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Effects of Timing of Bisphosphonate Treatment on Cleft Bone Grafting in an Animal Model

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Effects of Timing of Bisphosphonate Treatment on Cleft Bone Grafting in an Animal Model

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science in Oral Biology

by

Nicole Cheng

2014

ABSTRACT OF THE THESIS

Effects of Timing of Bisphosphonate Treatment on Cleft Bone Grafting in an Animal Model

by

Nicole Cheng

Master of Science in Oral Biology

University of California, Los Angeles, 2014

Professor Yeumin Hong, Co-Chair

Professor Sotirios Tetradis, Co-Chair

The aim of this study was to investigate the effects of BP on the success of bone grafts placed in palatal defects in rats. Bone was harvested and packed into palatal defects in recipient animals which were divided into four groups (n=8): (1) Saline, (2) BP at the time of surgery (T0), (3) BP one-week post-surgery (T1) and (4) BP three-weeks post-surgery (T2). All animals were euthanized at six weeks. Bone volume in the T1 BP (36.3 ± 8.8%) and T2 BP groups (36.9 ± 12.4%) was significantly greater than controls (24.8 ± 7.9%) and T0 BP group (22.1 ± 5.9%) ($p < 0.05$). H&E images confirmed increased bone in delayed BP groups. There was no difference in number of TRAP+ cells but BP groups showed abnormal morphology of 3.75% of TRAP+ cells. A single and delayed BP systemic injection can be considered a therapeutic drug to enhance cleft bone grafting.

The thesis of Nicole Cheng is approved.

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2014

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INTRODUCTION

Cleft lip and/or palate (CLP) is estimated to affect 1 in 700 births, representing one of the most commonly occurring congenital malformations to affect the orofacial region in the United States.[1] The associated problems in cleft patients are multifold such as nasal deformity, dental malocclusion, eating difficulty, speech disorders, ear infections, and a decline in psychosocial wellbeing.[2] The complexity in treating this anomaly necessitates multiple surgeries and frequent outpatient care throughout a patient's life, from childhood to adulthood.[3] Comprehensive treatment involves a team of specialists working closely together. In most cases, only after the completion of CLP surgical repairs, the patient is ready to undergo comprehensive orthodontic treatment (**Figure 1**).[4]

A major challenge for orthodontists in treating CLP patients is aligning the dentition surrounding the alveolar cleft where bone necessary for tooth eruption is lacking.[2] In CLP patients, orthodontic palatal expansion serves to eliminate any transverse discrepancy and widen the alveolar cleft region. Widening of the cleft volume facilitates the bone graft to "fill-in" the spaces.[5] Alveolar bone grafting is performed when one half to two thirds of the unerupted canine root has formed, known as secondary alveolar bone grafting. About six months following the graft surgery, the orthodontist begins to move the teeth into and around the grafted region.[6] This allows for alignment of the dentition in the upper arch for esthetic and functional purposes.

The process of orthodontic palatal expansion followed by alveolar bone grafting has become routine orthodontic treatment protocol for CLP patients.[7] However, it is well known that cleft bone grafting is subject to a high amount of bone resorption. Such bone resorption frequently results in insufficient bone volume in the cleft region necessitating additional bone graft surgeries and expansion procedures.[8] Repeated treatment 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.[2] It is, therefore, imperative to develop improved and innovative therapeutic modalities to obtain reproducible outcomes and to augment the clinical success of cleft bone graft surgery in CLP patients.

(A) Bone Graft Surgery in CLP Patients

During comprehensive orthodontic treatment, secondary bone graft surgery is performed 1) to close the alveolar cleft[2], 2) to prevent collapse and constriction of the dental arch[9], 3) to prevent escape of oral fluids through the oronasal fistula[10], and 4) to provide bony support for the cleft-adjacent erupting dentition (usually canines) to allow the teeth to erupt and stabilize the newly formed bone.[3] The gold standard for grafting in CLP patients is autograft.[11] The sources of autograft bone are commonly the iliac crest[6], calvaria[12] or tibia[13]. Autografts remain the gold standard because they are osteo-inductive, osteo-conductive and non-immunogenic.[14] Nonetheless, there is still an extremely high bone graft failure rate.

An imbalance of anabolic and catabolic activity during bone graft incorporation is a major contributor to bone graft failure.[15] Researchers have investigated different methods to increase the anabolic activity of osteoblasts as a way to increase graft stability. These methods include utilization of fibrin glue[16], platelet rich plasma (PRP)[17], and bone morphogenetic proteins (BMPs)[18]. However, the adverse effects of such methods, including hematoma formation, swelling, respiratory difficulties, and long-term growth effects are alarming and have prevented their routine use.[19-21]

Incorporation of a bone graft involves not only formation of new host bone, but also resorption of the donor bone. Slowing down graft resorption is particularly important for cleft

bone grafting, as studies measuring bone loss of grafts using CT scans report as high as 64% loss of bone volume after 1 year.[22] Bone grafting in the intraoral cleft is prone to high resorption tendency for several reasons: 1) tension developed from the mucoperiosteal flap and dehiscence[23], 2) bacterial infections specific to oral-nasal environments[10, 24, 25], and 3) the absence of mechanical stresses, which are required to increase bone formation and minimize bone resorption as stated in Wolff's Law[26, 27]. To date, there have been limited bone graft studies to determine whether new bone formation could be enhanced by slowing down the graft resorption process. If the bone graft scaffold can be retained for a longer period of time, this would allow for greater volume of bone to be deposited on, leading to a net increase in bone. Thus, limiting the increased resorption rate using an anti-resorptive agent is a promising new method that can significantly improve the clinical success of cleft bone graft surgery in treating CLP patients.

(B) Mechanism of Bisphosphonates (BPs)

Bisphosphonates (BPs) belong to a class of anti-resorptive drugs medically used to prevent loss of bone mass by binding to hydroxyapatite in bone and causing osteoclast dysfunction. Their chemical structure consists of two phosphonate groups and two side chains which differ depending on the type of BP used.[28] Previous studies have shown that BP therapy is associated with anti-catabolic activities during bone remodeling.

BPs' anti-resorptive function in bone grafting has been demonstrated in several studies. It has been shown that BPs can aid in bone graft incorporation when applied locally or in a single systemic injection. In a rat bone chamber model, it was shown that when bone grafts were soaked in the BP, Alendronate, they became resistant to resorption.[29] In another study, when morselized allografts, rinsed with Ibandronate, were given to patients undergoing total hip

replacement, there was significantly less bone resorption.[30] A single subcutaneous injection of Zoledronate protected against bone allograft resorption in bone chambers in rats.[31] However none of these previous studies have investigated the effects of BPs on the outcome of bone graft specifically in the oral cavity due to the difficult nature of the surgical procedure in the mouths of animal models.

(C) Existing Animal Models for Cleft Bone Graft Surgery

Genetic mouse models for CLP exist; however they die soon after birth, preventing the study of CLP post-natally.[32] Therefore, in order to study bone grafting in oral clefts, a critical-sized defect (CSD) needs to be created to mimic the cleft. A common CSD model used to study bone graft incorporation involves a circular defect creation in the calvaria as this design provides good surgical access and high reproducibility.[33] However it fails to accurately represent bone graft in CLP patients, as intraoral factors such as the oral bacterial flora, mastication forces and salivary components are not present in these models. Recently, a study has developed an intraoral CSD to mimic the cleft.[34] This design was recreated in our preliminary studies but we experienced severe incisal root damage. The defect cut through the tooth, causing unnecessary pain, introducing pulpal bleeding and cells to the defect. In addition, the design is an irregular shape, compromising reproducibility of the study. Therefore, better animal models are absolutely required to accurately mimic a cleft bone graft model.

(D) Specific Aims

This study was designed to:

1. Establish an animal model to study bone grafting in the oral cavity
2. Determine the effects of BP treatment on bone grafting in this animal model of a surgically created palatal cleft.

(E) Pilot Study & Rationale for Full Study

To investigate the potential physiological role of BP in limiting bone graft resorption *in vivo*, we first established an animal model for the intraoral cleft by creating a surgical defect to mimic the cleft **(Figure 2).** To determine the feasibility of the animal model and an adequate sample size, we first performed a pilot study, comparing results of BP versus Saline treatment after bone grafting in the palate.

For the full study, we examined the effect of timing of BP delivery on the clinical outcomes of oral cleft bone graft in rats. Previous studies have shown that a delayed BP administration can enhance clinical success of bone graft in femoral fracture and defect models.[35, 36] Contrasting these findings, a multi-center study found that there was no difference in fracture healing or incidence of complications with different timing of BP delivery. The timing of BP injection was compared at the time of surgery, one week post-op, one month post-op, and three months post-op.[37] Thus the effect of timing of post-operative BP injection remains controversial and additional studies are needed to determine its clinical significance. Furthermore, the effects of different timing of BP administration specific to intraoral cleft bone grafting are yet to be determined as these previous studies all examined the femur. In order to determine an optimal window for BP delivery, two delayed injections at one week and three weeks after surgery will be evaluated. We hypothesized that delayed BP injections will improve bone volume as compared to controls and injection at the time of surgery.

