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Effect of Bisphosphonate on Soft Tissue Contraction after Midpalatal Suture Expansion

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Effect of Bisphosphonate on Soft Tissue Contraction after Midpalatal Suture Expansion

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Oral Biology

by

Susan Bae

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ABSTRACT OF THE THESIS

Effect of Bisphosphonate on Soft Tissue Contraction after Midpalatal Suture Expansion

by

Susan Bae

Master of Science in Oral Biology

University of California, Los Angeles, 2018

Professor Yeumin Hong, Chair

Midpalatal expansion has been used in orthodontics to address maxillary deficiency in the transverse dimension. The separation of the palate causes micro tears along the suture and repair ensues in the resulting space. However, even after a period of stabilization, relapse can occur to adversely affect the orthodontic treatment outcomes. Currently, there is no consensus as to the cause of relapse; whether, it is mediated by the bony palatine shelves or the dense connective tissue covering them. Therefore, elucidating the molecular and cellular mechanism involved in the midpalatal suture expansion can be used as a foundation to explore ways to reduce relapse in orthodontic patients who undergo maxillary expansion. This is especially critical in many craniofacial patients, such as those with cleft lip and/or palate (CLP) malformations, in which maxillary expansion provides a sound foundation for dental development. The current therapeutic modalities do not provide a stable maintenance of the expansion, as relapse can occur in up to 45% of patients. In addition, prior research has shown that the anti-resorptive drug class of bisphosphonates (BP) can reduce the relapse ratio following expansion. However, the process

by which BP affect relapse is still unclear. The long-term goal of this study is to understand the etiology of relapse and to diminish relapse after maxillary expansion. This study hypothesized that oral myofibroblasts in soft tissue played a role in relapse following palatal expansion and that BP decreased relapse by inhibiting myofibroblast differentiation of normal human oral fibroblasts (NHOFs). The specific aims of this study were (1) to examine the changes that BP have on myofibroblasts and their differentiation of NHOFs; and (2) to establish a mouse model for studying palatal expansion using a modified expander. BP inhibited gene expression and protein expression of α-SMA in NHOF cells in a time-dependent and dose-dependent manner. A functional collagen matrix assay demonstrated the anti-contractile effect of BP on myofibroblasts. The establishment of the mouse model involved mice that underwent 7 days of palatal expansion using an expander with a helical spring and arms that loop around the central incisors. Midpalatal skeletal expansion was successfully achieved in mice. After expansion, the control group (n=15) were injected with physiologic saline solution (5mg/kg, 0.9% sodium chloride) and the experimental group (n=15) were subcutaneously injected with 0.1mg/kg of Zoledronate dissolved in physiologic saline solution. The expanders were then be converted to retention appliances and allowed 7 days of retention. At the end of retention, half from each group was immediately sacrificed. The other half was sacrificed after undergoing 7 days of relapse in which the retention device were removed. Data analysis conducted through micro-CT analysis and histology demonstrated that BP administration reduced relapse following expansion and that higher bone volume and density after BP injection enhanced post-expansion stability.

The thesis of Susan Bae is approved.

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INTRODUCTION

Midpalatal expansion has been used in orthodontics for decades in order to address the transverse discrepancy in the maxilla. This treatment modality actually first appeared in 1840, when E. C. Angell published a case study in which he expanded a 14 year old girls palate using a jackscrew. He noted a diastema that formed and claimed that the midpalatal suture separated (1). Today, we use palatal expansion to address a plethora of facial anomalies, from posterior crossbites, CLP, and crowding (2). In fact, according to one epidemiology study, 20% of all children presented with transverse skeletal discrepancies caused by maxillary deficiency (3).

In the 1970s, Melsen used histologic analysis to describe the changes in the palatal suture over time. During infancy, the suture is broad and Y shaped. As children get older the suture becomes more winding. And during adolescence, the suture is interdigitated and the connective tissue between the palatal shelves becomes narrower (4). When palatal expansion is performed, the suture is separated by small localized tears along the suture within blood vessels. Exudate fills the area; fibroblasts grow and proliferate and form a sort of net. Within three to four days, bone formation beings in the margin by osteoblast (5, 6).

Midpalatal Suture Development

Palatal shelf development is a culmination of several processes that include a variety of developmental pathways, such as cell proliferation, cell adhesion, apoptosis, and epithelial-mesenchymal interaction (7, 8). In humans, palatogenesis starts during the sixth week and palatal fusion is complete by 12 weeks of gestation (9). Mesenchymal cells from the neural crest migrate to the stomodeum where they merge with craniopharyngeal ectoderm to form the bilateral maxillary processes (8). The apparent primary palate first becomes morphologically visible as

the medial nasal processes fuses with the maxillary prominences in utero (9). From these maxillary processes arise the bilateral palatal shelves that grow first vertically down the sides of the tongue and then rapidly elevate to the horizontal position above the tongue (8). The palatal shelves fuse to form a midline epithelial seam that is disintegrated to allow for mesenchymal continuity (9).

The epithelial seam is formed when the epithelium of the medial edges of the palatal shelves adhere to each other by a sticky cell surface composed of glycoprotein (8, 10). The epithelial cells develop desmosomes and cell adhesion molecules and become the central cells of the seam (8). But almost as soon as the seam forms, it is quickly disintegrated. The epithelial seam reduces from the original 4-5 cell layered seam to one that is 1-2 cell layers thick (11). This is accomplished through the appearance of lysosomal bodies produced by the midline epithelial cells, indicating a type of programmed cell death (12). Acid phosphatases lead to the degeneration of cells, with the final phases of breakdown involving the disruption of the basal lamina between the epithelial and mesenchymal cells, and with the migration of tissue macrophages into this region with ensuing phagocytosis of degenerating epithelial cells (12, 13).

The postnatal development of the palatal suture and its growth activity are inconclusive, with various studies contesting the cessation of growth. Latham believed that the midpalatal sutural growth ceased between ages of 1 and 2 years, and Scott further reduced the midpalatal suture growth potential to no later than after age 1 (14, 15). On the other hand, other studies observed signs of growth at age 13 (4). Bjork's implant study pointed out that the sutural growth goes well into the pubertal spurt (16, 17).

Melsen's histologic study attempted to break down the postnatal development of the midpalatal suture into three stages: infantile, juvenile, and adolescent periods. Midpalatal sutural growth continued up to the age of 16 in girls and 18 in boys. Furthermore, the morphology of the suture changed. During the infantile stage, the suture was short, broad, and Y-shaped and only slightly sinuous. Then during puberty the suture was becomes more sinuous. Then during the later adolescent stage, the interdigitation of the palatal shelves was so heavy that separation would only be possible with the fracturing of the interdigitated processes (4).

In more recent studies, cone-beam computed tomography has been utilized to help define the midpalatal suture maturation. Angelieri and McNamara provide five stages of suture maturation based on radiographic analysis: stage A had a high density suture line with little or no interdigitation; stage B had a high density line with scalloping; stage C had 2 parallel lines that are separated by low-density space; stage D involved complete fusion of the palatine bones and no presence of a suture; and stage E with the final fusion in the anterior maxilla. Stages A and B were typically seen up to age 13. Stage C ranged from age 11 to 17 years. Fusion stages of D and E were only seen in girls after age 11 (18).

