Effect of Zoledronic Acid on Maxillary Alveolar Bone Coverage in Rice Rats
With and Without Dental Trauma

A Thesis

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Abstract

Bisphosphonates are a class of drug widely used in both human and veterinary medicine for the treatment of pathologies affecting bone remodeling. Currently they represent the most commonly prescribed therapy for osteoporosis, with over 5 million prescriptions filled in the United States every year. Over the last decade the use of high dose nitrogen-containing bisphosphonates (BPs) has become increasingly popular in the multimodal approach to oncologic conditions. This class of medication has been shown to slow the progression of certain tumor cells from penetrating healthy bone tissues as well as slowing tumor cell proliferation. In addition BPs have been shown to significantly reduce the discomfort associated with metastases in some patients. Unfortunately, as the use and dosing of BPs has increased, so has the clinical complication associated with their use. Most notably, a condition known as bisphosphonate related osteonecrosis of the jaw (BRONJ) has emerged over the last ten years. While this condition has been linked to multiple risk factors, high dose BP usage and trauma in the oral cavity have been shown to be the primary events that precede the development of BRONJ lesions. BRONJ is a disabling and painful condition affecting both the maxilla and mandible. To date this condition is poorly understood, and consequently current treatment options are inconsistently effective.
The overall purpose of this study was to develop a reliable animal model of BRONJ that can be used by researchers to advance the understanding of the pathophysiology of this disease. Because no grossly visible lesions were observed in these animals, we focused our efforts on exploring the role of two factors that have been implicated in the pathogenesis of BRONJ: periodontal disease and dental trauma. High-resolution micro computed tomography (µCT) was used to quantify the effects of a potent intravenous nitrogen-containing BP, zoledronic acid (ZA), on maxillary alveolar bone loss in an established rat model of periodontal disease. The role of trauma was determined by comparing data from sites adjacent to and remote from dental extractions. A statistically significant decrease in alveolar bone loss around maxillary molars both adjacent to and distant from dental extraction was identified. The magnitude of this effect was found to be associated with severity of initial bone loss. The more severe the initial degree of bone loss, the less of a protective effect BP has on disease progression. While no grossly visible BRONJ lesions were identified in our animal model, this study serves as a foundation for studying the effect of ZA on maxillary alveolar bone. Ongoing work on this project will include a detailed microscopic assessment of the tissue response in ZA-treated extraction sites and a comparison of histomorphometric data from the maxillary alveolar bone and trabecular bone in the appendicular skeleton.
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Chapter 1: Introduction

Bisphosphonates (BP) are a class of drug widely used in the treatment of disproportionate bone resorption. Examples of conditions include osteoporosis, osteitis deformans (Paget’s disease), hypercalcemia of malignancy, bone metastases from breast and prostate cancer and even early rheumatoid arthritis [1, 2]. Recently high dose BPs have been shown to slow the progression of certain tumor cells from penetrating healthy bone tissues as well as slowing tumor cell proliferation.

Examples of commonly prescribed BPs include pamidronate (Aredia), alendronate (Fosamax), ibandronate (Boniva), and zoledronic acid (Zometa). These compounds are potent inhibitors of bone resorption through the inhibition of osteoclasts [3, 4]. However, bisphosphonates (BPs) are not without adverse effects. Recent reports have found prolonged use of high doses to be associated with necrosis of the mandible and maxilla. This disease entity is now known as bisphosphonate related osteonecrosis of the jaw (BRONJ) [5, 6]. BRONJ is a disfiguring, disabling and painful condition for which only poor therapeutic treatment options are currently available. While several hypotheses have been reported, the overall pathogenesis is poorly understood [5]. Consequently, current treatment options are inconsistently effective.
Bone Remodeling and Bisphosphonates:

Bone is a dynamic and specialized type of connective tissue which undergoes constant remodeling and turnover [7]. For bone strength to be maintained, the remodeling process must be precisely regulated [7]. The skeleton is comprised of long and flat bones, of which cortical and trabecular are the two main types of osseous tissue. Cortical bone is very dense and primarily found in the outer shell of long bones and the surface of flat bones. Trabecular bone is less dense and is found at the end of long bones and the inner parts of flat bones [7]. Because it is more vascularized compared to cortical bone, trabecular bone is ideal for metabolic activity and thus greater turnover. In the oral cavity, alveolar bone is the portion of the maxilla and mandible that provides support to the tooth roots. It is composed of two parts: the alveolar bone proper and the supporting bone. The alveolar bone proper is a thin lamella of compact bone directly lining the tooth. The supporting bone is composed of a cortical plate and underlying cancellous bone.

Trabecular bone is estimated to remodel at a rate of 30% per year as compared to cortical bone with a remodeling rate of only 3% per year. As a result, the overall rate of bone turnover is highest in sites such as the vertebrae, where trabecular bone predominates, and lowest in sites such as the hip, where cortical bone is most prevalent [8]. The maxilla and mandible are sites of significant bone remodeling as a result of the large portion of trabecular alveolar bone present. When the bone remodeling equilibrium is altered, bone mass and density may become compromised. Even with normal aging processes, men lose an average of 20-30% of bone mass and women lose 30-40% during their lifetime [9].
Bone remodeling predominantly involves three types of cells including osteocytes, osteoclasts and osteoblasts. The activity of these cells is regulated by several variables including mechanical forces, bone kinetics, hormones, cytokines and local factors. The osteoclasts have a role in activating these three cells because they detect mechanical stress and respond to biomechanical stimuli. Their involvement in the activation process subsequently results in the endosteal surface cells being retracted and digested by matrix metalloproteinases [7]. Once osteoclasts are recruited and activated, they fuse to become multinucleated and mediate the resorption of the underlying bone.

Osteoblasts are subsequently recruited and lay down new bone called osteoid, which hardens by mineralization with calcium phosphate in the form of hydroxyapatite [7]. The osteoblasts become surrounded by the bone they are synthesizing and become osteocytes [7]. Recent research suggests osteocytes are not the static and inactive cells they have long been thought to be [8]. Osteocytes ensure a dynamic equilibrium between osteoblasts and osteoclasts because they are able to respond to stimuli from the central nervous system, bone marrow and immune system [8]. They translate mechanical strains into biochemical signals, which are sent to other cells and induce bone remodeling [8].

BPs are structural derivatives of pyrophosphate (P-O-P), with the bridging oxygen being replaced by carbon. Due to the lack of an enzymatic process capable of disrupting the P-C-P bond, they are poorly metabolized [3, 9, 10]. When given orally, only 1% of the BP is absorbed in the gut because of their large molecular size and hydrophilic property [3]. However this absorption is highly variable depending on specific drug and species [9]. Newer BPs such as zoledronic acid (ZA), are able to be administered intravenously (IV), greatly increasing their potency. Once in the blood stream,
approximately half of the BP is cleared within 24 hours by the kidneys due to its highly hydrophilic nature [3, 9]. The other half binds to the exposed hydroxyapatite bone mineral [3, 11]. These sites are most accessible at areas of bone resorption, giving the drug a non-uniform distribution pattern in the body. Consequently, a relatively larger proportion of BP is taken up by trabecular bone compared to cortical bone [3]. This is especially true in the alveolar bone of the jaw where remodeling rates have been shown to be 10-20 times higher than that in long bones of human subjects [12, 13]. After binding, subsequent bone formation covers the drug where it remains until it is released back into the circulation via the normal bone turnover process [3]. This results in BPs having a variable half-life once bound to bone inversely related to the particular bones turnover rate. For example, when BP binds to long bones, its half-life in humans is ten years [14] and in canines is three years [15].

BPs are composed of two classes, non-nitrogenous and nitrogenous [9, 16]. BPs inhibit osteoclast activity via different mechanisms depending on whether they are non-nitrogen-containing (clodronate and etidronate) or nitrogen-containing (alendronate, risedronate, ibandronate, pamidronate and ZA) [17]. While osteoclasts are the main cellular target, evidence has shown additional targets include osteoblasts, osteocytes, and gamma delta T-lymphocytes [12, 18].

