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AAO Foundation Final Report Form
(a/o 3/6/2015)

Please prepare a report that addresses the following:

Type of Award: Biomedical Research Award

Name(s) of Principal Investigator(s): Christine Hong

Title of Project: Preclinical Evaluation of Bisphosphonates in Stability of Cleft Bone Graft

Period of AAOF Support (e.g. 07-01-15 to 06-30-16): 07-01-14 to 02-29-16

Amount of Funding: $25,000

Summary/Abstract (250 word maximum)

Cleft bone graft during orthodontic treatment of craniofacial patients experiences high failure rate requiring multiple orthodontic expansions and bone graft surgeries and prolonging orthodontic treatment. The ability of bisphosphonates to control bone formation and removal makes it a drug with promising orthodontic applications. The long-term goal of this study is to develop a therapeutic modality using BPs to improve the clinical success of bone graft in CLP treatment. In order to optimize the use of BPs in enhancing the stability of cleft bone graft, we propose the following specific aims:

***AIM 1: To evaluate the timing of systemic BP delivery in enhancing the success of bone graft in the rat model*:**

AIM 2: To compare the effectiveness of local with systemic delivery of BPs in enhancing the success of bone graft in the rat model

Normal bone homeostasis relies on the balance between bone formation by osteoblasts and bone resorption by osteoclasts. Because the unpredictability of bone graft compromises the clinical outcomes of CLP treatment, new treatment modalities are absolutely required. **Utilizing a low dosage of therapeutic BPs in an effective manner, with regards to timing and method, is essential in enhancing bone formation and limiting bone resorption, thus paving the way for improved treatment outcomes for CLP patients.**

Response to the following questions:

1. Were the original, specific aims of the proposal realized? Yes

2. Were the results published? The manuscript has been submitted in December, 2015 and is currently under revision with The Cleft Palate-Craniofacial Journal.
    a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers
    b. Was AAOF support acknowledged? Yes
    c. If not, are there plans to publish? If not, why not?

3. Have the results of this proposal been presented? Yes
    a. If so, list titles, author or co-authors of these presentation/s, year and locations

    Hong C. "Preclinical Evaluation of Bisphosphonates in Cleft Bone Graft" Moyers Presymposium- International Conference on Craniofacial Research Ann Arbor, MI

    Cheng N, Park J, Hou L, Tetradis S, Ting K, **Hong C**. Effects of Systemic Bisphosphonate on Alveolar Cleft Bone Grafting, *J Dent Res* 92 (Spec Iss A):1486, 2013

    Cheng N, Park J, Tetradis S, **Hong C**. **"**Optimal Timing of Bisphosphonate Treatment on Cleft Bone Grafting" American Association of Orthodontists Annual Meeting, New Orleans, LA, April 27, 2014. **AAO Charley Schultz Scholar Award, 1st Place**

    Quach A, Cheng N, Park JY, Tetradis S, **Hong C**. "Effects of bisphosphonate treatment on cleft bone grafting in an animal model" 48th Scientific Congress of Korean Association of Orthodontists, Gwangju, Korea, Oct 15, 2015. **Excellent Poster Presentation Award**

    Olson J, Yu T, Kim R, **Hong C**. "The Effect of Single Dose Bisphosphonate on Tooth Eruption", UCLA School of Dentistry Research Day, Feb, 2016. **1st Place Award**

    Olson J, Yu T, Kim R, **Hong C**. "The Effect of Single Dose Bisphosphonate on Tooth Eruption", AADR annual meeting, Los Angeles, CA, March, 2016. **Oral Presentation**

    Quach A, **Hong C**. "Systemic Versus Local Delivery of Bisphosphonate Treatment in Alveolar Bone Grafting" American Association of Orthodontists Annual Meeting, Orlando, FL, May 1, 2016. **Oral Presentation**

    * Also, abstract is submitted for oral presentation at IADR 2016 entitled "Preclinical Evaluation of Bisphosphonate Administration in Cleft Bone Graft".
    b. Was AAOF support acknowledged? Yes, every time.
    c. If not, are there plans to do so? If not, why not?

