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Comparing Local and Systemic Delivery of Bisphosphonate in Enhancing Bone Graft Success and A Pilot Study on Evaluating Bisphosphonate's Effect on Tooth Eruption



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# Comparing Local and Systemic Delivery of Bisphosphonate in Enhancing Bone Graft Success and A Pilot Study on Evaluating Bisphosphonate's Effect on Tooth Eruption

A thesis submitted in partial satisfaction of the requirements

for the degree Master of Science in Oral Biology

by

Alison Jessie Quach

2016

ABSTRACT OF THE THESIS

Comparing Local and Systemic Delivery of Bisphosphonate
in Enhancing Bone Graft Success and
A Pilot Study on Evaluating Bisphosphonate's Effect on Tooth Eruption

by

Alison Jessie Quach
Master of Science in Oral Biology
University of California, Los Angeles, 2016
Professor Yeumin Hong, Chair

Cleft lip with or without palate (CLP) is the most common craniofacial congenital malformation to occur worldwide. CLP patients experience a combination of problems that require multi-disciplinary treatment, including orthodontics. In order to complete orthodontic treatment, up to 75% of cleft lip and/or palate patients require bone grafting. However, bone grafts are susceptible to failure due to loss of the graft material from excessive resorption. Insufficient bone volume in the cleft region then necessitates additional treatment which has a number of adverse consequences including surgical morbidity, lengthened overall orthodontic treatment duration and associated dental problems, decline in patient's mental health, and increased financial burden. New treatment modalities are needed to improve the clinical success of bone grafting in CLP treatment due to the unpredictability of current methods.

Bisphosphonates (BPs) have found a therapeutic role in treating osteoporosis and other bone-loss conditions owing to their main inhibitory activity on osteoclasts. In previous studies, BPs have shown to enhance bone volume fraction and graft incorporation, although, use in an intraoral model has not been studied extensively. In a bone-grafted, mid-palatal defect model, both local and systemic applications of Zoledronate resulted in higher bone volume fractions and bone mineral densities (BMD) compared to control (BV/TV: 69% and 63% vs. 39%; BMD: 0.63 and 0.59 vs. 0.41 g/cm^3, respectively). Increased bone graft retention and incorporation with new bone formation were observed in treated groups. However, there was no statistically significant difference between the systemic and local treatment groups. Although osteoclast numbers did not differ among the three groups, serum TRAP-5b levels confirmed that systemic administration, but not local delivery, had inhibitory effects on osteoclasts throughout the body. Furthermore, a single, low-dose, systemic application of ZA at 7 days of age delayed the eruption of the first and second molars in rat pups. Utilizing bisphosphonates in enhancing bone formation and limiting bone resorption proves to be a superior treatment method in achieving better outcomes in cleft lip and/or palate patients. In conjunction, local delivery may offer several advantages, such as ease of application and limiting systemic effects, over systemic administration.

The thesis of Alison Jessie Quach is approved.

Reuben Han-Kyu Kim

Sotirios Tetradis

Yeumin Hong, Committee Chair

University of California, Los Angeles

2016

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# 1. INTRODUCTION

## 1.1 Cleft lip ± palate (CLP): Definition, etiology, and incidence

Cleft lip with or without palate (CLP) is a craniofacial aberration that results from an interruption of lip and palate development during the eighth through twelve week of fetal development. Defective palatal shelf growth, delayed shelf elevation, or failure of fusion of the medial nasal processes, maxillary processes, or palatal shelves can lead to clefting[1]. Clefts may manifest as part of a syndrome, such as van der Woude, or more commonly, present as isolated cases in 70% of patients[2]. Despite the strong genetic basis of CLP with several specific gene loci having been identified, purely genetic causes account for only a number of cases. Additional studies have shown an association with a variety of environmental factors and teratogens, including exposure to drugs and chemicals during pregnancy and maternal health[2,3]. Thus, the aetiology of CLP is complex and thought to be multifactorial with shared interactions between both genetic and environmental components.

CLP rivals Down syndrome as the most frequently occurring birth defect. The prevalence of CLP varies greatly among geographical and ethnical origin, environmental exposure, and socioeconomic status. Furthermore, epidemiology studies quote a wide incidence rate of CLP due to differing data acquisition methods and whether cases were actually conveyed to the monitoring institutions. Most recent studies have stated worldwide rates range anywhere from 1/525 to 1/3195 live births with the highest incidence arising in the Asian and Native American populations[4]. In the United States, depending on the study, the average prevalence have been reported to range anywhere from 1 out of 700[2] to more recently 1 in every 1290 live births[4,5]. A multitude of problems are associated with clefts patients including poor facial growth and esthetics, feeding and swallowing difficulties, delayed speech and language development,

hearing loss, dental anomalies and malocclusion, and low psychosocial health[6]. Although rehabilitation is possible, patients with CLP are seen to have higher mortality rates, especially during the first years of life and in areas lacking access to health services, and suffer significant morbidities that lower their quality of health and place substantial financial burden on the families and society[7-9]. Comprehensive team care is essential to the rehabilitation process which includes multiple, intermittent surgeries and interdisciplinary care starting from birth through to adulthood[10-12].

### 1.2 Orthodontic treatment for CLP patients

The orthodontic treatment for cleft lip and palate patient has been continuously changing as new evidence emerges, but can be separated into four main time periods: neonatal/infancy, primary dentition, mixed dentition, and permanent dentition. Infant and early treatment continues to be controversial while contemporary treatment during when the mixed and permanent dentitions is well established[6,13,14]. Phase I orthodontic treatment, which is carried out between the ages of 7-12 years old[12,13], attempts to address considerable dental and skeletal issues and primarily prepares the patient for secondary alveolar bone grafting. Treatment objectives are to eliminate the anterior and posterior crossbites, manage space for the proper eruption of teeth, and align the dentition. In the transverse dimension, an expander appliance is used to widen maxilla to correct the posterior crossbites and reorient the maxillary segments prior to bone grafting. Even though expansion increases the cleft size, it improves surgical access and visibility to the area for bone graft placement[6]. Growth modification in the anterior-posterior relation may be utilized during this time or deferred until orthognathic surgery can be completed after growth has finished[13,14]. Phase I can include limited alignment of the maxillary incisors, which regularly erupt rotated and tipped, to prevent migration into the cleft region risking dental vitality and provide the child with

an increasingly esthetic smile and psychological benefits[14,15]. In order to achieve these Phase I goals, treatment is carefully coordinated around alveolar bone grafting to close the defect and stabilize the maxilla. Phase II treatment objectives are similar to non-cleft patients and may comprise of canine substitution or combine prosthetic treatment, such as dental implants, to replace missing teeth in the cleft region[6,13].

## 1.3 Alveolar bone grafting (ABG)

### *1.3.1 Introduction and purpose*

Alveolar clefting is seen in about 75% of cleft lip and palate patients[6] and is generally located between the lateral incisor and canine or between the central and lateral incisors. It is associated with a number of problems including reflux between residual oronasal fistulas, chronic periodontal inflammation and eventual teeth loss in the cleft area, limited bone for orthodontic movement or dental restorations, speech development, and esthetics[16]. With its introduction in the 19$^{th}$ century and continuous refinement in surgical techniques, alveolar bone grafting (ABG) has become routine treatment in the management of alveolar clefts[17]. Bone grafting, which includes reconstruction of the nasal floor and lateral piriform rim, in CLP patients helps to provide: 1) stabilization of the maxilla to help maintain palatal width and prevents collapse after expansion, 2) a scaffold for tooth eruption or future implant placement, 3) effective closure of oronasal fistulas, 4) support for the alar base of the nose and lip, and 5) improvement of esthetic results and overall facial symmetry[18-20]. The rationale for bone grafting is clearly evident, but the proper timing of graft placement and bone graft material has been long debated.

### *1.3.2 Timing of secondary ABG*

In the 1970s, along with the discontinuation of primary bone grafting due to impairment of maxillary growth, a series of studies by Boyne and Sands established a new bone grafting

procedure using autogenous, cancellous bone[17,21]. Secondary bone grafting is recommended to be performed between the ages of 7-12 years old when the majority of maxillary growth is complete and prior to canine eruption on the cleft side. Classic growth studies show that the maxilla completes growth in the transverse and sagittal directions by 8-9 years of age[22] and studies investigating the long-term effect of delayed grafting in CLP validate that no major detrimental effects on facial growth occur[23-25]. Presently, it is still the most widely accepted practice.

One principal goal of secondary bone grafting is the preservation of the permanent dentition without periodontal defects and, if possible, the elimination of prosthetic needs. An overwhelming majority agrees that the optimal time for grafting is before canine eruption, when the root of is one-half to two-thirds developed. During this time, the tooth exhibits accelerated and active eruption[26]. By grafting, the tooth-bearing function of the alveolar process is restored and enables spontaneous migration of the teeth through the graft[23,27-30]. Moreover, this can facilitate orthodontic space closure in cases where cleft-side lateral incisors are missing and canine substitution is the choice of treatment[29,31,32]. Equally, the eruption of the canine stimulates and stabilizes the graft as evident by the consistently higher bone levels described in countless studies compared to late, post-canine-eruption grafting[17,23,24,29,32-36,37]. In normal alveolar bone conditions, the supporting bone is brought along with an erupting tooth. But when a tooth erupts into a non-repaired cleft where there is insubstantial bone, a periodontal defect is often seen with permanent loss of supporting tissues[29]. Even with additional grafting, the bone level and periodontal support cannot be restored and the success of grafting decreases leading to an increase risk of tooth loss around the cleft and difficulty in orthodontic tooth movement and prosthodontic restoration[6,28]. Furthermore, external root resorption of the cervical third has been

demonstrated when grafts are placed after the eruption of the canine mainly due to contact of the grafted bone to the exposed root surface[23,24,38,39]. Though late secondary grafting is possible, it is more likely to fail. The bone undergoes extensive remodeling resulting in less alveolar height and oronasal fistulas relapse[24,40,41]. In general, bone grafting should not be based on chronological age but rather maxillary growth and dental development.

### *1.3.3 Bone graft sources*

Numerous graft materials and donor sites have been evaluated for use in alveolar bone grafting for CLP patients. Currently, the gold standard for alveolar cleft repair remains fresh, autogenous, cancellous grafts taken from the iliac crest because of its favorable outcome and predictability[23]. Autografts offer several advantages to other types of grafts in that they are osteoinductive, osteoconductive, osteogenic, and non-immunogenic. There is a consensus the use of particulate bone grafts over bone blocks as it is more readily incorporated, resistant to infection, and responsive to odontogenic demands of the alveolar bone. Small exposures or losses of particulate material may not compromise the entire graft[42,43]. The iliac crest, cranium, tibia, and mandibular symphysis have all served as donor sites with each having its own advantages and potential complications. The iliac crest is the preferred site for its abundance of pluripotent or osteogenic precursor cells, ease of access, and simultaneous harvest with cleft preparation thereby reducing surgical time. Though, drawbacks include post-operative pain with delayed ambulation, scarring, and damage to the cutaneous nerve[18,26,44].

### *1.3.4 Bone graft incorporation*

Incorporation of a bone graft follows a remodeling" cycle[45], and its success is dependent on the dynamic interplay of the biological function of the bone graft and host environment. Early phases consist of an initial inflammatory response with a migration of inflammatory cells and

fibroblasts and the release of cytokines and growth factors. Revascularization of the graft with the ingrowth of host vessels brings along with it osteoclasts that resorb on surfaces of the graft. Osteoinduction recruits osteoprogenitor cells to invade the graft and differentiate into bone-forming osteoblasts. The graft provides a scaffold for new bone formation to take place with osteoconduction continuing up to several years. Eventually, the original graft tissue is resorbed with replacement by new host bone[46-48]. An imbalance between the anabolic and catabolic activities during remodeling can influence the way the graft is incorporated and amount of graft loss[49,50]. Cancellous bone is more desirable than cortical bone[18,23,44,47,48,51-53]. Under optimal surgical conditions, simultaneous harvest and placement of the cancellous bone promotes the transplantation of viable osteogenic cells and rapid revascularization over three weeks allowing for faster bone healing. Osteoid is laid down surrounding cores of the necrotic bone graft. Initial bone formation takes the form of immature woven bone and is followed by resorption of graft bone in creeping substitution. Considerable resorption and new bone formation is accomplished by six months. On the other hand, cortical bone grafts undergoes reverse creeping substitution. Owing to its density, the rate of revascularization and remodeling is markedly diminished. The establishment of vascular flow through existing vessels or vascular ingrowth to receive nutrients can only commence after resorption of the cortical surfaces by osteoclasts. The lack of immediate blood supply results in the graft becoming non-vital followed by replacement by invading host bone cells[23,47,48].

