Title
Effects of Bisphosphonate Administration on Cleft Bone Graft in a Rat Model

Journal
The Cleft Palate-Craniofacial Journal, 54(6)

ISSN
1055-6656

Authors
Cheng, Nicole
Park, Juyoung
Olson, Jeffrey
et al.

Publication Date
2017-11-01

DOI
10.1597/15-356

Peer reviewed

Published in final edited form as:

Cleft Palate Craniofac J. 2017 November ; 54(6): 687–698. doi:10.1597/15-356.

Effects of Bisphosphonate Administration on Cleft Bone Graft in a Rat Model

Nicole Cheng, D.D.S., M.S., **Juyoung Park, D.D.S., M.S.**, **Jeffrey Olson, B.S.**, **Taewoo Kwon, B.S.**, **Deborah Lee, B.A.**, **Rachel Lim, B.S.**, **Sandy Ha, B.S.**, **Reuben Kim, D.D.S., Ph.D.**, **Xinli Zhang, M.D., M.S., Ph.D.**, **Kang Ting, D.M.D., D.Med.Sc.**, **Sotirios Tetradis, D.D.S., Ph.D.**, and **Christine Hong, D.M.D., M.S.**

Dr. Chen is Resident, Section of Orthodontics, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Dr, Park is master's student, Division of Oral Biology, University of California, Los Angeles, Los Angeles, California. Mr. Olson is dental student, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Mr. Kwon is dental student, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Ms. Lee is dental student, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Ms. Lin is dental student, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Ms. Ha is dental student, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Dr. Kim is Associate Professor, Section of Restorative Dentistry and Section of Oral Biology, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Dr. Zhang is Adjunct Professor, Section of Orthodontics, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Dr. Ting is Chair and Professor, Section of Orthodontics, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Dr. Tetradis is Chair and Professor, Section of Oral and Maxillofacial Radiology, School of Dentistry, University of California, Los Angeles, Los Angeles, California. Dr. Hong is Assistant Professor, Section of Orthodontics, School of Dentistry, University of California, Los Angeles, Los Angeles, California

Abstract

Objective—Bone grafts in patients with cleft lip and palate can undergo a significant amount of resorption. The aim of this study was to investigate the effects of bisphosphonates (BPs) on the success of bone grafts in rats.

Design—Thirty-five female 15-week-old Fischer F344 Inbred rats were divided into the following experimental groups, each receiving bone grafts to repair an intraoral CSD: (1) Graft/saline: systemic administration of saline and (2) systemic administration of zoledronic acid immediately following surgery (graft/BP/T0), (3) 1 week postoperatively (graft/BP/T1), and (4) 3 weeks postoperatively (graft/ BP/T2). As an additional control, the defect was left empty without bone graft.

Address correspondence to: Dr. Christine Hong, UCLA School of Dentistry, 63-014 CHS, 10833 Le Conte Ave, Los Angeles, CA 90095. chong@dentistry.ucla.edu.

Main Outcome Measures—Microcomputed tomography and histologic analyses were performed in addition to evaluation of osteoclasts through tartrate-resistant acid phosphatase staining.

Results—Bone volume fraction (bone volume/tissue volume) for the delayed BP treatment groups (graft/BP/T1 = 45.4% ± 8.8%; graft/BP/T2 = 46.1% ± 12.4%) were significantly greater than that for the graft/saline group (31.0% ± 7.9%) and the graft/BP/T0 (27.6% ± 5.9%) 6 weeks postoperatively ($P < .05$). Hematoxylin and eosin staining confirmed an evident increase in bone volume and fusion of defect margins with existing palatal bone in the graft/BP/T1 and graft/BP/T2 groups. The graft/BP/T0 group showed the lowest bone volume with signs of acute inflammation.

Conclusions—Delayed BP administration following cleft bone graft surgery led to significant increase in bone volume and integration compared with saline controls. However, BP injection immediately after the surgery did not enhance bone volume, and rather, may negatively affect bone graft incorporation.