4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

    With this AAOF BRA funding, we were able to discover the significant effects that local and delayed systemic administration of bisphosphonates can have in cleft bone graft treatment. As seen above, this funding allowed me to present at numerous national and international meetings and to acquire several awards. AAOF funding has enabled me to pursue orthodontic translational studies and my passion for developing revolutionary treatment solutions by bridging basic science with clinical knowledge. In addition, AAOF funding has been instrumental to establishing my academic career and fueling my development toward becoming an independent orthodontist-scientist with expertise in orthodontic patient care and research.

I. SPECIFIC AIMS

Cleft lip and/or palate (CLP) is the most common craniofacial congenital malformation occurring in 1 in 700 births in the United States alone. CLP patients suffer from a multitude of associated problems such as eating difficulty, speech disorders, hearing impairment, and social alienation due to physical appearance. The treatment of CLP requires a multi-disciplinary approach in which orthodontists play an essential and comprehensive role. The standard orthodontic intervention includes sequential treatment of maxillary expansion followed by cleft repair with bone graft. After sufficient width of maxilla is achieved through expansion, the widened cleft is repaired through autograft placement. This bone graft surgery is timed according to the eruption progress of the cleft-adjacent tooth, mostly canine, as it is critical that this tooth erupts into the grafted site to further stabilize and maintain the newly formed bone. However, orthodontists treating CLP patients have long struggled with a high failure rate of bone grafts, leading to repeated orthodontic and surgical treatments to achieve appropriate maxillary dimension and bone volume needed for maxillary alignment. Although cleft bone graft failure is of great concern in treating CLP patients at the clinical level, there is no appropriate preclinical animal model to evaluate different treatment modalities and treatment outcomes in a controlled manner.

Bisphosphonates (BPs) are anti-resorptive agents known to increase bone volume by inhibiting osteoclastic activity. To evaluate the effects of BPs on bone graft in the palate, we developed the rat intraoral cleft model by creating a critical sized defect in the mid-palate, placing bone graft, and administering a one-time systemic delivery of BPs at one-week after bone graft. Our study showed improved outcomes and enhanced clinical success of bone grafts as demonstrated by increased bone volume from the micro-CT analysis and histological analysis. Interestingly, histological examination revealed increased new bone formation and bone incorporation, suggesting that BP not only inhibited the function of osteoclasts but also induced osteoblastic activity.

The long-term goal of this study is to develop a therapeutic modality using BPs to improve the clinical success of bone graft in CLP treatment. In order to optimize the use of BPs in enhancing the stability of cleft bone graft, we propose the following specific aims:

***AIM 1: To evaluate the timing of systemic BP delivery in enhancing the success of bone graft in the rat model*:** BPs are known to be implicated in healing processes particularly in the oral cavity. Therefore, we will further examine, with increased sample size by the power analysis, how the timing of systemic BP delivery affects the clinical outcomes of bone graft incorporation in rats. Three time points of BP administration will be used: at the time of surgery, one week post-op, and three weeks post-op.

***AIM 2: To compare the effectiveness of local with systemic delivery of BPs in enhancing the success of bone graft in the rat model*:** Our preliminary study demonstrated the beneficial effect of BPs with systemic delivery in the rat cleft model. However, local delivery of BPs would be more clinically applicable due to the ease of the procedure. Therefore, we will evaluate whether grafting the BP-incorporated bone materials at the time of surgery would improve the outcomes of the bone graft procedure.

Normal bone homeostasis relies on the balance between bone formation by osteoblasts and bone resorption by osteoclasts. Because the unpredictability of bone graft compromises the clinical outcomes of CLP treatment, new treatment modalities are absolutely required. **Utilizing a low dosage of therapeutic BPs in an effective manner, with regards to timing and method, is essential in enhancing bone formation and limiting bone resorption, thus paving the way for improved treatment outcomes for CLP patients.**

II. STUDIES AND RESULTS

A. Studies conducted:

A.1. Original Aims

AIM 1: To evaluate the timing of systemic BP delivery in enhancing the success of bone graft in the rat model

AIM 2: To compare the effectiveness of local with systemic delivery of BPs in enhancing the success of bone graft in the rat model

Both **AIM 1 and AIM 2** have been completed. Assignment of groups and experimental timeline is shown in **Fig 1**.