Non-nitrogenous BPs inhibit bone resorption by creating a toxic analog of adenosine triphosphate which induces apoptosis of osteoclasts secondary to interference with mitochondrial function. More commonly utilized in medicine, however, are the nitrogenous BPs. This is because they have been shown to be more potent secondary to an increased binding affinity. Their primary mechanism of action is by inhibiting farnesyl
diphosphate synthetase, a necessary step in the pathway for cholesterol synthesis. This inhibition suppresses the process of protein geranylgeranylation that is required for basic cellular functions and therefore, for osteoclastic activity [17]. Consequently, the precursors for osteoclast formation and recruitment are inhibited and their ability to participate in bone remodeling is reduced [3, 9]. Nitrogen-containing BPs also inhibit osteoclast function by causing disappearance of the convoluted or ruffled membrane, which is hallmark for an active osteoclast. Recent observations have indicated that the disruption of the cytoskeleton is morphological evidence for osteoclast inactivation [19, 20]. This is a mechanism of inactivation that specifically occurs prior to the induction of apoptosis [21].

Reduced bone formation in the presence of BPs is mainly a consequence of remodeling suppression, thereby indirectly affecting the osteoblasts [12]. Osteoblasts arise from mesenchymal cells and secrete extracellular matrix (ECM) in addition to regulating its mineralization. Once the ECM is formed, 10-20% differentiate into osteocytes, which involves reducing cell volume, size and organelles [22, 23]. The cell shape changes from a round osteoblast to a dendritic osteocyte [24]. The cell membrane facing the bony matrix has short, thick processes and the membrane facing the vascular spaces contains long and thin processes, full of microtubules and microfilaments. The dendritic processes allow for cellular communication between osteocytes, osteoblasts and lining cells [25].

There is also recent strong in vitro evidence that BPs may indirectly mediate antiresorptive activity through their effect on osteoblasts. BPs’ mechanism of action on osteoblasts has not fully been defined, in part, because different BPs have distinct effects.
For instance, three different BPs have been found to inhibit endogenous prostaglandin E2 production and enhance phosphatase production and mineralization in vitro [26]. Additionally, etidronate and alendronate have been shown to stimulate the creation of osteoblast precursors and mineralized nodules in bone marrow cultures [27]. However, another study found ZA increases the activity of a RANKL sherdase, TACE (TNF-alpha converting enzyme), which decreases the transmembrane RANKL expression on osteoblasts [28]. The authors suggest this indicates in vitro evidence that BP may induce osteoblast apoptosis via TNF-alpha activation. These discrepancies suggest the effect and mechanism of action of BP on osteoblasts is likely drug specific.

Some studies suggest that BPs improve osteocyte survival and function. The support for this statement is based upon in vitro studies where BPs prevented apoptosis that should have been theoretically induced by etoposide, TNF-alpha or synthetic glucocorticoid dexamethasone [29].

**Use of Bisphosphonates in Medicine:**

BPs are most commonly used to treat osteoporosis, a disorder of skeletal fragility due to a combination of low bone mass, decreased bone quality, and deterioration of the bone microarchitecture [9, 30]. Currently over 5 million BP prescriptions are filled in the United States alone for the treatment of osteoporosis. In addition, BPs’ use in other bone degrading disorders such as Paget’s disease, hypercalcemia of malignancy, bone metastases from breast and prostate cancer, and even early rheumatoid arthritis has rapidly increased over the last decade [1, 2, 31].

The main clinical utility of this drug is to reduce fracture risk. Studies have demonstrated consistent results to this effect, as well as increasing bone mineral density
After using BPs for three years, the BMD increased in the spine by 5.4-8.8% [32, 34]; femoral neck by 1.6-5.9% [32, 34]; trochanter by 3.3-7.8% [32, 34]; and radius by 0.2% [34]. The risk of having a new vertebral fracture reduced by 41-49% [33-36]; non-vertebral fracture by 27-39% [33-36]; hip fracture by 53% [36], and wrist fracture by 30% [36]. Additionally, taking BPs has been shown to decrease the progression of vertebral deformities [32].

Glucocorticoids are frequently utilized to manage inflammatory and autoimmune diseases; however these treatments are the most common cause of secondary osteoporosis. Glucocorticoids increase osteoblast and osteocyte apoptosis, thereby slowing the rate of bone formation [37]. A study investigating the effect of BPs on patients to counteract the catabolic effects of glucocorticoids showed that bone mass density increased by 2.8% and 3.7% in groups of patients who received 5mg and 10mg, respectively, of alendronate. The placebo group’s BMD continued to decrease by 0.8% over the same 24-month period [38]. A study on the effect of risedronate treatment for patients on corticosteroid therapy had similar results, and concluded that there was a 70% reduction in vertebral fracture risk [39].

The use of high dose, high potency BPs for the treatment of oncologic conditions has steadily been gaining momentum in the medical community in both human and veterinary medicine. While standard dosing for the treatment of osteoporosis with ZA is a single 4mg annual dose [40], oncologic applications often administer 4mg every three weeks [41]. There are four primary applications for oncologic use. The first application is for the treatment of hypercalcemia of malignancy. In patients with multiple myeloma or another condition resulting in metastatic bone resorption, osteolytic lesions release high
levels of calcium in the blood. Bisphosphonates reduce osteoclastic bone resorption, thereby lowering the quantity of calcium released from the bone to the blood. The second application is to slow the progression of tumor cells from penetrating healthy bone tissue, thereby suppressing the formation of skeletal complications [42-48]. This application is heavily utilized in patients diagnosed with multiple myeloma, prostate, and breast carcinomas [49-51]. For example one study demonstrated a 35% reduction of bone complications in patients with prostate cancer treated with ZA [52]. The third application is to decrease pain associated with primary and metastatic bone cancer [41, 43, 47, 53]. One human study found a 8.3% median improvement in pain with the administration of ZA in patients with bone metastasis [41]. In canine oncologic patients, one study evaluating its use in ten dogs with appendicular osteosarcomas, found enhanced bone mineral density was achieved in groups treated with BP. Four out of the ten dogs had subjectively increased comfort levels noted by the owner [54].

The fourth application is currently highly controversial and involves a direct effect on slowing tumor cell proliferation and inducing tumor cell apoptosis [55]. In vitro studies have shown that nitrogen containing BPs have a direct effect on inducing tumor cell apoptosis and stimulating gamma-delta T cell cytotoxicity against tumor cells [56]. In addition, in vitro studies have recently demonstrated an increased uptake of the antineoplastic drug, doxorubicin, in malignant histiocytosis cell lines when given with ZA, significantly increasing cell death [57]. However the in-vivo drug concentration necessary to achieve these results is unknown, and it is questionable if these levels will be attainable in the living patient even with high level IV dosing as a result of BP being rapidly cleared from the blood stream.
Complications Associated with use of Bisphosphonates:

While generally well tolerated, BPs are not without adverse effects. Those most commonly noted include gastrointestinal intolerance, headache, pyrexia, myalgia, influenza-like symptoms and arthralgia [58]. With the more recent usage of high dose BPs in oncologic patients, more severe side effects have been recorded. These include renal toxicity, hypocalcemia, hepatotoxicity, atypical femur fractures, and BRONJ [58]. In a 2011 study, an association between prior alendronate treatment and a new entity of atraumatic fractures was identified. The radiographs in these individuals showed characteristic lateral cortex thickening of the femur with a beaked appearance at the site of the fracture [59]. In addition, BPs have been implicated in oesophageal cancer, atrial fibrillation, chronic musculoskeletal pain and impaired fracture healing [58]. However, a causal relationship for these complications is not currently supported in the literature.