METHODS

(B) Animals

For the pilot study (ARC # 2012-027-01), animals were divided into two groups: (1) **Control group:** graft with saline injection (n=3) and (2) **Systemic BP group:** graft with BP injection (n=4). Two animals served as negative controls where a defect was created but no bone graft was placed. Autograft bone was harvested from the iliac crest and femur of a Fischer F344 Inbred donor rat. One week after surgery, the control group was given a subcutaneous injection of saline while the experimental group was given an injection of the BP, Zoledronate (ZA) (0.1 mg/kg). All animals were sacrificed at 6 weeks post-op for μCT and histological analysis (**Figure 3**).

Based on our preliminary study, the power analysis was performed ($n = (z_{1-\alpha/2} + z_{1-\beta})^2(\sigma_1^2 + \sigma_2^2) / (\mu_1 - \mu_2)^2$) such that 8 rats will be required in each group to achieve a power level of 0.8 and α=0.05. The animal research protocol for the full study was then approved (ARC # 2012-027-02A) by the UCLA Animal Research Committee (ARC). A total of 40 female, fifteen-week-old Fischer F344 Inbred rats were purchased (Charles River Laboratories, Inc.) and housed in a light and temperature controlled environment. The rats were divided into four groups (n=8 for each group): (1) **Control group**: bone graft with systemic saline injection, (2) **Systemic BP injection (T0)**: Systemic ZA injection at the time of surgery (3) **Systemic BP injection (T1)**: Systemic ZA injection 1 week post-operative (4) **Systemic BP injection (T2)**: Systemic ZA injection 3 weeks post-operative. Two animals served as negative controls where the defect was created but the bone graft was not placed, and two animals served as positive controls where no surgery was performed. Four F344 Inbred rats were used as bone isograft donors (**Figure 4**).

(C) Surgical Procedure

(a) Harvesting bone graft from the donors: For donor animals, an incision was made on the lower back and the skin reflected. Soft tissue was separated through blunt dissection to gain

access to the pelvic bone. The corticocancellous bone from the iliac crest and femur was harvested. A glass mortar and pestle was used to manually grind the corticocancellous bone into fine particles with sterile saline. The minced bone was then placed on ice for immediate use.

(b) Creation of defect: Recipient animals were anesthetized initially with Isoflurane (4-5%) followed by intraperitoneal (IP) injection of 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 immediately after the induction of anesthesia to allow it to take effect before the first incision was made. The animals received 0.3 ml of 1% lidocaine with 1:5000 epinephrine submucosally along the alveolar ridge. A 1cm longitudinal mucosal incision was made along the junction between the hard palate and alveolus. The dentoalveolar periosteum was elevated to expose alveolar bone distal to the maxillary incisors (**Figure 5a**). Using a hand-operated, low-speed power drill, a 3mm midpalatal defect was created distal to the upper incisors, using a 3mm in diameter trephine bur, generating an accurate and consistent defect size for each surgery. Due to the long length of maxillary incisors of rats, a bi-cortical 3 mm circular defect was placed in the mid-palate to avoid dental root damage (**Figure 5b**). No puncture into the nasal mucosa was made. After adequate hemostasis using pressure and gauze, the harvested autograft was placed into the defect and the mucosa re-approximated with absorbable 5-0 Vicryl sutures (**Figure 5c**). Postoperatively, each animal received subcutaneous injections of Buprenex (0.05 mg/kg) two times a day for two days as analgesia. Trimethorprimsulfa (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.

(c) Placement of the bone graft: Harvested cancellous bone was placed into a 300µl syringe with the tip removed. The bone was packed into the syringe and saline blotted such that

there was dense concentration of bone without desiccating the graft material. 5µl was dispensed into each animal defect to ensure accurate consistency of volume and concentration.

(D) BP Delivery

A single 0.1 mg/kg subcutaneous injection of ZA was administered to the animals at either the time of surgery (T0), one week post-surgery (T1), or three weeks post-surgery (T2). For the control group, a single systemic injection of saline was administered one week post-surgery. Six weeks post-surgery, all animals were euthanized and the maxilla harvested.

(E) Data Analysis:

1. Micro-CT Analysis: The maxilla was dissected and fixed with 4% (w/v) paraformaldehyde in 0.1 M phosphate-buffered saline solution for 24 hours. The samples were scanned using a high-resolution micro-computed tomography (SkyScan 1172, SkyScan N.V., Belgium), at an image resolution of 9.87 µm, with 70 kV and 141 µA X-ray source and 0.5 mm aluminum filter. Then 3D image datasets were reconstructed from 2D X-ray images using NRecon software (SkyScan N.V., Belgium), which processes appropriate image correction steps including ring artifact correction, beam hardening correction and fine-tuning. After acquisition of the datasets, the images of samples were viewed and reoriented on each 3D plane with DataViewer software (SkyScan N.V., Belgium) to align the palatal defects parallel to the transaxial plane, which minimizes errors during analysis.

3D volumetric analysis was performed using CTAn software (SkyScan N.V., Belgium). To ensure the consistency of the results, all analyses were done twice at two separate time points by a single rater. The Region of Interest (ROI) was outlined with a 3mm circle on consecutive transaxial sections in order to create a uniform, cylindrical-shaped Volume of Interest (VOI), which encloses the entire defect area. Grey threshold values were determined by approximating

images to their true morphology. Bone volume (BV) and tissue volume (TV) were measured to calculate percent bone volume (BV/TV) of each graft site.

2. Histological Analysis: After Micro-CT analysis, the samples were decalcified for 20 days in 10% ethylenediaminetetraacetic acid (EDTA 0.1M, pH=7.1) solution. During decalcification, the solution was changed every two days. After decalcification, the samples were dehydrated through graded ethanol and embedded in paraffin. Then, the samples were sectioned coronally in 10 µm sections. Sections were stained with hematoxylin and eosin (H&E) by the UCLA Tissue Procurement Core Lab (TPCL) to visualize bone graft morphology.

Evaluating Osteoclasts: One section from each animal was stained for Tartrate-resistant acid phosphatase (TRAP), using TRAP staining kit (Sigma Aldrich, St. Louis, MO). The slides were de-paraffinized at 60^{o}C for 30 minutes, then rehydrated with xylene and graded ethanol solutions. The slides were then incubated in acetate buffer (pH 5.0) containing naphthol ASMX phosphate, Fast Red Violet LB Salt and 50 mM sodium tartrate. They were placed in humidity chambers at 37^{o}C for 2 hours. Slides were then counter-stained with Hematoxylin for 8 seconds to differentiate between soft and hard tissues, and then rinsed with tap water. Samples were mounted with aqueous mounting solution. Osteoclasts were defined as multinucleated TRAP-positive cells. For quantification, the number of TRAP+ multinuclear cells ($\geq$ 3 nuclei) on the external surfaces of the bone was counted using the surgical defect as the field of view. The number was presented as number of TRAP+ cells/mm^2 bone area. Quantification was performed with a single operator blinded to the clinical information at two separate time points.

3. Statistical Analysis: The data was expressed as means and standard deviations for each group. The data was analyzed using parametric statistical tests. One-way ANOVA analysis for statistical comparison among the groups: (1) Control, (2) T0 BP injection, (3) T1 BP injection,

and (4) T2 BP injection ($\alpha = 0.05$). Once a difference was detected with ANOVA analysis, post-hoc t-test by the Fisher least squares difference method (JMP, Cary, NC, USA) was used to compute the significant effects between the sub-groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Pilot study findings

The pilot study data confirmed that oral mucosal tissues at the surgical lesions were completely healed and closed without any complications up to six weeks post-surgery. Micro-CT analysis showed a significant increase in bone volume fraction (BV/TV) in the BP group (75.3 ± 6.8%) compared to that of the control group (43.1 ± 9.1%) ($p \leq 0.05$) (**Figure 6**). Similarly, H&E staining of coronal sections of the defect verified a clear increase in bone mass in the BP group (**Figure 7**).

Micro-CT findings

The percent bone volume in the positive control group was 80.3 ± 2.6% and in the negative control was 8.9 ± 1.5%, indicating this is a CSD model that does not heal completely over a six week duration. Delaying BP administration by one-week and three-weeks resulted in signficantly increased percent bone volume, 36.3 ± 8.8% and 36.9 ± 12.4% , respectively, compared to controls (24.8 ± 7.9%) ($p < 0.05$). There was no significant difference in bone volume between delay at one-week and three-weeks. Surprisingly, BP administration at the time of surgery did not increase percent bone volume (22.1 ± 5.9%) and was even slightly lower than that of saline controls (24.8 ± 7.9%) (**Figure 8**). The only significant difference in BMD was that between the positive control and the rest of the groups.

