateral healthy sites. Lastly, the presence of blood vessels was greatly reduced in ZA- versus vehicle-treated animals (Fig. 4A-E, G, *yellow arrows*).

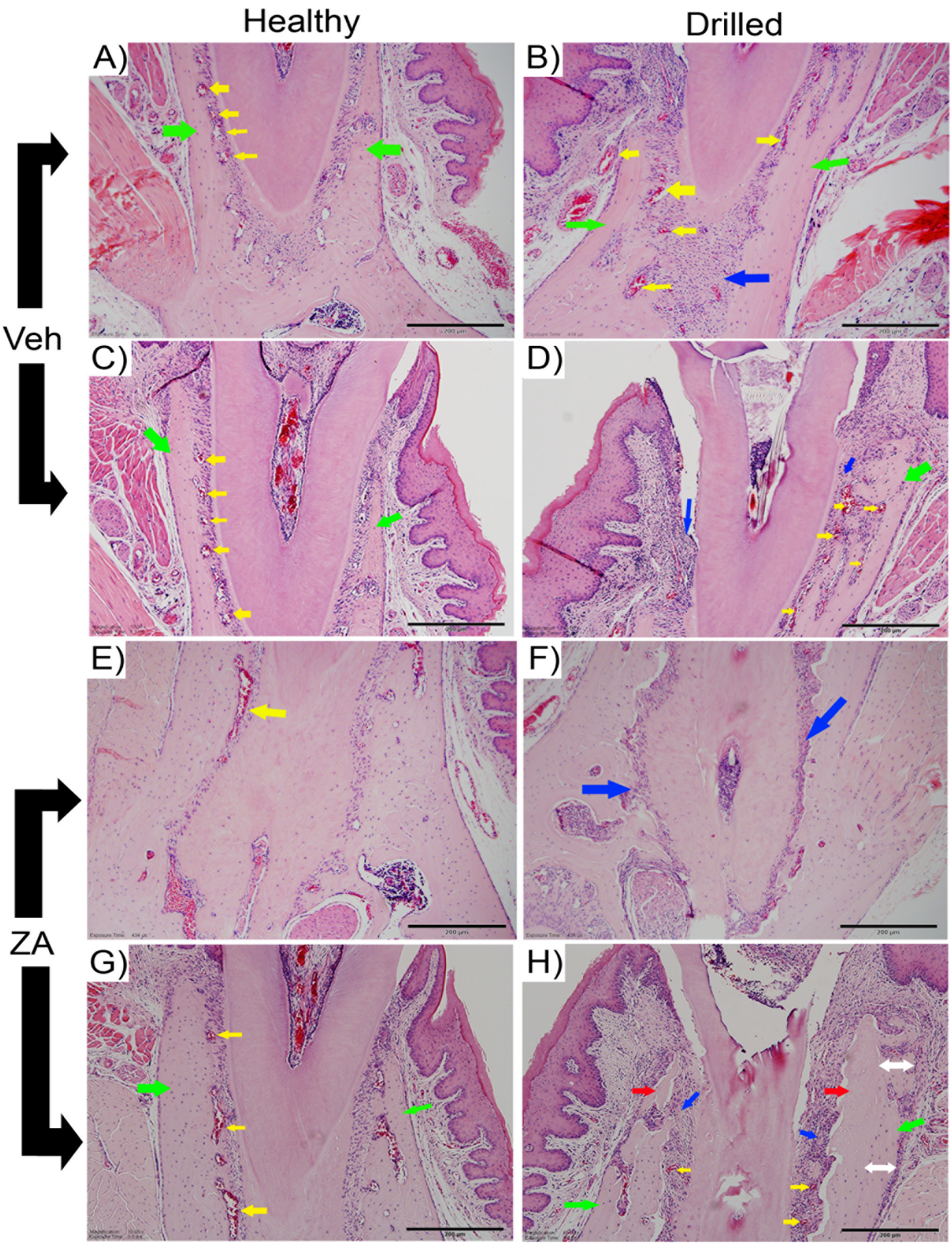

Healthy
Drilled
A)
B)
Veh
C)
D)
E)
F)
ZA
G)
H)

Figure 14. Histological analysis of periapical induced BRONJ.
Representative H&E images, (10X) of vehicle- versus ZA-treated first molars shown in the periapical region (A, B, E, F) and the periodontal region (C, D, G, H). *Green* arrows show viable bone; *yellow* arrows show blood vessels; *blue* arrows show inflammatory infiltrates; *red* arrows show necrotic bone and *double white* arrows show periosteal bone formation

Areas of osteonecrosis in al ZA-treated animals showed consistent increase in inflammatory response along with extensive areas of necrotic bone characterized by diffuse loss of osteocytes with empty lacunae (Fig. 5A-C, *red arrows*). To capture the extent of necrosis progression and development, Figure 6 shows three representative drilled site samples of different ZA-treated mice in the development of osteonecrosis. Areas of extensive osteonecrotic bone were largely surrounded by the presence of bacteria in some animals (Fig. 5A, *blue arrow*). ZA-treated animals also displayed extensive periosteal bone formation and in some cases epithelial rimming exposed periosteal bone (Fig. 5B, *white arrow*). Finally, consistent with all drilled sites, the presence by inflammatory infiltrates consisting of lymphocytes and neutrophils was observed around necrotic bone (Fig. 5C, *blue arrow*).