In 2001 the first case of a BRONJ was diagnosed via retrospective record analysis dating back to 1991 [6]. In 2003 the first prospectively diagnosed cases were documented in 36 patients receiving pamidronate or ZA [60]. In all but one case, the patient was receiving high doses for the treatment of a neoplastic condition. In 28 of the cases the patient initially presented with the complaint of a painful tooth. In these patients, the recognition of BRONJ lesions developed after surgical extraction of the tooth. In the remaining eight patients, the development of exposed bone occurred spontaneously [60]. Initial studies identified the overall incidence of BRONJ to be between 1 in 10,000 and 1 in 100,000 patients receiving BP for any reason [61]. However when high doses of ZA and/or pamidronate are administered to patients with multiple myeloma, an 11% incidence was observed [6]. This study found patients who received ZA had a 9.5 fold
greater risk of developing BRONJ when compared to patients who received pamidronate only, and a 4.5 fold greater risk when compared to those on pamidronate who later received ZA [6]. In addition geriatric patients and those with dental extraction were at higher risk for the development of lesions. Since then dozens of case reports have been described in the literature. While risk factors such as diabetes, smoking, and corticosteroid use have been identified, the most prevalent commonality is high dose IV BP and dental trauma, usually in the form of a tooth extraction [62].

BRONJ is characterized by alveolar bone exposure affecting the mandible, maxilla or both [60]. Symptoms include jaw or tooth pain, swelling, infection and loose teeth [63-66]. These patients endure considerable pain and distress and often progress to developing significant facial deformities [67]. While many hypotheses exist, there is a lack of knowledge regarding the pathophysiology of this condition [12]. Consequently, only poor therapeutic options are currently available.

The study of BRONJ can be broken down into soft tissue and bone. Argument for involvement of the gingival mucosa centers around two components: BPs’ antiangiogenic effects and a direct toxic effect on the mucosa secondary to BP released from the alveolar bone during injury [12]. It is hypothesized that failure of traumatized bone to reepithelialize results in the development of a necrotic foci. However, in a case study using topically applied BP gel to the oral mucosa of patients, no adverse effects were identified [68].

At the present time, the majority of BRONJ research focuses on the bone itself. At the core of this argument is BPs’ remodeling suppression of the osseous tissue. In humans, remodeling rates of the jaw have been shown to be 10-20 times higher than that
in long bones [12, 13]. Because the jaw is exposed to repeated trauma during mastication, a reduction in turnover rate may have a profound effect resulting in the formation of BRONJ lesions. Furthermore, individuals with genetic mutation affecting osteoclast activity have shown evidence of BRONJ lesions in the absence of BP administration [69]. However, it is not clear why this suppression results in the rapid progression of necrotic bone. In order to determine the underlying pathophysiology, more data is needed.

**Bisphosphonates and Animal Models:**

Because the pathogenesis of BRONJ has not been fully clarified, various researchers have aimed to create a reliable animal model for the purpose of progressing research capabilities. Mouse, rat and dog models have all demonstrated symptoms which are BRONJ-like [70-73]. In one mouse model for BRONJ-like disease, the development of necrotic bone and impaired soft tissue healing was shown to be dependent upon long-term high-dose BP use, immunosuppressants, chemotherapeutic drugs, as well as mechanical trauma [70]. Histological examination revealed suppressed angiogenesis within the extraction sockets with fewer capillaries in the provisional matrix, as well as a decreased prevalence of osteoclasts and osteoblasts. Based upon the authors’ observations, they hypothesized that the suppression of angiogenesis and bone remodeling may lead to the development of BRONJ-like disease in mice.

Rats injected with alendronate every 4 days for 14 injections, starting 2 days before tooth extraction showed a delay in new bone formation in the extraction site [71]. Similarly, Sonis et al. (2009) demonstrated that the administration of ZA and dexamethasone before dental extractions caused bony changes in the jaws of rats [72].
Specifically, there was histological evidence of ulceration overlying areas of necrosis, poor definition of the alveolar ridge with mixed radiodensity and increased inflammation.

A study was performed with the goal of establishing an animal model to replicate BRONJ in oncology patients [73]. Because patients with multiple myeloma are often given a regimen of ZA with concomitant dexamethasone, this study attempted to replicate this. When rats were treated with a sequence of ZA and dexamethasone during a 3-week period prior to a mandibular or maxillary extraction, they demonstrated BRONJ-like changes. They developed ulceration overlying areas of necrotic bone. The control animals, which were not given ZA nor dexamethasone, underwent predictable healing with rapid epithelialization.

While multiple groups have demonstrated the occurrence of BRONJ-like lesions in experimental models, it is important to note that currently no set standards exist for defining the requirements and parameters of a BRONJ lesion in an animal model. In addition while incidence of visible lesions range from 0-80%, no current studies have successfully demonstrated predictable lesions in all the experimental groups and none of the controls.

A study on 12 canines demonstrated IV administration of ZA prior to mini dental implant insertion was associated with significantly lower bone formation rates compared to the control group. Despite the reduction in bone formation, the remodeling rate was still substantially higher than it was for non-injured sites in the jaw [73]. However no patients receiving ZA had any lesions suggestive of BRONJ.

The overall goal of this group’s laboratory is to develop a reliable animal model of BRONJ that can be used by researchers to advance to understanding of the
pathophysiology of this disease. Because no grossly visible BRONJ lesions were observed in this study, we moved to evaluate the effect of the BP, ZA, on maxillary alveolar bone of aged female rice rats (*Oryzomys palustris*) when administered IV. Specifically, the study aims to answer the following 4 questions: 1) Are the maxillary molars in aged female rice rats equally affected by alveolar bone loss? 2) Does ZA affect the progression of alveolar bone loss in maxillary molars distant to dental trauma? 3) Is the effect of ZA altered by the amount of initial maxillary alveolar bone loss prior to treatment? and 4) Does ZA affect the progression of maxillary alveolar bone loss in teeth adjacent to dental trauma? Our hypothesis is that aged female rice rats treated with BPs will show a slower progression of maxillary alveolar bone loss compared to control rats both at sites adjacent to and distant from dental trauma.
Chapter 2: Materials and Methods

Thirty female rice rats (*Oryzomys palustris*) approximately five months of age were obtained through a non-commercial vendor (Indiana University South East, New Albany, IN). All animal procedures were performed under the guidelines approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC). The animals were obtained in solid bottom micro-isolator caging with corn cob bedding and given free choice Napa Nectar for transport. The animals were housed at The Ohio State University Laboratory Animals Resources (ULAR) satellite facility in ventilated racks. They were fed a high carbohydrate rat chow (Purina Feed 5001) and had access to automated reverse osmosis water *ad libitum*. The housing room was maintained on a 12:12 hour light:dark cycle, and temperature and humidity were maintained as per current ULAR guidelines. Certified polycarbonate mouse huts (Bioserv:Frenchtown, NJ) were provided for environmental enrichment. (Figure 1)
Figure 1: Female Rice Rat (Oryzomys palustris). These rats are a unique lab animal species previously shown to develop lesions similar to that of human periodontal disease. All rats were obtained through a non-commercial vendor (Indiana University South East, New Albany, IN).
The animals were in quarantine for 102 days. All rats tested negative via serology for RPV, H-1, RV, RMV, NS-1, Send, SCA/RCV, REO, MPUL, TMEV, LCMV, MRV, HTN, ECUN, CARB, and ROTA-B. Fur mites were identified via the presence of numerous eggs, however, no mite was able to be captured for positive identification. Treatment was initiated with dichlorvos placed in the bedding for three weeks, followed by two weeks of no treatment. Two rounds of therapy were required before the animals tested negative of fur mites and declared to pass quarantine by the ULAR veterinarians. No side effects were noted secondary to the dichlorvos treatment. Following quarantine the animals were transported to The Ohio State University Dorothy M. Davis Heart and Lung (H&L) Research Institute rodent facility.