### *1.3.5 Resorption in ABG*

Bone grafting in the intraoral cleft is prone to high resorption and may be due to several reasons including: 1) tension of mucoperiosteal flaps during surgery leading to wound dehiscence [18,22,23,42,54], (2) infections of the grafts, especially relating to oral hygiene and periodontal

health[31,35,36,55,56], and 3) absence of mechanical stressors and functional load, according to Wolff's Law[57-60]. Evaluation of bone volume is highly variable and dependent on the grading scale employed. Diverse scales have been proposed that look at different criteria such as interseptal bone height (Bergland), bony fill-in (Kindelan), and position of the bone (Chelsea)[26]. Assessment of bone was based on pre- and post-operative panoramic, occlusal, or periapical radiographs. With these methods, success rates range anywhere from 73% to 93%[61,62], suggesting that up to one out of four cases do not meet the adequate criteria for success. 14.8%[63] and 23%[55] of grafted clefts require repeated surgeries for revisions. Moreover, the lack of conformity in evaluation and the limitation of two-dimensional radiographs may not be an accurate representation of the actual bone present. None of the scales account for bone graft architecture or bone quality. With development and advances in radiographic technology, low-radiation cone-beam computed tomography (CBCT) is swiftly becoming the new approach for comprehensive, three-dimensional bone assessment post-grafting. In comparing dental radiographs and CT scans, Lee et al. (1995) found that 17% of the number of clinically successful bone grafts were overestimated[64], and Rosenstein et al. saw that root coverage may be overestimated by as much as 25%[65]. Studies using CT scans to assess the bone volume after grafting have reported up to 64% bone loss after the first year of grafting[54,66-68]. Hence, new treatment modalities are needed to augment the clinical success of bone grafting in CLP treatment due to the unpredictability of current methods.

## 1.4 Bisphosphonates

### *1.4.1 Introduction and mechanism of action*

Bisphosphonates (BPs), an anti-resorptive drug, have universally been used to effectively treat various bone diseases associated with excessive resorption, including osteoporosis, Paget's

disease, and tumor-associated osteolysis. Structurally, BPs are stable analogues of inorganic pyrophosphate consisting of a P-C-P backbone and two differing side chains that affect its binding ability and potency. They have a high affinity for hydroxyapatite crystals and preferentially bind to areas of active bone remodeling with high bone turnover. When ingested orally, their bioavailability is extremely limited (~1%) and highly affected by any source of calcium. Intravenous preparations have helped overcome these hurdles and reduced the frequency of dosing. BP not preserved in the skeleton (40-60% of absorbed amount) is rapidly cleared from the circulation and excreted, unaltered in the urine. However, once bound to bone mineral, they may stay embedded for a prolonged amount of time, with a half-life upwards of 10 years. Despite cited side effects, they are generally well-tolerated[69-71].

The effects of BP on inhibiting bone resorption have clearly been elucidated. Their main mechanism of action is the disruption of osteoclast function, thereby effectively suppressing bone resorption. Once bound to hydroxyapatite, BPs release may be facilitated by the micro-acidic environment created by osteoclasts in the resorptive phase, or become embedded within the bone as new bone tissue is laid down. The embedded BP remains inactive until it is recycled to the surface when osteoclast-mediated bone resorption transpires. Once release, it is internalized into osteoclastic cells through simple endocytosis. The different types of bisphosphonates are categorized by Drake et al. in Table 1[69]. First generation, non-nitrogen-containing bisphosphonates (NNBPs) are metabolized into analogues of ATP. The accumulation of these intracellular metabolites is cytotoxic and induces apoptosis of osteoclasts by inhibiting ATP-dependent cellular processes. Conversely, second and third generation BPs (NBPs) contain a nitrogen side chain that is responsible for their increased potency. Presently, Zoledronate (ZA) is the most the potent bisphosphonate available for clinical use allowing for intermittent

frequency of dosing, with treatment for osteoporosis scheduled once yearly[70]. These NBPs instead inhibit the activity of farnesyl pyrophosphate synthase (FPPS), a key regulatory enzyme in the mevalonic acid pathway for the production of cholesterol, other sterols, and isoprenoid lipids. The inhibition of FPPS prevents the downstream prenylation of small GTPases, which are important signaling molecules in regulating cell morphology, cytoskeletal arrangement, membrane ruffling, and trafficking of vesicles[69-71]. Consequently, osteoclast recruitment, differentiation, and resorptive activity are impaired and apoptosis ensues[70]. Biochemically, reduction of urinary excretion of deoxypyridinoline and pyridinoline, markers specific to bone derived from collagen degradation, could be detected in Alendronate (ALN)-treated animals indicating there was a suppression of bone turnover[72-74]. Histologically, studies have found a decrease in the number of osteoclasts and resorptive lacunae confirming biochemical results[72,73].

### *1.4.2 Bisphosphonate use in bone grafting*

Bisphosphonates have commonly been used for enhancing the recovery of bone graft material and preventing resorption. Bone allografts pretreated with Zoledronate before installation found a decreased amount of bone resorption with no negative effect on new bone formation. The average bone mineral density (BMD) was also found to be higher in BP-treated groups[75]. Similarly, in bone chamber studies, systemic or local application of BP showed less graft resorption and replacement by bone marrow with the chambers. More bone formation and retention of new bone with correlating higher BMDs was seen. However, the distance of bone ingrowth did not differ significantly suggesting that the rate of bone formation may not be affected by BPs in these studies[76-79]. In a study looking at maxillary sinus floor augmentation, rabbits that received daily Alendronate injections after grafting with either autogenous grafts or xenografts exhibited significantly greater bone area and formation and less fibrous tissue

formation than their saline counterparts[80]. BP use in dental implants placement with grafted bone illustrated improved graft healing, early stabilization, and better implant fixation[81,82]. These studies suggest that the use of bisphosphonates for bone grafting is promising in shifting the balance between bone resorption and formation towards an anabolic direction. A minimal number of studies regarding BP use in grafting in the oral environment exist, and no studies have previously examined its application in alveolar and palatal cleft grafting. Therefore, there is a need to identify whether BP use is a safe, effective, and feasible option for treating CLP patients who face higher rates of failure.

### *1.4.3 Systemic versus local delivery*

Both systemic and local applications of bisphosphonates are effective in inhibiting bone resorption[75,76,79,83,84,85] and enhancing bone formation[83,86]. However, the effectiveness of one method over the other has not been fully clarified. In comparing local and systemic treatment, Toker et al. concluded local application was as effective at increasing osteoblasts and bone formation as systemic BPs[87]. Still, others perceived that local delivery resulted in a stronger inhibition of resorption[50] and osteoblastic activity[88]. However, shortcomings of local treatment have been reported with impaired osteoconduction and decreased bone ingrowth in chamber models[50,89-91], and observed adverse effects with high dosages[83,84,89]. Still, local delivery offers several additional advantages over systemic delivery. Local delivery of BPs can easily be accomplished through the immersion of the bone graft material in the BP before grafting without the need for additional delivery vehicle[76,85]. With local delivery, the BP is highly concentrated in area of the graft only with little effect elsewhere[92]. McKenzie et al, showed that local elution of BP from porous implants had minimal systemic BP distribution[93]. At the same time, the higher local concentrations achieved have a greater effect allowing for smaller and less frequent

dosing[50,93]. Another important advantage of local delivery of BP is decrease risk of adverse effects that accompanies systemic delivery due to its widespread distribution[69-71]. Up to 42-60% of patients experience an acute-phase response (APR) within the first few days following the first systemic infusion of highly potent Zoledronate have been reported[94,95]. APR events include fever, musculoskeletal, gastrointestinal symptoms, eye inflammation, palpitations, and other general symptoms. Highly relevant to dental professionals is the potential risk of bisphosphonate-related osteonecrosis of the jaw (BRONJ) associated with systemic BP treatment. In a rat BRONJ model, where systemic BP treatment lead to ONJ-like lesions in all cases, local Zoledronate treatment improved implant fixation without inducing ONJ-like lesions[96]. Local delivery offers more advantages then systemic use of BPs, but its clinical efficacy remains to be determined in an intraoral cleft bone-grafting model.

### *1.4.4 Bisphosphonates' effect on tooth eruption*

As mentioned previously, one key reason of alveolar bone grafting is to establish a functional alveolar ridge for orthodontic or prosthodontic treatment. Grafting prior to canine eruption plays a dual role; the bone graft provides a scaffold through which the canine can erupt with healthier periodontal attachments post-eruption, while the canine serves as a mechanical stressor resulting in improved bone retention, as explained by Wolff's law. Tooth development is a complex process involving a closely intimate relationship between the dental follicle and periodontal tissues. An essential part of this process is the remodeling, specifically the action of osteoclasts, of the surrounding alveolar bone to enable normal tooth development and eruption[97-99]. Therefore, it is necessary to evaluate whether the use of bisphosphonates, through inhibiting osteoclastic action, will interfere with the eruption of the developing dentition. Previous studies have confirmed that the use of bisphosphonates in rabbits and rats resulted in delayed eruption

and exfoliated of deciduous molars[100], and delayed permanent tooth eruption with associated dental deformities in the developing crowns and roots[100-103]. Still, these studies differed in the type of BP used, method of BP intake, dosage, and frequency of dosing. In the majority of these studies, Alendronate was administered with repeated doses (daily or weekly). The effect of a single, low dose of ZA has yet to be determined.

### 1.7 Specific Aims

The long-term goal of this study is to develop a therapeutic modality to improve the clinical success and survival of bone grafting in CLP treatment. Our preliminary study demonstrated the beneficial effect of BPs with systemic delivery in the rat cleft model. However, the benefits of local delivery such as the ease, convenience, and prevention of systemic side effects would be more clinically applicable.

*Aim 1:* To evaluate the effectiveness of local delivery of BPs in enhancing the success of bone grafting in the rat cleft model.

*Aim 2:* To compare the effectiveness of local and systemic delivery of BPs in enhancing the success of bone grafting in the rat cleft model.

*Aim 3:* To evaluate the effect of BP on tooth eruption in a pilot study

## 2. METHODS

All experimental procedures involving animals were conducted according to the protocol approved by the UCLA Institutional Animal Care and Use Committee (#: 2014-047-03).

**Aims 1 and 2: Systemic versus local bisphosphonate delivery**

### *2.1 Experimental animals (Figure 1)*

In order to achieve a power level of 0.80 and $\alpha = 0.05$, a power analysis ($n = (z_{1-\alpha/2} + z_{1-\beta})^2(\sigma_1^2 + \sigma_2^2) / (\mu_1 - \mu_2)^2$) determined that 8 rats per treatment group is required based on our lab's preliminary study[104]. A total of 28 female Fischer F344 Inbred rats (16-20 week old; average weight of 180 g) were purchased (Charles River Laboratories, Wilmington, MA), housed in light- and temperature-controlled facilities, and given food and water ad libitum. 24 rats were randomly assigned into three treatment groups (n = 8 per group): 1) Control: graft with saline injection, 2) Systemic: graft with BP injection, 3) Local: graft pre-treated with BP prior to placement. The remaining four rats served bone isograft donors (Figure 1). All animals were humanely euthanized at 6 weeks post-operatively and the maxilla dissected.

### *2.2 Surgery*

*(A) Bone Graft Harvest:* Donor rats were euthanized and surgically prepped with isopropyl alcohol and Betadine solution (Purdue, Stamford, CT). An incision was made on the lower back and skin reflected. Blunt dissection of the subcutaneous tissue and muscle was performed to gain access to the pelvic bone. The corticocancellous bone from the iliac crests and femurs were harvested bilaterally, and any cartilaginous tissues removed. A bone mill (G. Hartzell & Son, Concord, CA) was used to manually reduce the bone size to deliver consistent and uniform particles. The fine bone particles were then placed on ice for immediate use.

*(B) Palatal Defect Creation (Figure 2):* Our group previously established a novel, critical-sized palatal defect animal model for studying the effects of different medications on bone remodeling[104]. General anesthesia was initially achieved with isoflurane (4-5%) followed by a combination of intramuscular (IM) Ketamine (40 mg/kg) and Xylazine (10 mg/kg). Bland ophthalmic ointment was applied to prevent corneal desiccation. The first dose of analgesic, Buprenorphine (0.01-0.05 mg/kg), was given subcutaneously after the induction of anesthesia to allow it to take effect before the first incision was made. Aseptic surgical procedures were followed. With animals supine, a 1cm longitudinal mucosal incision was made from behind the maxillary incisors posteriorly down the middle of the palate. The periosteum was elevated to expose palatal and alveolar bone (Figure 2A). 3mm-diameter mid-palatal defects were created with a low-speed, hand-operated power drill and trephine bur under constant irrigation and with care to avoid injury to the adjacent bone. These defects were established as critical sized defects due to the lack of spontaneous bone healing in our lab's previous studies. To avoid injury to the incisor roots, the defect was placed in the middle of the palate. In all cases, preservation of an intact nasal mucosa was attempted. Hemostasis was achieved using pressure and cotton tip applicators. The harvested cancellous autograft was placed into the defect and slightly overpacked beyond the margins of the defect (Figure 2B). The oral mucosa was re-approximated with multiple interrupted sutures using absorbable 5-0 Vicryl (Figure 2C). Postoperatively, each animal received twice daily subcutaneous injections of Buprenorphine (0.01-0.05 mg/kg) for two days as analgesia. Trimethoprimsulfamethoxazole (TMS) was placed in the drinking water (5ml TMS/500ml water) for a period of two weeks post-surgery, beginning the day before surgery, to prevent infection. In addition to the normal diet, a soft diet was provided for the first three days after surgery.