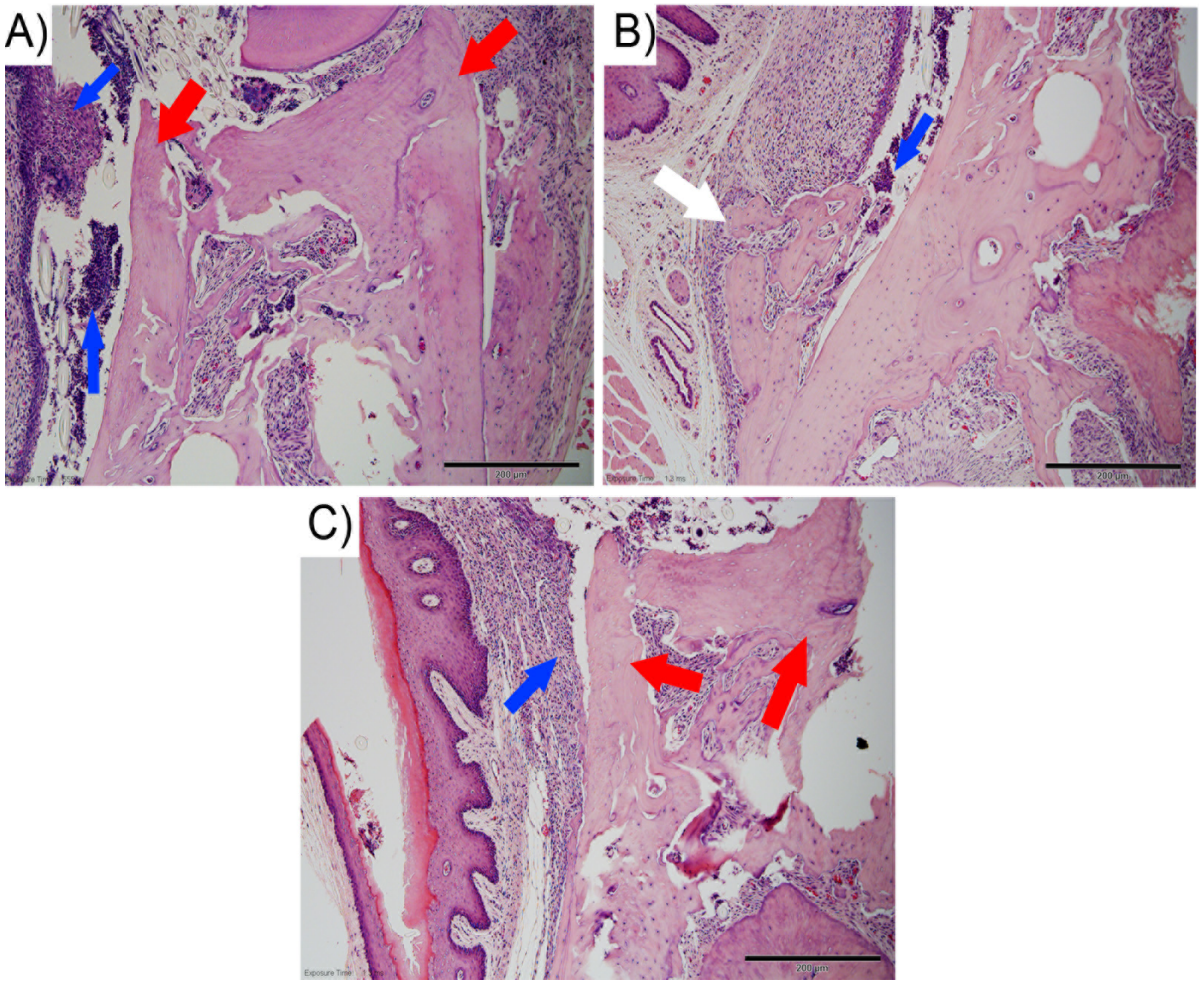
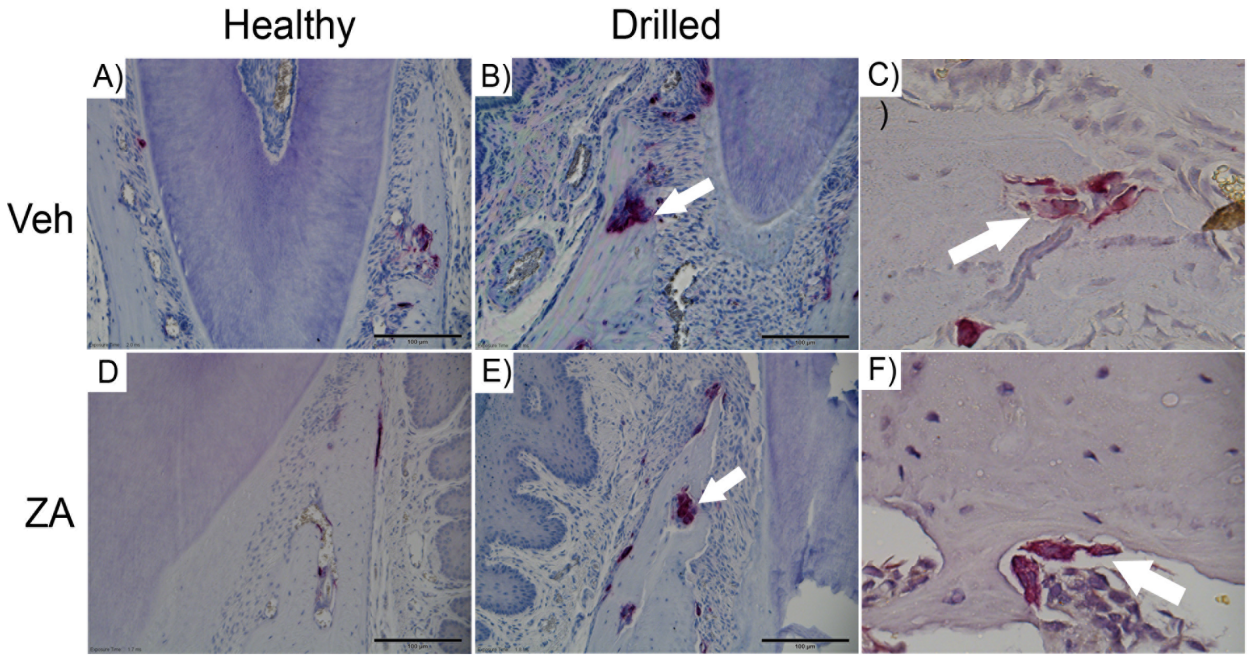


Figure 15. Histologic BRONJ development in different animals.
Representative H&E images of different ZA-treated mice on the drilled site. Extensive necrotic bone areas, characterized by empty osteocytic lacunae were seen in all drilled sites for ZA-treated animals (A and C, *red* arrow). Periosteal bone formation and bone exposure was present in some mice (B, *white arrow)*. Inflammatory cells were seen throughout drilled sites (A-C, *blue arrows)*.

Since BPs have direct effects on osteoclasts [22,23], we performed TRAP staining to evaluate changes in osteoclast number and morphology in vehicle- versus ZA-treated animals. Both drilled sites of vehicle- and ZA-treated animals showed increase multinucleated TRAP+ staining in comparison to healthy sites (Fig. 6). Vehicle-treated animals displayed TRAP+ cells

in direct attachment to adjacent alveolar bone with clearly defined resorption pits (Fig. 6B, *white arrow*). ZA-treated animals showed a significant qualitative difference in hyperchromatic, flattened (Fig 6D) and detached osteoclasts (Fig 6E, *white arrow*). Higher magnification shows a clear detachment of multinucleated TRAP+ cells of ZA- versus vehicle-treated animals (Fig. 6C and F, respectively). Multinucleated TRAP+ cells were further quantified and showed significant increase in numbers on drilled sites for both vehicle- and ZA-treated animals in comparison to their corresponding healthy sites (Fig 6G). No significant difference was observed between drilled sites of vehicle- versus ZA-treated animals.



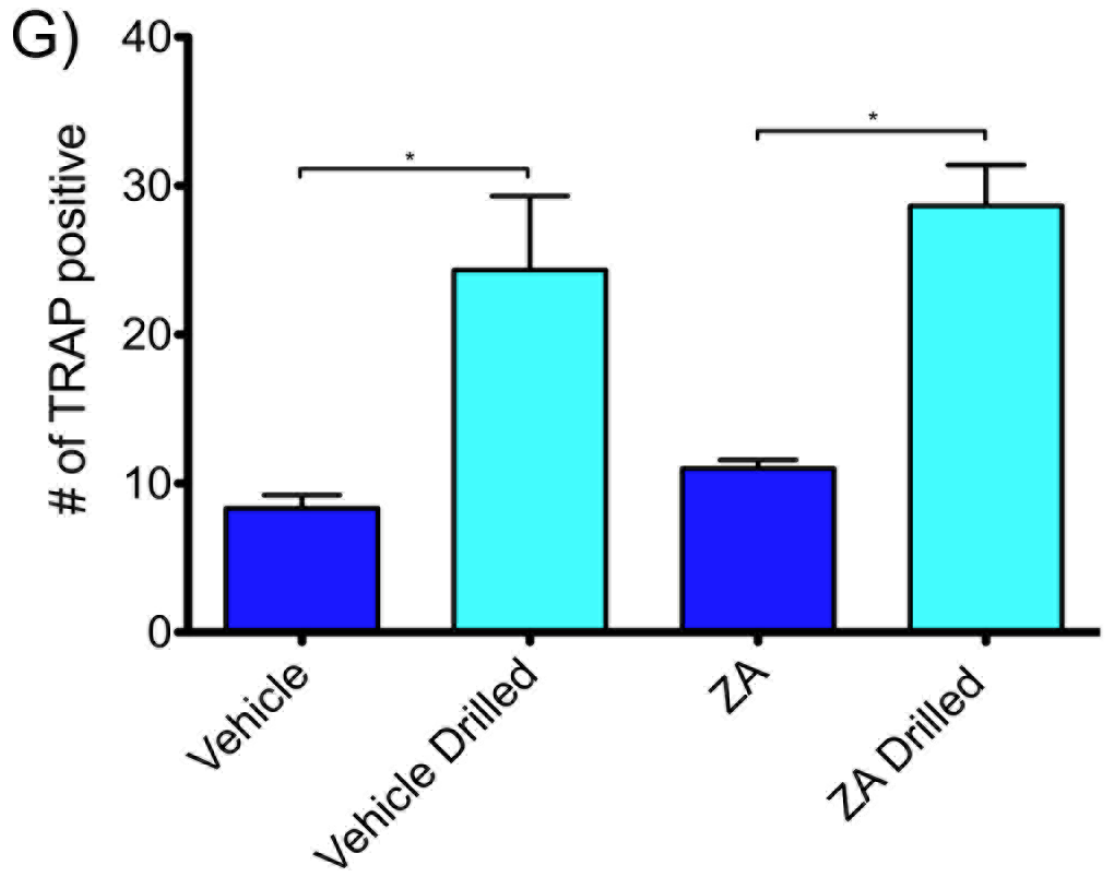


Figure 16. Qualitative and quantification of TRAP+ multinucleated cells.
Multinucleated TRAP+ cells in vehicle-treated mice display direct bone attachment and resorption pits (A and B, *arrow)*. Increased TRAP+ numbers are seen in drilled sites. In contracts, ZA-treated mice show detached, hyperchromatic and flattened osteoclasts (D and E, *arrow)*. Higher magnification shows clear attachment of vehicle-treated animals (C, arrow) while ZA-treated animals shows clear detachment of multinucleated TRAP+ cells. While drilled sites for both vehicle- and ZA-treated animals showed a significant increase in TRAP+ cells in comparison to their respective control sites, no significant difference was observed between vehicle versus ZA-treated animals in the treated sites.