Palatal Expansion and Relapse

Maxillary palatal expansion is a commonly used treatment modality that increases the transverse dimension. Maxillary expansion is used for patients with discrepancies that result in posterior crossbites, anteroposteior discrepancies, cleft lip and palate patients with collapsed maxillae, and even for crowding in the dental arches (19). Separation of the suture can be accomplished in different ways: (1) rapid expansion with a jackscrew device, such as a Haas or Hyrax, attached to the maxillary posterior teeth, typically at a rate of 0.5-1mm/day; (2) slow

expansion with a similar device at about 1mm/week; (3) implant-supported expanders that directly applied force to the bone and no pressure to the teeth; and (4) surgically-assisted expansion (20).

However, one of the problems with palatal expansion is the occurrence of relapse, regardless of the type of expansion or device. Some studies show that palatal expansion relapse was up to 45% of the time (21). Even with a period of retention, there can be up to 25% relapse after expansion. Furthermore, these statistics go up when addressing CLP patients. And whether the expansion was accomplished non-surgically (such as with a tooth-borne expander) or surgically (SARPE), the concern for relapse does not dissolve (19, 22-24). Currently, however, there is no consensus as to the etiology of the relapse that occurs frequently in an oft utilized orthodontic procedure.

When the midpalatal suture is expanded, the soft tissue surrounding the palatal bones is subsequently altered to support the new transverse dimension. As early as 1894, Matteson wrote that "although the two maxillary bones are separated following the application of mechanical forces, a concomitant stretching of the soft tissue of the palate takes places… (and) the tension of the soft tissue would cause a relapse of the maxillary bones to the original position" (25). Furthermore, after surgical closure of a cleft, wound contraction and scar tissue largely contribute to growth disturbance (26). When distraction osteogenesis is performed, scar tissue formation can be a cause of relapse. In cases of hemifacial macrosomia, when distraction osteogenesis is used, up to 50% of patients relapse, possibly due to the failure of the soft tissue matrix to accommodate the bony changes brought about by distraction (24, 27). Hence, the concept of the soft tissue perhaps causing relapse exists; however, the molecular mechanism behind is still not well understood.

Wound Healing and Myofibroblasts

When palatal expansion is performed, the suture is separated by small localized tears along the suture within blood vessels. Exudate fills the area; fibroblasts grow and proliferate and form a sort of net of fibrous connective tissue. Platelets are attracted to the site and release various growth factors, such as transforming growth factor-β (TGF-β) (28). TGF-β1 is expressed in gingival fibroblasts and is involved in their proliferation (29). Within three to four days, bone formation beings in the margin by osteoblast (5, 6). Palatal expansion can be considered as a form of damage to the suture that the body subsequently repairs by generating new tissue, or in essence, a scar. And wound contraction and scar tissue formation is largely mediated by myofibroblasts, which are found in the extracellular matrix (ECM).

Myofibroblasts were first described by Gabbiani in 1971 in the granulation tissue and was identified to have a role in wound contraction. They described the structure of these cells as a modified fibroblast with smooth muscle like features, as they showed bundles of microfilaments, with dense bodies scattered in between them and showed gap junctions. They have a transient appearance: forming from fibroblasts during contraction and then disappearing by apoptosis once wound healing is accomplished. Various factors and proteins induce its formation in the extracellular matrix. When mechanical tension is created, such as resulting from a cut, the mechanical stress leads to the formation of proto-myofibroblasts from the fibroblast. During wound healing, fibroblasts first migrate toward the area and lay down collagen and fibronectin in the ECM that help with the closing of the wound. As tension increases, fibroblasts differentiate into myofibroblasts. The differentiation is what drives the wound healing process. Myofibroblasts express α-smooth muscle actin (α-SMA), which is a contractile protein, and in wound healing, the myofibroblasts mediate wound contraction and development of the collagen-

rich ECM (30). TGF-β1 has been shown to have a direct effect on cell phenotype, such as the contractile phenotype through the upregulation of α-SMA both in vitro and in vivo conditions (31). Furthermore, research has shown that mesenchymal cells have been involved in contributing to the myofibroblast population during wound healing (32, 33).

The TGF-β ligand family is involved in various cellular functions such as cell growth, cell proliferation, and apoptosis. TGF-β ligands bind to their respective type I and type II threonine kinase transmembrane receptors. The type I and type II receptor-ligand bound compounds into a terameric complex whereby the type II receptor phosphorylates the type I receptor (34). Then this activated type I transmits the signal through phosphorylation of R-Smads (receptor-activated Smads) of the canonical Smad pathway (35). There are three different groups of Smad proteins. The first group comprises of R-Smads, of which Smad2 and Smad3 are activated by TGF-β-specific type I receptors, and of Smad1, Smad5, and Smad8, which are activated by BMP-specific type I receptors (28). The second group contains co-Smad group (common mediator) and the third comprises of inhibitory Smads (I-Smads, such as Smad6 and Smad7) (35). The activated R-Smads combine with Co-Smad, after which this complex then enters the nucleus and alters the transcription of specific target genes (28). TGF-β induces expression of type I collagen and α-SMA, which is a myofibroblast marker and induces the differentiation of fibroblasts into myofibroblasts in a Smad-dependent pathway (36).

Bisphosphonates

Previous studies have shown how bisphosphonates (BP), such as zoledronic acid, promote bone formation and decrease relapse ratio after expansion (21). Bisphosphonates are anti-resorptive drugs with carbon-substituted phosphate groups that have high affinity for

hydroxyapatite crystals found in bone (37, 38). Liberated during bone resorption, bisphosphonate are taken up by osteoclasts, which have a ruffled border. The engulfed bisphosphonate leads to changes in the ruffled border and the osteoclast become inactive, subsequently preventing further resorption by osteoclastic activity. The newer generation of bisphosphonates contains nitrogen that facilitates in the inhibition of farnesyl pyrophosphate synthase (FPPS), which is a key enzyme in the mevalonate pathway. This pathway is important in the production of cholesterol, which is a component of the cytoskeleton. Nitrogen-containing BPs prompts changes in the osteoclast cytoskeleton—or raffled border, and leading to inactivation and possibly apoptosis (39).

Specific Aims

We hypothesize that inhibition of oral myofibroblast function improves post-expansion stability and that bisphosphonate inhibits myofibroblast differentiation and contraction mediated by TGF-β. The long-term goal of this study is to better understand the etiology of palatal relapse after expansion and to develop new therapeutics to improve clinical outcomes of expansion, which would be especially valuable for CLP patients.

Specific Aims:

1. Examine the effects of BP on myofibroblasts in vitro
2. Establish a mouse model to study soft tissue changes after palatal expansion
3. Examine the effects of BP on soft tissue relapse in vivo

MATERIALS AND METHODS

All experimental procedures involving animals were conducted under the guidelines provided by UCLA Animal Research Committee (#2016-009-01).

Study subjects

Animals: 30 6-week old male C57BL/6J mice were purchased (Jackson Laboratory, #JAX000664), housed in light-and temperature-controlled facilities, and given a soft diet and water. The mice were divided into two treatment groups (n=15 per group): 1) Control: saline injection; 2) Experimental: BP injection. Both groups underwent 1 week of expansion. Then the expanders were converted to retainers. At this point, mice were injected with either BP or saline. The control group was injected with physiologic saline solution (5mg/kg, 0.9% sodium chloride) and the BP group was injected with (0.1mg/kg of Zoledronate) subcutaneously. After 1 week of retention, half from each group was sacrificed. The other half was allowed to undergo 1 week of relapse, after which the mice were euthanized post-relapse.