Histological findings

H&E images confirmed increased amount of a combination of new bone and bone graft in delayed BP injected animals at T1 and T2. The positive control showed an intact palatal structure consistent with normal rat maxillary anatomy. The negative control confirmed the surgical defect and absence of bone in the defect site with only soft tissue. Interestingly, all animals with BP at T0 showed significantly decreased bone volume with signs of acute inflammation. These findings demonstrate that if BP is administered immediately following surgery, it can have a negative impact on bone healing (**Figure 9**). There was no significant difference in TRAP-positive cells between BP and control groups. Nine out of 240 TRAP+ cells (3.75%) of the BP-treated animals did exhibit abnormal morphology of TRAP-positive cells which could be described as rounded and detached from the bone surface (**Figure 10**).

DISCUSSION

Animal Model for Intraoral Bone Grafting Studies

CSDs were originally defined as the smallest intra-osseous wound which does not spontaneously heal during the lifetime of an animal.[38] More recently, another study has redefined CSD as a defect that will not heal over the duration of the study.[39] While researchers have long utilized CSD animal models to study bone generation *in vivo*, there remain differing data in regards to the exact dimensions of a CSD in rats. Studies have created CSDs in different strains of rats ranging from 2-8mm, and it has been determined that 3mm or less calvarial defects are subcritical while 5mm and greater are considered CSDs.[40] According to these previous studies, the 3mm defect created in the palatal region is considered a subcritical size defect (sCSD), which is defined as a defect in which complete bone regeneration is expected in the animal lifetime. The sCSD can be advantageous in that it provides an environment to study the "natural" process

of bone healing and thus can assess the effects that various drugs and conditions can have on the physiological process of bone healing.[41]

However, all these previous studies analyzed the healing of defects in the calvaria, and thus these predetermined values for sCSD and CSD, may not be applicable to healing in other bones such as the maxilla. The added variables in the maxilla are numerous and include the presence of load stress, graft mobility, oronasal bacteria[42], salivary components and differences in vascularity. Furthermore, the specific rat strain, age, and weight which are unique to our study will affect healing of the defect. For example, it has been shown that animal age is crucial to bony healing and that younger animals demonstrate an increased healing capacity.[43] In our study we determined that the 3mm in diameter palatal defect does not heal completely over the six-week duration of this study and up to a 10-week observation period. This animal model is considered a CSD if defined as a defect that does not heal over the duration of the study. A total of 43 animals from both the pilot study and full study underwent surgical creation of this palatal defect. All animals survived without post-operative complications demonstrating the surgical predictability and reproducibility of this procedure representing the successful establishment of intraoral CSD in this study.

These findings are significant in that our established animal model is instrumental in evaluating the clinically challenging procedure of cleft bone grafting, at the preclinical level. This defect would circumvent lethal problems associated with genetic mouse models for CLP while accurately representing a typical bone graft procedure in CLP patients by incorporating intraoral confounding variables. This animal model may have further implications in studying other agents and conditions in relation to cleft bone grafting.

Future Application to Human Clinical Trials

In addition to being able to study other osseous drugs, this model was designed such that it could be applied for human trials. This study was created to closely mimic the alveolar cleft surgery in humans. Since the gold standard for cleft bone grafting remains the autograft, we chose to use Fischer F344 Inbred rats due to genetic consistency from breeding and elimination of the host immune response.[44] This allows for bone isografts to be transferred between rats without triggering an immunological reaction, and mimics autografts in humans. Similarly, the type and dosage of BP was determined based on what is currently used clinically. Zoledronate (ZA) was chosen because its higher potency allows less frequent administration compared to other BPs, and patients use ZA as infrequently as a single injection per year, making it one of the more convenient BPs in the market.[45] The concentration of 0.1 mg/kg ZA is calculated for rats based on the commonly used therapeutic low dose given annually to humans for treatment of osteoporosis.[35] This concentration of ZA is proven to be clinically applicable and safe for use by the Food and Drug Administration (FDA). In addition, we have injected ZA at this concentration only one time, which is even lower dosage than giving injections yearly in osteoporotic patients. Effectively, this concentration of ZA can be more easily applicable to human trials in future studies of cleft bone grafting pending confirmation of results with further animal studies.

Significance of Results within the Current Body of Literature

The significant increase in percent bone volume seen with the delayed BP injection groups at T1 and T2 correlate with previous findings studying the variation of timing for BP injection in femoral fracture and defect animal models. A study examining bone fracture healing in the rat femur, demonstrated that delaying BP injection by one and two weeks produced a larger and stronger callus.[35] Another study by the same group demonstrated that BP injection two weeks after creating a femoral defect increased the bone volume and strength compared to BP injection

at the time of defect creation.[36] More recent studies have utilized these findings and delayed BP injections in their experiments. In a femoral osteotomy model, BP injections were delayed by 2 weeks and results showed an increased callus volume and denseness and higher peak force with mechanical testing.[46, 47] Their results further confirmed that delayed BP injection can improve treatment outcomes.

There could be different reasons as to why this delayed BP can increase volume and strength of bone. It is possible that delaying BP injection can allow the endogenous anabolic and catabolic responses to establish themselves before administering the drug. As such, when BP is administration is delayed there is a higher rate of bone turnover present. This higher rate of bone turnover can allow for greater binding of BP to the grafted region, leading to a higher anti-catabolic response. It has been shown that BPs have a strong affinity for bone mineral, particularly at sites of increased bone turnover. Carbon-14 labeling of BP has shown that there is higher concentration in delayed injection groups in a femoral fracture model.[35]

These findings of higher BP binding in delayed injections in a femur model can be applied to grafting in the maxilla and may even be amplified. Maxillary bone is unique in that the bone remodeling rate is much higher. In a study examining the jaws and femur of young dogs, it was determined that there is a three to six fold difference in bone turnover rate between the maxilla and femur.[48, 49] While the higher rate of remodeling in alveolar bone is unclear, it has been postulated that functional and eruptive forces from the dentition could be responsible. This high bone remodeling rate in the maxilla may allow for even greater binding of BP and thus a higher anti-catabolic effect in cleft bone grafting. Future studies with radioactive or fluorescent labeling of BP can be conducted to determine the bio-distribution in this intraoral cleft model.

The differences in bone volume fraction between the groups may also be associated with soft tissue healing. Allowing enough time for complete primary wound closure before drug administration can reduce the risk for post-operative infection and/or BP-related osteonecrosis of the jaw (BRONJ). From our pilot study, we confirmed that there was complete primary soft tissue healing by one week after the bone graft surgery. We suspect that when BP administration is delayed by at least one week, oral soft tissue is allowed to achieve primary wound closure, thereby reducing the likelihood of secondary infections.

A study found that BP accumulation in bone can be directly toxic to the oral epithelium.[50] Studies have shown that BPs can impair epithelial hemeostasis by slowing the re-epithelialization ability in wound healing. The epithelial adhesion, terminal differentiation and proliferation of keratinocytes were reduced in a study examining the effects on the oral mucosa of women taking BP for osteoporosis.[51] *In vitro* studies have also demonstrated that fibroblast and oral keratinocyte proliferation is reduced after BP administration.[52, 53] Another *in vitro* study examining keratinocytes found that BPs suppress epithelial cell growth through inhibition of the cholesterol synthesis.[54] In line with these findings, our results show that BP injection at T0 demonstrated significantly lower percent bone volume and all animals had signs of acute inflammation on histological examination (**Figure 9**). An inadequate soft tissue primary healing mechanism may another reason why BP T0 percent bone volume was significantly lower than that in the delayed injections.

Our histological results demonstrated no difference in TRAP-positive cells between BP and Control groups. Tartrate-resistant acid phosphatase (TRAP) is an enzyme whose expression is used as a marker for osteoclasts. It is found in within osteoclast lysosomes and resorptive hemivacuoles,[55] and is secreted by osteoclasts into the resorptive bone area. It has been shown

that TRAP knockout mice have increased mineral density and mild osteopetrosis, indicating the important role it plays in maintain *in vivo* bone homeostasis.[56] On the opposite end of the spectrum, transgenic mice created to over-express TRAP have demonstrated decreased levels of trabecular bone.[57] Interestingly, in these mice with TRAP over-expression, there was no significant difference in osteoclast number despite a significant decrease trabecular bone. These findings suggest that TRAP expression is not directly associated with osteoclast number and/or activity, and can explain why there was no difference in TRAP numbers in our study between BP and Control groups.