DISCUSSION

Since the start of BRONJ clinical reports in 2003, several studies have utilized common contributing factors in developing animal models to understand the pathophysiology of BRONJ.

There are two necessary factors to all of these animal models, which include the use of a bisphosphonate and a form of dental injury. To study the pathophysiology of BRONJ, we created an animal model in a proof of principle concept by using continuous BP treatment at a superphysiological high dose in mice that had received periapical lesions to induce BRONJ. From this model, we looked at the changes to alveolar bone in the mandible through radiology, histology and TRAP staining.

It has recently been reported that the use of endodontic treatment to treat periapical infection with BP administration caused ONJ development in humans [148]. In this report, two male individuals who were treated with intravenous ZA treatment for skeletal complications associated with prostate cancer developed ONJ after endodontic treatment [148]. This clinical finding as well as other reports involving extraction procedures and periodontal disease contributions to the development of ONJ, led us to inducing periapical disease in ZA-treated animals to promote similar results.

Previous animal models have employed different dental disease models with BP treatment to recreate BRONJ [71,96,134,149]. In addition, clinical reports have shown that there is a preference of BRONJ development in the mandible [8]. Allen and Burr et al., used dynamic histomorphometry and showed that intracortical bone remodeling in the mandible of untreated dogs were 10 times higher than the tibial cortex [71]. In these studies, specifically analysis demonstrated that the alveolar portion of the mandible had the highest rate of intracortical turnover, being 8 times higher than non-alveolar parts of the mandible [73]. Finally, it was shown that in a mature skeleton, the alveolar mandible has higher turnover than the alveolar mandible [73].

Reports have linked BPs and dental disease in exacerbating BRONJ development due to increased bone turnover rate at a sevenfold increased prevalence [150]. Bone turnover rates in extractions sites of the alveolar bone have been accentuated to be 400% greater than other skeletal sites [151]. In agreement with these studies, we have shown that increased turnover from periapical disease can contribute to exacerbated BRONJ development through infection and inflammation in the mouse.

Intravenous BPs have been well documented in their potent inhibition of osteoclast function. For this reasons, BPs have been instrumentally used to treat pathologies to reduce bone lysis and osteolytic metastases. Importantly, BPs slow the remodeling process and increase bone mineral density and have been commonly used in osteoporotic patients to reduce fractures and prevent bone loss [152]. These effects were clearly seen in our periapical-induced ZA-treated animals where a significant increase in bone density both of the mandibular alveolar bone was observed. Most notably, stark differences in the thickness of the lamina dura in ZA-treated animals were seen in contrast to vehicle-treated animals. On the other hand, radiographic observations showed the effects of periapical disease in causing bone loss in vehicle-treated animals.

Periapical disease has been well characterized by studies conducted by Stashenko et al. [144,145,147,153]. In these investigations, periapical lesions were induced in a rat model between 0 and 15 days and characterization and analysis of the pathogenesis of the disease were clearly studied. Balto et al., used μCT to quantify periapical bone destruction using a similar method of inducing periapical disease and showed a significant increase in bone loss in those teeth with pulp exposures over control. Similarly, to ensure the establishment of periapical disease we

measure the linear distance from the apex of drilled molars to the periapical alveolar bone. We should there was a significant increase in linear distance in molars that had pulp exposure over controls, suggesting the development of periapical disease in vehicle-treated mice. These effects were reversed in animals that were treated with ZA.

Interestingly, periosteal bone formation was seen in ZA-treated animals in both radiographic and histology samples. Lingual bone thickness was significantly different between ZA- versus vehicle-treated animals in drilled sites. Although altered bone remodeling is expected due to the suppressive effects of BPs on osteoclasts, it is still unclear about BPs effects of remodeling, specifically on the periosteal surface [154-156]. Reports have been conflicting between the suppression and formation of periosteal bone showing that age and different skeletal site have variable effects [157]. Pre-clinical studies in dogs and pigs show no effect of BPs on periosteal surfaces while rodent models have been more variable [154,156,158]. In our results, we observed periosteal bone formation 100% of the time in ZA-treated animals only on drilled sites. This suggests that BPs do have significant effects in deregulating normal bone remodeling where osteoclast function is little to none. Histological samples of effected animals showed periosteal bone formation consistently adjacent to necrotic bone. BPs could suppress osteoclast function to the extent where necrotic bone is unable to be removed from the effected sites, and osteoblastic activity continues in the mandible. Periosteal reaction then ensues with continual bone formation and little to none elimination.

Histological analysis showed large areas of necrotic bone characterized by diffuse loss of osteocytes with confluent areas of empty lacunae in all of our ZA-treated animals. Similar results were observed from other investigators when employing different forms of dental injury

[71,96,149,159,160]. A most striking observation was the medullary spaces of necrotic bone that were filled large areas of inflammation in both vehicle- and ZA-treated animals in drilled sites. These inflammatory cells consisted of lymphocytes and neutrophils. Periapical lesions were reduced in animals that were received ZA treatment although inflammation persisted.

Numerous studies have associated the pathogenesis of BRONJ development with inflammation. In a clinical study, necrotic bone from 30 BRONJ patients treated or malignant diseases and osteoporosis was analyzed histologically [94]. In the study, the investigators saw medullary space of the necrotic bone filled with bacteria along surrounded with inflammatory cell infiltration in the bone marrow. By counting the number of inflammatory cells between control and ONJ patients, the researchers were able to discriminate two ONJ subgroups and showed osteocyte apoptosis and empty osteocyte lacunae increased with the degree of inflammation [94]. Yu et al., characterized inflammatory cell types as contributors to periapical lesion pathogenesis in pulp exposed teeth of rats and found that lymphocytes were the predominant cell type, along with leukocytes and macrophage-monocytes presence [147]. Also, in agreement with previous studies, Stashenko et al., attributed proinflammatory cytokines including interluekin-1α, IL-1β and tumor necrosis factor alpha enveloped in the periapical region in the root at day 15 leading to significant amount of periapical alveolar bone loss [145]. It would be interesting to use genetic mouse models that are immunosuppressed in our ZA-induced periapical disease model to see whether the development of BRONJ is reduced or even reversed.

Table 1. Radiographic and histologic findings in vehicle- versus ZA-treated mice

Treatment	Number of animals	Periosteal Bone Formation	Histologic Osteonecrosis	Bone Exposure
Vehicle	17	0 (0%)	0 (0%)	0 (0%)
ZA	18	18 (100%)	18 (100%)	6 (33%)

Based on the clinical definition of BRONJ, gingival and bone interactions have led to hypotheses of compromised wound healing in the pathogenesis of the disease. We observed bone exposure in 33% of our ZA-treated animals, when histologically analyzing the oral epithelium. Thin epithelial rimming and attachment to periosteal bone formation was observed in ZA-treated animals in drilled sites. Epithelial attachment to necrotic bone was never seen.