Cells: Normal human oral fibroblasts (NHOFs) in early passages were prepared from oral mucosal tissues according to methods previously described (40). Briefly, oral mucosal tissue was collected from normal dental patients. The oral mucosal tissue was then cut into 25 mm^2 × 0.5 mm sections and incubated in 2.5 mg/ml dispase solution (Cat. no. 17105; Gibco, Grand Island, NY, USA) in 37°C for 1 h, after which the epithelial and connective layers were separated. The connective tissue layer was minced and digested with collagenase (Cat. no. 17100017; Gibco) in 37°C for1 h to harvest the NHOFs.

Cell culture

NHOFs were cultured in Dulbecco's modified Eagle's medium (DMEM/199) (4:1) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Zoledronate (ZOL) was obtained from LKT Laboratories, Inc. (St. Paul, MN, USA). TGF-β1 was purchased from PeproTech (Rocky Hill, NJ, USA).

Contraction Assay

To examine the anti-contractile properties of bisphosphonate treatment, a cellular collagen contraction matrix assay was used. Type I bovine collagen (Advanced Biomatrix Purecol Collagen, Fisher Scientific, Waltham, MA, USA) was mixed with 10x MEM w/ Earle's salts (Lonza, Walkersville, MD, USA) , L-glutamine (Gibco, Fisher Scientific, Waltham, MA, USA), FBS (Invitrogen, Carlsbad, CA, USA), sodium bicarbonate (Lonza, Walkersville, MD, USA) to induce polymerization. To this mixture NHOF early passage cells suspended in DMEM w/ 10% FBS were added. 3mL of this solution were distributed to each well in a 12-well plate. After 1 hour at room temperature, the plate was then incubated for 1 hour at 37 °C and 5% CO_2. Once the collagen turned pink and firm, enough media (containing either TGF-β and/or 5μM ZOL) was added to fully submerge the collagen lattices. After 2 days incubation, a sterile spatula was used to gently scrape the periphery of the collagen gel from the inner wall of the well. The wells were monitored for 5-7 days for contraction, after which the wells were photographed with a digital camera.

RNA Isolation and Real-time Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cell cultures using Trizol Reagent (Invitrogen). A cDNA pool was synthesized from the RNA using Superscript Reverse Transcriptase II (Invitrogen).

Quantitative reverse-transcription polymerase chain reaction was completed in triplicates with LC480 SYBR Green I (Roche, Indianapolis, IN) using the LightCycler 480 II real time PCR system (Roche). A total of 55 cycles were completed and Cq value determination method was used to compare fold changes. The primers for α-SMA were forward, 5'-CTGTTCCAGCCATCCTTCAT-3' and reverse, 5'-TCATGATGCTGTTGTAGGTGGT-3'.

Western Blot Analysis

Whole cell extracts were isolated from cultured cells and lysed and then separated by centrifugation at 13,000rpm at 4°C for 20 minutes. The collected supernatant was fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis and then transferred to Immobilon membrane (Millipore, Billerica, MA). Membrane was blocked with 5% non-fat milk for 1 hour at room temperature. After blocking, the membrane was incubated with primary antibody and probed with secondary antibodies conjugated with HRP. Chemiluminescence signals were detected using HyGLO Chemiluminescent HRP antibody detection reagent (Denville Scientific, South Plainfield, NJ) using the ChemiDoc XRS System (Biorad, Hercules, CA). Antibodies against α-SMA (Abcam, Cambridge, MA, USA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

Midpalatal Expansion and Relapse Model for Mice

Expander design: A novel mouse palatal expander as previously described (but with modifications) was fabricated using 0.012-inch stainless steel wire with an intraoral helical spring and extended arms that loop around the central incisors (41). The dimensions of the appliance are as follows: helical spring is 1.3mm in diameter and the extension arms are 6.3mm long. The force of 40 g was exerted by compressing on the helical part of the spring.

Expansion procedure: The animals were anesthetized with ketamine/xyalzine (75-85mg/kg, 5-10mg/kg) intraperitoneally. An expansion force of 40 g was calibrated using a force gauge (GAC Dentsply, York, PA). Transbond Plus self-etching primer (3M Unitek, Monrovia, CA) was applied to the maxillary incisors prior to bonding of the expander at the most gingival surface of the incisors with acrylic resin (Transbond Supreme LV Low Viscosity Light Cure Adhesive, 3M Unitek, Monrovia, CA). The mice underwent 7 days of expansion.

Bisphosphonate delivery: After seven days of expansion, saline or BP were administered. One dose of 0.1 mg/kg of Zoledronate were injected subcutaneously to the BP group after the 7 day expansion period. The concentration of 0.1mg/kg was based on previous study (42). Physiologic saline solution (5mg/kg, 0.9% sodium chloride) was injected for the control group.

Retention procedure: Following the injections, the expanders were converted into retention devices by halting the force provided by the helical loop. Acrylic resin was placed on the loop to achieve that result. Then both control and BP groups underwent 7 days of retention. At the end of the 7 days, half of the mice were sacrificed.

Relapse: The other half that was not sacrificed after the 7 days of retention underwent a period of relapse in which each group (BP and control) was allowed to relapse for 7 days. Then after that period, the relapse mice were sacrificed. The relapse ratio was calculated from direct measurements using the following time points and formula:

T1: Pre-expansion

T2: Post-expansion

T3: Post-retention

T4: Relapse

$$\text{Relapse ratio} = (T4 - T3) / (T3 - T1) \times 100 \quad \textbf{(21)}$$

Micro-computed tomography (μCT) scan and three-dimensional volumetric analysis

The harvested maxillae were fixed with 10% formalin at room temperature for 24 hours and then stored in 70% ethanol solution. The fixed maxillae were scanned using a high-resolution micro-computed tomography (SkyScan 1172, SkyScan N.V., Belgium). Then 3D image datasets were reconstructed from 2D X-ray images with the NRecon software (SkyScan N.V., Belgium). 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.

Histology

After μCT scanning, the tissues were decalcified in 5% EDTA, 4% sucrose, and PBS solution (pH 7.4). Decalcification was for 2 to 3 weeks at 4°C and the decalcification solution changed daily. Tissue samples were sent to UCLA Translational Procurement Core Laboratory (TPCL) and processed for paraffin embedding. 5-μm-thick sections were cut and processed for H & E staining by TPCL.

Statistical analysis

The experimental results were expressed as means ± standard deviation. To compare the data, the outcome measurements were compared to using the Student's t-test. p-values of <0.05 were considered significant.

RESULTS

Inhibition of α-SMA expression with BP treatment

Early passage NHOFs were incubated with BP and induced with TGF-β1. ZOL was added 2 hours before the start of the experiments (Fig. 1). Quantitative real time analysis was performed after 1 day of incubation and Western blot analysis was completed after the cells were incubated in the induction media for 2 days. NHOFs were treated with increasing ZOL, 0μM, 1μM, or 5μM and with our without TGF-β1 (10ng/ml) induction media. Published data indicate that there are no cytotoxic effects of bisphosphonates on NHOFs up to 10 μM (43). α-SMA mRNA levels quantified by qPCR demonstrated that BP significantly inhibited gene expression in myofibroblasts (Fig. 2A). The slight increase detected between 0μM and 1μM was not significant at a p=0.06. Detection of α-SMA by Western blot indicated that increasing levels of BP decreased transcription of α-SMA, which is a marker for myofibroblasts (Fig. 2B). BP inhibits α-SMA expression mediated by TGF-β1.

BP effect on contraction

Collagen lattice contraction demonstrated the effect of BP on myofibroblast function. NHOF cells were incorporated into collagen lattices and allowed to contract for 7 days in the presence of BP and TGF-β1 induction media. Visual representation of the wells demonstrated less contraction in BP-treated samples (Fig. 3A). Percent contraction was measured as gel area/plate area. BP inhibited gel contraction by 3-fold as compared to control (Fig. 3B).