While there was no difference in TRAP-positive cells, there were 3.75% of the TRAP+ cells with abnormal morphology of being rounded and detached from the bone surface in BP-treated groups (**Figure 10**). Similar to our findings, other studies examining high dosage BP treatment in animals have found no difference in TRAP-positive cells,[58, 59] but abnormal morphology of TRAP-positive cells in BP-treated groups.[58] This morphological aberration may indicate a disruption of osteoclast function. Apoptosis of osteoclasts has been described as presence of chromatin condensation and nuclear fragmentation with a loss of adhesion to the bone surface.[60] The BP treatment in our study does appear to impair the resorptive activity of osteoclasts but is only seen in a few cells. Since TRAP activity and osteoclast function are not directly correlated, a mild disruption of osteoclast function seen in our study may not be enough to detect a significant difference in TRAP numbers. Future studies coupling apoptosis assays in conjunction with TRAP staining could help to explain number of non-functioning to functioning osteoclasts.

Alternatively, this data can be interpreted in that osteoclast function may have been initially significantly disrupted. However at the time of sacrifice of six weeks the activity may

have been almost fully restored. Studies have shown that the osteoclast lifespan is short lived with the range from a few days[61] and maximally up to six weeks.[62] It is possible that at the time of sacrifice at six weeks in our study, most of the dysfunctional osteoclasts affected by BP had already undergone apoptosis, while the newly generated osteoclasts show normal resorptive activity. Future studies sacrificing animals soon after BP injection with multiple shorter intervals of sacrifice, could demonstrate osteoclast activity levels as a function of time.

Complications Associated with BP to Consider

After alveolar cleft bone grafting in humans, the developing canine is expected to erupt into the grafted region and stabilize bone levels. A possible concern regarding the application of BP in cleft patients is that it may inhibit or delay permanent tooth eruption. Bone resorption is needed during tooth development such that there is space in the alveolar process for the tooth to erupt into the oral cavity. It has been shown that injection of Pamidronate 1.25 µg/g daily for up to 15 days results in delayed tooth eruption in newborn rats.[63] A more recent study examined the development of molar buds from the day of birth up to 30 days, where they injected 2.5 mg/kg of Alendronate daily. By day 30 the control molars erupted to the level of the occlusal plane while the molars of the BP-treated animals remained surrounded by bone and did not erupt.[64] These previous studies injected daily doses of BP at high concentrations for up to one month, giving a much higher dosage of BP than used for our study. It may be possible that ZA will not interfere with tooth eruption at a low dose, single injection of 1.0 mg/kg. Future studies in our laboratory will examine the effects of our one-time low dosage injection of BP on newborn rat tooth development.

Another concern is that these patients often need dental implants when they are finished growing, as they are often missing teeth in the cleft region. Previous studies demonstrated that

dental implants are not a contraindication in patients taking oral bisphosphonates, particularly if they have not been taking BPs for more than 5 years.[65] However studies have shown that dental implants are contraindicated in patients taking BP through intravenous (IV) injection. These studies however examined BP injections given for cancer therapy, which utilizes much higher dosage, frequency and duration of BP treatment than that used to treat osteoporosis.[66] There have not been clinical studies examining dental implant placement in patients taking lower dosage IV BP, however recently there was a case report published where they placed a dental implant in a 58-year-old male taking ZA for osteoporosis.[67] They followed up the patient up to 6 months after implant placement, and there was successful implant osseointegration with no clinical signs of pathology. More studies are needed to determine potential risks associated with dental implant placement in patients taking IV ZA once yearly for osteoporosis.

The application of BP in our study will be used specifically in growing patients. As such, there are concerns regarding the long-term effects on bone remodeling and maturation. There are studies demonstrating BP usage in children and adolescents to treat a myriad of bone pathologies most commonly including Fibrous Dysplasia (FD) and Osteogenesis Imperfecta (OI).[68] In a retrospective study utilizing BP to treat FD in nine children, they found sclerotic bands at the epiphyseal and metaphyseal growth plates,[69] however after cessation of BP new bone forms at the growth plate and the sclerotic bands disappear, indicating that this is a reversible phenomenon. In a larger study treating Fibrous Dysplasia in 58 patients, 17 of them being under 18 years of age, they used IV Pamidronate. These patients were followed for an average of 50 months, and it was concluded that long-term treatment with Pamidronate was safe.[70] BP therapy for OI in children has also become a safe and established protocol to increase BMD and reduce fracture incidence.[71-73] However, long term effects on growth after BP treatment still need to be

determined. A study examining long bone changes after BP treatment in OI patients found that after discontinuation of BP, there was lower bone density at the metaphysis of the radius than that of bone created during BP treatment. The authors suggest that this may produce a localized region of bone fragility after treatment is stopped in growing patients.[74] In contrast, a case report has shown that extremely high doses of BP during treatment can lead to osteopetrosis[75] and ongoing remodeling defects that continue to persist for up to six years after treatment.[76] However in this patient the amount of Pamidronate used was more than four times the dosage typically used to treat OI. Conclusively, the literature shows that BP use in growing patients is justified however there should be caution in regard to transient but possibly reversible changes at bony growth plates. The dosage of BP should also be limited to reduce side effects and possibility for osteopetrosis. Further long-term studies in children are still needed since BP is known to accumulate in bone for many years.[77]

The risk for BRONJ with IV BP treatment is the complication most concerning to dental professionals, with the incidence ranging from 0.8-12% when used to treat hypercalcemia and bone metastases.[78] In 2007, IV ZA was FDA approved to treat osteoporosis, with the concentration and frequency of administration much lower than that of IV BP used to treat malignancies. A randomized clinical trial of over 8,000 patients demonstrated that a once a year 5mg IV ZA treatment over three years had only two cases of ONJ, one in each BP and placebo groups.[79] This study demonstrates the risk for BRONJ with IV BP for osteoporosis is significantly lower than reported data for IV BP treatment for bone malignancies, with nearly 0% incidence. BP use particularly in children has been reported as well. A case report of a child receiving BP for Osteogenesis Imperfecta demonstrated that multiple primary teeth extractions did not cause BRONJ.[80] Confirming these findings a study examining 64 children treated with

BP for OI found 0% incidence of BRONJ.[81] Another difference to note is that alveolar cleft bone grafting is done in the maxilla, and it has been shown that BRONJ is twice as likely to occur in the mandible than in the maxilla.[82] Based on these previous clinical studies, the concentration of ZA used in our palatal defect animal model poses a minimal risk for developing BRONJ.

Conclusions and Study Significance

We have developed a novel palatal defect animal model, which can be applied to study analysis of different agents in regulation of endogenous metabolic activities during bone graft incorporation. We demonstrate that a single, delayed systemic injection of ZA significantly increases percent bone volume six weeks after bone graft surgery. While overall bone volume was increased, TRAP staining revealed no difference in osteoclast numbers but a small, insignificant change in morphology of TRAP-positive cells. The anti-catabolic properties of ZA could offer promising new clinical applications towards cleft treatment.

FIGURE 1

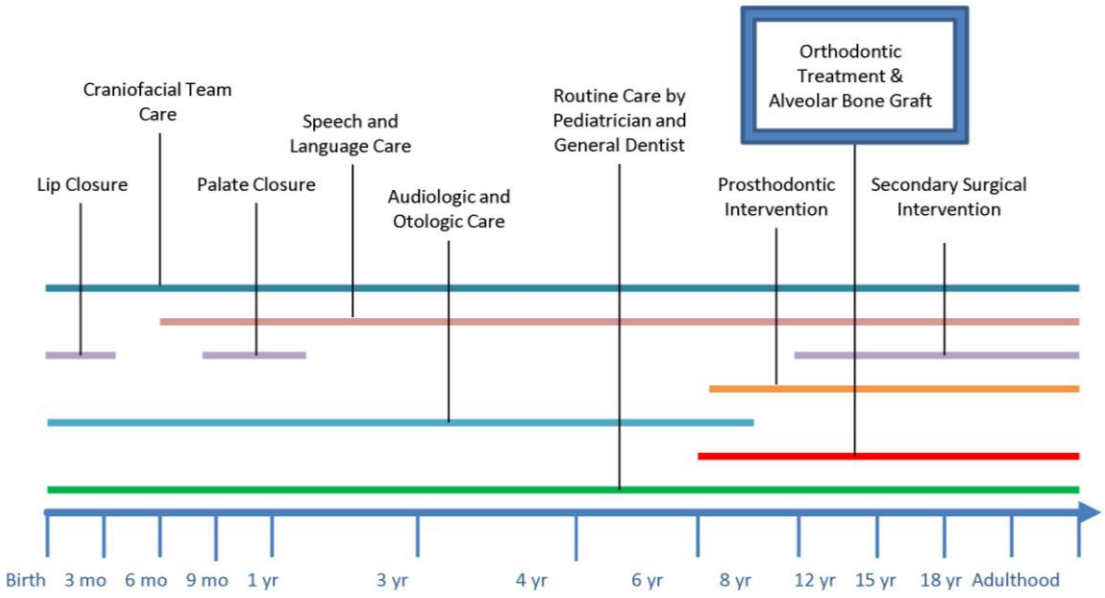


Figure 1. Cleft lip and/or palate treatment timeline. Treatment of CLP requires a team of specialists working together up until adulthood. Surgical closure of the lip and palate are complete during the first year of life. Repair of the alveolar cleft occurs during early adolescence with orthodontic maxillary expansion and alveolar cleft bone grafting (red line). This timeline is adapted from the Saint John's Cleft Palate Center annual conference.