BP toxicity on epithelial cells have been well documented [161-165]. These reports show the significance in BP-induced cell death and senescence of epithelial cells *in vitro*. However, the question remains how does the epithelium get exposed to BPs *in vivo*? It has been postulated that oral epithelium exposure to BPs are derived from the underlying bone when remodeling occurs [161]. Kobayashi et al., used an extraction model in mice to show delayed would healing of extraction sockets with the use of ZA [162]. Although the investigators demonstrated a delay in wound healing for animals that were BP-treated and had extractions, the study looked at only a single time point of one-week post dental injury. It would be difficult to postulate the order of pathogenesis as either a primary or secondary event based on this study. From our results, epithelial changes secondary to primary bone necrosis can be considered since bone necrosis was seen 100% of the time while only 33% of these samples had histologic bone exposure. Moreover, if BP had a direct effect on the gingiva of these animals, intravenous administration of

BP would affect epithelium of both the drilled and non-drilled sites. Also, it should be noted that exposure of necrotic bone is predominantly seen after dental intervention. Consideration of direct effects of BPs versus ischemia should be taken into consideration. A reduced presence of blood vessels was observed in ZA-treated animals. Nevertheless, the effects of BRONJ in epithelial rimming exposing necrotic bone still remains unknown**.**

TRAP staining revealed a significant increase in TRAP+ multinucleated cells in both our vehicle- and ZA-treated animals. Interestingly, there was no significant difference between the two groups. Roelofs et al., showed that fluorescent risedronate uptake in osteoclasts *in vivo* [166] suggesting the possibility that BPs may effect the function of these cells. Although reports of the mechanism of BP-induced osteoclast functional inhibition are unclear, results including increased apoptosis through TUNEL staining, caspase activity and DNA fragmentation have been reported. It has been clearly shown that nitrogenous BPs inhibit osteoclasts through the deregulation of small GTPase signaling cascades that lead to the disruption of the cytoskeleton effecting osteoclasts transport and sealing zone [7,30,167]. As a result, attachment of osteoclasts to bone surfaces is attenuated and BP-induced detachment is seen.

Similar results were seen in a long-term BPs therapy in mice where no difference in TRAP+ cells were seen in ZA- versus vehicle animals over a 13 month period [34]. In contrast, we observed nicely defined resorption pits in our vehicle-treated animals of multinucleated TRAP+ cells attached to the alveolar bone. Moreover, recent reports have shown that long-term use of nitrogenous BPs are associated with formation of large osteoclasts detached from the bone surface [168]. Since osteoclasts have proteases, such as TRAP and cathepsin-K they could play a role in osseous inflammation [34].

New therapies that target the RANKL/RANK/OG pathway have illuminated osteoclast suppression as a major factor to the pathogenesis of ONJ. Denosumab, a human monoclonal antibody to RANKL, has demonstrated efficacy for decreasing vertebral, non-vertebral and hip fractures in post-menopausal osteoporotic women [169]. In some reports, Denosumab was shown to have greater efficacy for increasing bone mineral density in the hip, femoral neck, trochanter and lumbar spine while also decreasing resorption markers compared to alendrontate treatment for 12 months [170]. Clinically, Denosumab has been shown to induce ONJ specifically in non-BP-treated patients [171]. It has even been shown that ONJ can be reversed through the cessation of Denosumab treatment [172]. This drug has alluded to the direct effects on inhibiting osteoclastic function and differentiation as a key factor in the pathogenesis of ONJ.

Taken together, these results suggest BPs have a role in directly inhibiting the function of osteoclast in the normal bone remodeling process. Since the half-life of nitrogenous BPs have been estimated to be greater than 10 years, it regimented infusions of these BPs should be carefully administered due to their long lasting effects in patients [173,174]. Although BP treatment for patients could be halted for extended periods of time, prevalence of ONJ development could still remain high because of BPs ability to remain in the bones for long periods of time.

Reduced blood vessel presence was observed in ZA-treated animals. Other animal models showed similar results of reduced vascularity following tooth extraction and alendronate treatment. Previous studies have supported the relation between osteoclasts and angiogenesis. Cackowski *et al.,* showed that osteoclasts contributed to angiogenesis *in vitro* and *in vivo* by a mechanism requiring matrix metalloproteinase-9 (MMP-9) [175]. In conditions such as multiple myeloma, bone metastases and rheumatoid arthritis, osteoclastogenesis and angiogenesis have

shown to be enhanced [176,177]. In addition, it has been shown that a blood vessel is present at every osteoclast [178]. Osteoclast conditioned media have been reported to be angiogenic *in vitro* [179,180]. ZA indirectly impairs angiogenesis targeting MMP-9-expressing macrophage [77]. Although we did not see a change in the number of osteoclasts in vehicle- versus ZA-treated animals, these findings suggest that osteoclast formation and activity and angiogenesis may be linked in bone remodeling. Understanding temporal effects of BPs on angiogenesis could shed light to suppression of recruitment of osteoclast precursors in diseased sites.

Finally, this study confirms that ONJ is drug and dose dependent. Our study utilized a superphysiological high dose of 200 μg/kg dose that is approximately 3.5 higher than the human equivalent dose of 4 mg/month[181]. This high dose was to ensure that all our BP-treated animals rapidly received enough BP to reconstitute the disease. It must be taken into consideration that high concentrations of BPs have been shown to have direct cytotoxic effects on cells. It is possible that prolonged exposure to high local concentrations could have had cytotoxic effects to osteocytes. However, since we administered the drug intravenously, bone necrosis would have been expected at other skeletal cites. When examining these other sites, bone density had significantly increased, but clinical representations of osteonecrosis were not seen (data not shown).

Various dose studies have shown differences in the progression of BRONJ in animal models. Investigators have used doses of 35 μg/kg to 125 μg/kg of ZA in mice seeing a progression in severity development of BRONJ development suggesting a direct effect of BRONJ development in the drugs administration [96,149,159]. However, it must be noted that these animal models have all incorporated different types of dental intervention to supplement their BP

dosing including vitamin D deficient diets and the use of dexamethosone and docetaxel. We only incorporate two variables in the development of ONJ suggesting that dental disease and BPs are sufficient and necessary in creating the disease. In addition, ours is the first model to show a natural development of periapical disease to contribute towards BRONJ development. Other animal models have predominately used extraction models

In summary, we demonstrate that ZA administration and periapical disease can induce osteonecrosis of the jaws in mice. Changes in alveolar bone with ZA treatment and periapical disease include a suppression of bone loss, areas of extensive necrotic bone formation, increase periosteal bone formation and loss of functional osteoclast. BRONJ in a multifactorial disease that is attributed to infection, inflammation, changes in vascularity, epithelial and osteoclasts. Understanding each of these factors will help bring forth therapies that can help patients with the disease.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

BRONJ is a multifactorial pathology that is dependent on numerous variables for its development. Although the side effect of BP administration is relatively low, increasing clinical reports have been reported as our understanding of the pathophysiology of BRONJ increases.

Our lab has established two BRONJ animal models utilizing periodontal and periapical disease with BP treatment to induce osteonecrosis. The novelty of our approach lies in the use of only two variables to establish ONJ. Our results suggest that BP and dental disease is sufficient and necessary for BRONJ development. In addition, we have considered employing dental disease in a manner that naturally progress in pathology. Unlike other extraction models, our analysis of examining ONJ development through a natural disease progression translate our findings clinically. Although we have observed many changes in the establishment of ONJ, we recognize the need to further analyze confounding factors to elucidate clear mechanistic pathways in BRONJ pathogenesis.

We have shown through μCT and radiographic images changes in alveolar bone in both periodontal- and periapical-induced BRONJ. Using a ligature-induced periodontal disease rat model, we showed a significant increase in alveolar bone loss in vehicle-treated animals. Careful consideration was taken in the placement of these wire ligatures to eliminate any ischemic or

mechanical alveolar bone destruction during surgeries. In ZA-treated animals, increased bone density was seen throughout the maxilla of the rat. Periosteal bone formation and sequestrum formation of the maxillary alveolar bone was seen in ligature-induced ZA-treated animals in 32% of our animals, while 21% displayed exposed necrotic bone.