Establishment of novel midpalatal expansion and relapse model for mice

The timeline for the animal study was derived and modified from a previous study (Fig. 4A) (41). Several preliminary experiments were conducted to determine the appropriate expander for mice. After examining various compositions and designs, the established expander

comprised of a 0.012 stainless steel wire, which was formed to consist of a 1.3mm helical spring and 6.3mm extension arms (Fig. 4B). The arms hooked around the central incisors, with the helical spring facing the oral cavity. The expanders were cemented on 30 6-week old male C57BL/6J mice and they experienced 40g of force from the expanders. For T1, the mice underwent 7 days of expansion and successful separation was noted by direct observation and by μCT imaging. After the 7 days, the expanders were converted to retention devices at T2 by deactivating the helical spring with a ball of rigid acrylic resin (Fig. 4C).

BP administration decreased relapse in vivo

At T1, the incisors were touching and the distance measured zero (Fig. 5A). At T2, post-expansion, there is clear separation of the incisors (Fig. 5B). The rate of decrease in the distance between the incisors was measured to calculate the relapse ratio. The relapse ratio was significantly reduced in the BP group when compared to the control group (Fig. 5C). With BP treatment, the relapse ratio in the BP group was 14.9%, which was significantly less than that of the control group, which had a relapse ratio of 44.5%.

Micro-computed tomography (μCT) scan and volumetric bone analysis

Micro-computed tomography analysis was completed to visualize successful suture separation and to evaluate the effect of BP on bone formation and mineralization in the palatal suture. Three-dimensional volumetric images were generated and analyzed to quantify bone volume and bone mineral density (BMD). The μCT images visually demonstrate the marked bone formation at the expanded region at T3 and T4 in the BP group as opposed to the control group (Fig. 6A). These findings are further corroborated by the volumetric analysis. Bone volume/tissue volume (BV/TV) was significantly greater in the BP-treated group compared to

the control group after 7 days of retention and after 7 days of relapse (Fig. 6B). Furthermore, the BP group demonstrated significantly increased BMD compared to the control group at T4; however, there was no statistical difference between the control and BP groups at T3 (Fig. 6C).

Histology

To further confirm the volumetric data, histologic analysis of the suture region was performed with hematoxylin and eosin (H&E) staining (Fig. 7 A and B). The black rectangles demarcate the midpalatal sutures of these coronal sections. The control sections demonstrated a region of unorganized pattern of connective tissue while the bisphosphonate sections indicated a suture area that appeared to have more organized such as found in bone. There appeared to be cells encapsulated in a space, much like osteocytes trapped in lacunae. Argument could be made that the bisphosphonate sections are simply more organized connective tissue. However, all bone formation starts with connective tissue so the suture may well in fact be in the midst of bone formation. Although μCT data of increased bone density in the BP suture may lean toward the bone formation, this must be confirmed by more specific histologic findings.

DISCUSSION

The number of murine expansion models that exist are limited and do not emphasize the midpalatal suture, which is the suture of interest in orthodontic palatal expansion. There are invasive surgical expansion protocols which involve cranial osteotomy for the placement of sagittal expanders in mice (21, 44). Such studies also suggest how bisphosphonates decrease relapse ratio possibly due to increased bone formation. Other studies bond palatal expanders to molars molars, but require a unique custom –made restrainer and led to midpalatal expansion detected in micrometers and invisible to direct inspection (2, 45). In contrast, the present study's palatal expansion mouse model involves the attachment of the self-activated expander around the two incisors, facilitating efficient skeletal expansion that replicates expansion found in orthodontic patients (Fig. 5). In addition, the expansion observation and analysis is similar to what is done in orthopedically expanded patients; this includes measuring the diastema formed between the incisors and using radiographic images, such as computed tomography, to evaluate the suture separation.

Bisphosphonates were first synthesized in the 1800s, but have only been more recently introduced into clinical use (46). In fact, it was only until the 1990s that their biochemical pathways were discovered (47). There are numerous articles in literature that addresses the effect of bisphosphonate on orthodontic therapies. Topical or systemic administration of bisphosphonates decreases orthodontic tooth movement, reduces skeletal relapse after maxillary expansion, and reduces relapse after mandibular distraction (48, 49). The existing relapse models evaluate the effects of bisphosphonate on rat sagittal suture after expansion (21, 44). And they conclude that bisphosphonate administration decrease relapse after expansion due inhibition of bone resorption and increased bone formation at the suture (21, 44). For the first time, the

present study examined the effects of bisphosphonates in the reduction of post-expansion relapse using the midpalatal suture in mice, rather than the sagittal suture in rats, which is more representative of the expansion protocol used in orthodontic patients. In concordance with other relapse studies, this study also demonstrated the positive effect on bone formation after bisphosphonate treatment (Fig. 6). Furthermore, in addition to the evidence of increased bone levels as an explanation for the reduced relapse, this study demonstrated the role of soft tissue wound contraction as a possible mechanism contributing to relapse after palatal expansion.

Surgical repair of orofacial clefts have been associated with maxillary growth impairment and adverse dentoalveolar changes. Excessive wound contraction and scar tissue formation contribute to such growth disturbances and suggest the influence of connective tissue on the adaptation of the body to changes in dimension. The possibility of decreasing the contraction carries significant clinical importance in perhaps minimizing the impairment. Fibroblasts start wound healing and then transiently form myofibroblasts, which have the contractile α-SMA proteins that generate the force for contraction. And once wound closure is complete, the myofibroblasts disappear by apoptosis (26, 50).

Along the same lines, orthodontic palatal expansion disrupts the soft tissue surrounding the suture and elicits a wound healing response, which involves the contraction of the palatal tissue (25). While the majority of bisphosphonate studies focus on its effect on bone, there are studies that examine the effect of bisphosphonates on the soft tissues of the gastrointestinal tract, from the oral cavity to the intestines; however, they focus on the epithelium (51, 52). When intestinal epithelial cells are exposed to BP, there is widening in intercellular spaces and cell death (53). When oral keratinocytes are exposed to BP, cell proliferation decreases and wound healing is suppressed (51). Apoptosis is prevented when BP-induced inhibition of

farnyldiphosphate (FPP) synthase—an enzyme in the mevalonate pathway—is avoided by the addition of Geranylgeraniol, which is a downstream prenylation molecule in cell membrane synthesis (51). Aside from the epithelial tissue, the existing studies on the connective tissue examine cytotoxicity of bisphosphonates to fibroblasts (43, 54). High concentrations of bisphosphonates can lead to apoptosis, however Zoledronate concentration of up to 5μM, which reflects our data's physiological reproduction, is the local BP level in bone in patients taking BP (55, 56). Currently, there is no research published on the effect of BP on myofibroblasts, which contain the contractile properties crucial to wound healing. Considering the use of non-toxic levels of BP, this study's findings suggest that BP can modulate myofibroblastic differentiation thereby inhibiting their contractile function, rather than simply causing cell toxicity.

Komatsu et. al. discovered that BP, specifically Zoledronate, reduced wound closure by inhibiting the granulation tissue formation by gingival fibroblasts stimulated with TGF-β1, probably through the suppression of Smad2/2 signaling transduction (28). The expression of myofibroblast markers, such as α-SMA and type I collagen were reduced (28). Our results also indicated the reduction in α-SMA after BP treatment (Fig. 2). BP inhibited gel contraction by 3-fold as compared to control (Fig. 3B). As expected, the addition of TGF-β1 significantly increased contraction and decreased gel area compared to control, possibly due to activation of more myofibroblasts. However, when comparing BP-treated samples with or without TGF-β1, the same decrease in gel area is not significant, indication that BP may have had more of an anti-contractile effect over TGF-β1. Furthermore, the relevance to orthodontics comes with the animal study results, which suggest that the post-expansion relapse is possibly due to the soft tissue contracture associated with wound healing.