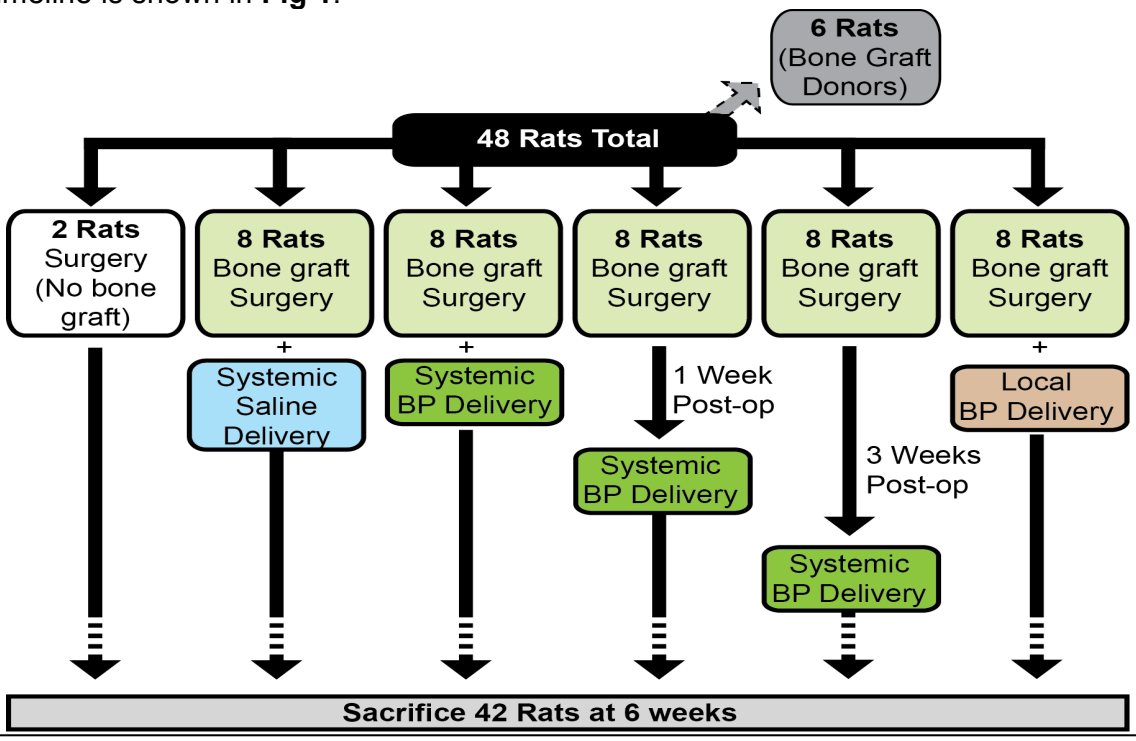


Figure 1. **Assignment of groups and experimental timeline**

Based on our preliminary study, power analysis was performed and 8 rats were required in each group to achieve a power level of 0.8 and $\alpha = 0.05$. A total of 48 female fourteen-week-old Fischer F344 inbred rats was purchased and were divided into five groups (n=8 per group) (1) **Graft/Saline**: bone graft with systemic saline injection (2) **Graft/BP/T0**: systemic BP injection at the time of surgery (3) **Graft/BP/T1**: Systemic BP injection 1 week post-operative (4) **Graft/BP/T2:** Systemic BP injection 3 weeks post-operative. (5) **Graft/Local**: bone graft with local BP. Two animals served as negative controls (**Negative Control)** where the defect was created but the bone graft was not placed. Six F344 Inbred rats were used as autogenous bone graft donors **(Fig. 1).** The use of Fischer F344 Inbred rats was most appropriate for this study due to genetic consistency from breeding and elimination of the host immune response. This allowed for bone grafts to be transferred between rats without triggering an immunological reaction.