The rats were housed for two weeks prior to beginning the study to allow for acclimation to the environment, as well as animal handling and injection training of the investigator (JC) by ULAR. The animals were randomly numbered via a random number generator. All procedures and treatments were performed on rats 1-15 on a given day followed by rats 16-30 the subsequent day. Performing all procedures/treatments on a single day was not possible due to the time required. As a result rats 16-30 were all one day behind on the study compared to rats 1-15. At the time the study began, the rats were approximately 9 months of age.

On day one of the study, the rats were anesthetized using isoflurane (1-5%) delivered via a face mask. The rats were approximately 9 months of age at this time. The animals were weighed and the ears were notched for identification. Sequential fluorochrome labels (20 mg/kg of alizarin red, administered as a 7.5% w/v solution in
sterile saline) were administered by intraperitoneal injection with an interval of 7 days between injections (see Appendix A for timeline).

On day 17 the animals were transported to the OSU Biomedical Research Tower where micro-computed tomography (µCT) scans of their skull were obtained using a Siemens µCT scanner (Siemens Inveon Preclinical microCT, Knoxville, TN). All images were acquired under the following protocol: 36.15 µm pixel size, 401 projections, 675 ms exposure, 500µA, 80 kVP, with a cone angle of 14.6104 degrees. This initial µCT will be identified as T1.

All rats were induced under general anesthesia with isoflurane and maintained at 3% v/v isoflurane in oxygen for the duration of the scan. During the procedure the animal was monitored via a live video feed and a respiratory sensor (Figure 2).

Figure 2: Preparation for µCT. The animal was maintained under anesthesia utilizing 3% isoflurane and respirations were monitored via a pressure sensitive pad.
The µCT images were reformatted and evaluated using Dolphin imaging 11.0 Premium. The 3-D reconstructions of each animal were subjectively evaluated by one member or the team (SH) with extensive experience within the field of periodontal disease. The rats were classified as mild, moderate or severe maxillary periodontal disease. No measurements were taken at this time for definitive classification. A random number generator was then used to divide the rats into two groups of 15 with each group containing equal representation of rats with mild, moderate, and severe periodontal disease. Group 1 was labeled as the control rats and consisted of rat numbers: 3, 4, 7, 8, 9, 11, 15, 16, 19, 22, 23, 24, 25, 29 and 30. Group 2 was labeled as the experimental rats and consisted of rat numbers: 1, 2, 5, 6, 10, 12, 13, 14, 17, 18, 20, 21, 26, 27, and 28.

The control group was given IV saline injections through the tail vein and the experimental group was administered 0.4 mg/kg zoledronic acid (ZA) (Zometa; Novartis, Stein, Switzerland) by IV injection through the tail vein. Immediately before injection, the ZA was diluted with saline to a final concentration of 0.25 mg/ml for injection. Each rat was anesthetized with inhaled isoflurane and body weights were recorded. A warmed DeltaPhase™ isothermal pad was placed on the tail vein for 30 seconds to allow for venous dilation.

Prior to the first injection of ZA, 200 microliters of blood was obtained via the tail vein. The serum was separated and frozen for later use. The initial set of injections occurred at one week intervals on days 32, 39, 46, 53, 60, 67, 74, and 81.

Surgical Extraction and Post-Surgical Care:

Prior to dental extractions, three formalin fixed rice rat cadaver heads were obtained. Surgical extraction of maxillary teeth was performed on the specimens by a
Diplomate of the American Board of Orthodontics (SH), A Diplomate of the American Board of Periodontology (DT), and a veterinary surgical resident (JC). Two of the team members had performed similar extraction on rice rats during the pilot study (SH & DT). Incisions around the tooth were performed using a number 11 scalpel blade. Extractions were performed using standard dental extraction forceps, as well as a custom made dental extraction forceps.

Surgical extraction of maxillary teeth was performed on day 85. The anesthesia and surgery protocol was based upon data from the previous pilot study. Animals were administered an intraperitoneal injection of ketamine (70 mg/kg) and dexmedetomidine (0.235mg/kg). All patients were administered oxygen as needed and were maintained on a warming pad with a trough (Carex Health Brands) during the procedure. After the completion of the procedure, the animals were reversed with an intramuscular injection of atipamezole at a dose of 2.5 mg/kg. They recovered on a warmed DeltaPhase isothermal pad and heat lamp until they were able to ambulate. This protocol reliably produced a plane of anesthesia that allowed the surgical extractions to be performed.

Each extraction was performed by two members of the study (SH and DT) who were blinded to the control and experimental groups. The maxillary extraction sites were randomly selected by SH. The only information available was access to the T1 µCT to allow for visualization of the root structure to aide in extraction. The buccal and palatal mucosa was incised using a number 11 scalpel blade. Dental explorer and extraction forceps were used to extract the tooth (Figure 3). Hemostasis was achieved using cotton tip applicators, dilute epinephrine (1:100,000) and Gelfoam (Pharmacia and Upjohn Company, Kalamazoo, Michigan). All teeth were saved for further analysis. Post-
operatively animals were given ibuprofen water at a concentration of 47 mg per 100ml water and the food was made into a gruel utilizing the same water. This was the only food and water made available during the seven day post-operative period.

Figure 3: Surgical extraction of maxillary molar. The animal was placed in dorsal recumbency. Buccal and palatal mucosa was incised using a number 11 scalpel blade. A dental explorer and extraction forceps was used to extract the tooth. Hemostasis was achieved using a cotton tip applicator, dilute epinephrine 1: 100,000 and an absorbable gelatin sponge (Gelfoam®).
Post-extraction µCT scan (T2) of the maxilla and mandible of all rice rats remaining in the study was obtained seven days after the surgical procedure (day 92). The rats were approximately 12 months of age at this time. Anesthesia and image acquisition was consistent with the previous description. All animals recovered without complications.

After the µCT, the remaining control and experimental animals continued to be injected with saline and ZA, respectively as previously described. These injections occurred at day 95, 102, 109 and 116. In addition a pair of calcein fluorochrome bone labels (20mg/kg) were administered IP to all animals on days 109 and 116.

On day 128, blood samples were obtained from each rat followed by euthanasia via carbon dioxide narcosis. 200ul of blood was separated and stored as previously described. The remainder of the blood was utilized for complete blood counts and biochemical profiles. The final µCT (T3) was immediately performed on the animal, after which the jaw and surrounding soft tissue were dissected out, photographed, and placed in a 10% neutral buffered formalin solution for preservation. The µCT acquisition protocol was as previously described. The rats were approximately 13 months of age at the conclusion of the study.

**Measurements:**

The reformatted DICOM images were evaluated using orthodontic 3-D imaging software (Dolphin Imaging 11.5 Premium, Chatsworth, CA). For animals completing the duration of the study, all three µCT time points were included in their portfolio. An individual not related to the study utilized a random number generator to reassign the images, in order to blind the evaluator (JC) to the control and experimental animals. 3-D
images of the maxilla were rendered for each animal (Figure 4). A single evaluator (JC) was responsible for obtaining all measurements.