One puzzling result in this model was the development of histological osteonecrosis in a single vehicle-treated animal. It should be noted that ligature-induced animal models has been well accepted to study periodontal disease. However, ligature models have ranged from using silk-sutures to sterile wires. Mechanical mastication of these animals could be attributed to harsh movements of the wire during animal feeding. It is therefore a possibility that the wire itself broke a piece of the alveolar bone, while periodontal infection persisted eventually leading to bone death. Furthermore, since ZA was never administered to these animals, severe osteomyelitis could be suspect in promoting detached bone necrosis. It should be noted that other contributing factors of exposed bone or periosteal bone formation was not present in this vehicle treated animal.

We took advantage of the vast cases of clinical BRONJ development in patients to compare with our animal model. Clearly, radiographic images showed parallel results between the rat and human in observations of increased bone density, periosteal bone formation and sequestrum formation were consistently seen in patient and animal samples.

Using the same concept, we moved to a mouse model in hopes to take advantage of genetic mouse models and immunohistochemical evaluation tools that were lacking in the rat system. Since, reports were utilizing dental injury as an essential factor with BP administration for BRONJ development, we created periapical disease in mice by pulp exposure in the

mandibular molars. Radiographic and μCT analysis showed significant increase in bone loss in vehicle- versus ZA-treated animals. ZA-treated animals showed increase bone density and drilled sites showed periosteal bone formation. Our most significant result was all ZA-treated animals developed periosteal bone formation.

Histological analysis between both animal models showed similarities in ONJ development. ZA-treated animals showed extensive areas of osteonecrotic alveolar bone characterized with diffuse empty osteocytes with confluence loss of lacunae. These areas resulted in TUNEL+ osteocytes and were significantly different in comparison to the contralateral sites of ZA- and all sites of vehicle-treated animals. Inflammation surrounded these affected areas in diseased sites. A significant increase in the number of polymorphonuclear neutrophils was present in both diseased sites of vehicle- and ZA-treated animals. Increases in intraepithelial lymphocytes were significantly increased on in diseased sites of ZA-treated animals. At times, bacteria were present with inflammatory cells surrounding osteonecrotic areas. Periosteal bone formation was seen adjacent to necrotic areas. In cases of exposed bone, epithelial rimming to viable periosteal bone was consistently seen. Periapical disease induced ZA-treated animals developed histologic osteonecrosis 100% of the time.

Finally, TRAP staining showed qualitative differences in ZA- versus vehicle-treated animals in drilled sites. We observed a significant increase in TRAP+ multinucleated cells in drilled sites versus non-drilled sites. However, no significant difference was observed in the number of TRAP+ multinucleated cells. Similar results have been observed in other studies. Although the number of TRAP+ multinucleated cells did not differ, significant qualitative

differences in were observed in the ZA treated group including detached, hyperchromatic osteoclasts with flattened ruffled border.

One interesting result was the development of necrotic bone in the periodontal region of animals induced with periapical disease. Localization of necrotic bone has previous been identified in the region of infection. Following this logic, periapical disease should have developed periapical necrotic bone. However, in all our periapical diseased induced animals, we only saw necrotic bone in the periodontal regions. This result may be due to the fact that even within the mandible, there are areas that have different rates of bone remodeling. Allen et al., showed these differences in dog mandibles using fluorescent light showing highest remodeling within the cortex, periosteal surface and areas adjacent to the periodontal ligament [182]. Considering these results may explain observing necrotic bone only in the periodontal region of these animals.

In conclusion, we have developed two animal models that employ two of the most common dental diseases: periodontal and periapical disease. Using ZA, we demonstrated that BPs and dental disease are necessary and sufficient to create BRONJ in the rat and mouse. We refined our rat model, with a more robust mouse model that showed consistent development of BRONJ.

BRONJ has been proposed as a disease that occurs by a combination of multiple factors. With our robust animal model, we believe focused studies on pathophysiological contributors to BRONJ development can be studied extensively and comprehensively. Specifically, using the vast genetically engineered mice will be beneficially in discovering targeted therapies for translatable benefits. With the number of BRONJ cases on the rise, understanding the

pathophysiology of the disease is greatly needed to find potential treatments and cures for adversely affected patients.

FUTURE DIRECTIONS

ANTIBIOTIC TREATMENT ON THE EFFECTS OF INFLAMMATION AND BACTERIAL INFECTION

Guidelines for the prevention and management of BRONJ have emphasized the associated risk of disease progression due to poor oral health and management. Approximately 80% of BRONJ is due to invasive dental procedures that stem from poor oral health management. These procedures range from invasive dental extractions to prevalent periodontal or periapical disease. In addition, several reports have analyzed the microbial biofilms that may play a role in the etiopathogenesis in the oral cavity. Currently, non-surgical, conservative therapies such as oral rinses, antibiotic, analgesics or even discontinuation of BP therapy have been proposed and followed.

In our study of BRONJ development through BP administration and periodontal disease, we showed a significant increase in polymorphonuclear neutrophils and lymphocytes in ZA-treated animals. Similarly, histological analysis of our periapical disease BRONJ model showed an increase number of neutrophils and lymphocytes. It would be interesting to see if antibiotic treatment could alleviate or even reverse the progression of BRONJ progression. It is important to note that understand the effects of antibiotic treatment would be most effective in reference to

BRONJ establishment. Since the cumulative dosage and time frame of BP treatment correlates with BRONJ progression and severity, antibiotic treatment, if effective would only be useful when the possibility of BRONJ pathogenesis is the weakest.

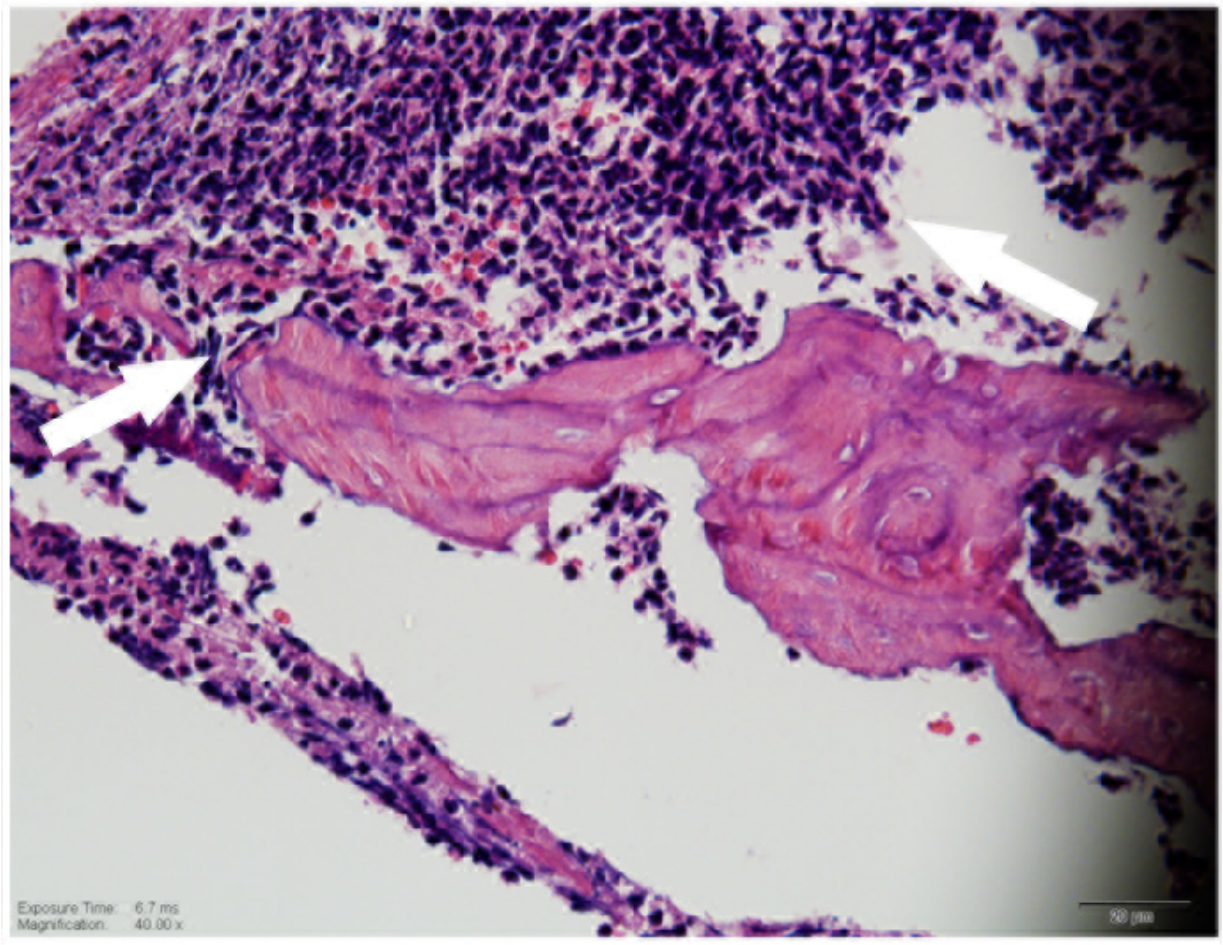


Figure 17. Bacterial presence in ONJ animals.
Significant presence of aggregate bacteria was observed around necrotic bone in ZA-treated animals in drilled sites.