Relapse is a significant clinical problem in orthodontics, and it is especially critical to cleft lip and palate patients, who are in treatment from birth to well into their adulthood, and who have the highest relapse rate after expansion. This study attempts to elucidate the mechanism behind relapse by introducing the novel concept of soft tissue contraction as a source for post-expansion relapse. By understanding and controlling the relapse, we can help reduce the obstacles faced by these craniofacial patients.

CONCLUSION AND FUTURE DIRECTION

The present study's findings suggest that bisphosphonate administration can decrease relapse ratio in palatally expanded mice and that bisphosphonate reduces α-SMA expression and contraction:

- Midpalatal skeletal expansion was successfully achieved in mice with a tooth-borne expander hooked around the central incisors.
- BP administration reduced relapse following expansion
- Micro-CT and histological analysis demonstrated higher bone volume after BP injection, indicating enhanced post-expansion stability with BP administration
- In NHOF, BP inhibited gene expression, and protein expression of α-SMA mediated by TGF-β1 in a time-dependent and dose-dependent manner.
- BP inhibited the functional contractile mechanism of myofibroblasts.

The study could have been further enhanced with the addition of immunohistochemical data on the localization of α-SMA in situ in order to establish a connection between *in vivo* and *in vitro* data. The exact composition of the sections would need to be further examined. Future experiments, such as alizarin red stain for bone and picrosirius red staining for connective tissue, would help elucidate the suture composition. With the establishment of the mouse model, we can now move forward with transgenic mice in order to better elucidate the mechanism behind palatal relapse. We would predict that knocking out α-SMA reduces the relapse potential after expansion. Furthermore, we could examine other myofibroblast markers, such as smooth muscle myosin heavy chain (Smemb), ED-A fibronectin, and paxillin (57, 58). Future studies would also

consider the administration of BP at different time points and the retention of the device for longer periods of time. In addition, distinctions could be made between male and female mice as maturation stages may vary between these two groups and thus have an effect on expansion.

APPENDIX

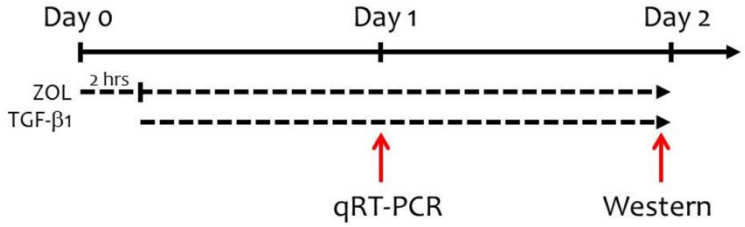


Figure 1. *In vitro* experiment timeline. Normal human oral fibroblasts (NHOF) were incubated with BP and induced with TGF-β1. Quantitative real time analysis was performed after 1 day and Western blot analysis after 2 days.

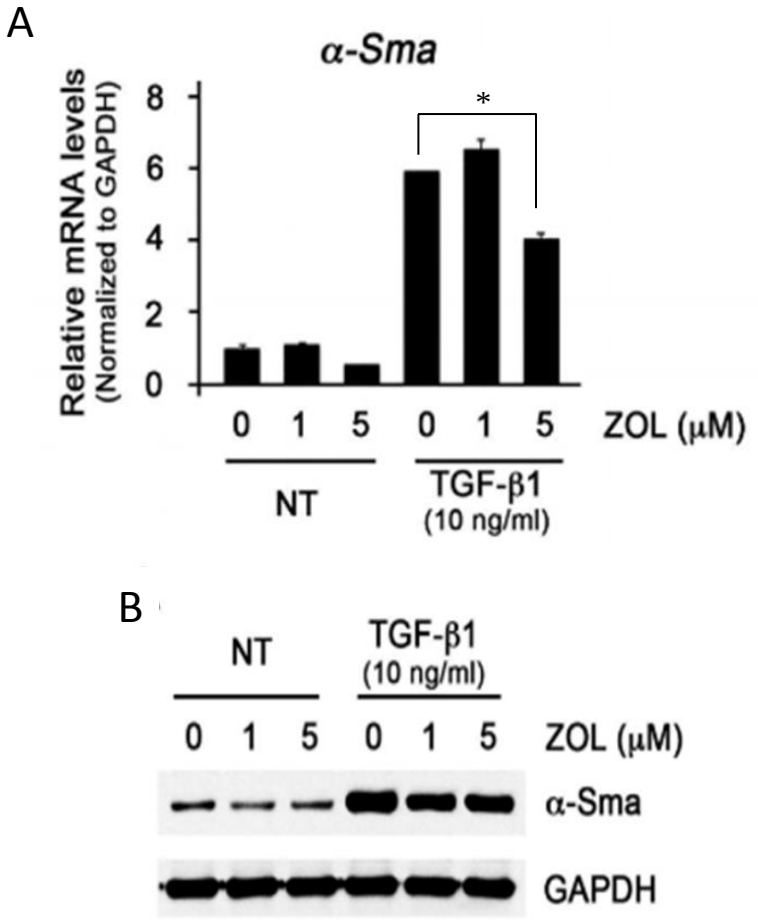


Figure 2: BP inhibits α-SMA expression mediated by TGF-β1. (A) NHOF cells were treated with BP at the indicated concentrations for 1 day and α-SMA mRNA quantified by qPCR. BP inhibits gene expression of α-SMA mediate by TGF-β1, $p < 0.05$. (B) Detection of α-SMA by Western blot from cell lysate treated with BP at the indicated concentrations for 2 days. Increasing levels of α-SMA decreased protein expression of α-SMA.