FIGURE 2

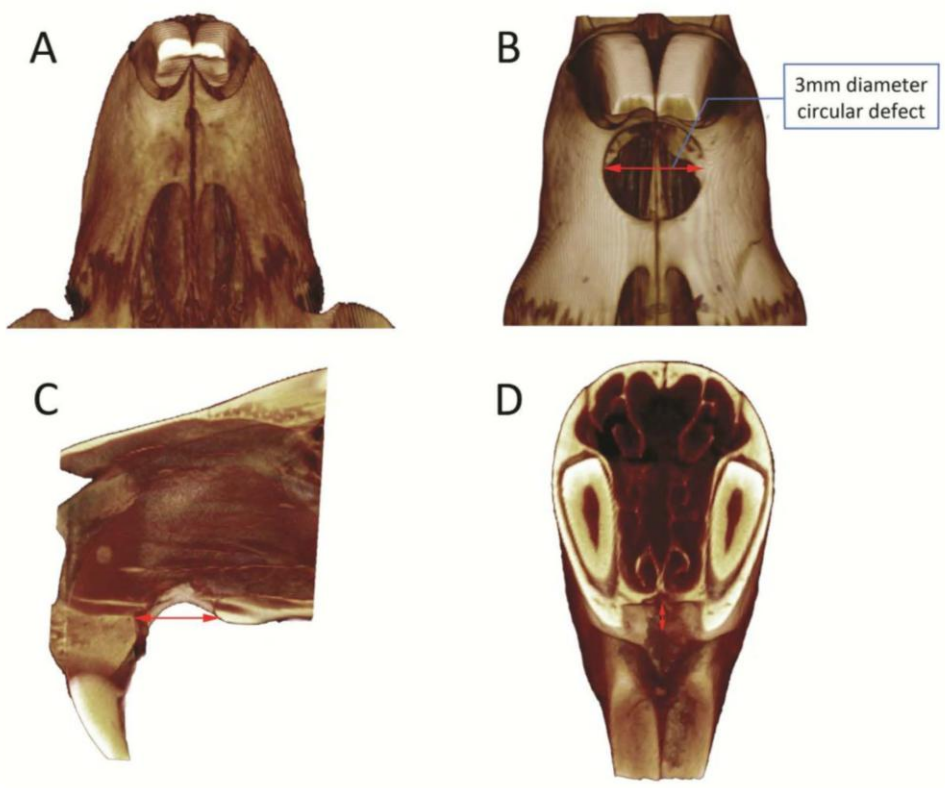


Figure 2. Critical-size rat mid-palatal defect model viewed by micro-CT 3D imaging. (A) Anatomy of rat anterior palate (B) Circular defect with 3mm in diameter (C) Sagittal slice indicating 3mm in diameter (D) Coronal slice, indicating 0.95mm depth of the defect, without damaging the incisal roots.

FIGURE 3

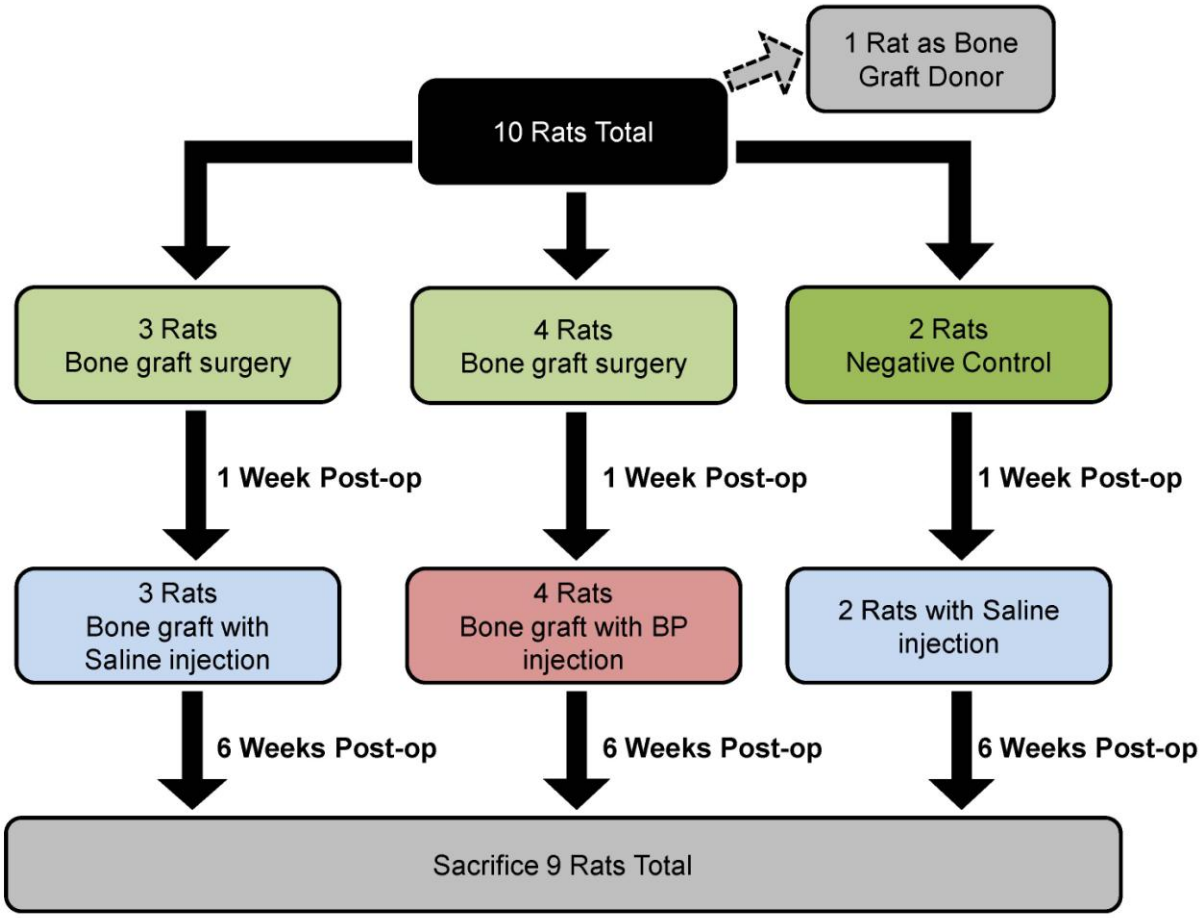


Figure 3. Experimental design flow chart for pilot study. Bone isograft was harvested from the iliac crest and femur of one donor animal (Fischer F344) and placed into the surgical defects of recipients. Animals were injected with BP (red) or Saline (blue) one-week post-surgery and sacrificed at six weeks. Two animals served as negative controls.