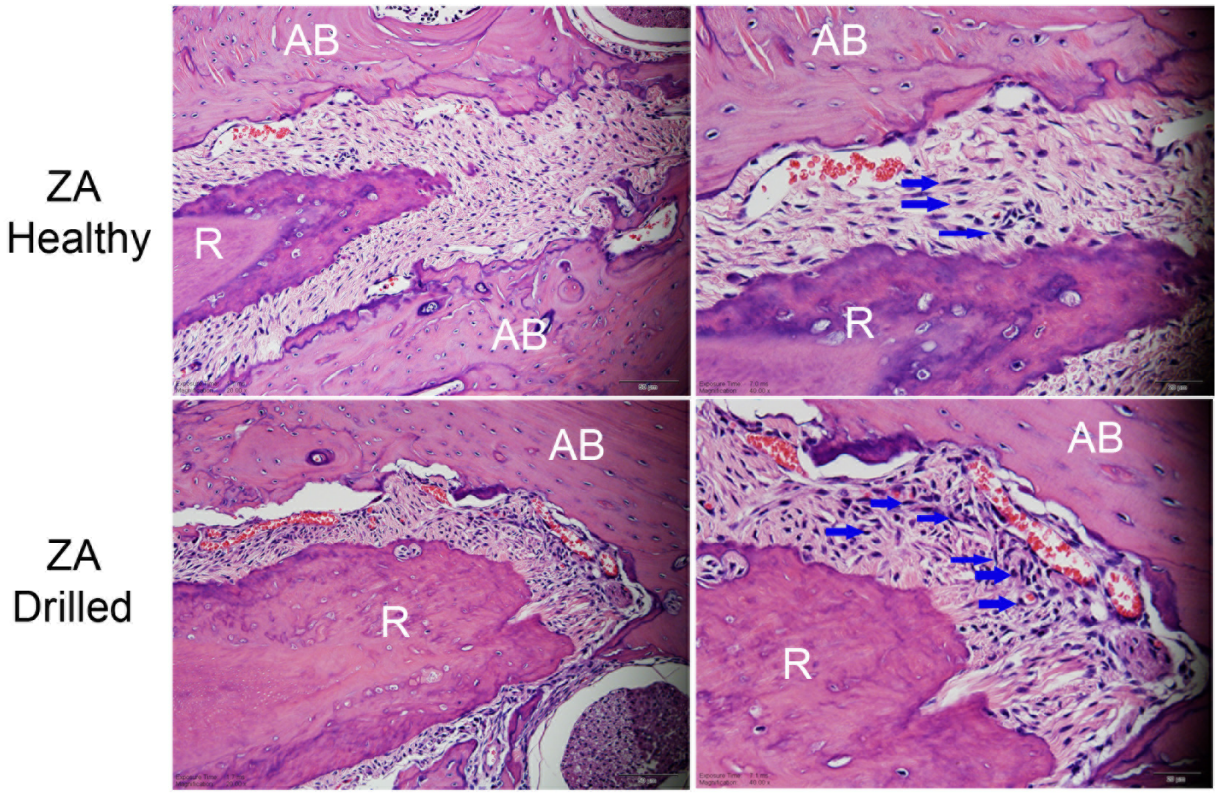


Figure 18. Inflammatory response with ZA treatment.
Increased inflammatory infiltrates composed of neutrophils and lymphocytes were observed in all drilled versus non-drilled sites (*blue arrows*). Qualitatively, increase inflammatory recruitment was seen in ZA- versus vehicle-treated animals. AB: alveolar bone, R: apex of root.

TIME COURSE CHARACTERIZATION

There have been a plethora of animal models that have been developed to study contributing factors of the pathophysiology of BRONJ. While, most of these animal models follow an extraction model, many of the time points in studying the disease are evaluated when BRONJ has been established. These animal models are certainly useful in study the contributing factors of BRONJ, but are limited in understanding disease progression.

There is a constant debate of researchers between the 'inside-out' theory versus the 'outside-in' theory. Since a multitude of pathophysiological hypothesis has arisen, investigators are constantly at odds of BRONJ either starting from necrosis of the alveolar bone first or from the epithelium. Although it is generally agreed upon that BPs have direct effects on suppressing osteoclast function, and thus affecting normal bone remodeling, it is intriguing to speculate the effects BPs may have of oral epithelium. By using our periapical animal model at different time points, we could evaluate which changes occur first.

In our preliminary data, we have used our periapical model to look at BRONJ development at 2, 4 and 6 weeks post periapical lesion induction. From our initial results, we have seen a time-point dependent development of osteonecrotic area. Further analysis is needed to elucidate BRONJ development.