A.2. Additional Aim

AIM 3: To evaluate the effects of a single clinical dose of systemic BP on the formation and eruption of teeth in rats

As this bone graft surgery is timed according to the eruption progress of the cleft-adjacent tooth, mostly canines, it is critical that this tooth erupts into the grafted site to further stabilize and maintain the newly formed bone. In addition to the proposed two aims, we studied the effect of our systemic BP dosing (0.1mg/kg) on tooth eruption and development.

AIM 3 has been completed.

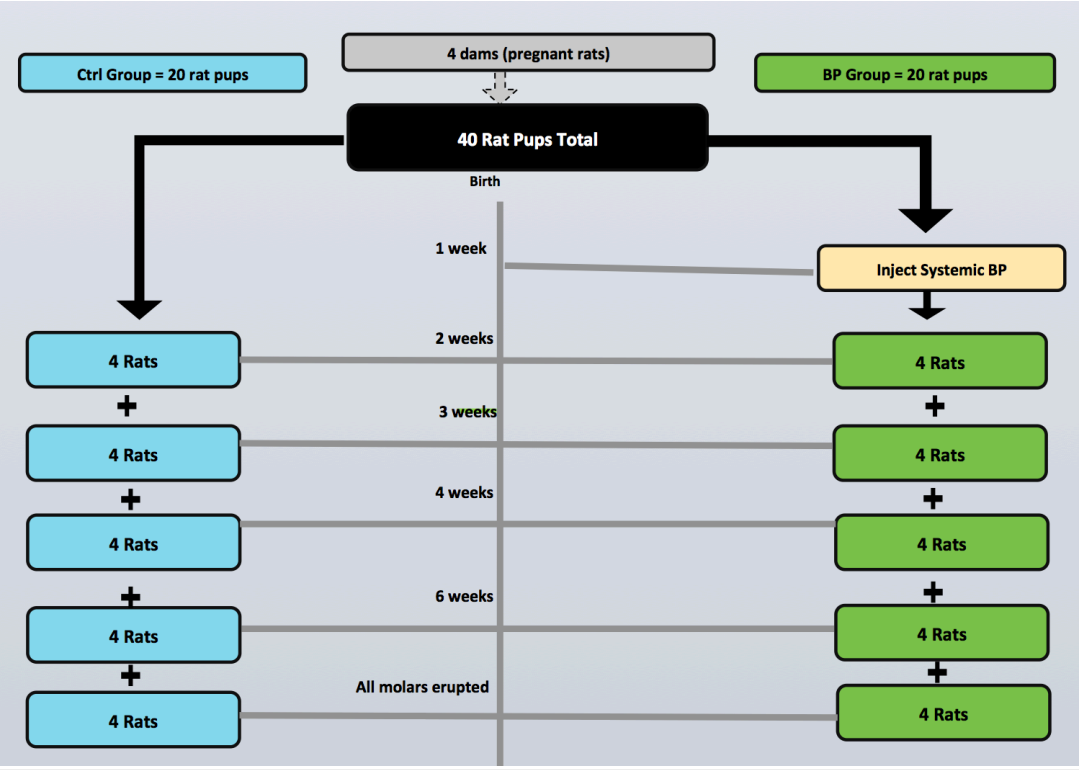
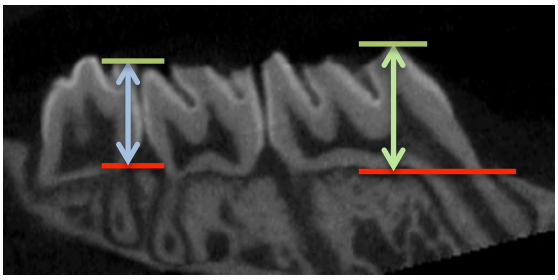