FIGURE 4

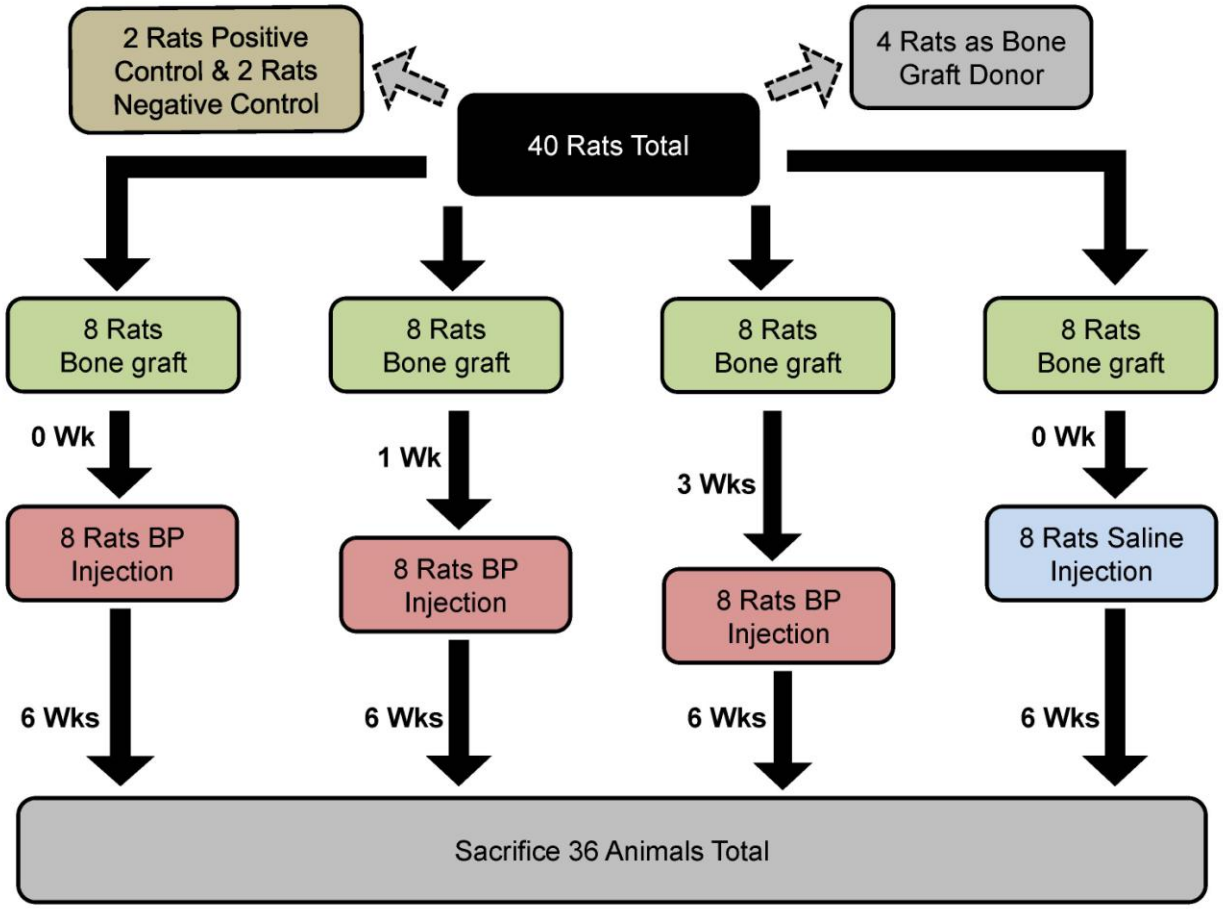


Figure 4. Experimental design flow chart for full study on BP injection timing. Eight animals were assigned to each group as determined by the power analysis from data from the pilot study. Bone isograft was harvested from the iliac crest of four donor animals and placed into the surgical defects of recipients. Animals were injected with BP (red) at the time of surgery (T0), one-week after surgery (T1) and three weeks after surgery (T2). Control group animals were injected with Saline (blue) at the time of surgery. There were two negative and two positive controls and all animals were sacrificed at six weeks.

FIGURE 5

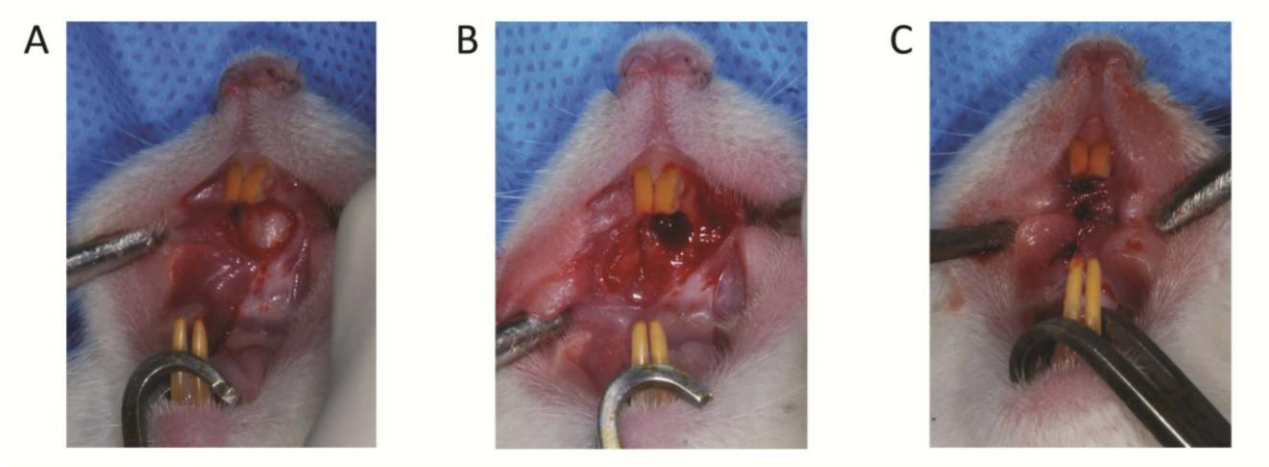


Figure 5. Surgical procedure of the intraoral critical-sized defect in rats. (A) 1cm longitudinal mucosal incision along the junction between the hard palate and alveolus (B) Placement of 3mm midpalatal defect with a trephine bur (C) Soft tissue suturing.

FIGURE 6

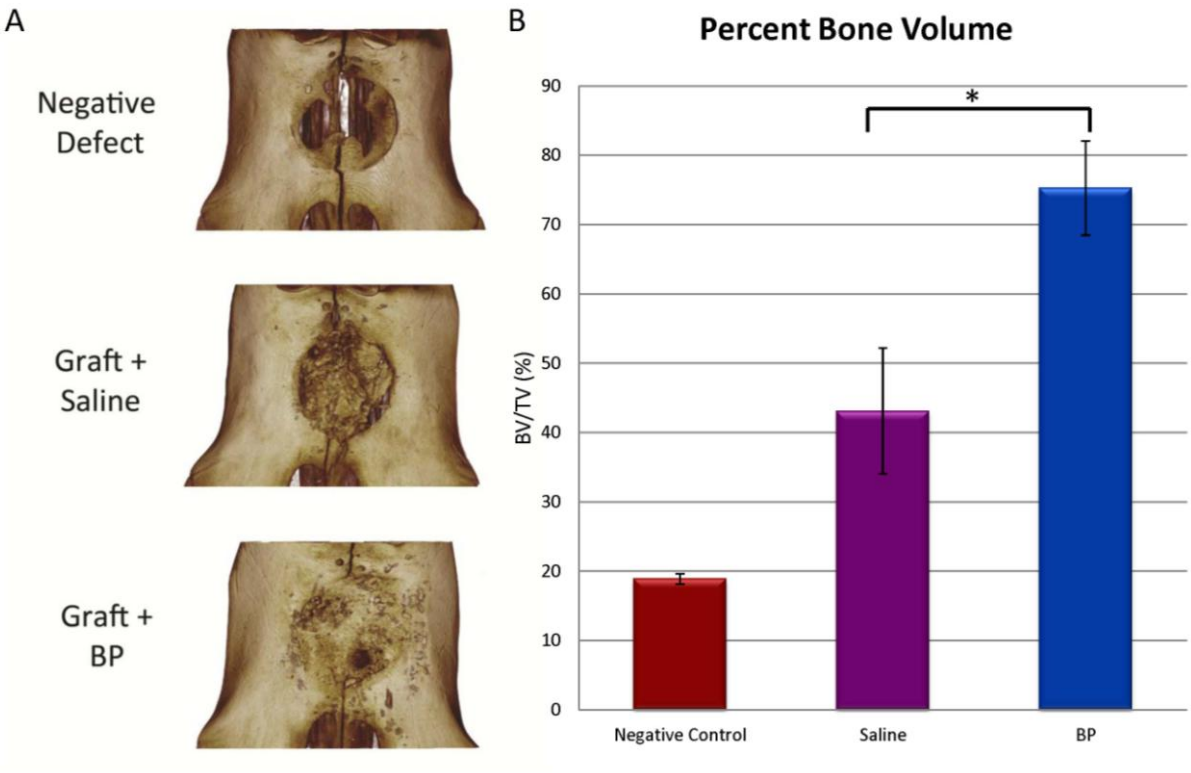


Figure 6. Pilot study micro-CT data. (A) 3D micro-CT images of the 3 groups demonstrated a clear increase in bone volume and integration in the BP group (B) Quantification of bone volume by micro-CT analysis showed statistically significant increase in bone volume. *Statistically significant, $p<0.05$. Student's t-test was used to calculate differences. Error bars show Standard Error.