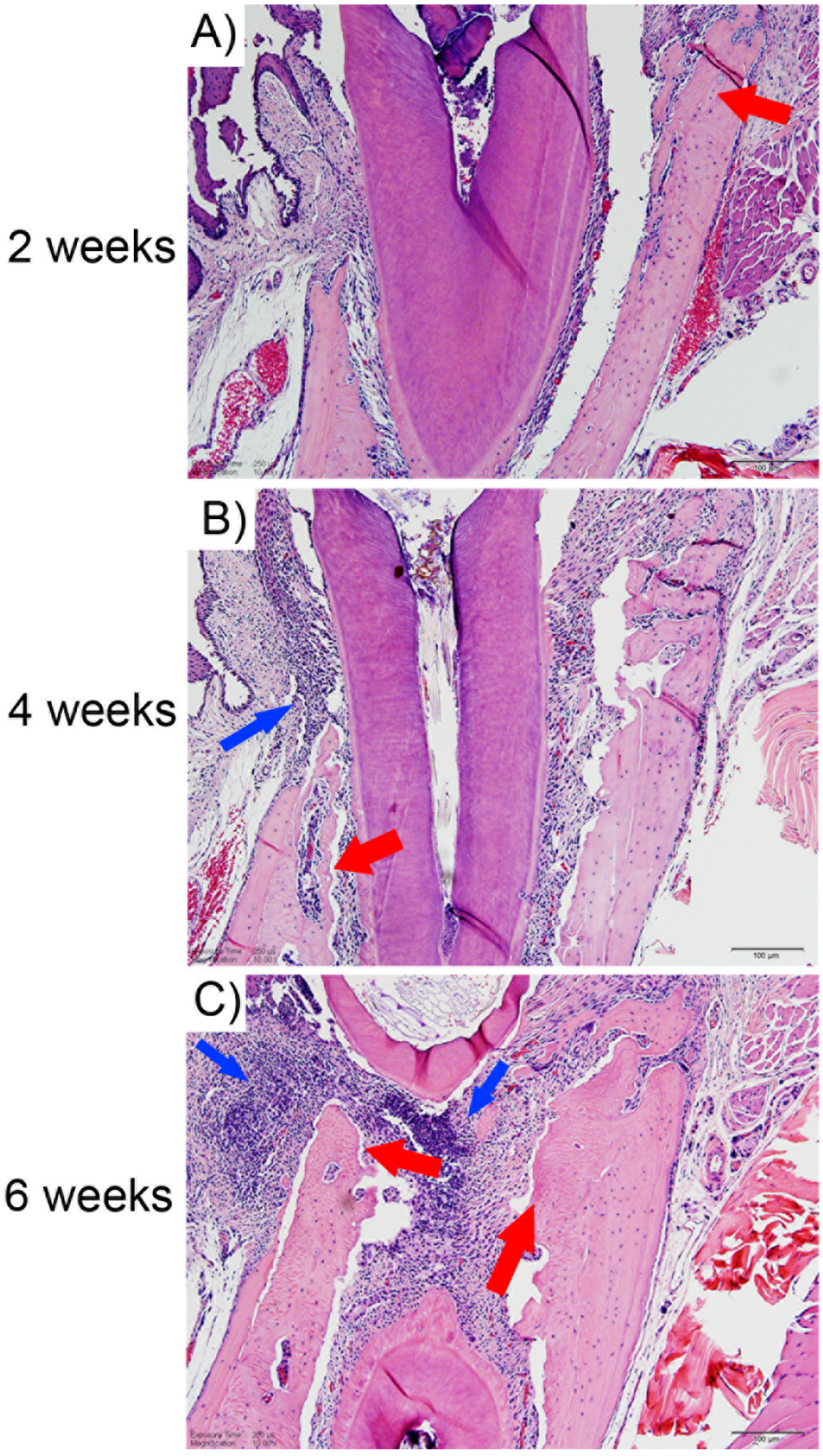

A)
2 weeks
B)
4 weeks
C)
6 weeks

Figure 19. Effects of necrotic bone development 2, 4 and 6 weeks post drilling.
Increased necrotic bone development was observed in a time-dependent manner post 2, 4 and 6 weeks of tooth drilling (A, B and C, *red arrows*). Increased inflammation was also seen in a time-dependent manner (A, B and C, *blue arrows*).

BISPHOSPHONATE LOCALIZATION IN DIFFERENT SKELETAL SITES

BPs have been readily used for its antiresportive properties. It has been shown that these drugs have differing affinities for bone mineral. The specificity of ONJ development to the jaws suggest a unique side effect to a particular skeletal site with BP therapies

In study done by Roelofs et al., three different conjugates of risedronate that were fluorescently labeled were compared to evaluate the localization at in tibias of rats. Results showed that fluorescently labeled compounds showed higher degree of labeling at forming endocortical surfaces compared with resorbing periosteal surfaces. In contract, there was no difference seen in quiescent endocortical and periosteal surfaces, suggesting that differential labeling relates specifically to the type of surface undergoing remodeling, rather than anatomical site. The investigators continue to suggest that newly formed mineral crystals available for risedronate to bind probably due to the large surface area provided higher degree of labeling at forming surfaces. Other investigators used radiolabeled BP to show preferential binding to resorbing surfaces. Despite contradictory results, it should be noted that whether using fluorescent labeling or radiolabeling, BP preferentially localized in areas of greater remodeling.

Similarly, we have started to use fluorescently labeled zoledronate (FAM-ZOL) in differing dental disease mouse and rat models. Our preliminary data of rats that have developed periodontal disease through our ligature model qualitatively show increased BP labeling in

infected sites compared to animals with FAM alone. We are currently adapting the use of FAM-ZOL in a mouse model for both periodontal, periapical and extraction dental injuries. We expect to see in increased uptake of FAM-ZOL in these areas of remodeling in the jaws versus other skeletal sites of the body such as the long bones.

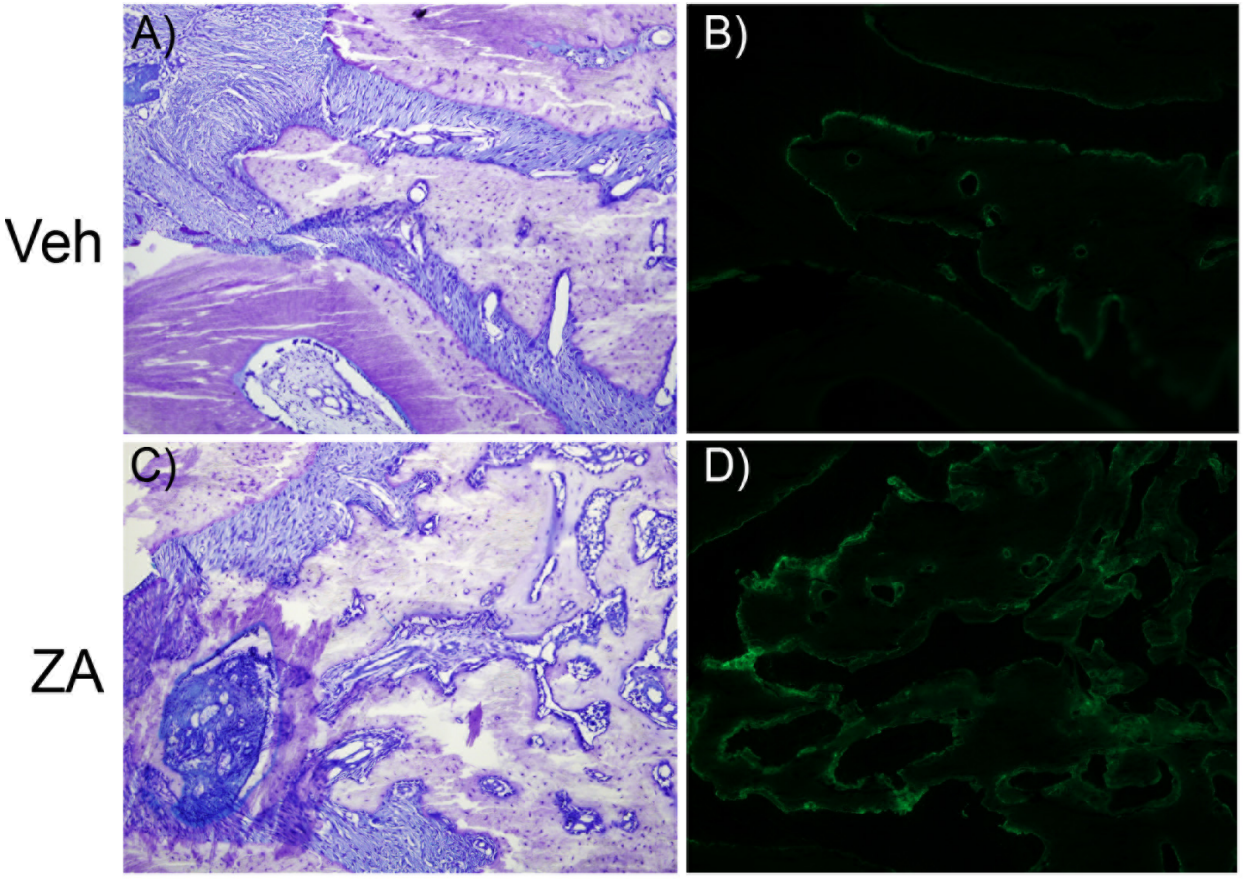


Figure 20. FAM-Zol localization in diseased sites.
Qualitative toluidine blue staining of vehicle- versus ZA-treated ligature-induced periodontal disease rats (A and C). Significant localization of fluorescent ZA in periodontal disease rats was seen in comparison to vehicle-treated animals (B and D).

EFFECT OF CHEMOTHERAPY AND CORTICOSTEROIDS

BPs are proven to be effective in direct anti-tumor activity. The overall effects of nitrogen containing BPs on tumor cells appear to be mediated via diverse pathways, such as apoptosis, angiostasis and immunomodulation. *In vivo* models have shown ZA to decrease the release of tumor-promoting growth factors from bone to delay further progression of bone metastases. Researchers have shown that when human breast cancer cells are inoculated into immunodeficient animals to induce bone metastases, ZA inhibit the formation and progression of osteolytic lesions and promote tumor cell apoptosis in bone lesions and reduce skeletal burden.