### *2.3 BP delivery*

*(A) Control:* A single subcutaneous injection of saline was administered to the animals one-week post-surgery.

*(B) Systemic:* A single 0.1 mg/kg subcutaneous injection of Zoledronate (Reclast ®, Novartis, Hanover, NJ) was administered to the animals one week post-surgery.

Based on regularly used therapeutic doses used in humans, a concentration of 0.1mg/kg of ZA was chosen for systemic administration[105]. Moreover, this dosage proved to be more effective than alendronate in the osseointegration of implants and positive effects of BMD[106].

*(C) Local:* Zoledronate was diluted in saline to a concentration of 0.005mg/ml. Prior to graft insertion, the bone graft particles were pretreated with one 3-minute immersion in ZA followed by three one-minute saline washes with gentle agitation to remove excessive unbound ZA. In a study exploring dose-response by Jakobsen et al., this concentration was shown to maximize new bone formation, whereas, the highest dose of ZA (0.5mg/ml) resulted in toxic effects and decreased new bone formation[83]. A number of studies emphasize the importance of removing unbound BP as it could interfere with new bone formation[89,107], while others did not find that to be the case[50,108].

**Data Analysis**

### *2.4 High resolution microCT imaging*

Rat maxillae were fixed with 4% (w/v) paraformaldehyde in 0.1M phosphate-buffered saline (PBS) for 48 hours, and then transferred and stored in 70% ethanol. Samples were scanned with high-resolution computed tomography (SkyScan 1172, SkyScan N.V., Belgium), at an image resolution of 15µm, with 70 kV and 141 µA X-ray source and 0.5 mm aluminum filter. 3D image reconstructions were performed using NRecon software (SkyScan N.V., Belgium), with

image correction steps for beam hardening correction, ring artifact correction, and fine-tuning. 3D image visualization was performed with Dolphin Imaging V 11.7 software (Chatsworth, CA). In order to quantify bone within the defect, 3D volumetric analysis was completed with CTAn software (SkyScan N.V., Belgium) and all analyses were repeated two separate times by a single, trained, operator. Samples were oriented such that the floor of the defect (nasal side) and the anterior palatal were perpendicular to each other in the coronal and sagittal planes, respectively. A cylindrical Volume of Interest (VOI), enclosing the defect, was demarcated by the defect edges in the transaxial view and extended the full depth of the defect (palatal surface to the floor of the defect at nasal mucosa). Using a grey threshold that approximated images to their true morphology, bone volume (BV), tissue volume (TV), as well as bone mineral density (BMD) were measured and bone volume fraction (BV/TV) was calculated.

### *2.5 Histomorphometry*

After micro-CT analysis, the maxillary specimens were decalcified in in 14.5% ethylenediaminetetraacetic acid (EDTA 0.1M, pH=7.4) solution, changed every 2-3 days, for 28 days. Samples were washed and then dehydrated in 70% ethanol. Cuts were made coronally through the center of the defect and both anterior and posterior sections were embedded in paraffin and sectioned by the UCLA Tissue Procurement Core Lab (TPCL). 5μm-thick coronal sections were made starting from the center of the defect.

To visualize and analyze bone morphology and remodeling, the sections were de-paraffinized, rehydrated, and every fourth section used for hematoxylin and eosin (H&E). All photomicrographs were generated using Olympus BX51 microscope at x40 and x100 magnification and captured with Olympus DP72 digital microscope camera with cellSens software (Olympus, Center Valley, PA). Bone area (BA) and total tissue areas (TA) were

analyzed using H&E stained slides. A rectangular Region of Interest (ROI) measuring 1mm x 0.5mm (length x width) was outlined directly below the nasal cavity at x40 magnification, and the fraction of bone area/total area (BA/TA) was calculated with Advanced SPOT 4.6 software. The bone fraction for each treatment group was an average measured from four representative samples.

### *2.6 TRAP staining*

Tartrate-resistant acid phosphatase (TRAP) staining was performed as previously described (Verron et al). Decalcified tissue sections were de-paraffinized at 60$^{o}$C for 30 minutes, then rehydrated through xylene and graded ethanol. Slides were incubated for 1 hour at 37°C with TRAP staining solution, according to manufacturer's protocol (Leukocyte Acid Phosphatase Kit; Sigma-Aldrich, Inc., St. Louis, MO). The slides were counterstained with hematoxylin solution for 2 minutes and mounted with aqueous mounting solution (Permount; Fisher Scientific, Tustin, CA). Osteoclasts were defined as multinucleated (≥ 3 nuclei) TRAP-positive cells attached to bone surfaces. To quantify osteoclast activity in the bones, the number of mature osteoclasts was counted within the defect area at x100 and x400 magnification and bone surface was calculated using ImageJ software (National Institute of Health, Bethesda, MD). Osteoclast counts are expressed as number of OCs/mm bone surface. Numbers in treatment group were averages from four representative samples. A single operator at two separate time points performed the quantification.

### *2.6 Biomarker analysis: TRAP-5b ELISA assay*

To study the systemic activity of osteoclasts, ~300µl of peripheral blood was collected from the tail vein of each animal at 2, 4, and 6 weeks post-operatively. Samples were centrifuged at 3,000 rpm for 15 minutes at 4°C to obtain blood serum. The supernatants were collected and stored at

−20°C until needed for analysis. Serum TRAP-5b levels were quantified using the commercially available RatTRAP-5b ELISA kit (Immunodiagnostic Systems, Gaithersburg, MD). The ELISA procedures were performed according to the manufacturer's instructions. All samples were triplicated and the results were averaged in each group.

**AIM 3: Pilot study: Effect of Zoledronate on tooth eruption**

### *2.7 Experimental animals and injections (Figure 3)*

Two, timed pregnant, female Sprague-Dawley rats (E18 weeks on arrival) were purchased from Charles River Laboratories (Wilmington, MA) to yield a litter of 16 rat pups. The 16 rat pups were divided into two groups (n = 8): 1) control with saline injection, 2) systemic BP injection. At one-week old, 100μL of saline was injected subcutaneously into the control group and 100μL solution, with a concentration of 0.1mg/kg Zoledronate, was injected into the systemic group Pups were weaned at 3 weeks old. In addition to free access to their regular feed, all animals received a daily supplemental soft diet composed of the regular hard pellets crushed and softened with water. Four rats (n=2 from each group) were euthanized at time points 2, 3, 4, and 6 weeks after birth.

### *2.8 Clinical oral examination*

Only molar teeth were observed due to the fact that rodent incisors continuously erupt throughout their lifetime. The typical eruption timeline for rodent teeth are as follows: incisor at day 8-10, first molars at day 19, second molars at day 21, third molars at days 35-40, molar grow slows greatly at day 125[109]. Animals were placed under inhalation anesthesia with isoflurane (2-3%) during the oral exam. Visual observation and physical palpation using a periodontal probe of the first and second molar teeth in the maxilla was conducted and recorded daily, starting at age 15-days post-natally.

### *2.9 microCT imaging*

After euthanasia, the maxilla was dissected and prepared for microCT imaging as described above. Samples were scanned at 20μm, and reoriented along the mid-palatal suture in both coronal and transaxial views with the anterior palate perpendicular in the sagittal view. Two-dimensional images were generated in the sagittal plane at the most convex point on the mesial surface of the molar crowns when seen in the transaxial view (Figure 4A). For the first molars, the degree of eruption was quantified as the distance from the top of alveolar bone crest, measured on the mesial side of the molar, vertically to the maximum height of the mesial cusp tip. For the second and third molars, a reference line (RL) was first drawn along the height of the alveolar bone connecting bone mesial of the first molar to bone distal to the third molar. Vertical measurements were made from the tip of the distal cusp of the second molar and the middle cusp of the third molar to the RL (Figure 4B).

**Statistical analysis**

Statistical analysis was performed using a commercially available software program, GraphPad Prism 6 (GraphPad Inc., San Diego, CA). The mean values and standard deviations (SD) were derived for each group. Shapiro-Wilk test was carried out to test for normal distribution and Q-Q plot and histogram of frequency distribution graphed (Figure 5A-C). Normality was accepted, and data were analyzed using parametric statistical tests for aims 1 and 2. One-way ANOVA was employed for multiple comparisons between the three groups followed by Tukey's post-hoc methods to adjust for Type I errors. In aim 3, due to the small sample size and failure to pass the normality test, the nonparametric Mann-Whitney U test was performed to compare the eruption distance between the two groups at each time point. Values of $p \leq 0.05$ were considered to be statistically significant.

# 3 RESULTS

## 3.1 Effect of bisphosphonate on bone grafting

Postoperative healing was considered generally uneventful in all animals. No complications such as premature exposure of the augmented sites or infections were observed throughout the study period.

*MicroCT imaging and bone quantification*

To evaluate the effect of Zoledronate on bone graft retention and bone regeneration in a cleft model, high-resolution microCT was used to obtain 3-dimensional data for qualitative and quantitative analysis after 6 weeks. Both systemic and local delivery of ZA displayed significant bone regeneration compared with control defects (Figure 6A). Volumetric analysis confirmed a statistically significant higher bone volume fraction (BV/TV) in both systemic (63 ± 14%) and local (69 ± 13%) groups relative to control (39 ± 10%). The local delivery group had the greatest percentage bone volume, however, only a minimal difference was noted between the two ZA-treated groups (Figure 6B). Similarly, the bone mineral density (BMD) was greater in both systemic and local groups by a comparable amount, 0.59 ± 0.12 and 0.63 ± 0.12 g/cm$^3$, respectively, compared to the control of 0.41 ± 0.09 g/cm$^3$ (Figure 6C).

*Histomorphometric analysis*

Conventional H&E histology showed overall increased amounts of retained bone graft particles within the defect in ZA-treated groups along with enhanced bone graft incorporation. The presence of blood vessels suggests revascularization of the graft, an essential step in survival of the graft, had occurred (Goldberg). Immature woven bone and osteocytes indicate there is active bone remodeling and new bone formation. Bony bridging was observed between the bone graft particles and defect margins. Control groups largely showed minimal bone in the defect areas

(Figure 7A). Quantification of bone tissue through SPOT software confirmed increased bone tissue fraction in ZA-treated groups (control: 20 ± 19%, systemic: 79 ± 18%, local: 90 ± 5%) (Figure 7B). This indicates that Zoledronate is effective in preventing bone graft loss.

*Osteoclast activity*

Bisphosphonates prevent bone resorption through the inhibition of osteoclast function and apoptosis. To evaluate this effect in our study, TRAP staining performed and serum TRAP-5b levels were analyzed. Overall, the local group had the greatest number of osteoclasts out of the three groups. The control group exhibited the fewest number of osteoclasts, possibly be due to the lack of bone present. However, when comparing the relative number of OCs per bone surface, differences were non-significant (Figure 8A and B). As expected, TRAP-5b levels were consistently low in the systemic group most likely indicating that Zoledronate entered the circulation and was affecting osteoclasts throughout the body (systemic at weeks 2, 4, 6: 0.460 ± 0.065, 0.77 ± 0.526, 0.863 ± 0.489 U/L). In contrast, the local ZA-delivery group had minimal effects on peripheral osteoclasts, as serum levels were closer to those of control animals (local at weeks 2, 4, 6: 2.944 ± 1.005, 1.711 ± 0.320, 2.672 ± 0.992 U/L; control at weeks 2, 4, 6: 2.110 ± 0.467, 2.740 ± 1.581, 3.459 ± 0.971 U/L) (Figure 8C).

### 3.2 Delayed tooth eruption with bisphosphonate use

*Tooth eruption timeline*

Molars were considered erupted when all cusp tips were fully emerged through the soft tissue and clinically visible in the oral cavity (Figure 9A,B). In the control group, the eruption of the first molars was first observed at age day 17 postnatal and the second molars erupted around days 20-22. Although third molars were not followed, they were present by the time of sacrifice at day 42 (Table 2). In BP-treated animals, neither the first nor the second molars were clinically

visible within the oral cavity by day 42. Interestingly, it was noted that the third molars erupted at day 35. The eruption time was rather consistent among the animals within each group. In addition, the eruption pattern of the molars in the mandible followed those in the maxilla.