FIGURE 7

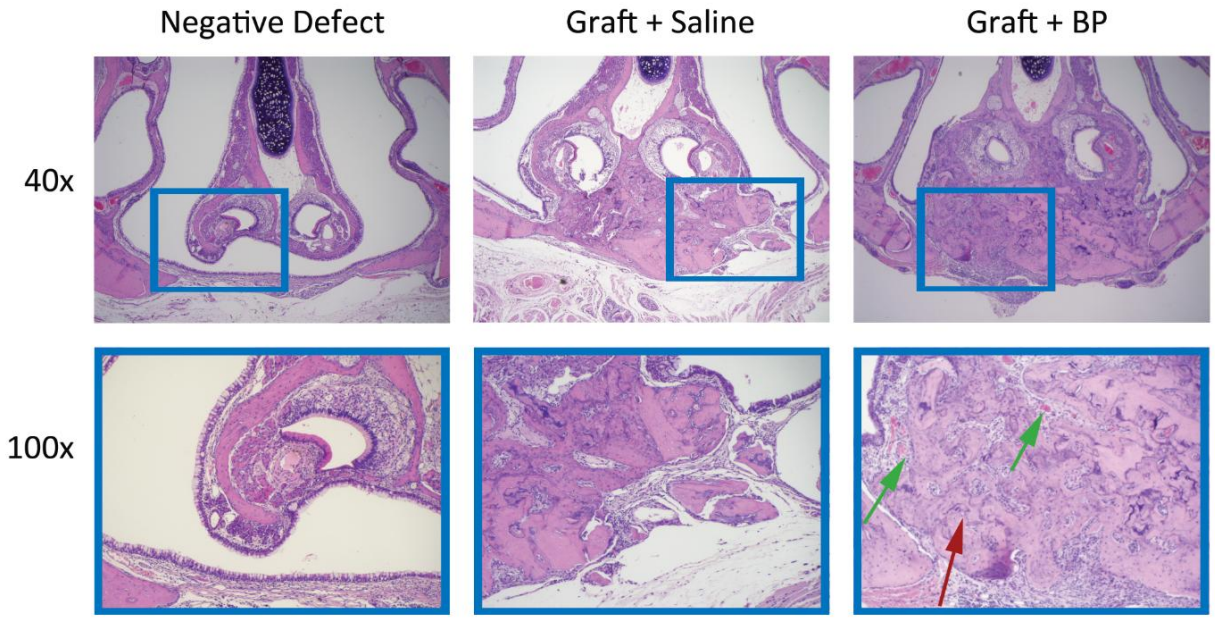
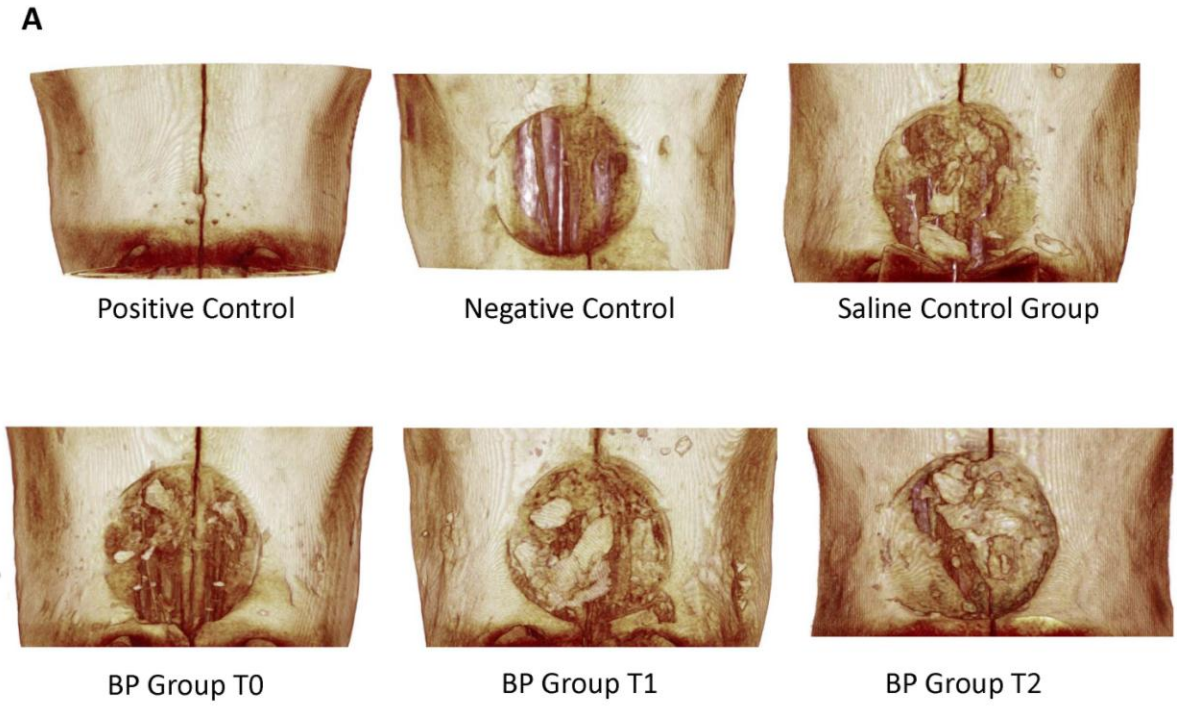


Figure 7. Pilot study H&E images. H&E stained coronal sections confirmed increased bone volume in the BP group. The BP group exhibited presence of angiogenesis (green arrows), osteocytes (red arrows) and bone integration. There was bony union of defect margins with the existing skeletal structures indicating clinical success of bone graft procedure.

FIGURE 8


A
Positive Control
Negative Control
Saline Control Group
BP Group T0
BP Group T1
BP Group T2

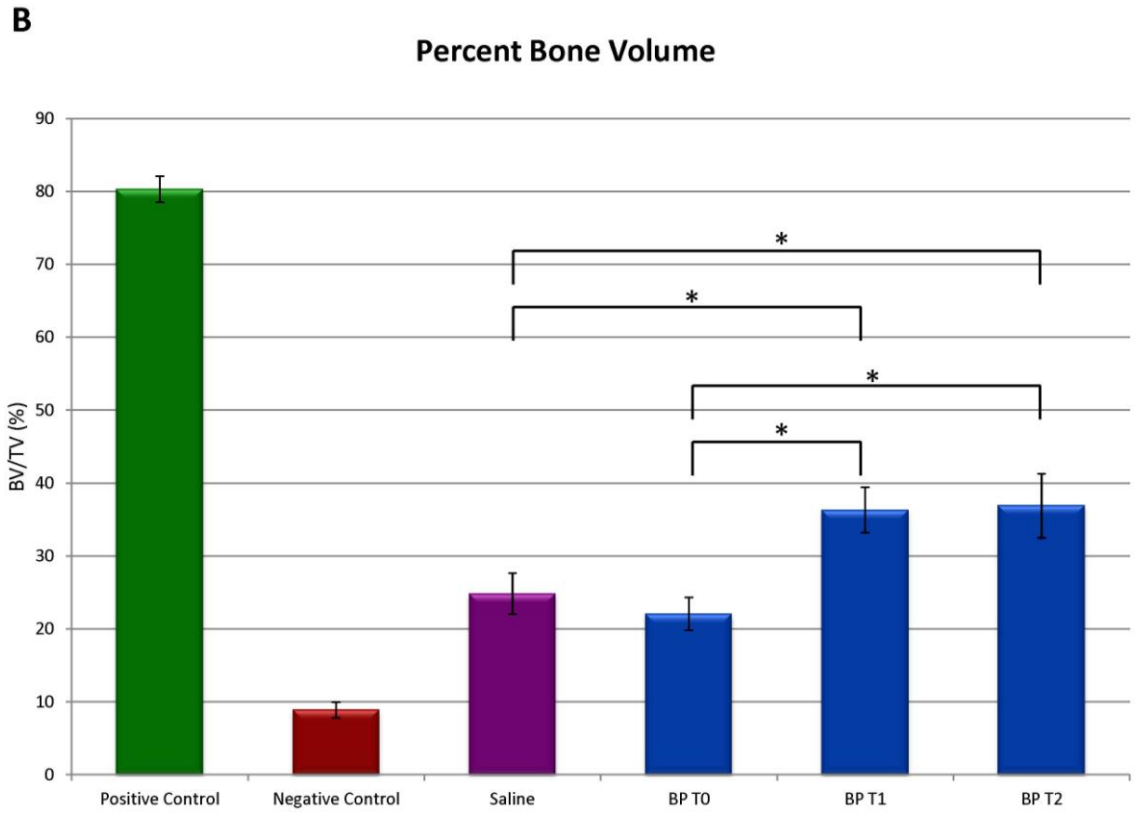

B
Percent Bone Volume
BV/TV (%)
0
10
20
30
40
50
60
70
80
90
*
*
*
*
Positive Control
Negative Control
Saline
BP T0
BP T1
BP T2

Figure 8. Full Study on BP Timing micro-CT Data. Bone volume (BV) and tissue volume (TV) was measured to calculate percent bone volume (BV/TV) of each graft site. (A) 3D micro-CT images of the six groups demonstrated a clear increase in bone volume in the BP T1 and T2 groups. (B) Quantification of bone volume by micro-CT analysis showed statistically significant increase in bone volume in BP T1 and T2 groups. BP T0 group had similar percent bone volume to the Saline group. ANOVA with post-hoc t-tests was used to calculate differences. *Statistically significant, $p<0.05$. Error bars show Standard Error.