Although the effects of BP therapy reduce burden in cancer patients, epidemiological data strongly associated concurrent chemotherapy as potential factors that exacerbate BRONJ incidence. Patients with multiple myeloma are at a significantly higher risk of BRONJ development osteoporotic patients. Differences in treatment of patients include therapies such as thalidomide and glucocorticoids.

Bi et al., were the first to report a mouse model of exposed bone in the jaws following administration of ZA and dexamethasone post tooth extraction. These areas of ONJ-like lesions resulted in significantly larger areas than pervious animal models that solely employed the use of a dental disease and BP alone.

Using our ligature-induced periodontal model, we have administered daily injections of thalidomide and dexamethasone to mimic multiple myeloma treatments in patients. Our results show a significant amount of osteonecrotic area development in the rat. Most interesting is the exuberant amount of periosteal reaction that is extensively seen throughout the diseased area. Although it is established that BPs are sufficient to cause BRONJ, simultaneous administration of chemotherapy drugs increase the severity of the disease in a qualitatively significant increase

in periosteal bone formation development and necrotic bone area. Further analysis of soft tissue healing, vascularity and cellular differences in this model is needed to compare results and BRONJ risk to our established animal models.

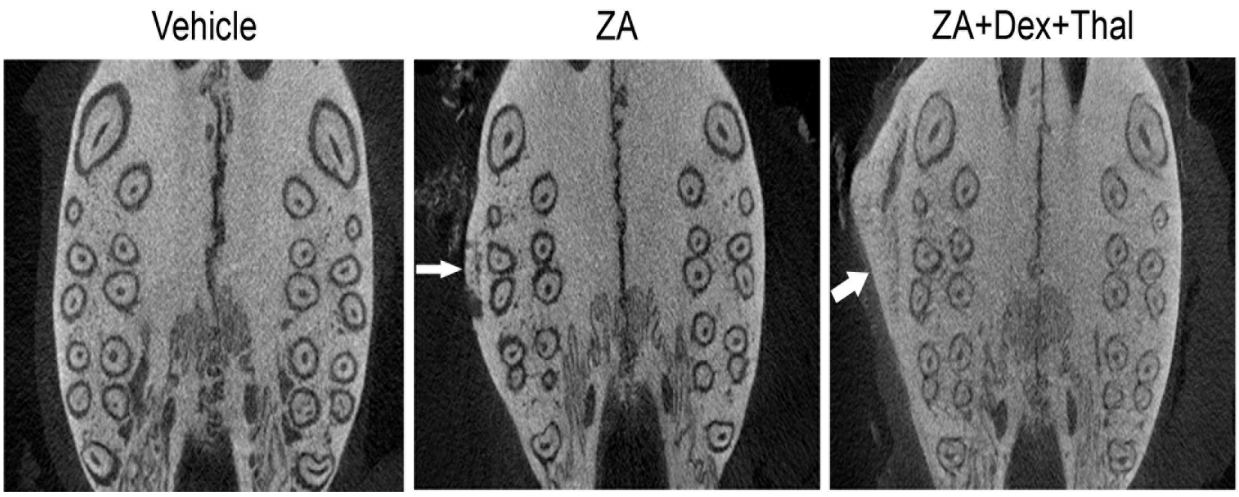


Figure 21. Effects of dexamethasone and thalidomide in ONJ severity.
Dexamethasone and thalidomide significantly increase periosteal bone formation in ZA-treated animals.

EFFECTS ON WOUND HEALING

Direct effects of BP toxicity to epithelial cells have been extensively shown *in vitro* [183]. In fact, gastric side effects and oral ulceration development has been well documented of orally administered BPs [163,184]. Concentrations of BPs in the soft tissue have been attributed to the release of BPs from the alveolar bone during remodeling. Specifically, for intravenous BPs, studies have postulated that concentrations of 1 nmol/ml of ZA in the oral cavity could have toxic effects on the oral cavity. Therefore, daily doses of 4 mg/month of ZA in long-term treated oncology patients could pose a significant risk for prolonged exposures of ZA in the oral cavity leading to mucosal breaching and bone exposure.

In our periapical disease induced mouse model, we performed trichrome staining to see the qualitative effects ZA had on Sharpey's fibers in the periosteum. Initial results show a qualitative thinning of collagen fibers in ZA-treated animals versus the vehicle-treated animals in drilled sites. Furthermore, H&E images of ZA-treated animals show a discontinuity in attachment of the periodontal ligament between the alveolar bone and tooth in all drilled sites.

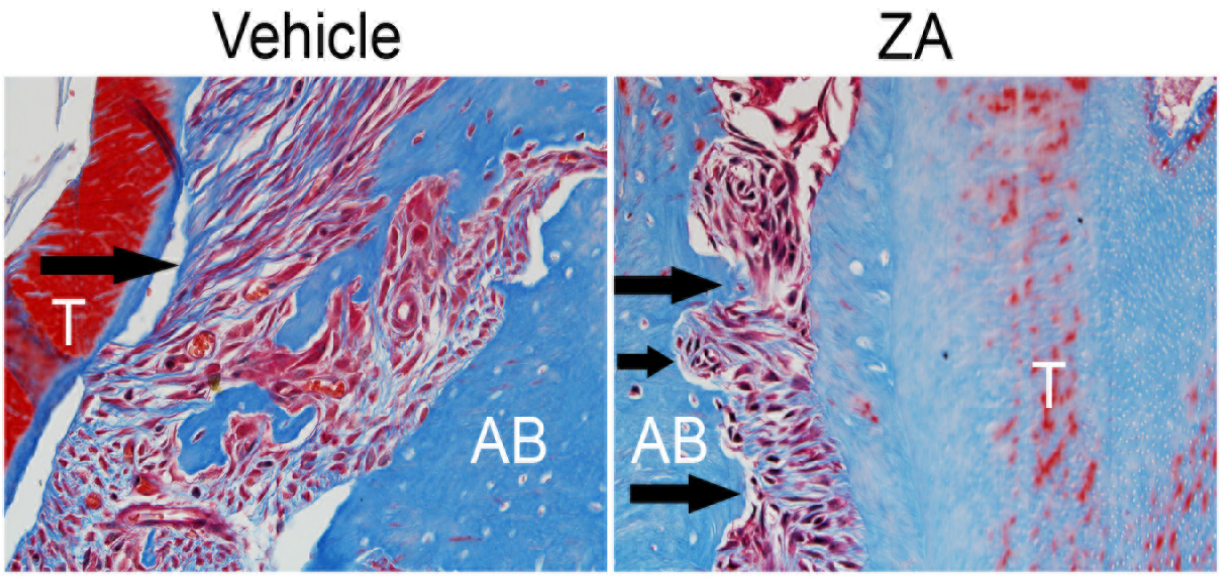


Figure 22. Trichrome stain of collagen fibers.
Qualitative observations of collagen fibers in the periodontal space show attachment in vehicle-treated animals in drilled sites (*black arrow)*. ZA-treated animals showed collagen fibers that were fragmented and discontinuous in attachment between alveolar bone and tooth in drilled sites (*black arrows*). AB: alveolar bone; T: tooth.

GENETIC FACTORS IN BRONJ

Increasing reports of genetic predispositions have been investigated to confer susceptibility or resistance to developing BRONJ. Since only a minority of BP users develop ONJ, it is conceivable that individual genetic variations to drug metabolism or skeletal

homeostasis could be a factor. Sarasquete et al., conducted a genome-wide associate study, typing half a million SNPs in two groups of multiple myeloma patients who had received BP therapy [185]. Among the several SNPs related to the presence of BRONJ, the investigators identified four within cytochrome P450, subfamily 2C polypeptide 8 gene (CYP2C8). These findings demonstrated that CYP2C8 influenced the likelihood of BRONJ development in multiple myeloma patients receiving BP therapy.

Similarly, using our animal models, we propose investigating candidate genes through microarray analysis of alveolar bone tissue in BRONJ animals. Analysis of these genes could give probably mechanistic pathways to investigate as contributing factors to BRONJ development. In addition, since we have established a mouse model of BRONJ, we could use the numerous genetic knockout models to target development and progression of BRONJ. These models can target potential genes for therapeutics in alleviating or potential reversing BRONJ development.

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