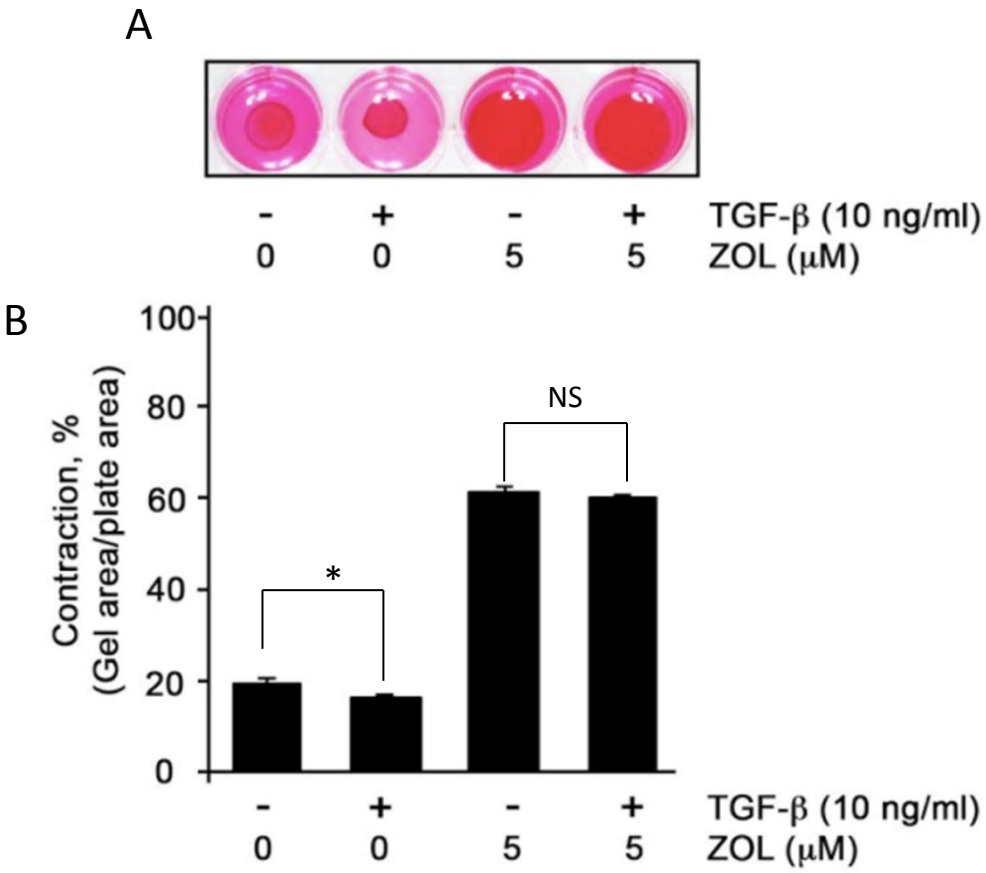


Figure 3: Collagen contraction inhibited by BP. NHOF cells were incorporated into collagen lattices and allowed to contract for 7 days in the presence of BP and TGF-β induction media. (A) Photograph of the collagen gels. The right two gels with BP showed less contraction. (B) BP inhibited gel contraction by 3-fold when measured by the gel area to plate area, $p<0.05$.

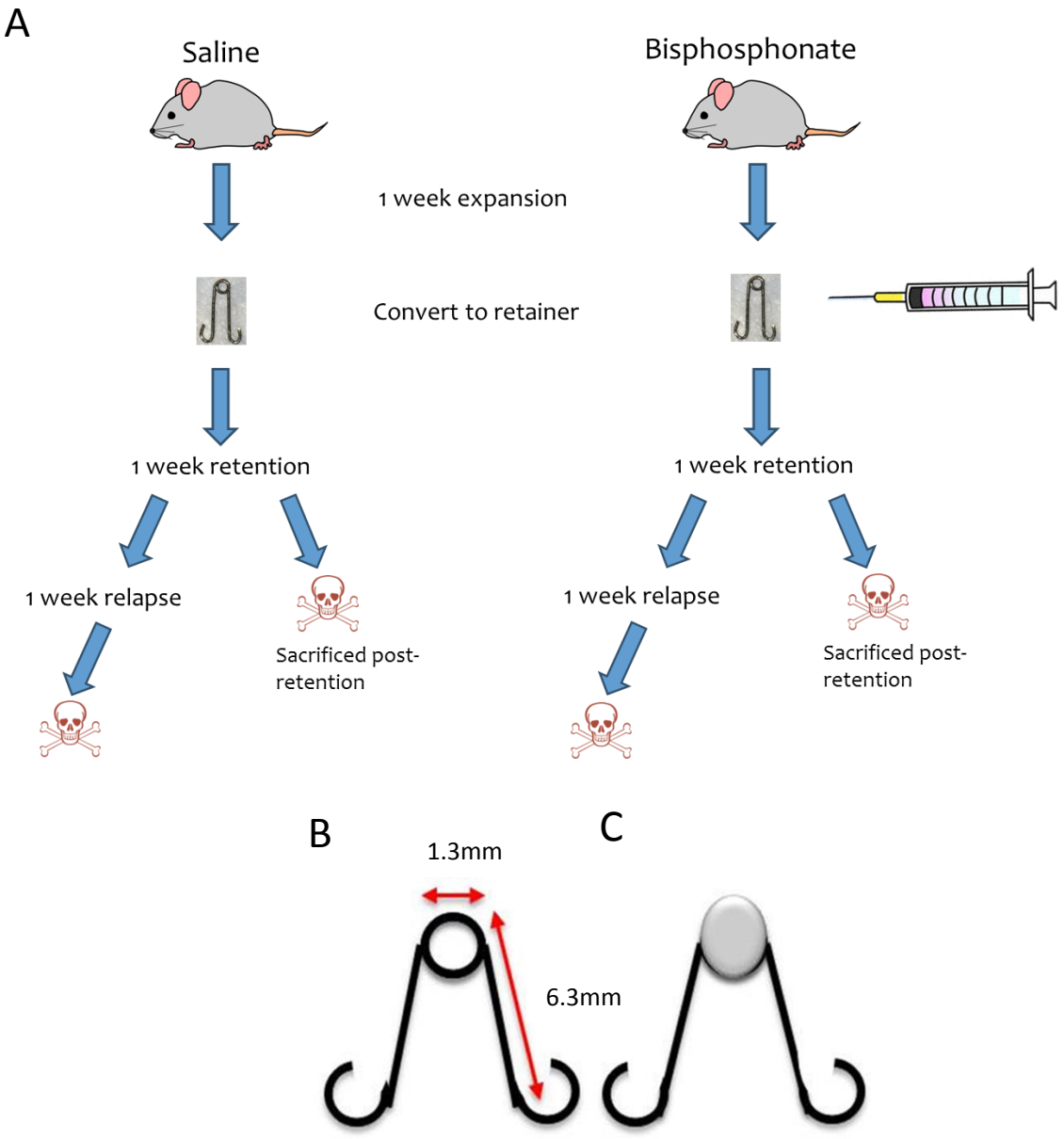


Figure 4: Study design. (A) 6-week-old C57BL/6J mice underwent 1 week of expansion. Then the expanders were converted to retainers. At this point, mice were injected with either BP or saline. After retention half was sacrificed. The other half sacrificed after relapse. The control group was injected with physiologic saline solution (5mg/kg, 0.9% sodium chloride) and the BP group (n=10) was injected with (0.1mg/kg of Zoledronate) subcutaneously. (B) Visual representation of the intraoral self-activated expander with the active helix 1.3 mm in diameter and extension arms 6.3 mm in lengths bilaterally. The force of 40 g was exerted by compressing on the helical part of the spring. (C) Visual representation of the expander converted into the retention device at T2 by deactivating the helical spring with rigid acrylic resin.