Figure 2: Assignment of groups and experimental timeline

A total of 40 seven-day old Sprague-Dawley newborn rats were split into two groups (n=20 per group) (1) **Control**: saline injection (2) **BP**: 0.1 mg/kg BP systemic injection. The eruption of their molars was observed clinically on a daily basis under isoflurane anesthesia. Groups of rats were sacrificed at five time points for further analysis, with the last group sacrificed as the final molars erupted through the gingiva.

a



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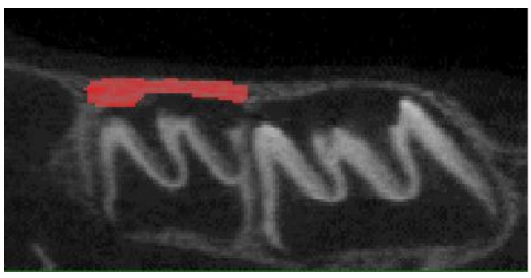


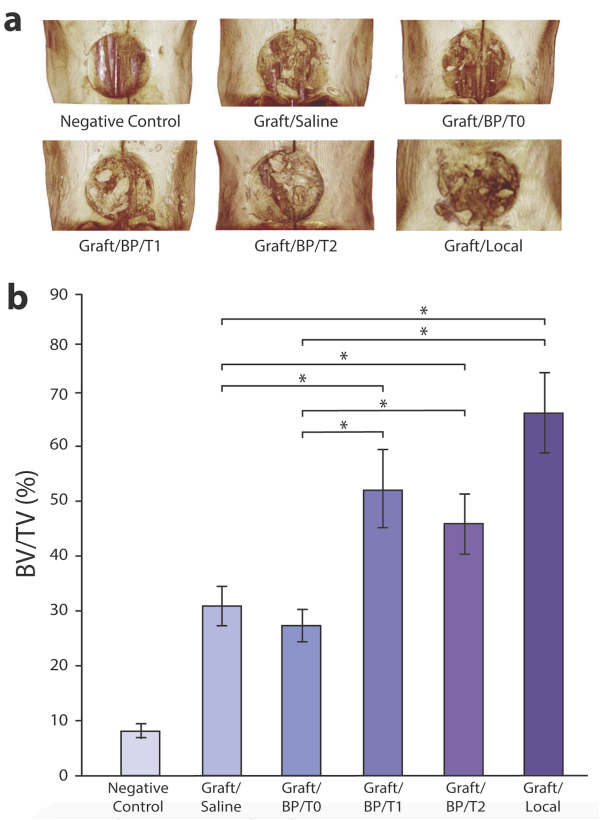
Micro-CT Analysis

A) Micro-CT images were used to measure the eruption of each molar using the distance from the mesial cusp tip (green) to the height of the alveolar crest immediately mesial to the tooth (red).
B) For 3D volumetric analysis, CTAn software (SkyScan 1172, SkyScan N.V., Belgium) was used to create a custom region of interest enveloping the alveolar bone coronal to unerupted 2nd molars and calculate the ratio of bone volume to tissue volume (BV/TV).

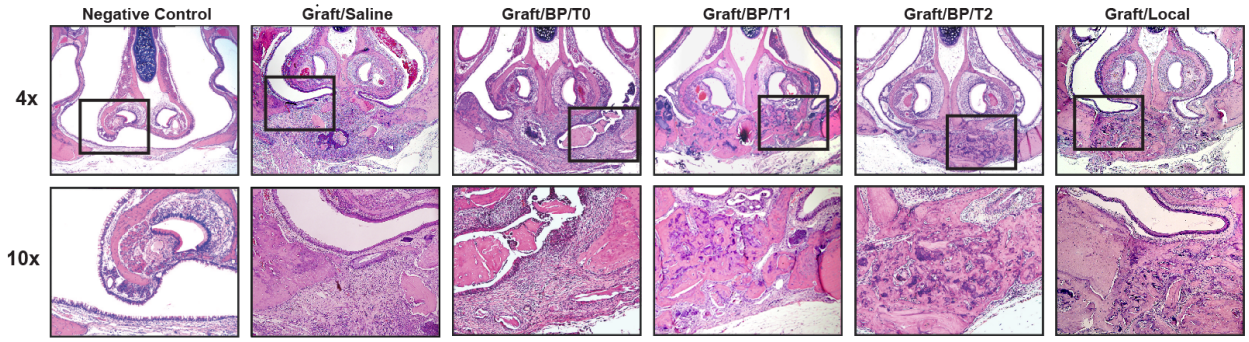
B. Results:

B.1. Original Aims

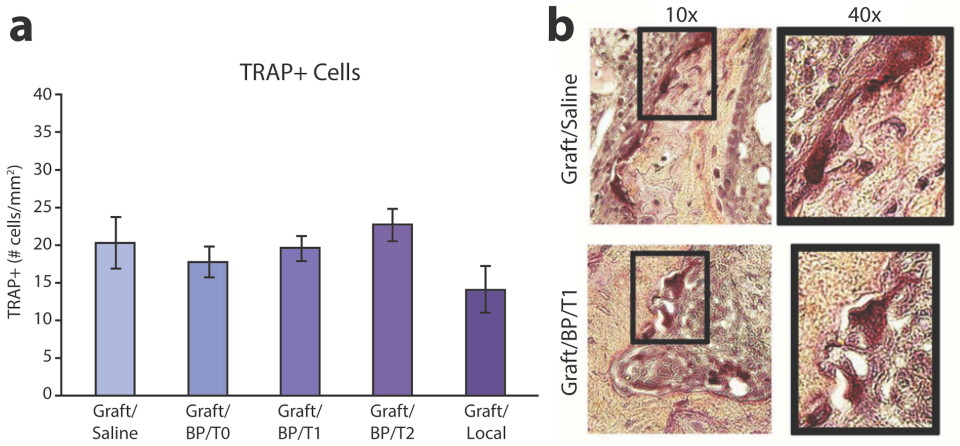
(1) Micro-CT 3D reconstructed images of defect area re-confirmed the non-healing defect in the Negative Control group. Delayed BP administration by 1 week and 3 weeks (Graft/BP/T1 and Graft/BP/T2) results in increased percent bone volume. BP administration at the time of surgery (Graft/BP/T0) did not increase percent bone volume. Local BP administration (Graft/Local) increased percent bone volume. Results also revealed integration of defect margins with existing palatal bone in the Graft/Local, Graft/BP/T1 and Graft/BP/T2 groups, indicating clinical success of the bone graft procedure. Indeed, one of the Graft/Saline rats showed severe bone resorption and lacked new bone formation entirely, indicating the clinical unpredictability of oral cleft bone graft surgery. Interestingly, all animals in the Graft/BP/T0 group showed significantly decreased bone volume with signs of acute inflammation. Graft/BP/T0 H&E images demonstrated extensive infiltration of inflammatory cells in the entire defect and high resorption of bone graft along with lack of new bone formation.



(2) H&E stained coronal sections confirmed increased bone volume in Graft/Local, Graft/BP/T1 and Graft/BP/T2 compared to Graft/Saline. Graft/BP/T0 animals all exhibited signs of acute inflammation and a clear decrease in bone mass. In these delayed BP groups and local BP group, more exuberant angiogenesis and new bone formation was evident with prominent vasculature and a greater number of osteocytes in mineralized tissues. In addition, evident amplification of osteoblasts and integration of new bone with bone graft was visualized. Interestingly, in these three groups, complete fusion of defect margins with existing palatal bone was present histologically while most of the Graft/Saline and Graft/BP/T0 groups had non-union.



(3) TRAP+ cell number did not show significant differences across the groups. However, some of TRAP+ multinucleated osteoclasts in BP-treated rats (Graft/BP/T0, Graft/BP/T1, Graft/BP/T2, Graft/Local) exhibited abnormal morphology, appearing rounded and detached from the bone surface.