For each 3-D rendering, the image was orientated to align the maxillary occlusal surface parallel to the axial slice in the front, right and left views (Figure 4). In addition, the image was aligned in the midsagittal plane. The dental formula of the maxillary teeth in rats consists of one incisor and three molars on both the right and left hemimaxilla. Each of the maxillary teeth have three roots: one mesial buccal, one palatal and one distal buccal. For purposes of this study, alveolar bone coverage of each root was determined at each time point. Both the coronal and sagittal slices were aligned to run through the long axis of the root being evaluated. This reorientation is critical for consistency of measurements between the three time points (Figure 5).
Figure 5: A) Proper orientation prior to measurement of the mesial crest of the mesiobuccal root (T1, M1 rat number 16). Reorientation of the sagittal and coronal slice through the long axis of the tooth must occur prior to the measurement of each root. Failure to do so prevents accurate measurement from being obtained. B) Screen overlay to ensure measurements are parallel to the long axes of the root.
Measurements were obtained at the mesial crest of the mesiobuccal roots, the
distal crest of the distobuccal root, and the mesial crest of the palatal roots on the sagittal
slice. Linear measurements were taken to the hundredth of a millimeter from the
cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) at each of the
aforementioned root locations. In the same orientation, the root length (RL) was
measured in hundredths of a millimeter from the CEJ to the root apex (RA) parallel to the
long axis of the tooth. A transparent overlay with perpendicular lines was used to
enhance accuracy of measurements (Figure 6). The percent of bone coverage was then
calculated by the following equation for each root: ((RL-distance from CEJ to
ABC))/RL x 100.

In order to maximize accuracy, measurements were obtained for all three roots on a
given tooth for T1, T2, and T3 in consecutive order without interruption. For each tooth,
the percent of alveolar bone coverage on the sagittal slice was calculated by averaging the
three roots.
Figure 6: Measurements: A) The root tip, alveolar bone crest and cementum enamel junction (CEJ). B) Root length obtained by measuring from the CEJ to the root tip. C) Alveolar bone loss is measured from the CEJ to the alveolar bone crest.
**Measured Initial Alveolar Bone Loss:**

This effect of BPs on periodontal disease was further evaluated to determine if the initial severity of periodontal disease had an effect on outcome. Initial periodontal disease was determined based upon the T1 µCT scan and were divided into three categories. Class one consisted of more than 75% alveolar bone coverage and was referred to as mild periodontal disease. Class 2 consisted of 75-50% alveolar bone coverage and was referred to as moderate periodontal disease. Class 3 consisted of less than 50% alveolar bone coverage and was referred to as severe periodontal disease.

**Image Resolution:**

All measurements were evaluated using 3-voxel slice thickness with the program set to high resolution. All measurements were obtained using the same high resolution computer screen with the resolution set at 1920 x 1080. Images were acquired with a voxel size of (36.15 µm)³. No down sampling of data occurred during reformatting of the DICOM images. In order to determine intra-observer reliability, alveolar bone coverage was measured 15 separate times for two teeth.

**Post Study Oral Evaluation:**

At the time of the study conclusion, all maxilla were disarticulated from the mandible and evaluated for oral lesions by a member of the team (SH) with experience in BRONJ oral lesion identification in rice rats. In addition all maxilla were photographed for future evaluation.

**Statistical Evaluation:**

Statistical Analysis was performed using commercially available software (SPSS v. 20, IBM, Armonk, New York). Intra-class correlation coefficient was calculated to
determine intra-observer reliability. Normality was tested using the Kolmogorov-Smirnov test. Change in weight and percentage alveolar bone coverage was determined using the non-parametric Mann-Whitney Rank sum test where the p-values were adjusted using the Holm’s procedure to conserve the type I error at 0.05 due to multiple comparisons. Wilcoxon Signed-Rank test was used to evaluate change in weight during the course of the experiment. The distribution of extraction sites amongst molar teeth in the rice rats were evaluated using the Fisher exact test. Data is reported in the following format: mean (standard deviation). A p-value <0.05 was considered significant for all tests.
Chapter 3: Results

Thirty aged female rice rats were enrolled in the study and divided into 15 control and 15 experimental animals. 11 controls and 7 experimental animals survived to the conclusion of the study. Only those rats that survived the duration of the study were included in the analysis. Teeth too severely damaged during the extraction to obtain measurements were not included in the analysis. There were no statistically significant differences in the location of teeth extracted or remaining (Table 1).

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<td>5</td>
<td>9</td>
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</table>

Table 1: Distribution of extraction sites amongst maxillary molar teeth in rice rats. There was no statistically significant differences in the ratio of teeth extracted to teeth remaining between the control and experimental rats.
On day 1 of the study the mean weight of the animals was 54.7 grams with a range from 40-65g. Weight was not used when assigning groups and did not significantly differ between the two groups, control: 54.6g, experimental: 55.0g (p=0.928). During the duration of the study, mean weight gain in the control and experimental groups was 4.1g and 3.6g, respectively. These values were not significantly different (p=0.964). Only one control and two experimental rats lost weight during the study time period.

Prior to surgery none of the control rats experienced any complications other than rat four which had increased respiratory efforts on day 81. In the experimental group, rat number 18 experienced cardiac arrest one minute post injection of zoledronic acid on day 53. The cause of the cardiac arrest was not apparent. Additionally, mild to moderate necrosis of the tail at and distal to the injection site was observed on all experimental rats at some point during study time period. This necrosis was not observed in the control group (Figure 7).

Figure 7: Example of tail necrosis in a rat treated with zoledronic acid.
Four control and seven experimental rats died during recovery from the surgical tooth extraction. No excessive hemorrhage was noted, and in all but one control rat there was no apparent cause for their deaths. Increased respiratory effort in rat number 4 had previously been observed and this progressed likely resulting in the rat’s death during the recovery period.

Intra-observer reliability was calculated by measuring the percent alveolar bone coverage of the right M1 and M2 on the first scan of rat number 22. Each of these two measurements was taken a total of 15 times. Intra-observer reliability was found to be excellent with an intra-class correlation coefficient (95% confidence interval) of 0.965 (0.828, 1.000). The relative uncertainty was 0.635%.

All extraction sites healed without complication based upon visual evaluation at the time of study conclusion. Further evaluation of photographs by a member of the team experienced in identifying BRONJ lesions in rice rats (SH) failed to identify any mucosal defects in either the experimental or control group. (Photographs in Appendix B)

For the initial evaluation of alveolar bone coverage of maxillary molars, 36 first molars (M1), 35 second molars (M2), and 36 third molars (M3) were available for evaluation. One M2 was unable to be evaluated as a result of the tooth being fractured prior to the beginning of the study. The cause of this fracture was unknown. Mean (SD) for the percentage of alveolar coverage of maxillary molars was as follows: M1: 67.7% (14.7%); M2: 48.2% (27.6%); and M3: 48.0% (27.8%). M1 was found to have significantly less periodontal disease compared to M2 (p= 0.006) and M3 (p=0.006). However, M2 and M3 were not found to statistically differ (p= 0.859).
The effect of BPs on the progression of maxillary alveolar bone loss in sites distant from dental extraction was evaluated by comparing the T3 to T1 µCT scan. A total of 27 control teeth and 21 experimental teeth were available for analysis. The control rats were found to have a mean (SD) decrease in alveolar bone coverage of 6.95% (5.74%). The experimental rats were found to have a mean (SD) increase in alveolar bone coverage of 0.61% (3.40%). These values differed statistically at p<0.001 (Figure 8).
Figure 8: Box and whisker plot for change in alveolar bone coverage of maxillary molars distant to dental extraction from T1-T3 μCT. The two groups were found to statistically differ. For each box, the diamond represents the median value and the lower and upper boundaries represent 25th and 75 percentiles, respectively. Whiskers represent the most extreme values.
This effect of BPs on alveolar bone loss associated with maxillary molars was further evaluated to determine if the initial severity of periodontal disease had an effect on outcome. Initial periodontal disease was determined based upon the T1 µCT scan and classified according to the previous description. Change in alveolar bone coverage is as follows: 12 control, mean (SD): -8.20% (4.06%), and seven experimental, mean (SD): 2.66% (2.34%) teeth were identified to have class 1 periodontal disease. The difference between groups was found to be statistically significant with p<0.001. Class 2 periodontal disease was observed in six controls, mean (SD): -7.64% (9.07%) and 8 experimental, mean (SD): 0.35% (4.07%), which were not statistically different (p=0.06). Class three periodontal disease was present in 9 controls, mean -5.96% (4.85%) and 6 experimental, mean (SD): 0.75% (4.04%). These values were not found to statistically differ (p=0.078) (Figure 9). Unfortunately, insufficient sample size did not allow for statistical analysis of periodontal disease of alveolar bone associated with molars adjacent to extraction sites.
Figure 9: Box and whisker plot for change in alveolar bone coverage of maxillary molars distant to dental extraction as a function of initial alveolar bone coverage. µCT from T1 to T3 was compared. A significant difference was found between the control and experimental groups with an initial periodontal disease of class 1. For each box, the diamond represents the median value and the lower and upper boundaries represent 25th and 75 percentiles, respectively. Whiskers represent the most extreme values.
The final component of our study evaluated the effect of BPs on the progression of alveolar bone loss in sites adjacent to dental extractions. Because adjacent extractions could result in direct damage to the alveolar bone on the remaining tooth itself, this effect was evaluated by comparing the µCT scans at T2 and T3. A total of 13 control maxillary molars, mean change in coverage (SD): -3.12% (5.51%) and six experimental maxillary molars 7.26% (3.75%) teeth were available for evaluation. These values were found to statistically differ with p= 0.006. In order to compare the impact of the adjacent extraction, this data was compared to teeth not adjacent to extraction sites from the T3 to T2 µCT. For this set of data, control molars were found to have a mean decrease of 1.81% (3.20%) in coverage and experimental molars were found to have a mean increase of 1.87% (2.41%) in alveolar coverage. Molars distant to extraction sites and adjacent to extraction sites were compared for both controls and experimental rats. While there was no significant difference between the controls (p=0.623), animals given ZA were found to have significantly more alveolar bone associated with molars adjacent to dental trauma compared to alveolar bone associated with molars not adjacent to trauma (p=0.025) (Figure 10).