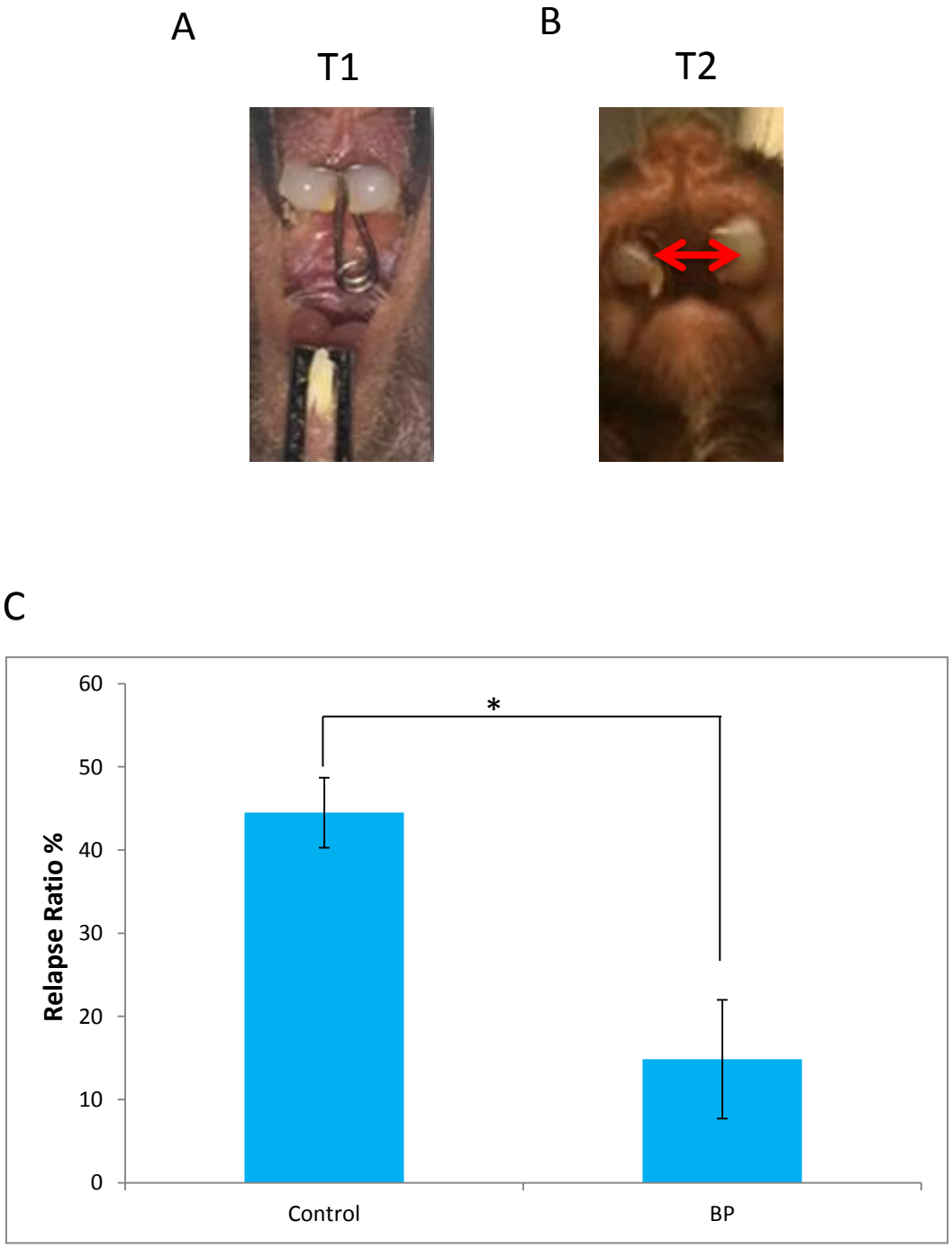
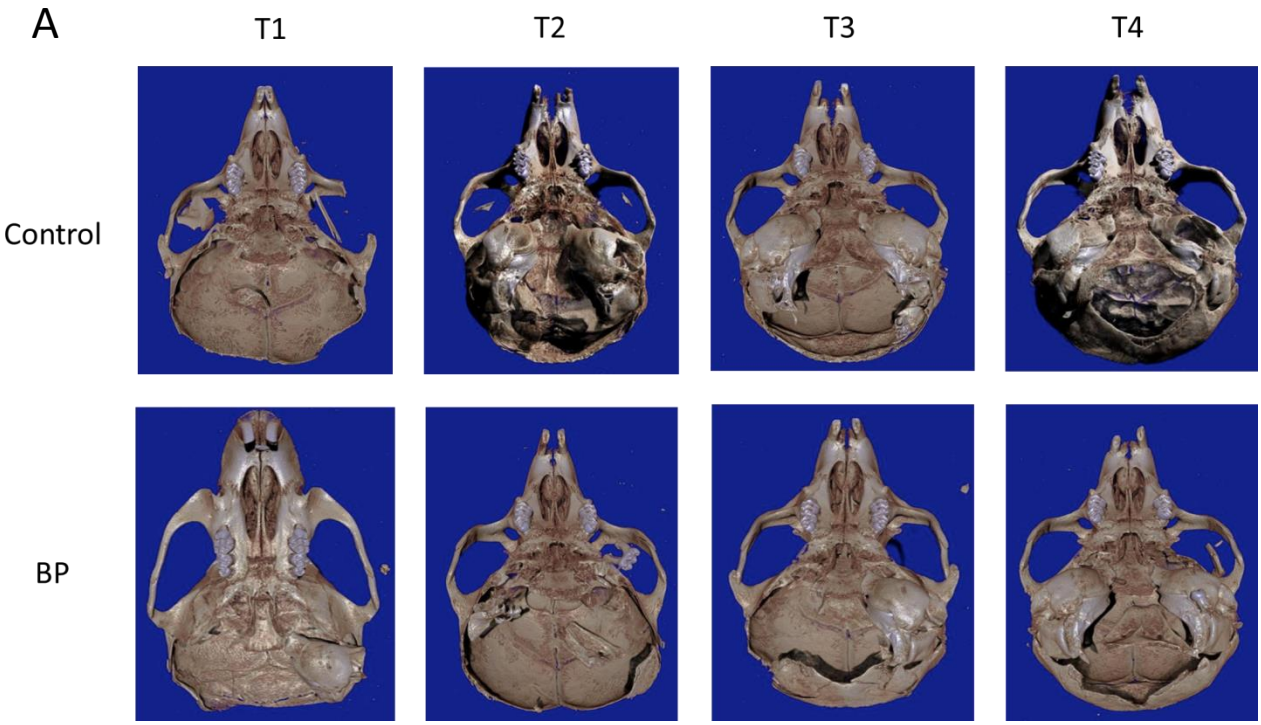
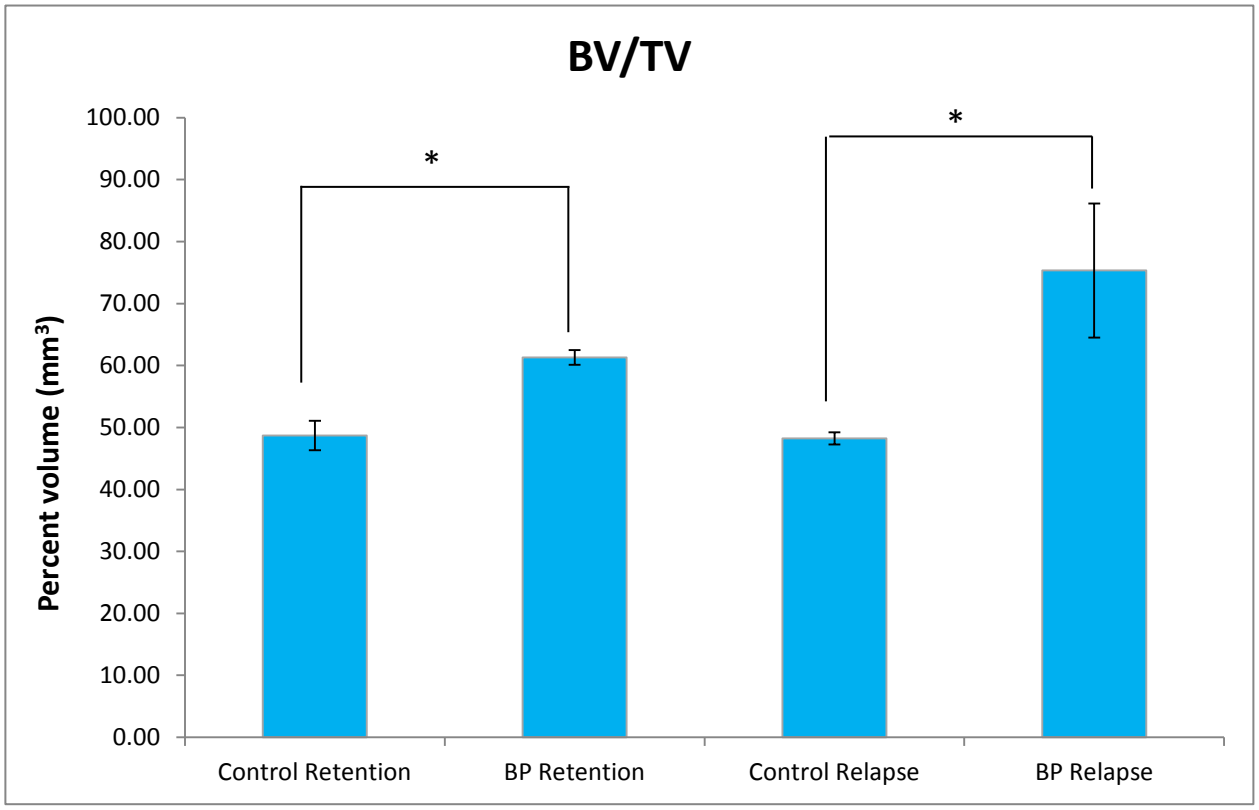