B.2. Additional Aim

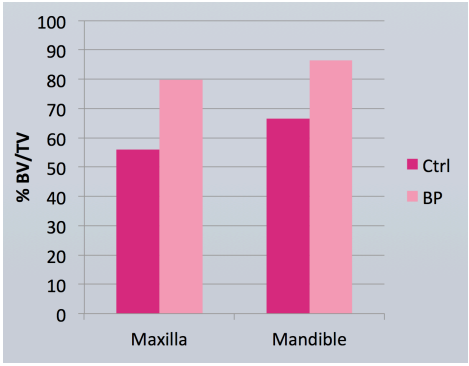
(1) Clinical eruption was determined to be when all cusp tips had completely emerged through the gingiva. Eruption of the 1st molars was delayed by 44 days and eruption of 2nd molars was delayed by 18.5 days in BP groups as compared to the control. 3rd molar eruption was not affected.

	Control	BP
1st Molars	Day 18	Day 62
2nd Molars	Day 22.5	Day 41
3rd Molars	Day 36	Day 37

(2) Micro-CT analysis revealed normal root development with an absence of abnormal morphology of the crown and/or root. A continuous periodontal ligament space was observed and no ankylosis was detected. Further, the first group revealed that the 1st and 2nd molars had already begun the eruption process, while the 3rd molar bud was still under development.

(3) Micro-CT analysis revealed that 1st molars in BP-treated groups at each time point had traveled significantly less distance along the eruption pathway compared to controls. Conversely, there was not a significant difference between the degree of eruption between the two groups for the 3rd molars.

(4) The ratio of bone volume to tissue volume (BV/TV) in the alveolar bone above unerupted molars in week 2 was calculated using CTAn software by creating a custom region of interest enveloping the unresorbed alveolar crest. BV/TV was significantly higher in the alveolar bone coronal to unerupted teeth in the BP group than in the control group.



III. FINAL REPORT

A. Original Aims

Both **AIM 1 and AIM 2** have been fully answered. In **AIM 1**, the preliminary findings were confirmed through an expanded study with a larger sample size determined by power analysis using the pilot study data. In addition, the effect of timing of BP delivery on the clinical outcomes of oral cleft bone graft in rats was examined. Although previous studies showed that delayed BP administration enhanced the clinical success of bone grafts by allowing for soft tissue healing and increased drug binding and potency, the effect of the

timing specifically in intraoral cleft grafts was still unknown. In order to determine an optimal window for BP delivery, two delayed injections at one week and three weeks after surgery were evaluated. The results showed that delayed BP administration at 1 week and 3 weeks significantly increased bone volume, confirming our hypothesis. BP administration at T0 did not increase bone volume compared to the control group and exhibited signs of inflammation. These results suggest that initial bone and soft tissue healing is critical in cleft bone graft.

In **AIM 2**, local BP delivery was compared with systemic BP delivery in its effectiveness in enhancing the clinical outcomes of bone graft surgery in rats. Local administration of BPs has many advantages over systemic injection, including ease of delivery and decreased adverse effects associated with systemic delivery. While previous studies showed improved clinical success with local delivery, the study on intraoral defect is lacking. Initially, we expected to find that local BP treatment would have similar effects on bone graft incorporation as systemic BP treatment because we chose dosages for both local and systemic BP delivery that have been shown to enhance anabolic and reduce catabolic bone graft responses. However, our micro-CT and histological analysis results showed that local BP delivery significantly enhanced bone formation and incorporation in the defect compared to the systemic BP delivery (at all three time points).

B. Additional Aim

In **AIM 3**, a one time systemic administration of BP (equivalent to the clinical pediatric dose) substantially delayed the eruption process in the molars of rats. However, teeth erupted eventually although delayed and completed normal development, indicating that BPs' beneficial effects may outweigh potential adverse effects for administration in children at this dosage. The degree to which BPs inhibited molar eruption depended on the tooth's stage of development. 3rd molars, whose buds had only just begun developing, were mostly unaffected, while 1st molars, which were in the most active eruption stage, were the most affected. BV/TV was significantly higher in the alveolar bones of BP-treated rats, suggesting that BPs were incorporated into the bone, disrupting osteoclasts, resulting in a relative increase in bone volume and delayed tooth eruption. Caution should be used with administration of BPs in children, as it may delay eruption of the developing dentition.