Keywords

cleft lip and palate; critical-sized defect; bisphosphonates

Cleft lip and/or palate (CLP) is one of the most commonly occurring congenital malformations in the orofacial region (Dixon et al., 2011) and leads to significant clinical problems, such as nasal deformity, dental malocclusion, eating difficulty, and a decline in psychosocial well-being (Hupp et al., 2008). A major challenge for orthodontists in treating patients with CLP is aligning the dentition surrounding the cleft where bone necessary for tooth eruption is lacking, thereby preventing proper dental development (Hupp et al., 2008). Also, these patients have limited maxillary transverse growth as the maxillary and nasal processes do not fuse developmentally, thereby requiring orthodontic maxillary expansion (Nicholson and Plint, 1989, Long et al., 2000; Lewis et al., 2008). Maxillary expansion not only eliminates transverse deficiency but also widens the cleft region and allows for subsequent bone graft surgery in patients with CLP to fill in the spaces (Tindlund, 1994, Lidral and Vig, 2002). Unfortunately, cleft bone grafting often results in insufficient bone volume in the cleft region due to a high amount of bone resorption, necessitating additional expansion procedures and bone graft surgeries (Jia et al., 2006). This repeated treatment has a number of adverse consequences, including surgical morbidity, lengthened overall orthodontic treatment duration and associated dental problems, decline in the patient's mental health, and increased financial burden (Hupp et al., 2008). 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 patients with CLP.

An imbalance of anabolic and catabolic activity during bone graft incorporation is a major cause of bone graft failure (Burchardt and Enneking, 1978). Researchers have investigated different methods to increase the anabolic activity of osteoblasts as a way to achieve successful clinical outcomes. These methods include utilization of fibrin glue (Segura-Castillo et al., 2005), platelet rich plasma (Marukawa et al., 2011), and bone morphogenetic proteins (Nguyen et al., 2009). Although early results are promising (Ayoub et al., 2016), the adverse effects of such methods, including hematoma formation and long-term growth

effects, are alarming and have prevented their routine use (Smith et al., 2008, Alonso et al., 2010, Argintar et al., 2011).

Incorporation of a bone graft involves not only formation but also resorption of bone in the grafted area. Decreasing the rate of graft resorption is particularly important for cleft bone grafting, as studies measuring bone loss of grafts using computed tomography (CT) scans report loss of bone volume as high as 64% after 1 year (Feichtinger et al., 2006). Bone grafting in the intraoral cleft is prone to high resorption rates for several reasons: (1) tension developing from the mucoperiosteal flap and dehiscence (Kortebein et al., 1991; Rossell-Perry, 2015), (2) bacterial infections specific to oral-nasal environments (Johanson et al., 1974, Enemark et al., 1985, Lilja et al., 1987, Rossell-Perry, 2015), and (3) the absence of mechanical stress, which is required for increased bone formation and decreased bone resorption as stated in Wolff's law (Bergland et al., 1986, Frost, 2004). To date, there have been limited cleft bone graft studies to determine if new bone formation could be enhanced by limiting graft resorption, particularly with consideration of the intraoral environment. Limiting the increased resorption rate using antiresorptive agents is a promising new method that can significantly improve the clinical success of cleft bone graft surgery in treating patients with CLP.

Bisphosphonates (BPs) are promising antiresorptive drugs medically used to prevent bone loss by binding to hydroxyapatite in bone and leading to osteoclast dysfunction (Russell et al., 2008). Previous studies have shown the protective role of BPs against bone allograft resorption by either soaking bone graft materials in BPs (Aspenberg and Astrand, 2002; Kesteris and Aspenberg, 2006) or administering a single injection subcutaneously (Tagil et al., 2006). However, these methods have not yet been established for bone grafting in CLP models. A numbers of non-intraoral critical-sized defect (CSD) animal models have been used to study bone graft incorporation at the preclinical level; nonetheless, these models fail to accurately represent bone grafting in patients with CLP as intraoral factors such as the oral bacterial flora, mastication forces, and salivary components were not present (Springer et al., 2005; Aghaloo et al., 2010).

In this study, we set our aims to (1) develop a novel animal model to accurately mimic a cleft bone graft in the oral cavity and (2) examine the role of BPs in preventing bone resorption following cleft bone grafting with an emphasis on different time points of single BP administration. Specifically, in order to determine an optimal window for BP delivery, BP systemic administration was given at the time of surgery and at 1 week and 3 weeks after surgery. We hypothesized that a delayed single dose of BP administration would improve bone volume as compared with controls and administration at the time of surgery.

Materials and Methods

Animals

To first establish a novel intraoral CLP model, animals were divided into two groups: (1) control: defect without bone graft (n = 3) and (2) graft: defect with graft (n = 4). Isograft bone was harvested from the iliac crest and femur of a Fischer F344 Inbred donor rat. All animals were euthanized at 6 weeks postoperatively for micro-CT and histologic analysis.