Biochemical profiles of all rats at the study conclusion were within normal limits for both the control and experimental animals. A trend was observed for a slightly higher blood urea nitrogen in the experimental rats, mean 25.1mg/dl (3.48mg/dl) as compared to the control rats 19.5mg/dl (7.01mg/dl), however statistical significance was not found (p=0.065). Appendix C represents an overview of alveolar bone coverage for each maxillary tooth over the study time period.
Figure 10: Box and whisker plot for change in alveolar bone coverage of maxillary molars from µCT T2 to T3. No difference was found between non-extracted and extracted molars in the control group. A significant difference was identified between non-extracted and extracted molars in the experimental group. For each box, the diamond represents the mean value and the lower and upper boundaries represent 25th and 75 percentiles, respectively. Whiskers represent the most extreme values.
Chapter 4: Discussion

The purpose of this study was to develop an animal model to further evaluate the uncommon, but serious condition known as BRONJ. Although animals were treated with very high concentrations of the potent bisphosphonate, zoledronic acid, we did not identify gross lesions suggestive of BRONJ in any of these rats. For the purpose of this thesis, we have therefore elected to focus our attention on better characterizing the effects of BP on maxillary alveolar bone in rice rats. Our primary aim was to determine whether ZA modulates the progression of alveolar bone loss in the face of two factors that have been shown to impact the incidence of BRONJ in humans: periodontal disease and dental trauma [62, 74].

Rice rats were selected as they have been previously shown to develop lesions similar to that of moderate periodontitis in humans [75, 76]. In 1981, a rice rat model was utilized to determine if subcutaneous injection of the non-nitrogen containing BP, clodronate, could slow the progression of periodontal disease [77]. This study found a reduction in alveolar bone loss as well as an abnormal morphologic pattern in the alveolar bone of the treated animals. While BRONJ lesions are currently not considered to be associated with non-nitrogen containing BPs, it is possible that these observations may have been the initial accounts of experimentally induced BRONJ lesions. In addition, a recent study successfully utilized oncologic doses of ZA to induce lesions characterized
by areas of exposed necrotic alveolar bone, osteolysis, honeycomb-like appearance of the alveolar bone, presence of bacterial colonies and periodontal tissue destruction [78]. The study further found decreases in blood vessel numbers in alveolar bone in the treated rats. The authors suggested that the ZA exacerbates the inflammatory response and periodontal tissue damage in rice rats resulting in lesions that resemble BRONJ. Our study differs from the previous mentioned study as well as other in several aspects. First, rice rats in this study were not placed on a special high sucrose diet. The occurrence of periodontal disease was naturally occurring in this species. Secondly, we utilized aged female rice rats. Third, no adjunctive therapy in addition to the ZA was administered. Our goal was to develop a model where the effects can be directly linked to ZA administration and tooth extraction in a naturally occurring model of human periodontal disease.

Four control and eight experimental animals died unexpectedly before the end of the study period. All but two (one experimental and one control) expired at the time of surgery with no known underlying pathology. The experimental rat expired within one minute after an injection of ZA on day 53. No apparent health abnormalities were noted and the cause of this death was not apparent. One suggestion for such death is the accidental injection of air resulting in an air embolism. The control rat developed increased respiratory effort on day 81 that progressed over the following four days and likely was the result of death at the time of surgery. A necropsy was not performed and thus the cause of the death could not be definitively determined. The remaining rats expired during the 30 minute recovery from surgery. Clinical signs consisted of animals beginning to awaken followed by increased respiratory effort then respiratory arrest. One
hypothesis for these deaths includes excessive hemorrhage. While this was not reported by the surgeons, exact blood loss was not quantified. Another hypothesis is the aspiration of blood, as the airway was not protected during the procedure or recovery. The presence of an underlying pathology in these rats is also a possible contributing factor, and coincident with these deaths in our colony another investigator experienced animal losses due to a respiratory pathogen. However, we were not able to confirm this in our animals.

The nature of this evaluation was focused on \( \mu \text{CT} \) analysis to evaluate the effect of alveolar bone associated with maxillary molars. The first part of our study confirmed the presence of naturally occurring periodontal disease in rice rats. Unlike previous publications, these changes were naturally occurring and not induced by feeding a high sucrose diet. In addition we found that the degree of naturally occurring periodontal disease was less in M1 compared to M2 and M3. This variance within a single animal serves as an excellent internal control for evaluating the effect of initial periodontal disease.

We found administration of ZA prevented alveolar bone loss in sites distant to dental extraction. This finding is consistent with ZA’s primary mechanism of action involving the inhibition of osteoclasts \([3, 4]\). In this situation normal alveolar trabecular bone turnover is in a catabolic state, thus suppressing this activity would slow down resorption. Additionally, we found the animals treated with ZA had a tendency to lay down additional alveolar bone. The exact effect of BP on osteoblasts is still highly debated. Evaluation of the microstructure of this new bone is needed to evaluate its integrity as it has been shown that BRONJ-like lesions often have empty osteocyte
lacunae [78]. In addition this new bone may contain high levels of BP, resulting in a
direct toxic and antiangiogenic effect on the mucosa [12].

The characterization of periodontal disease into three classes was used to assess
the effect initial periodontal disease had on ZA’s ability to alter maxillary alveolar bone
coverage. In this species of animal, the naturally occurring space from the CEJ to the
ABC in non-disease teeth is currently unknown. As a result our classification scheme was
based solely on comparative evaluation between three degrees of alveolar bone coverage
in rice rats. Utilizing our classification scheme, we found that ZA was most effective in
slowing the progression of periodontal disease in the presence of mild periodontitis
(>75% alveolar bone coverage). While molars with moderate and severe periodontal
disease did not show a significant reduction in the progression of disease with ZA, a trend
for reduction was present. Since BPs serve to protect bone from being resorbed, it is not
surprising that we confirmed the efficacy of ZA was most evident in situations where
there is significant residual bone stock at the time of treatment. Once bone has been lost,
as is the case with advanced periodontal disease, it is unlikely that any anti-resorptive
therapy would be effective. Anabolic therapy would be needed to restore bone mass in
this situation.