*Eruption development*

Only maxillary molars were evaluated. At week 2, only the crowns of the first and second molar teeth were present with all molars were unerupted and located beneath soft tissue. A layer of cortical bone is seen overlying all second molars and the first molars in the ZA-treated group at this time. The third molars were in the initial stages of development with only an outline of the follicle present. In weeks 3 and 4, the first and second molars of the control group have erupted. In the ZA-treated group, the molars appear to have emerged through the overlying bone but not soft tissue. Additionally, it appears root development of these teeth is suppressed. Normal third molar buds are developing in both groups. At week 6, all molars have erupted to the level of the occlusal plane in control animals, whereas, only the third molars have exhibited normal development and erupted in ZA-treated groups. Continuous and uniform PDL spaces without ankylosis or dental abnormalities were noted on microCT (Figure 10). Measuring the distances from the height of the cusp tip to alveolar bone level, both first and second molars showed a significant delay in eruption in ZA-treated groups at weeks 3, 4, and 6. Conversely, there was not a significant difference between the degree of eruption between the two groups for third molars at all time points (Figure 11).

## 4. DISCUSSION

Alveolar bone grafting is a common and necessary procedure for a majority of cleft lip and/or lip patients[6]. As with all bone grafts, partial resorption of the graft occurs resulting in lower bone volumes than initially grafted with a chance of graft failure. The limited amount of bone may compromise functionality, subsequent treatment, or esthetic outcomes, and may require repeated surgery. Graft incorporation is further complicated in CLP patients due to additional complexities[22,24,36,58,59]. The use of a wide variety of anabolic agents has been applied with mixed outcomes. Researchers have looked at the application of fibrin glue, platelet rich plasma (PRP), a variety of growth factors such as TGF-beta and bone morphogenic proteins (BMPs), as well as systemic factors such as parathyroid hormone injections[49,71]. Few may show potential results, however, the limited studies on long-term effects and adverse problems of such methods including hematoma formation, swelling, respiratory difficulties, and long-term growth effects have prevented their routine use[110-112]. Bisphosphonates are known to inhibit osteoclast action. However, it has been shown that bisphosphonates also stimulate differentiation, maturation, and proliferation of osteoblasts in vitro[113-116]. Thus, our lab investigated the use of bisphosphonates in enhancing bone graft success. In our previous study, it was determined that a single injection of Zoledronate given one week or three weeks after surgery equally maximized the amount of bone retained within the defect compared to saline controls and immediate BP injections[104]. This study further examined whether systemic administration or local delivery was a superior method. The higher BV/TV, BMD, and MB/TA seen in ZA-treated groups affirmed our previous conclusion that BPs are effective in preventing bone resorption in a palatal defect model and can encourage new bone formation and graft incorporation.

No significant differences were observed between the systemic- and local- treated groups.

One explanation could be the local tissue concentration of ZA in the local delivery group was too high resulting in an over-suppression of bone healing. In two different studies, the omission of rinsing unbound BP from the graft is suggested to have blocked new bone formation due to the excessive BP[89,107]. Conversely, a study comparing the effects of rinsing or omission of rinsing in a bone chamber model found no difference in bone ingrowth distance despite having ~150x higher concentration in the unrinsed group as determined through radioactive labeling[108]. Another group supported that conclusion, implying that the amount of bound BP was a more important because the presence of unbound BP is most likely diluted into surrounding tissues. Instead, the affinity of BP to the bone, concentration of the BP solution, and soaking time are critical factors[50]. Still, Jakobsen et al. investigated a dose-response effect of ZA, and found that the lowest dose (0.005 mg/ml) increased new bone formation in the allograft but had the less inhibition of graft resorption. The inverse was seen in the highest dose (0.5mg/ml) group, with lack of new bone formation but greatest inhibition of allograft resorption[83]. At high concentrations, bisphosphonates have a negative effect on osteoblasts by inducing apoptosis and restricting proliferation[117,118]. At our dose, the concentration may still be too high causing toxicity and thereby, blocking bone metabolism. Because the unbound bisphosphonate was rinsed away to reduce any possible toxic effects, the exact dose at which the autograft was treated and implanted is unknown in our study. Moreover, BP adsorption to bone surfaces is dependent on the rate of bone turnover. Further dosing studies to include lower concentrations and various immersion times are needed to determine the optimal therapeutic window. Investigation of carriers to slow drug release may help avoid potential toxicity issues. Elution analysis to evaluate the release kinetics in a bone remodeling model as well as labeling of ZA could also prove useful in determining accurate concentrations of bound BP and explore the

biodistribution of bound and unbound BP once the graft is implanted.

Another reason why systemic and local groups appear similar may be a result of tightly overpacking of the graft. Revision joint replacements typically employ impacted, moralized allografts for initial stability. In a study investigating the use of Alendronate to facilitate implant fixation and graft incorporation, they found decreased biomechanical implant fixation for all impacted grafts soaked in ALN and blocked formation of new bone[91]. As explained above, cancellous graft incorporation undergoing a process known as creeping substitution, where bone formation and resorption occur concurrently. The density of the graft may not provide enough space for ingrowth of tissue or blood vessels[91,108]. Consequently, it assumes a cortical graft architecture, where new bone formation is primarily dependent on bone resorption. Because ZA inhibits bone resorption, it may have secondarily inhibited new bone ingrowth to some degree in our study.

The role of BPs in activating anabolic bone formation has been proposed but the mechanism of action is unclear. Altundal et al. investigated the effects of systemic, repeated ALN injections on bone formation after autogeneous grafting in rats. Serum urinary biomarkers for bone formation, osteocalcin and bone alkaline phosphatase, were analyzed and were found to be increased. In addition, histopathology confirmed biochemical results revealing increased numbers of osteoblasts and greater areas of osteoid and bone formation[119]. Accompanying this, others found mRNA expression of osteocalcin and alkaline phosphate was significantly higher than controls[73]. Other groups refute this theory and attribute the increase in bone mass to bone formation rates relatively surpassing resorption rates[120,121]. An in vitro study saw no stimulation of osteoblastic activity at BP levels adequate to inhibit the resorptive capacity of osteoclasts by 50%[122]. Acceptable bone formation was observed in our BP-treated samples but whether this is a

product of increased anabolic events is undetermined. Serum biomarkers (osteocalcin, alkaline phosphatase, and procollagen type I), specific histological stains for mineralized or calcified tissues, or immunohistochemistry would aid in estimating rate and quantity of new bone formation. An interesting future direction would be researching the use a combination of both pro-anabolic and anti-catabolic additives to minimize adverse effects and optimize outcomes of bone grafting for alveolar clefts. Recent studies show favorable results[123-125], but evaluations of bone grafting in the oral environment and long-term follow up are needed.

The action of Zoledronate on osteoclasts was supported by the observation that more bone was retained in BP-treated groups compared to control. Surprisingly, the number of osteoclasts observed on histological analysis did not decline in BP-treated groups. Instead, there was an increase in numbers, though not significant. This conflicts most literature that reports decreased osteoclast counts[69-72,126] and resorption lacunae size[72]. On the other hand, our results support findings by Kaynak et al. Only the morphology of osteoclasts evaluated diverged statistically, but not the difference in number of osteoclasts between BP-treated and control groups[121]. Morphological changes in apoptotic osteoclasts can be described as presence of cytoplasmic contraction, chromatin condensation, and nuclear fragmentation in detached cells[126]. Studies indicate that there is toxic elimination of osteoclasts only at high doses of BP[127,128]. Another study tested the mechanism of action of NNBPs and NBPs by inhibiting the function of caspases, enzymes vital to the process of apoptosis. At doses tenfold lower than those that induce osteoclast apoptosis, treatment with NBP along with an anti-apoptotic caspase inhibitor maintained osteoclast number but inhibition of bone resorption activity continued. When geranylgeraniol, an intermediate metabolite within the mevalonate pathway was supplemented, bone resorption levels returned to normal. This is in contrast to NNBPs where resorption levels

were restored when treated with the caspase inhibitor[129]. This indicates that NBPs' inhibitory action on osteoclasts does not require apoptosis. Our concentration of ZA exposure to osteoclasts may not have been high enough to stimulate apoptosis and diminish OC counts but continue to affect osteoclastic activity. Still, others observed an increase in the number of osteoclasts[130,131]. Features of apoptosis, such as enlargement, hypernucleation, pyknotic nuclei, and detachment, were detected in a large number (20-37%) of OCs proposing that these cells were undergoing protracted apoptosis[131]. Atypical morphology (round, detached TRAP+ cells) was also noted in our BP-treated groups indicative of disrupted OC ultrastructure and function. Since TRAP staining was examined at only one time point, initial osteoclasts may have already undergone programmed cell death, either from BP toxicity or reached the limits of their natural lifespan and cleared by phagocytes. Whereas, newly generated OCs may show normal activity, early signs of BP toxicity, or delayed apoptosis. Proliferation, migration, and differentiation of osteoclasts from precursors require 1-2 weeks[132], with average lifespans ranging from ~2 weeks[133] to 6 weeks[132]. Establishment of vascular ingrowth is also required for OC access to graft surfaces. In conjunction with TRAP staining, apoptosis assays can help identify nonfunctional OCs.

To evaluate the osteoclastic activity as a function of time, serum TRAP-5b levels were assayed through ELISA. Normally used resorption markers consist of urinary and serum biomarkers, but the heighten sensitivity to errors and laboriousness of urinary markers place preference on serum markers. TRAP is highly expressed by osteoclasts and activated macrophages. In serum, two distinct isoforms exist with TRAP-5b being specific to osteoclasts[134], and therefore, is a sensitive measure of osteoclastic activity. Serum was taken at time points 2, 4, and 6 weeks. Substantial reductions in the level of TRAP-5b were seen and maintained in the systemic group across all time points denoting that a single subcutaneous

injection of ZA was able to affect osteoclasts throughout the body for an extended period of time. As expected, levels of TRAP-5b did not differ statistically between control and local groups. By pretreating the graft with ZA through soaking, the action of BP is confined to the defect area with minimal influence systemically decreasing the potential for adverse effects in other organs. In local delivery, bone analysis of other anatomical structures as well as labeling of BP can detail the extent of systemic distribution. Serum markers provide a convenient method to identify and quantify overall functional activity across time in live patients but little is known about local activity. Coupled with the fact that serum TRAP-5b level may not accurately reflect resorption activity, as recorded by one group who saw higher levels with ZA treatment[130], histological analysis is still required.

**Considerations of bisphosphonate treatment**

One goal after cleft repair is restoration of the dentition at the cleft site whether by moving teeth through the site with canine substitution or the placement of a dental implant. Bisphosphonates are known to delay the eruption of teeth[100-103]. Even with a one-time systemic dose, inhibition of molar eruption was confirmed in our study. Unexpectedly, the eruption of the third molar proceeded normally with no delay or impaired root formation with BP treatment. At 2 weeks, both the first and second molar crowns were observable on microCT, while only the follicle of the third molar could be detected with no signs of the developing tooth bud. This suggests that the effect of BP on tooth eruption is correlated to the timing of BP injection in relation to the development stage of the teeth. This verifies the finding by Hiraga et al who also demonstrated a dose dependent response. Our dosage closely correlated to their middle dosage. At one week, formation and mineralization of the first and second molar crowns were in progress. With early treatment of ZA given at one week, none of the molars erupted by 12 weeks of age. In the late

treatment group, ZA was given rat pups two weeks old. Here, they saw that the first molar had already partially erupted into the oral cavity, and consequently, normal eruption persisted. In contrast, both the second and third molars did not erupt[103]. Third molar tooth buds were present by week 2 in our rats. A longer evaluation period is needed to evaluate whether eruption is just delayed or completely inhibited as it could be contraindicated for cleft patients where eruption of the canine plays a role in stabilizing the graft. Other tooth eruption studies using higher doses also depicted deformities in the enamel[101,135], ankylosis[103], and other dental abnormalities[100,103]. Histological analysis can be an important tool in assessing evidence of structural deformities in our samples.