FIGURE 9

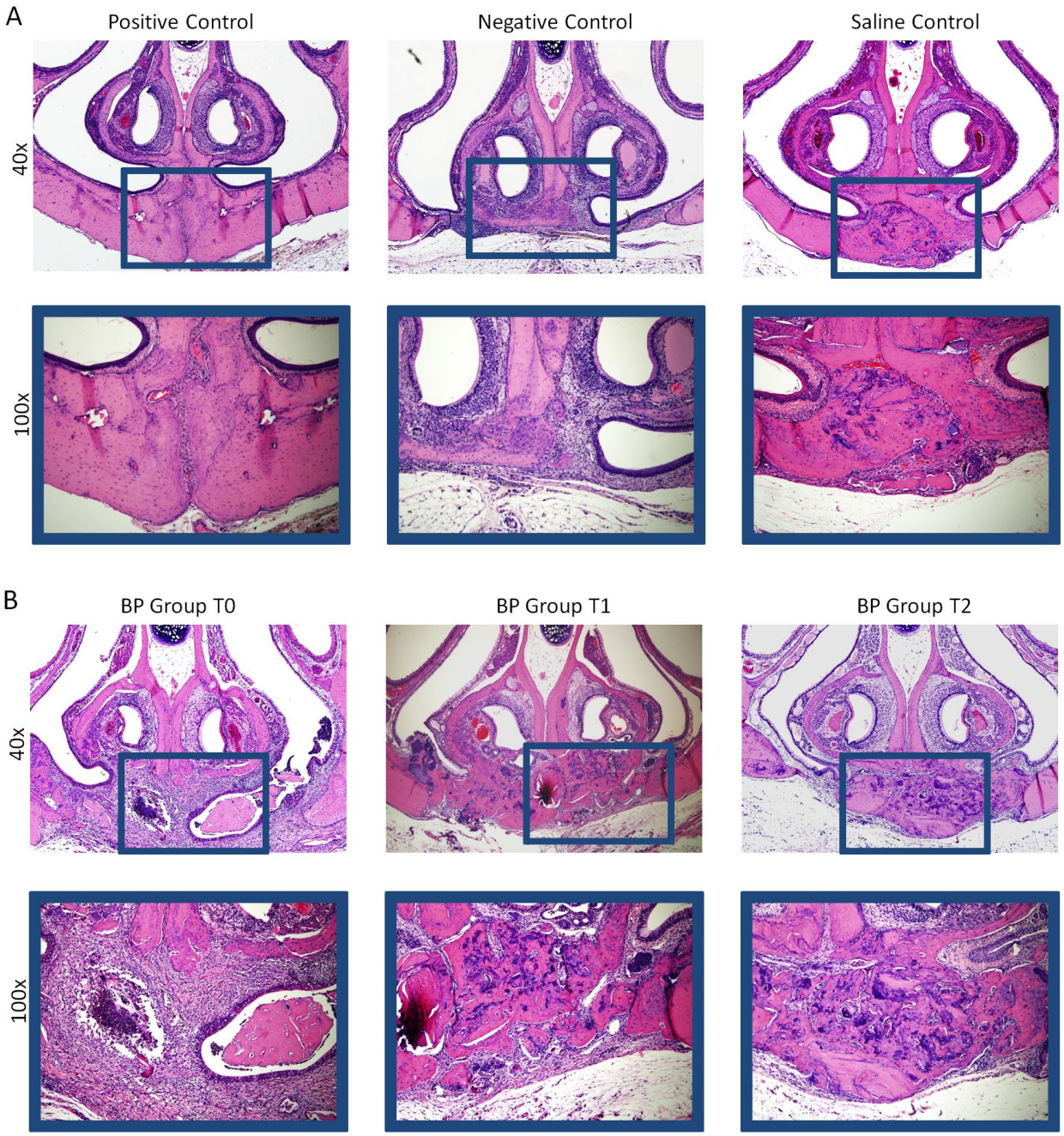


Figure 9. Full study on BP Timing H&E images. (A) Coronal sections of CSD region of controls confirmed presence of normal palatal bone anatomy on the positive control and lack of bone present in the negative control. Saline control group exhibited bone graft incorporation but

significantly less bone present. (B) Coronal sections of CSD region in BP treatment groups confirmed increased bone volume in the T1 and T2 delayed BP injection groups as compared to controls. The BP T0 group animals all exhibited signs of acute inflammation and a clear decrease in bone mass with presence of unresorbed bone graft.

FIGURE 10

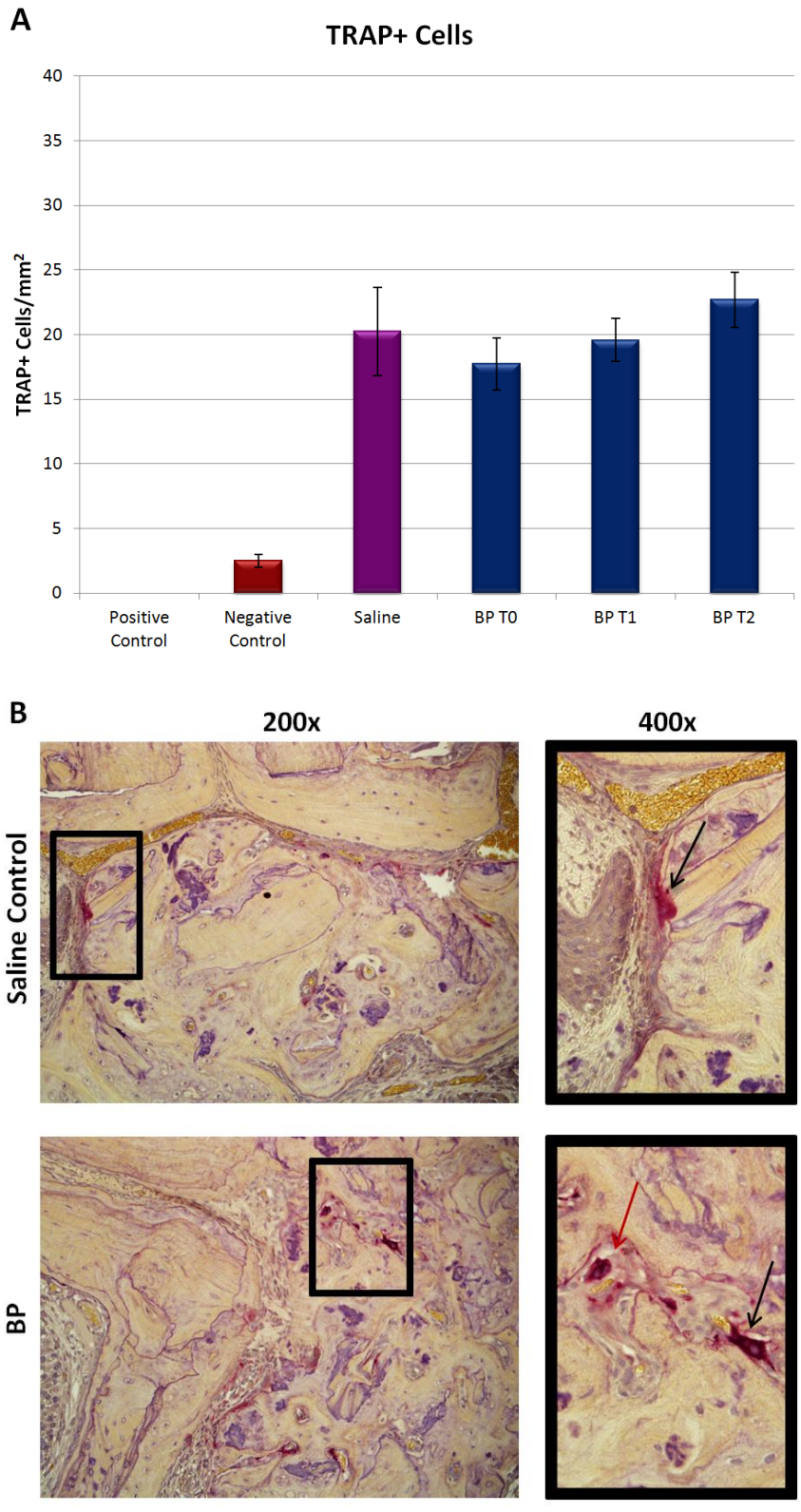

A
TRAP+ Cells
TRAP+ Cells/mm²
40
35
30
25
20
15
10
5
0
Positive Control
Negative Control
Saline
BP T0
BP T1
BP T2
B
200x
400x
Saline Control
BP

Figure 10. Full study on BP Timing TRAP images. (A) There was no significant difference in the number of TRAP+ cells between control and BP groups. Positive and negative controls demonstrate zero to few osteoclasts present, significantly less than treatment groups. ANOVA with post-hoc t-tests was used to calculate differences. Error bars show Standard Error. (B) The morphology of nine out of 240 (3.75%) of the TRAP+ cells in the BP groups was disrupted (red arrow) as compared to normal osteoclast morphology (black arrows). This abnormal morphology (red arrow) could be described as rounded and detached from the bone surface.

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