As previously discussed, dental trauma mainly in the form of tooth extraction is
currently considered the primary event that precedes the development of BRONJ [79]. To
investigate this, we evaluated the effect of ZA on alveolar bone coverage associated with
molars adjacent to extraction sites. Our data demonstrated that BPs suppressed the
progression of alveolar bone loss in this situation. This information is important in the
development of an animal model of BRONJ because it evaluates the effect in an anabolic
environment with tissue healing. Previous studies evaluating ZA on bone healing subsequent to mini-implant insertion in a canine model found bone remodeling to be elevated compared to baseline, but diminished compared to implant sites in control dogs [79]. These results are comparable to that seen in our study.

In the current study, maxillary teeth were evaluated. This was utilized as surgical extraction of mandibular teeth is challenging secondary to the divergent nature and formation of an osseous attachment of the adult teeth to the surrounding alveolar bone. As a result, fracture of the mandible itself is common and necessitates the rat being removed from the study. While both human and rat model studies have demonstrated an approximately two-fold greater occurrence of BRONJ in the mandible compared to the maxilla, our pilot study suggested formation of BRONJ lesions could be reliably created in the maxilla of our rice rats [80]. Further analysis comparing the maxillary and mandibular alveolar bone in rice rats may serve as a basis for understanding this disparity. However, the degree of injury and lack of extraction sites in our animal model did not allow for this comparison.

This study had several limitations. First was the observation of mild to moderate tail necrosis associated distal to the site of injection in rats treated with ZA. While the exact nature of injury was unknown, the lesion appeared to be secondary to vascular compromise. In human a total does of 4 mg is recommended to be given as a dilute constant rate infusion, in no less than 15 minutes. It is possible that our rapid infusion of a high concentration caused direct injury to the endothelium itself, inhibiting normal blood flow to the tip of the tail. Two alternatives can help to reduce this occurrence in future studies. First the placement of an intravenous access port would have helped to
minimize perivascular administration as well as reduced or possibly eliminated the need for anesthetic events to administer the injection. Alternatively, recent studies have demonstrated safe and effective subcutaneous dosing in juvenile rice rats [78]. This too would hopefully eliminate the need for repeated anesthetic events. It should be noted that despite the potential concerns over the efficiency of drug absorption following IV injection, preliminary reviews of histology sections prepared from the tibiae of these animals confirmed that there was a robust anti-resorptive effect in the ZA-treated animals. Definitive proof of the extent of this effect will come from detailed histomorphometric analysis of these sections, and this work is currently ongoing.

A second important limitations lies in the unanticipated loss of over 50% of the experimental animals. We did not see these losses in the original pilot study and do not anticipate that it relates to the surgical model per se. We will continue to refine the anesthetic and surgical procedures for dental extraction in rice rats. We will also ensure that necropsy examinations are performed on any rat that dies unexpectedly.

Another important potential limitation with this study relates to the selection of the drug dose and duration of therapy. Although based on encouraging preliminary results from a pilot study, the optimal dose, frequency and duration of ZA therapy required to induce BRONJ lesions in rice rats have not been determined. In stark contrast with other studies using immature, growing rats [78] this study was performed in skeletally mature animals. Additional work should be performed to determine whether age-related differences in bone turnover play a role in determining the sensitivity of alveolar bone to ZA therapy. In addition, there may be important differences between mandibular and maxillary alveolar bone in this regard.
By clinical definition, BRONJ refers only to an area of exposed bone in the maxillofacial region. However, substantial clinical reports in humans have documented additional features including osteolysis, osteosclerosis, presence of woven bone, honeycomb-like appearance of affected areas of jaw bone, and pathologic fracture [78]. In the current study, no grossly visible lesions were present at the conclusion. There are several reasons this may have occurred. First the current study utilized female rice rats as compared to the pilot study which utilized male rice rats. It is possible that sex related differences resulted in the suppression of the development of gross lesions. Secondly it is possible that we did not allow enough time post extraction to allow for the development of grossly visible oral lesions. However in addition to grossly visible oral lesions BRONJ has been characterized by many other histological factors. Clearly the next critical step in this project is to perform detailed histomorphometric analysis of the extraction sites. This evaluation is currently underway, but not available for inclusion into this thesis.

In conclusion this study confirmed rice rats (*Oryzomys palustris*) are a good animal model of periodontal disease. We demonstrated the administration of ZA resulted in a significant reduction in the rate of maxillary alveolar bone loss associated with the molars. This effect was present at sites both adjacent to and remote from extraction sites. However, as the baseline severity of periodontal disease increased, the effect of the BP decreased. While this data suggests rice rats serve as an effective model for studying changes in alveolar bone turnover, additional evaluation such as immunohistochemistry will be needed to determine if subclinical BRONJ lesions occurred with ZA administration. The documentation of such subclinical disease would be extremely
helpful in the development of early BRONJ detection and may lead to the ability to apply early therapeutic intervention.
Bibliography


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Appendix A: Study Timeline
Study Timeline

Acquisition of Animals:

- **arrival**
  - 30 female rice rats arrive to quarantine (approx 5 months of age)

- **Week 14**
  - Animals released from quarantine.

- **Week 16**
  - Begin Study (approx 9 months of age)

Study:

- **Day 1**
  - Alizarin bone label given IP

- **Day 7**
  - Second Alizarin bone label given IP

- **Day 17**
  - First μCT scan performed (approx 9.5 months of age)
Study Cont.

Day 32: Randomize 30 rats to control and experimental groups (N=15 per group). Begin weekly injection of saline or ZA for 8 weeks.


Day 92: Post-operative μCT scan (T2) (approx 12 months of age)

Day 95: Resumed weekly injection of saline of ZA for 4 weeks. Calcein bone labels also given on days 109 and 116.

Day 109: Calcein bone label given IP

Day 116: Second calcein bone label given IP

Day 128: Study conclusion. Euthanasia and final μCT (T3) (approx. 13 months of age)
Appendix B: Photograph of Maxilla at Study Conclusion
Control Rats Conclusion

Rat 3

Rat 7

Rat 8

Rat 9

Rat 11

Rat 15

Photo Not Available
Control Rats Conclusion

Rat 16

Rat 19

Rat 22

Photo Not Available

Rat 23

Rat 30

Photo Not Available
Experimental Rats Conclusion
Experimental Rats Conclusion

Rat 17
Appendix C: Overview of Alveolar Bone Coverage
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<th>Right M2</th>
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### Rat 3

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T1: M1: 71.75%, M2: 66.78%, M3: 73.36%
T2: M1: 71.60%, M2: 57.54%, M3: 72.84%
T3: M1: 68.35%, M2: 59.74%, M3: 75.40%
Overall | Left M1 | Left M2 | Left M3 | Right M1 | Right M2 | Right M3
---|---|---|---|---|---|---
T3-T1= | 6.61% | -4.11% | 0.19% | 3.45% |
T3-T2= | 7.69% | 0.70% | 4.64% | 6.53% |
Rat 7

Control

T1

M1: 68.53%
M2: 18.28%
M3: 25.99%

T2

M1: 62.46%
M2: Extracted
M3: 24.93%

T3

M1: 64.13%
M2: Extracted
M3: 24.65%

Overall

<table>
<thead>
<tr>
<th></th>
<th>Left M1</th>
<th>Left M2</th>
<th>Left M3</th>
<th>Right M1</th>
<th>Right M2</th>
<th>Right M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3-T1=</td>
<td>-4.40%</td>
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<td>-1.34%</td>
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<td></td>
<td>-21.27%</td>
</tr>
<tr>
<td>T3-T2=</td>
<td>1.67%</td>
<td></td>
<td>-0.28%</td>
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<td></td>
<td>-0.65%</td>
</tr>
</tbody>
</table>