Closely related is the effect of BP on orthodontic tooth movement (OTM). Mechanical stressors, such as orthodontic forces, promote bone remodeling. Tooth movement is carried out by alveolar bone resorption by osteoclasts on the compression side and new bone formation by osteoblasts on the pressure side[136], so BP's interference with osteoclastic function could definitely cause a decreased in efficacy and effectiveness of orthodontic treatment. A limited number of studies have explored the effects of both systemic[137,138] and local[139,140] BPs on tooth movement in rodent models, all of which conclude that that tooth movement is inhibited to varying degrees[141,142]. In the only study looking at Zoledronate, systemic administration inhibits excessive tooth movement up to ~50%[138]. Local application of BP showed inhibition of tooth movement in a dose dependent manner with an accompanying decrease of osteoclasts[140], and prevention of tooth relapse[139]. Likewise, root resorption, an undesirable side effect of orthodontic treatment due to odontoclast activity, was prevented[136,140]. In a paper that summarizes case reports of orthodontic movement in patients receiving BP treatment, orthodontic treatment was

not contraindicated but complications such as delayed tooth movement, incomplete space closure, poor root parallelism, and longer treatment duration should be expected[142].

If orthodontic space closure is not possible after alveolar cleft grafting, dental implants are the first line choice for restoring edentulous areas. Recent literature shows the incidence of implant failure and risk of BRONJ is minimal for individuals who have taken oral bisphosphonates[143-145] for less than four years[146]. However, implant placement has been contraindicated in those taking IV BPs. Numerous studies have shown early fixation and better osseointegration implants placed in the tibiae[82,147] or femurs[81] in BP-treated, osteoporosis animal models. But studies evaluating the outcome of dental implant placements in the presence of BP are lacking. One animal study demonstrated initial dental implant stability but impaired long-term healing at 8 weeks around the dental implant with systemic BP exposure[148]. In the only clinical prospective study, BP-coated dental implants saw improved implant fixation with no loss at 6 months post-placement[149]. A case report documenting dental implant placement in a 58-year-old male, who received a single injection of IV ZA therapy for osteoporosis, showed adequate osseointegration with no signs of clinical pathology at 6 months[150]. Dental implant placement may not be absolutely contraindicated in BP use, but a detailed history of BP use and a risk-benefit analysis should be considered. Insufficient data with long-term outcomes in IV BP patients exist, but it appears that the chances of success are improved when using low doses and local delivery to minimize BP exposure.

Of greatest concern to dental professionals is the risk of BRONJ. This increases with the use of third generation BPs, IV injection, high concentration doses, and extending duration of medical therapy. The incidence of BRONJ in cancer patients receiving high doses of ZA range from 0.7-6.7%, with systematic reviews and RCTs reporting a 1% risk as compared to those

receiving placebo (0.019% risk). However, BPs used in treating osteoporosis patients are given in much lower doses and less frequently, which translates in prevalence and risk of BRONJ. When compared to cancer patients, osteoporotic patients exposed to anti-resorptive medications have a risk that is 100 times smaller[146]. Prevalence rates have been recorded as anywhere from 0.004-0.21% in long-term oral BP use. In studies analyzing IV ZA given yearly, and followed up to six years, found a prevalence rate of 0.017% which approximated the risk of ONJ in patients enrolled in placebo groups (0-0.02%)[151,152]. So, though the risk of BRONJ is real in those exposed to BP, it remains low. The concentration and one time injection in our study, mimicking low dose treatment for osteoporosis, has minimal risk of inducing BRONJ. Several of our samples exhibited sequestered bone tissue surrounded by dense inflammatory cells on H&E demonstrating necrotic bone, however, it is unlikely related to bisphosphonate use as they were found in both control and BP-treated groups.

The pathophysiology of BRONJ has not been fully elucidated but potential hypotheses explaining localization to the jaws include altered bone remodeling, inhibition of angiogenesis, suppressed immunity, vitamin D deficiency, soft tissue BP toxicity, and inflammation/infection[146]. With local delivery, the levels of BP are concentrated in the area of interest possibly exacerbating some of these mechanisms. Inhibition of angiogenesis has been a leading factor in ONJ as interruption of vascular supply has typically characterized osteoradionecrosis[153], with experiments illustrating a reduction in angiogenesis[129] and decrease in VEGF levels in response to ZA[154,155]. This would theoretically affect the bone graft incorporation process, which relies on revascularization of the graft. Yet, one study found no effect changes in angiogenesis-related genes[156], and another observed no changes in vascular density and total length of vessel network in a bone chamber model from a single injection ZA at

clinically relevant dosing regimens[157]. The appearance of blood vessels in all three groups on histological analysis support these findings.

Impairment of wound healing is not only a concern to the risk of BRONJ[158] but also of graft loss. Toxic effects on oral epithelium and wound healing have been published with inhibited adhesion[159] and proliferation with[160] or without apoptosis[161] of oral keratinocytes. But in studies involving an oral wound healing model, palatal mucosa was denuded to observe spontaneous healing in ZA-injected rodents. Although healing appeared hindered at the connective tissue level[130], epithelialization was unaffected with no exposure of bone[130,156]. On gross observation, the palatal mucosa healed without complications in all of our animals. No wound dehiscence or infections of soft tissue were noted. This may be due to the small concentration of BP exposed to the soft tissue and rapid diffusion into other surrounding tissues, but histological examination of the layers of oral mucosa can yield a more in-depth analysis.

Besides dental implants, cleft patients often require extraction of teeth or surgical exposure of impacted canines putting them at risk for BRONJ with BP use. But few studies regarding the risk of BRONJ in the pediatric population exist. In a systemic review that included 5 retrospective studies examining the incidence of BRONJ in IV BP-treated children, zero cases of BRONJ were documented. Sample size ranged from 15 to 278 with an average BP duration of 4.5-6.8 years. Dental treatment included surgical or non-surgical extractions, and manipulation of the bone (i.e. exposing impacted teeth)[162]. From these previous studies, it appears that the risk for BRONJ may even be lower in the pediatric population receiving long term IV BP than in adults. A more complete investigation is needed, but the low incidence rates reinforce the use of BP in bone grafting cleft patients.

Alveolar cleft repair is predominantly performed on adolescents so concerns regarding the impact of BPs on growth and bone remodeling are imperative. The role of BP in the pediatric population is primarily in the management of osteogenesis imperfecta (OI), but has extended beyond to fibrous dysplasia and disorders characterized by impaired mobility leading to bone density loss[163]. BP use during growth is reflected in the epiphyseal and metaphyseal growth plates as sclerotic bands, but once treatment is discontinued before the growth plate closed, the bands gradually disappear suggesting that this phenomenon is reversible [164]. Along with that, another study saw the discontinuation of therapy lead to lower BMD in subsequently deposited bone tissue, which may create focal areas of bone weakness[165]. BP can also affect morphological bone shape by interfering with the normal process of periosteal resorption[166], with associated retention of the cartilaginous matrix at the chondro-osseous junction leading to minor losses in bone length[167,168]. Another study of BPs on a growing murine skeleton show no adverse effects on skeletal growth[169]. One particular fear is over-suppression of modeling and remodeling. Excessive doses can result in osteopetrosis[170] and persistent remodeling defects even six years after discontinuing the drug therapy[171]. No effect on spontaneous fracture healing but delayed healing after osteotomy procedures was reported[172]. Data on standard treatment guidelines and long-term effects in children are insufficient. Currently, treatment should be reserved for moderate to severe cases only and limited to reduce side effects. The use of local application can mitigate these effects.

## 5. SUMMARY AND CONCLUSION

Bone grafting incorporation follows a remodeling cycle with a balance between new bone formation and graft bone resorption. Here, we attempted to tip the balance towards new bone formation by inhibiting osteoclast-mediated resorption through the use of a bisphosphonate. We demonstrate that a locally delivered application of Zoledronate significantly increases the bone volume and bone mineral density in a palatal defect bone-grafting model with evidence of new bone formation. When compared to systemic administration, local application was slightly more effective in increasing bone volume, though not significantly. The number of osteoclasts was relatively higher in both systemic- and local- treated groups compared to control, but was not significant. Only subcutaneous injection of ZA resulted in a body-wide depression of TRAP-5b levels. Furthermore, a single, low-dose injection of ZA effectively delayed tooth eruption. The degree of inhibition of eruption is dependent on the development stage of the teeth at time of administration. Local delivery of ZA offers an effective method in enhancing bone grafts for cleft treatment with minimal impact on the peripheral body. However, additional studies are needed to evaluate whether tooth eruption is completely blocked or just delayed, as this plays an important part in the orthodontic treatment of cleft lip and/or palate patients.

**APPENDIX**

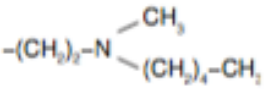
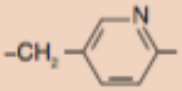
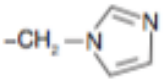
| Bisphosphonate | $-R_1$ | $-R_2$ | Relative potency |
|---|---|---|---|
| Etidronate | –OH | $-CH_3$ | 0.01 |
| Clodronate | –Cl | –Cl | 0.1 |
| Tiludronate | –H |  | 0.1 |
| | | | |
| Pamidronate | –OH | $-(CH_2)_2-NH_2$ | 1 |
| Alendronate | –OH | $-(CH_2)_3-NH_2$ | 5 |
| Ibandronate | –OH |  | 10 |
| Risedronate | –OH |  | 20 |
| Zoledronate | –OH |  | 100 |

**Table 1:** Bisphosphonate Structures and Relative Activities (reprinted from Drake et al., *Bisphosphonate Therapeutics in Bone Disease*)[69]

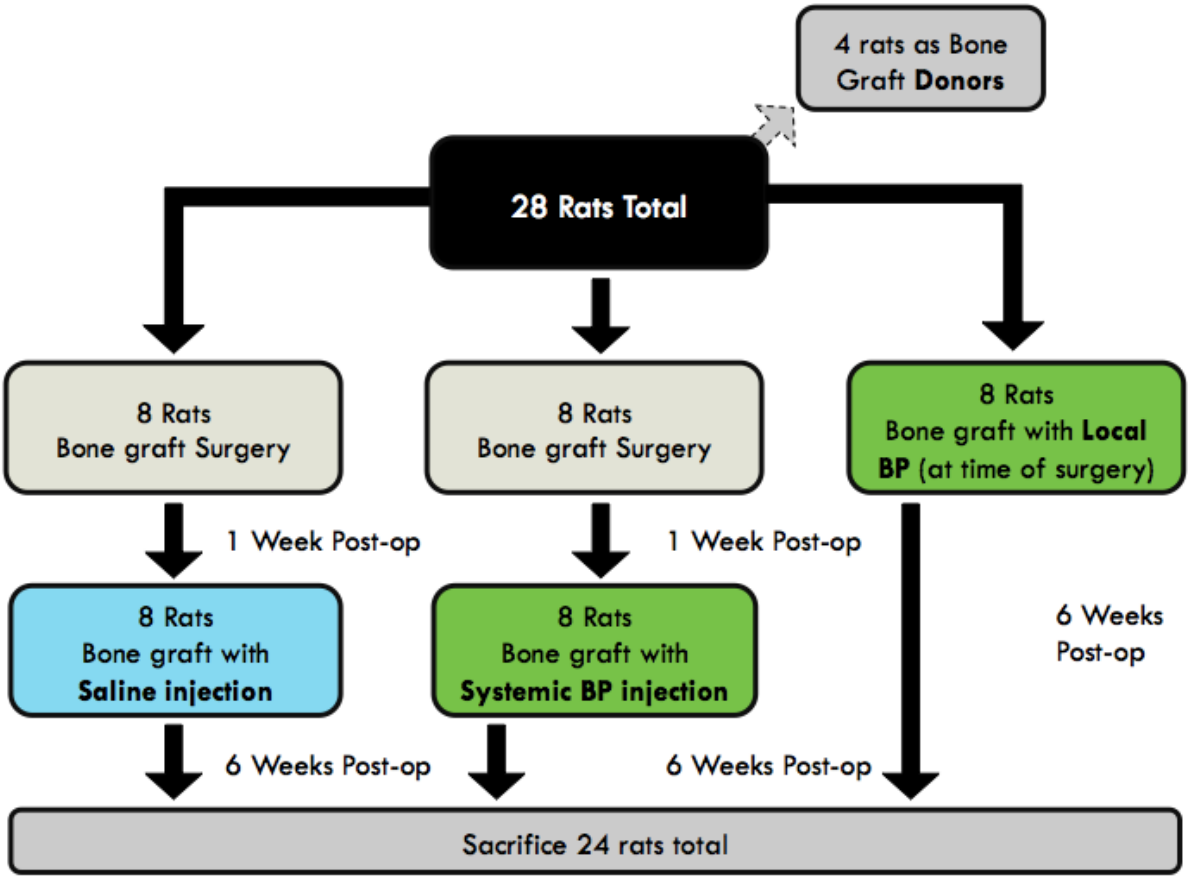



**Figure 1:** Experimental design for study of systemic versus local delivery of Zoledronate and bone grafting of a palatal defect model. 4 rats will serves as isograft donors. 24 rats underwent bone grafting, and were divided into three treatment groups (n=8): 1) control with saline injection one-week post-surgery, 2) systemic administration with 0.1mg/kg Zoledronate one-week post-surgery, 3) local delivery of 0.005mg/kg Zoledronate. Animals will be euthanized after 6 weeks for analysis.