Figure 5: BP results in significant decrease in relapse following palatal expansion. (A) Intraoral view of expansion appliance cemented onto maxillary incisors at time T1. Note the lack of space between the incisors. (B) Intraoral view of expansion appliance cemented onto maxillary incisors at time T2. Diastema measurement is indicated by the red arrow. (C) Relapse ratio was significantly reduced in the BP group compare to control, $p<0.05$.

A


T1
T2
T3
T4
Control
BP

B


BV/TV
Percent volume (mm³)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
*
*
Control Retention
BP Retention
Control Relapse
BP Relapse

C

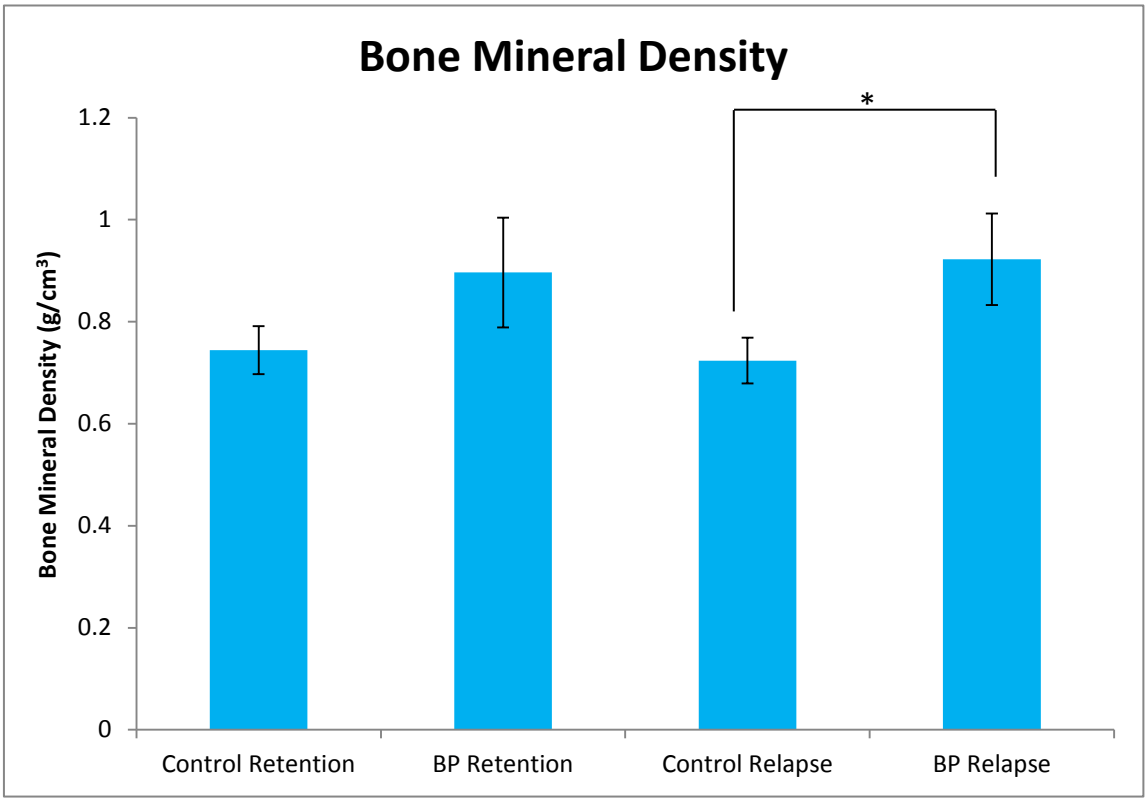


Figure 6: BP increases Bone Mineral Density and Bone Volume. (A) Three-dimensional volume rendering of maxillae among different time points. (B and C) Volumetric analysis of BV/TV at T3 and T4. The BP group had higher bone volume and density compared to the control group at both T3 and T4, $p < 0.05$.

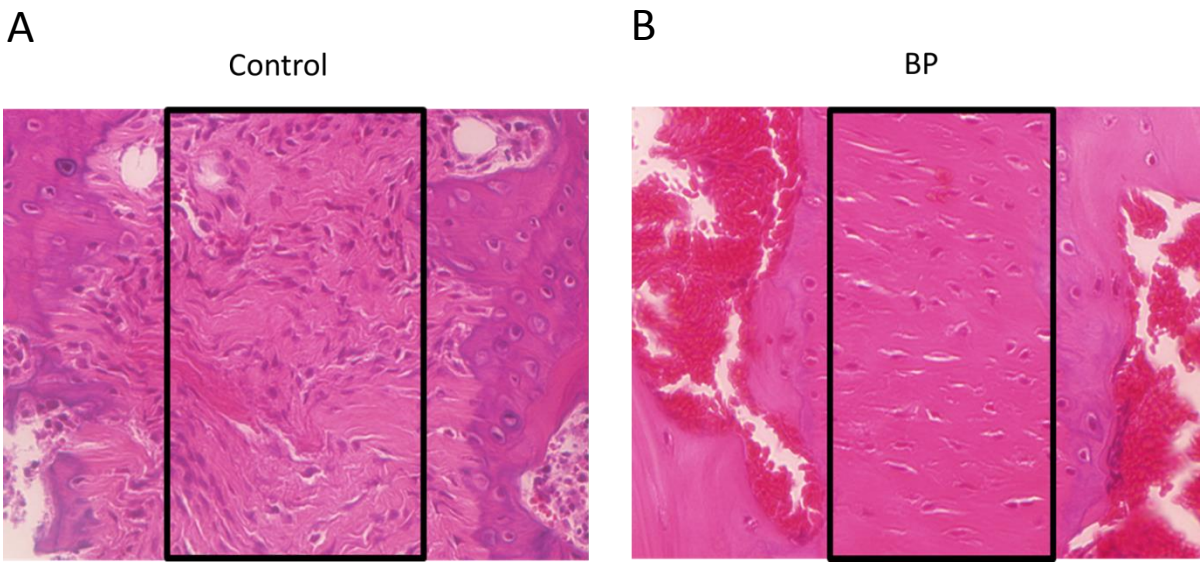


Figure 7: Histologic analysis at T4. H&E images of frontal sections of the midpalatal sutures which is outlined by the black rectangle. (A) Unorganized pattern of connective tissue while (B) has organized matrix that may include developing bone.

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