66
<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Left M1</th>
<th>Left M2</th>
<th>Left M3</th>
<th>Right M1</th>
<th>Right M2</th>
<th>Right M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3-T1=</td>
<td>-3.62%</td>
<td></td>
<td></td>
<td></td>
<td>-2.21%</td>
<td>-9.50%</td>
<td>-2.24%</td>
</tr>
<tr>
<td>T3-T2=</td>
<td>-4.20%</td>
<td></td>
<td></td>
<td></td>
<td>-3.32%</td>
<td>-2.45%</td>
<td>-2.27%</td>
</tr>
<tr>
<td>Overall</td>
<td>Left M1</td>
<td>Left M2</td>
<td>Left M3</td>
<td>Right M1</td>
<td>Right M2</td>
<td>Right M3</td>
<td></td>
</tr>
<tr>
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<td>---------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>T3-T1=</td>
<td>-8.33%</td>
<td>-18.49%</td>
<td>-5.71%</td>
<td>-7.26%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3-T2=</td>
<td>-4.78%</td>
<td>-5.98%</td>
<td>-5.08%</td>
<td>-4.65%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Overall Left M1 Left M2 Left M3 Right M1 Right M2 Right M3
<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>T3-T1=</td>
<td>-1.05%</td>
<td>-1.83%</td>
<td>-4.20%</td>
<td>-1.66%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3-T2=</td>
<td>-0.43%</td>
<td>2.24%</td>
<td>-3.46%</td>
<td>1.38%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Rat 10**

**Experimental**

**LEFT**

**Cranial**

- **T1**
  - M1: 74.52%
  - M2: 72.29%
  - M3: 61.93%

- **T2**
  - M1: 73.90%
  - M2: 68.23%
  - M3: 61.19%

- **T3**
  - M1: 73.47%
  - M2: 70.46%
  - M3: 57.73%

**CAUDAL**

- **T1**
  - M1: 50.14%
  - M2: Extracted
  - M3: Extracted

- **T2**
  - M1: 51.52%
  - M2: Extracted
  - M3: Extracted

- **T3**
  - M1: 51.52%
  - M2: Extracted
  - M3: Extracted
### Rat 11

#### Control

<table>
<thead>
<tr>
<th>Time</th>
<th>Overall</th>
<th>LEFT</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>RIGHT</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td></td>
<td></td>
<td>30.60%</td>
<td>0.00%</td>
<td>4.23%</td>
<td></td>
<td>64.04%</td>
<td>14.97%</td>
<td>0.00%</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
<td>32.02%</td>
<td>0.00%</td>
<td>Extracted</td>
<td></td>
<td>46.09%</td>
<td>10.28%</td>
<td>Extracted</td>
</tr>
<tr>
<td>T3</td>
<td></td>
<td></td>
<td>28.96%</td>
<td>0.00%</td>
<td>Extracted</td>
<td></td>
<td>3.82%</td>
<td>40.28%</td>
<td>7.69%</td>
</tr>
</tbody>
</table>

#### Summary

<table>
<thead>
<tr>
<th>Time</th>
<th>T3-T1</th>
<th>T3-T2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1.64%</td>
<td>-3.06%</td>
</tr>
</tbody>
</table>

---

**Cranial vs. Caudal**

- **Cranial**:
  - Left: M1: 30.60%, M2: 0.00%, M3: 4.23%
  - Right: M1: 64.04%, M2: 14.97%, M3: 0.00%

- **Caudal**:
  - Left: M1: 32.02%, M2: Extracted, M3: Extracted
  - Right: M1: 46.09%, M2: 10.28%, M3: 0.00%
### Rat 12

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Overall</th>
<th>Left M1</th>
<th>Left M2</th>
<th>Left M3</th>
<th>Right M1</th>
<th>Right M2</th>
<th>Right M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3-T1=</td>
<td>3.85%</td>
<td>8.15%</td>
<td>-1.90%</td>
<td>1.63%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3-T2=</td>
<td>2.63%</td>
<td>3.33%</td>
<td>2.02%</td>
<td>2.19%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cranial and Caudal views are shown for each timepoint (T1, T2, T3). The percentages represent the distribution of certain features or measurements in each view.
### Rat 13

**Experimental**

#### LEFT

- **M1:** 72.37%
- **M2:** 39.49%
- **M3:** 27.76%

#### RIGHT

- **M1:** 78.03%
- **M2:** 49.98%
- **M3:** 48.82%

### Overall Changes

<table>
<thead>
<tr>
<th></th>
<th>Left M1</th>
<th>Left M2</th>
<th>Left M3</th>
<th>Right M1</th>
<th>Right M2</th>
<th>Right M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3-T1=</td>
<td>3.46%</td>
<td></td>
<td></td>
<td>1.97%</td>
<td>1.57%</td>
<td>-3.85%</td>
</tr>
<tr>
<td>T3-T2=</td>
<td>5.02%</td>
<td></td>
<td></td>
<td>4.05%</td>
<td>2.91%</td>
<td>-1.35%</td>
</tr>
</tbody>
</table>

72
Overall | Left M1 | Left M2 | Left M3 | Right M1 | Right M2 | Right M3
---|---|---|---|---|---|---
T3-T1= | -18.01% |
T3-T2= | -14.40% |
### Rat 16

**Control**

<table>
<thead>
<tr>
<th>Time</th>
<th>Overall</th>
<th>Left M1</th>
<th>Left M2</th>
<th>Left M3</th>
<th>Right M1</th>
<th>Right M2</th>
<th>Right M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3-T1</td>
<td>-13.19%</td>
<td></td>
<td></td>
<td>-4.75%</td>
<td>-1.64%</td>
<td>0.19%</td>
<td></td>
</tr>
<tr>
<td>T3-T2</td>
<td>-7.74%</td>
<td></td>
<td></td>
<td>-5.38%</td>
<td>-1.98%</td>
<td>-0.09%</td>
<td></td>
</tr>
</tbody>
</table>

---

![Diagram of Tooth Development](image-url)
Rat 17

**LEFT**

M1: 75.44%
M2: 76.82%
M3: 82.89%

**T1**

M1: 76.73%
M3: 82.17%
M2: 77.24%

**T2**

M1: 75.44%
M3: 82.89%
M2: 76.82%

**T3**

M1: 78.88%
M3: 86.11%
M2: 84.20%

**RIGHT**

M3: 27.83%
M1: 69.95%
M2: 30.62%

M3: Extracted
M1: 69.76%
M2: Extracted

M3: Extracted
M1: 75.47%
M2: Extracted

<table>
<thead>
<tr>
<th>Overall</th>
<th>Left M1</th>
<th>Left M2</th>
<th>Left M3</th>
<th>Right M1</th>
<th>Right M2</th>
<th>Right M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3-T1=</td>
<td>2.15%</td>
<td>6.96%</td>
<td>3.95%</td>
<td>5.51%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3-T2=</td>
<td>3.44%</td>
<td>7.38%</td>
<td>3.22%</td>
<td>5.71%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>Left M1</td>
<td>Left M2</td>
<td>Left M3</td>
<td>Right M1</td>
<td>Right M2</td>
<td>Right M3</td>
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<td>---------</td>
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<td>----------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>T3-T1=</td>
<td>2.09%</td>
<td>-11.50%</td>
<td>-8.55%</td>
<td>-1.62%</td>
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<td></td>
</tr>
<tr>
<td>T3-T2=</td>
<td>8.71%</td>
<td>-1.32%</td>
<td>-1.75%</td>
<td>2.96%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>Left M1</td>
<td>Left M2</td>
<td>Left M3</td>
<td>Right M1</td>
<td>Right M2</td>
<td>Right M3</td>
</tr>
<tr>
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<td>---------</td>
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<td>-0.22%</td>
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<td>0.29%</td>
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</table>

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