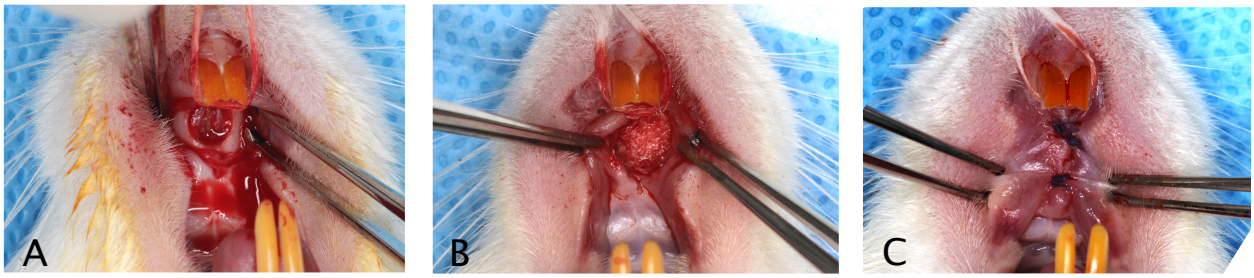



**Figure 2:** Surgical defect creation. (A) A longitudinal mucosal incision was made down the middle of the palatal and periosteum elevated for access to the palatal hard tissue. A 3mm palatal defect was surgically created using a slow-speed handpiece and trephine bur. (B) Bone graft particles from isograft donors were slightly overpacked into the defect. (C) Palatal soft tissue was approximated and simple, interrupted sutures placed for wound closure.

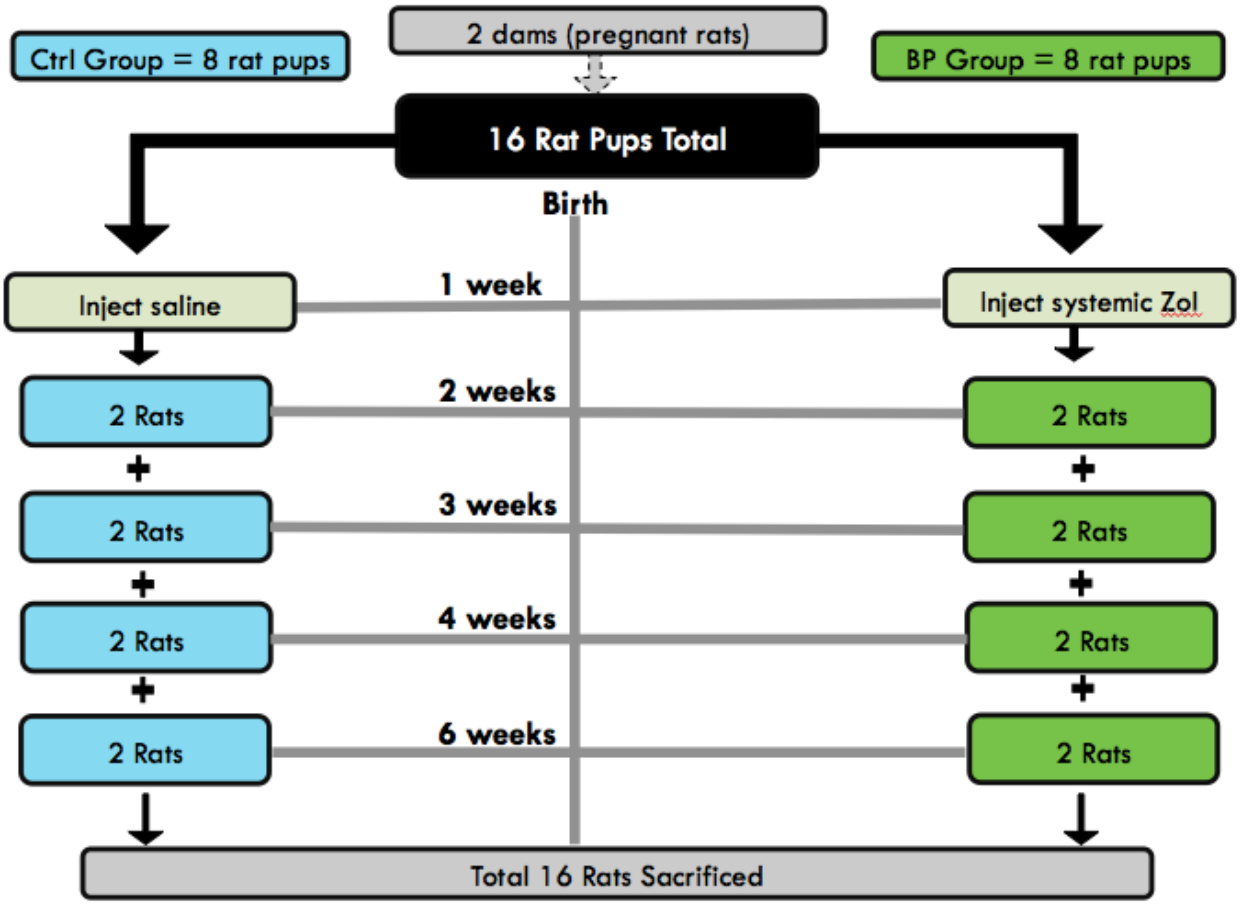



**Figure 3:** Experimental design for study of a one-time, low dose of Zoledronate on molar eruption in rats. Sixteen rat pups were divided into two treatment groups (n=8): 1) control with saline injection and 2) Zoledronate (0.1mg/kg injection). Injections were given to one-week old pups. At weeks 2, 3, 4, and 6, two rats from each group were sacrificed for microCT analysis.

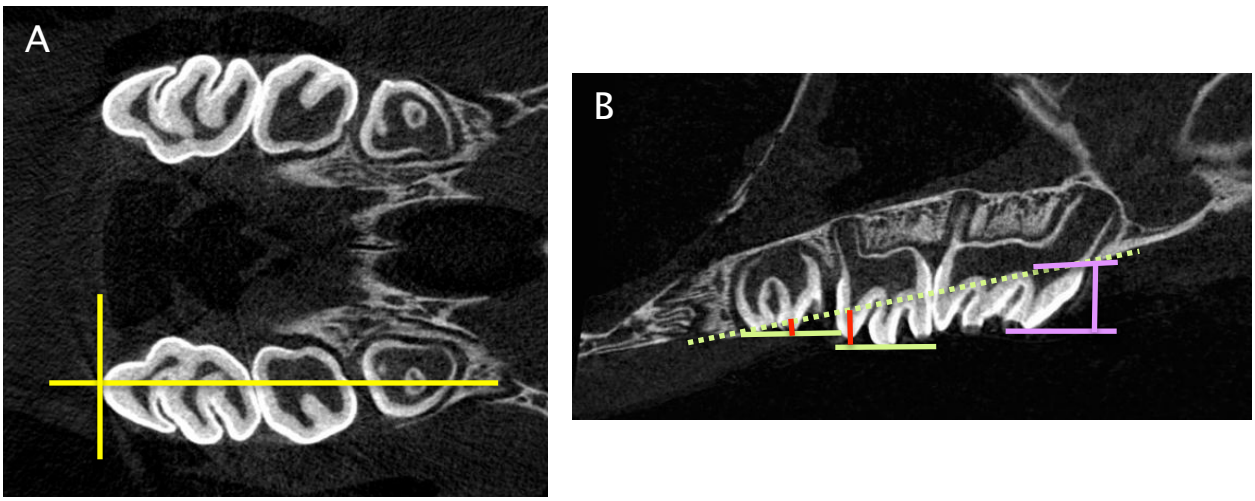
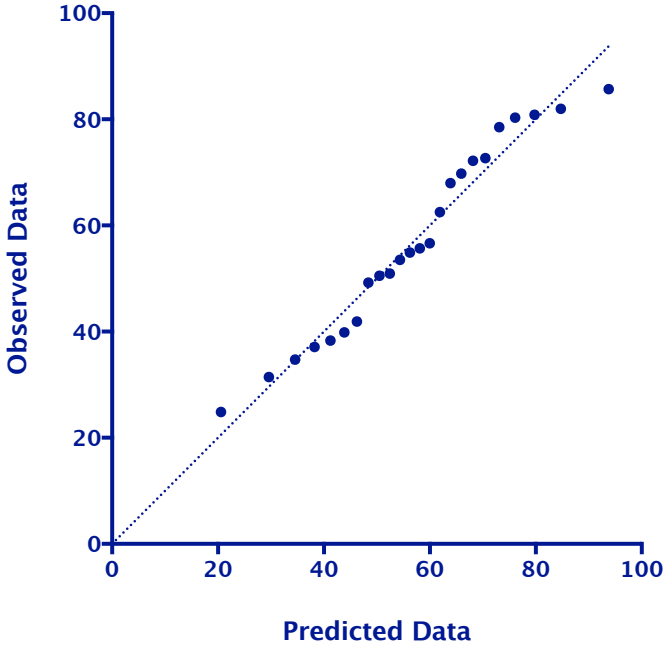



**Figure 4:** Reference planes for measuring degree of tooth eruption. (A) In the transaxial view, the most convex point on the mesial surface of the crown was used as reference for the sagittal view. (B) Sagittal view. The vertical distance first molars erupted was calculated from the maximum height of the mesial cusp to the level of the alveolar bone crest on the mesial side of the tooth (purple line). For second and third molars, first, a reference line (RL) was drawn connecting the mesial and distal bone levels (dotted line). From there, the distance up to the distal cusp tip of the second molars and the middle cusp tip of the third molars were measured (red lines).

A

| Shapiro-Wilk test | |
|---|---|
| W (observed) | 0.9550 |
| P-value | 0.3460 |
| Alpha | 0.05 |
| Reject | Yes |

B




C

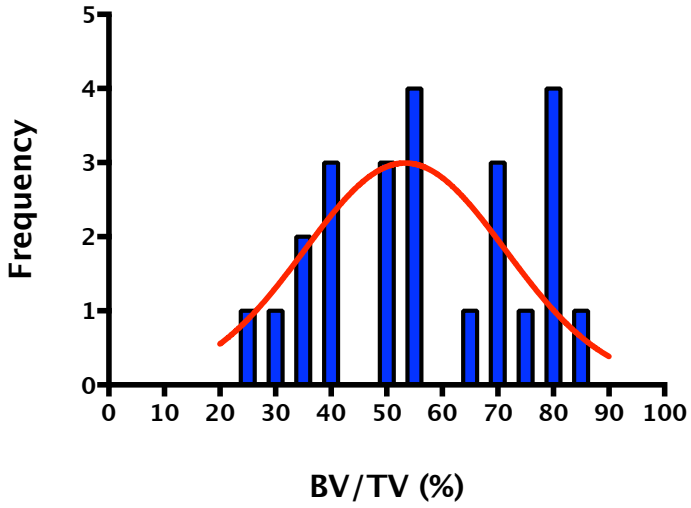
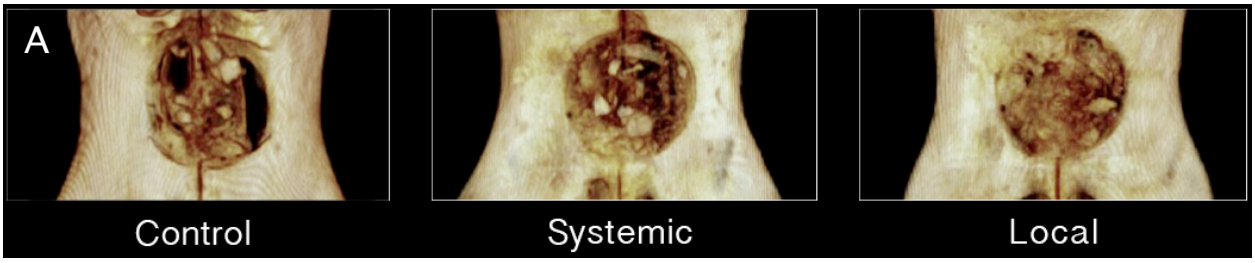
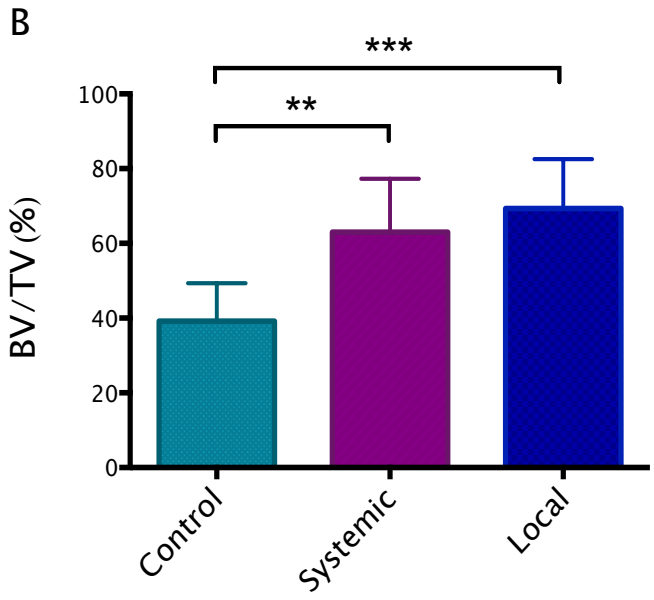



**Figure 6:** Testing for normality. (A) Shapiro-Wilk test (W), $p<0.05$. (B) Q-Q plot. (C) Histogram of bone volume fraction.



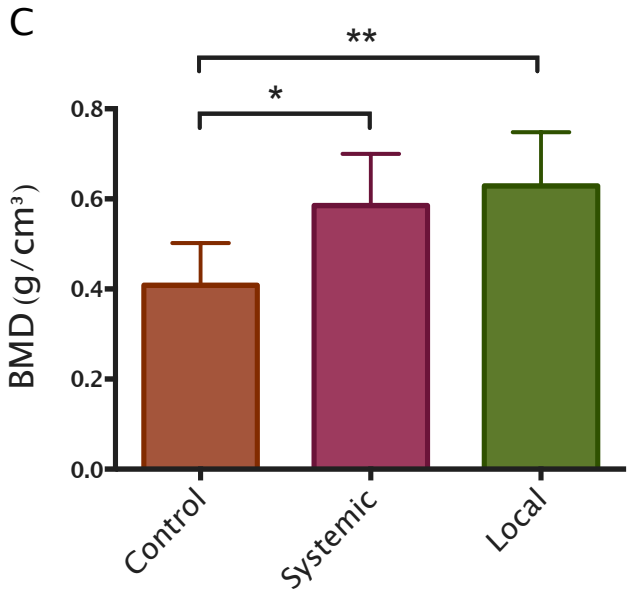
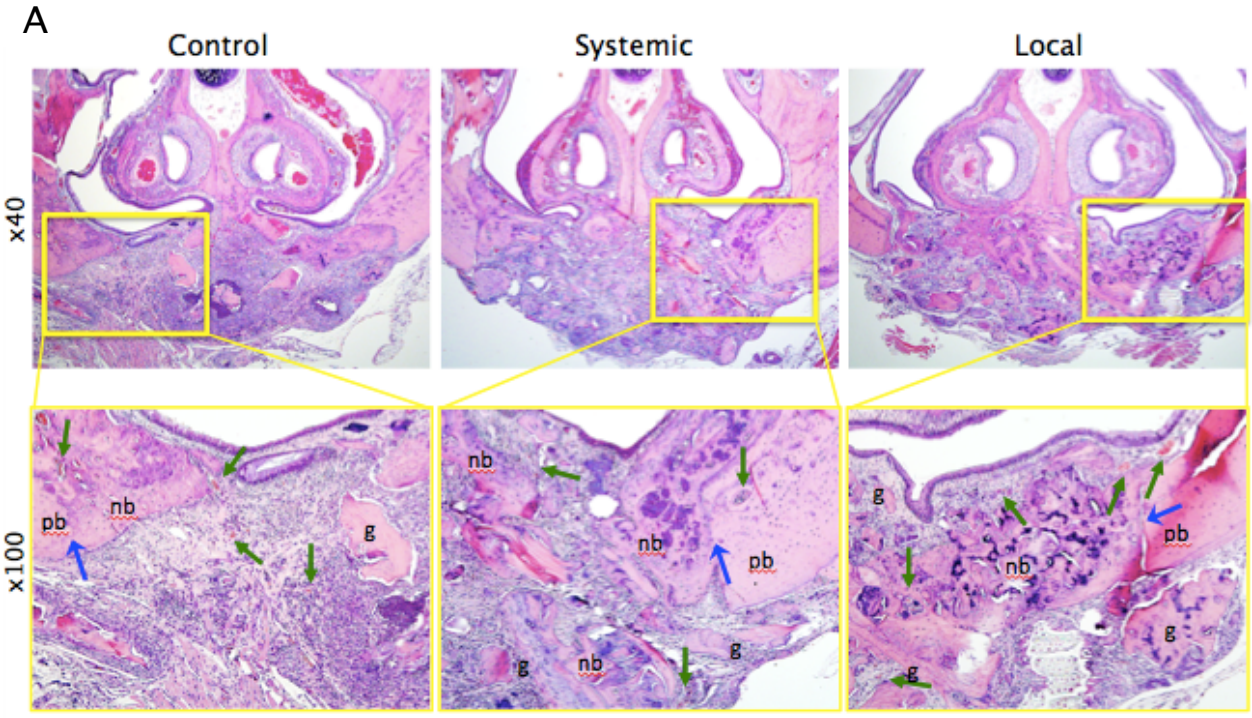




**Figure 6:** MicroCT imaging and 3D volumetric analysis. (A) 3D image reconstruction of palatal defect and grafting six weeks after surgery. Both systemic and local groups demonstrate a clear increase in bone volume compared to control. (B) Bone volume fraction (BV/TV). Both systemic and local groups had statistically significant increased bone volume compared to control, however, there was no significant difference between the two treated groups. (C) Bone mineral density (BMD). Similarly, both BP-treated groups had a higher BMD measurement than control, but did not differ between the two. *Statistically significant, $p<0.05$. Error bars show standard error.



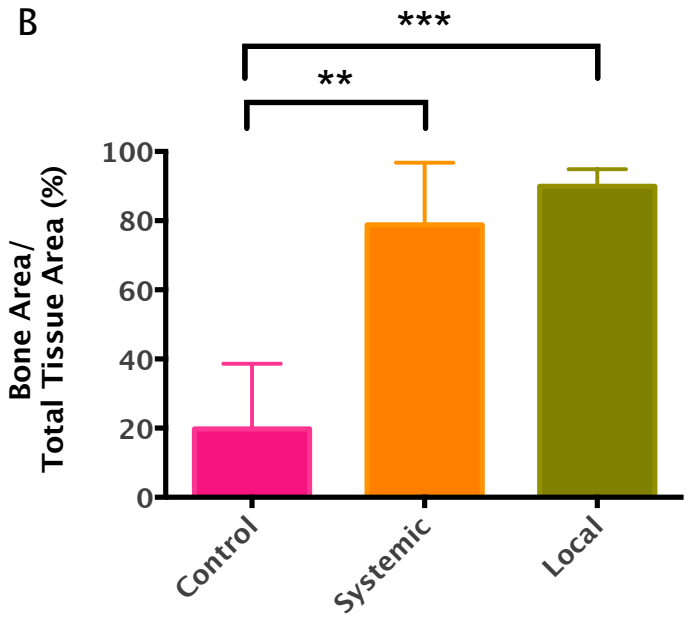




**Figure 6:** Histomorphometric analysis. (A) H&E stained coronal sections at x40 and x100 magnifications. New bone appeared as unorganized woven bone with the blue arrows demarcating bony union between the host palatal bone and graft bone. All three groups expressed angiogenesis (green arrows). g = graft, pb=palatal bone, nb= new bone, green arrows = blood vessels, blue arrows = border demarcating host and graft. (B) Percent bone area analyzed on H&E. The amount of bone tissue/total tissue area confirmed that BP-treated groups retained more bone tissue, graft material or newly formed, within the defect area. *Statistically significant, $p<0.05$. Error bars show standard error.

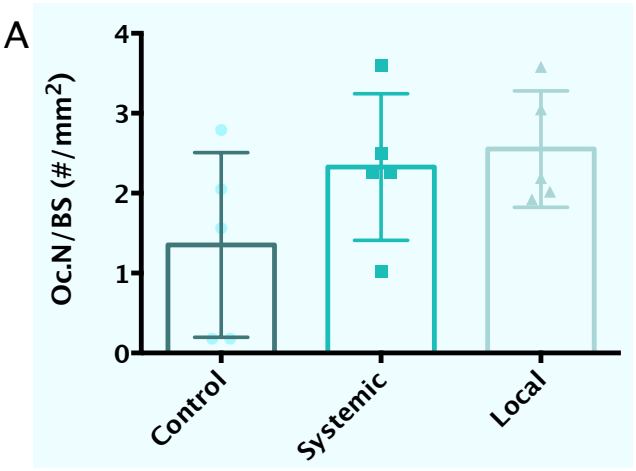
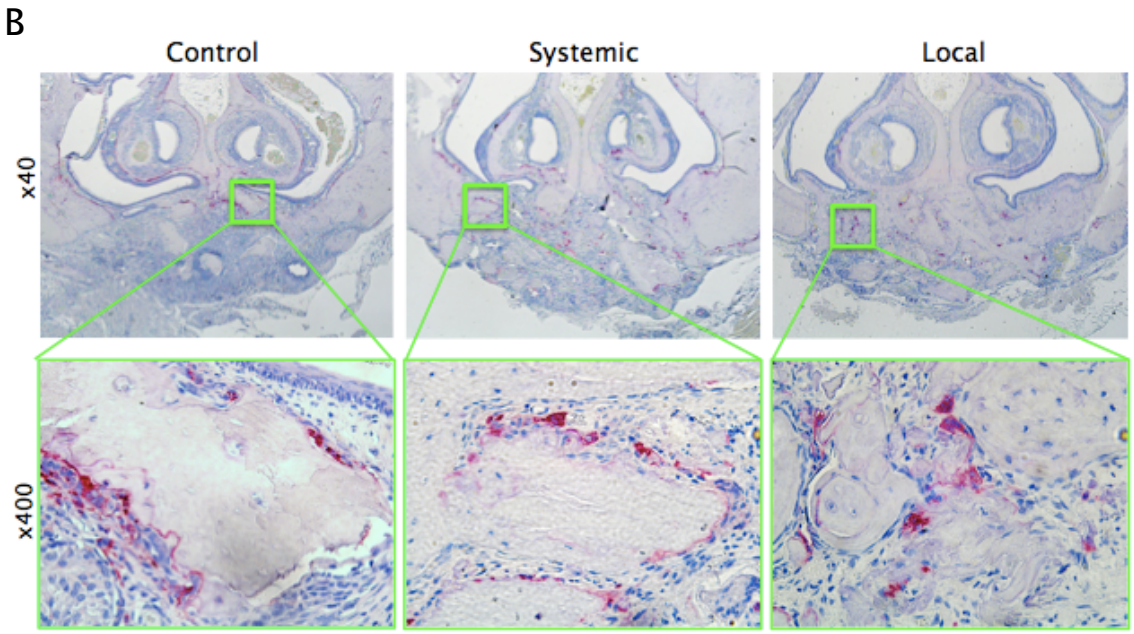
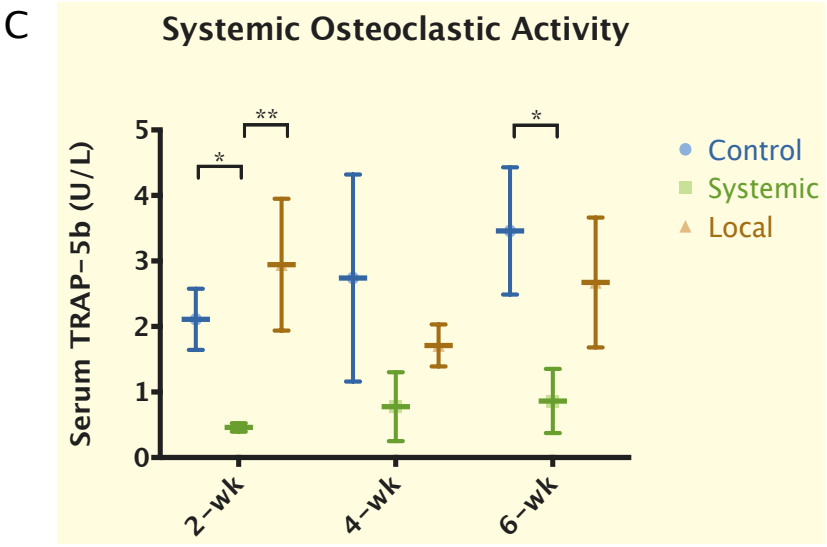



**Figure 8:** Osteoclast activity. (A) The difference in number of TRAP+ cells (osteoclasts) per bone surface was non-significant between all three groups. (B) TRAP staining at x100 and x400 magnification. (C) Serum TRAP-5b levels assayed by ELISA detected a significant reduction of osteoclastic activity in the system group compared to either the control or local groups. *Statistically significant, $p<0.05$. Error bars show standard error.



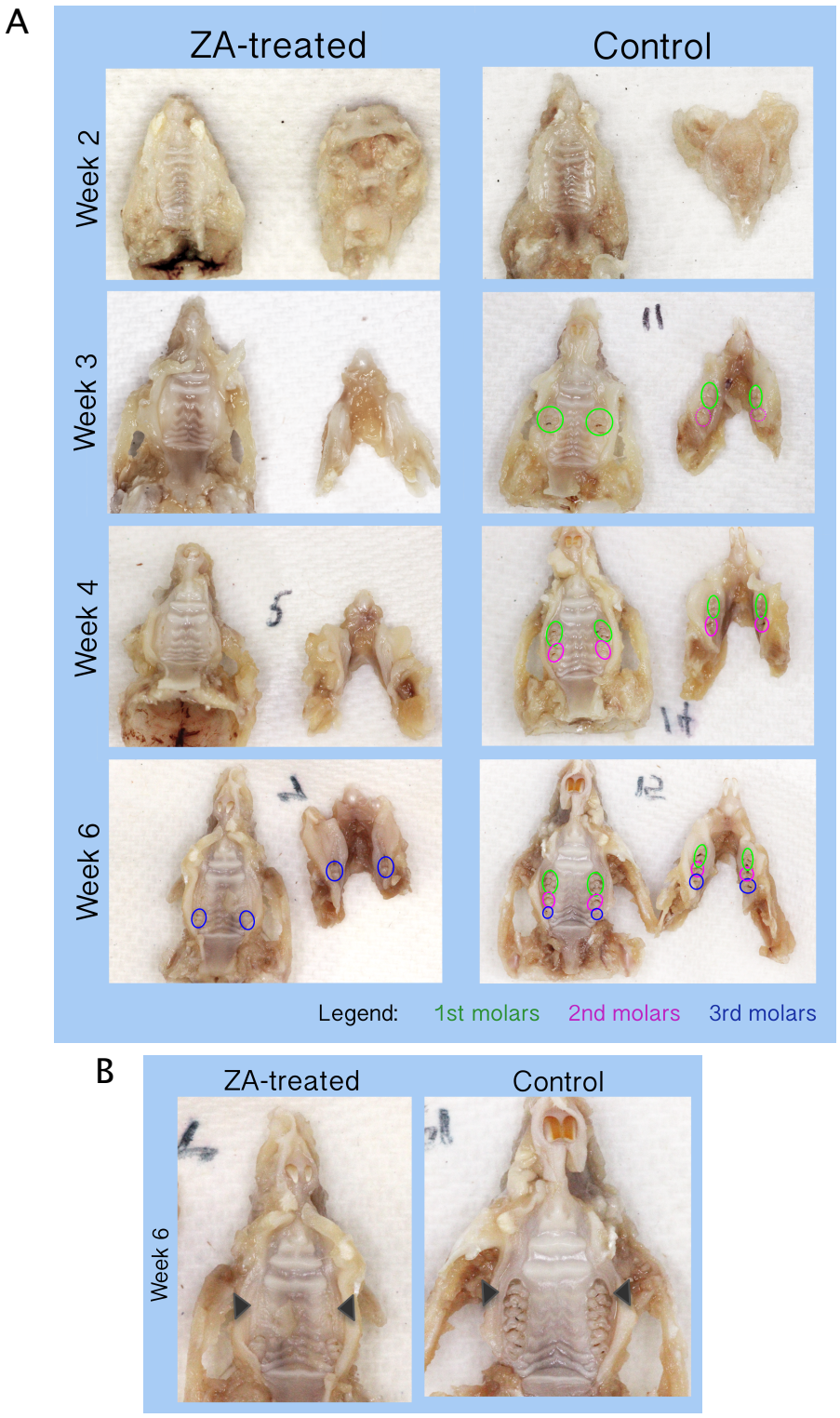



**Figure 9:** Clinical observation of molar eruption at weeks 2, 3, 4, and 6. (A) Eruption pattern in the mandible also closely mirrored that of the maxilla. Green = 1$^{st}$ molars, purple = 2$^{nd}$ molars, blue = 3$^{rd}$ molars. (B) Enlarged image of maxillae at week 6. Arrowheads indicate position of maxillary first molars.

| | Control | BP-treated |
|---|---|---|
| 1st Molars | Day 17 | Not visible by day 42 |
| 2nd Molars | Day 22 | Not visible by day 42 |
| 3rd Molars | Not reported (Visible by day 42) | Day 35 |

**Table 2:** Tooth Eruption Timeline. Age (in days) when molars were clinically detected as fully erupted (all cusps emerged through oral cavity). The third molars were not originally followed. However, molar eruption in the Zoledronate-treated group initially appeared at day 35, which was later confirmed by microCT to be third molars. Third molars in the control group erupted between days 28-42.

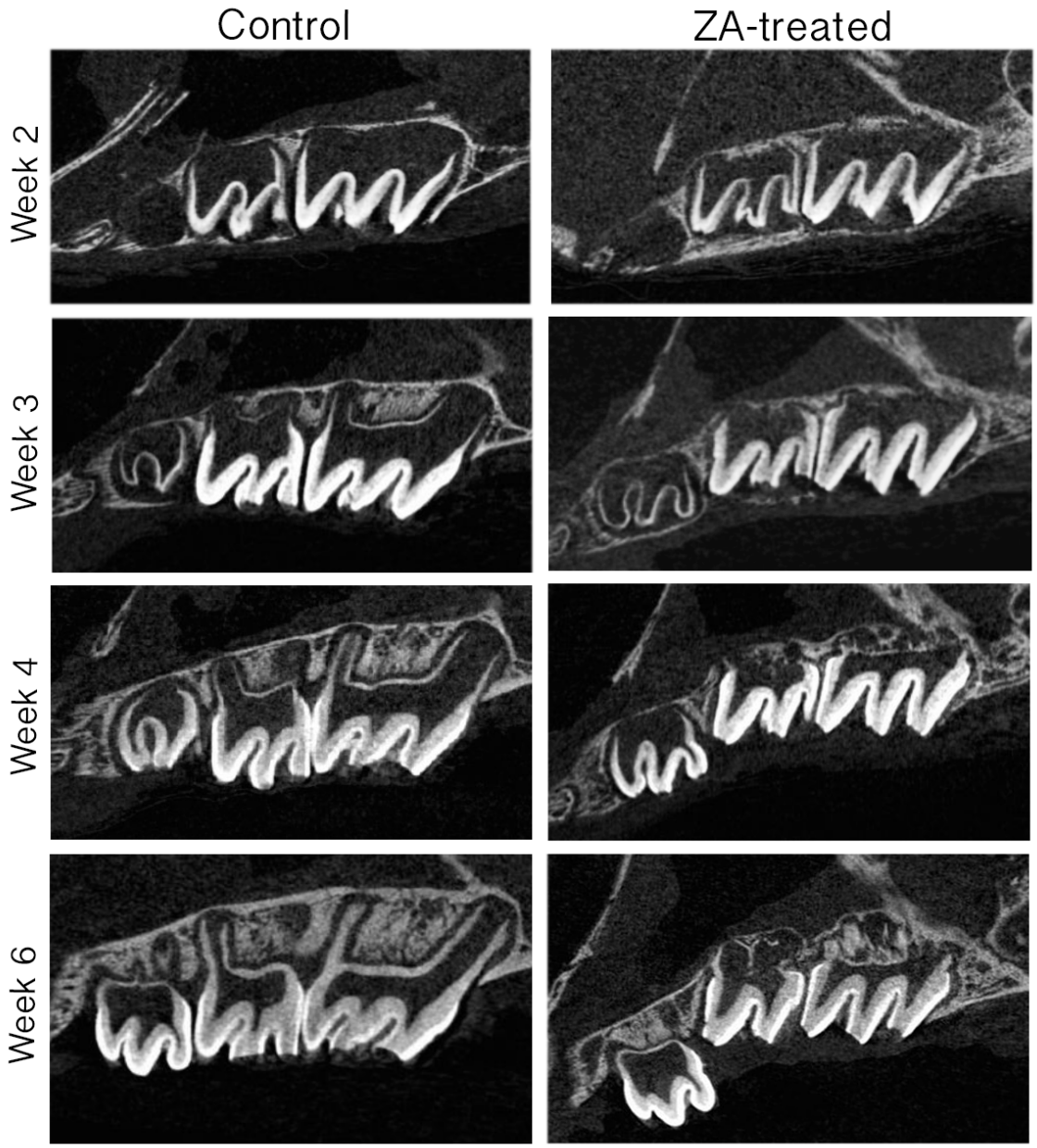



**Figure 10:** MicroCT imaging of molars. First and second molar crowns, but not third molar crowns, were present by week 2. Control group showed first and second molars eruption through bone starting at week 3, whereas, ZA-treated groups still show bone overlying the molars at week 3. Third molars erupted normally by week 6 in both groups.

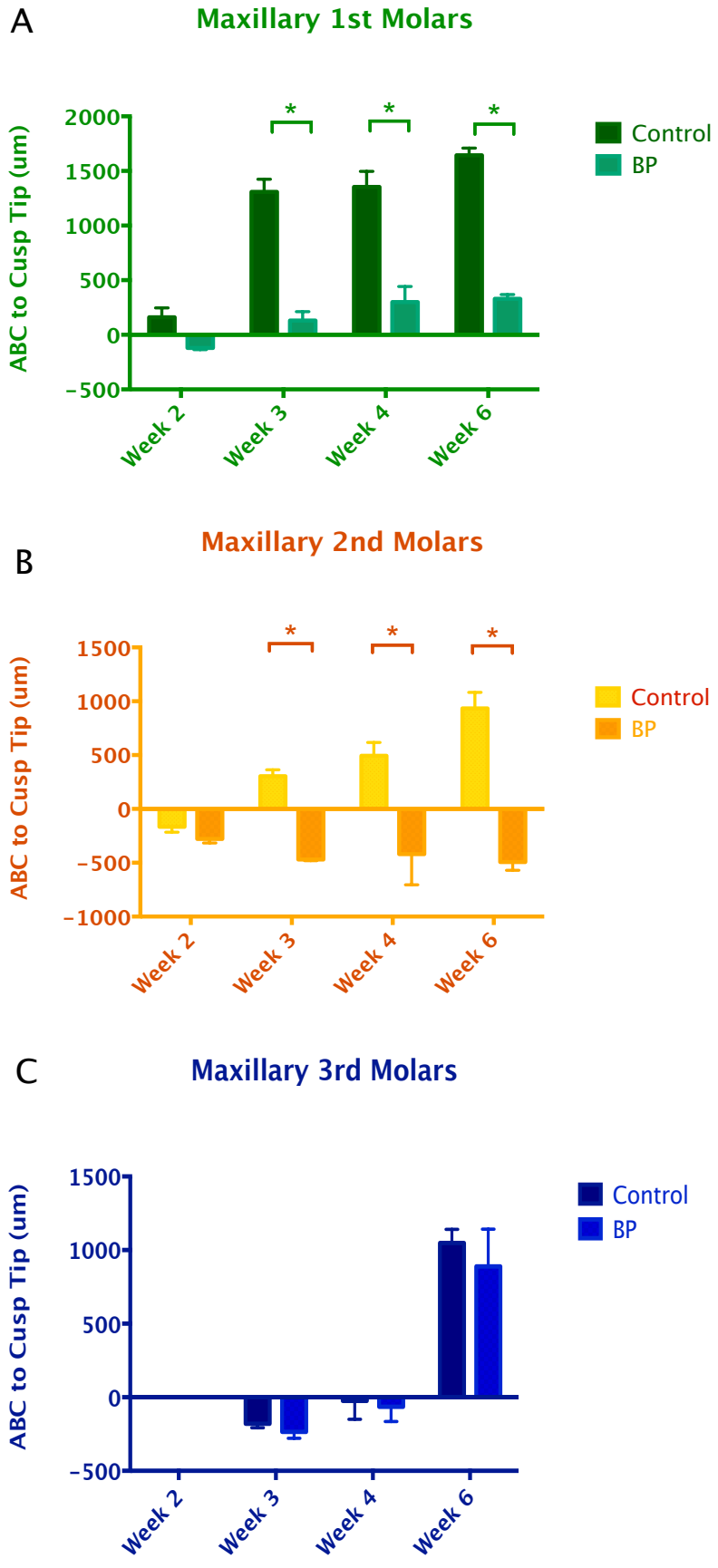



**Figure 11:** Degree of molar eruption. (A,B) The eruption of all first and second molars of ZA-treated animals was significantly delayed. Neither the first or second molars had erupted into the oral cavity by day 42. (C) In contrast, the third molars eruption normally and traveled approximately the same distance in both groups. *Statistically significant, $p<0.05$. Error bars show standard error.

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