Based on a feasibility study, a 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 eight rats would be required in each group to achieve a power level of 0.8 and $\alpha = 0.05$. The animal research protocol for the main experiment was approved (ARC 2012-027-02A) by the UCLA Animal Research Committee. A total of 39 female 15-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 five groups: (1) graft/saline: bone graft with systemic saline injection (n = 8); (2) graft/BP/T0: systemic BP injection at the time of surgery (n = 8); (3) graft/BP/T1: systemic zoledronic acid (ZA) injection 1 week postoperative (n = 8); (4) graft/BP/T2: systemic ZA injection 3 weeks postoperative (n = 8); (5) control: the defect was created but no bone graft was placed (n = 3). Four F344 Inbred rats were used as bone isograft donors.

Surgical Procedure

Bone Graft Harvest—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 were used to manually grind the corticocancellous bone into fine particles with sterile saline, which were then placed on ice for immediate use.

Creation of a Critical-Sized Bone Defect

Recipient animals were anesthetized with isoflurane (4% to 5%), followed by intraperitoneal 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 to 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 1-cm 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. Using a hand-operated, low-speed power drill, a circular 3-mm midpalatal defect was created distal to the upper incisors using a 3-mm diameter trephine bur, generating an accurate and consistent defect size for each surgery. Due to the length of the maxillary incisors, the defect was placed in the midpalate to avoid dental root damage (Fig 1). After adequate hemostasis using pressure and gauze, the harvested isograft was placed into the defect and the mucosa reapproximated. Postoperatively, each animal received subcutaneous injections of buprenex (0.05 mg/kg) two times a day for 2 days as analgesia. Trimethorprimsulfa was placed in the drinking water (5 mL trimethorprimsulfa per 500 mL water) for a period of 2 weeks postsurgery, beginning the day before surgery, to prevent infection.

Placement of the Bone Graft—Harvested cancellous bone was placed in a 30-μL syringe with the tip removed. The bone was packed into the syringe and saline blotted for dense concentration of bone without desiccating the graft material. Then, 7 μL was dispensed into each animal defect for consistency of volume and concentration.

BP Delivery

A single 0.1 mg/kg subcutaneous injection of ZA was administered to the animals at 1 week postsurgery for the pilot study. For the graft/BP groups, ZA was administered at various time points: at the time of surgery (T0), 1 week postsurgery (T1), or 3 weeks postsurgery (T2). For the graft/saline group, a single systemic injection of saline was administered 1 week postsurgery. Six weeks postsurgery, all animals were euthanized and the maxilla harvested.

Data Analysis

Micro-CT Analysis—The maxilla was dissected and fixed with 4% (weight/ volume) paraformaldehyde in 0.1 M phosphate-buffered saline solution for 24 hours. The samples were scanned using a high-resolution micro-CT (SkyScan 1172, Sky-Scan 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 three-dimensional (3D) image datasets were reconstructed from two-dimensional x-ray images using NRecon software (SkyScan N.V), 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.) to align the palatal defects parallel to the transaxial plane to minimize analysis errors.

A 3D volumetric analysis was performed using CTAn software (SkyScan N.V.). To ensure consistency of results, all analyses were repeated at a separate time point by a single rater. The region of interest was outlined with a 3-mm circle on consecutive transaxial sections in order to create a uniform, cylindrical-shaped volume of interest, 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, and bone mineral density (BMD) was also calculated.

Histologic Analysis—After micro-CT analysis, the samples were decalcified for 20 days in 10% ethylenediaminetetraacetic acid (EDTA) (0.1 M, pH=7.1) solution. During decalcification, the solution was changed every 2 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 to visualize bone graft morphology. Mineralized area/total area of mineralized sections was calculated using Advanced SPOT 4.6 software at 4× magnification.

Evaluating Osteoclasts—One section from each animal was stained for tartrate-resistant acid phosphatase (TRAP), using a TRAP staining kit (Sigma Aldrich, St. Louis, MO). The slides were de-paraffinized at 60°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 AS-MX phosphate, Fast Red Violet LB Salt, and 50 mM sodium tartrate. They were placed in humidity chambers with no light exposure at 37°C for 2 hours. Slides were then counterstained 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+ cells. For quantification, the number of TRAP+ multinuclear cells (≥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 using Advanced SPOT 4.6 software.

Statistical Analysis—The data were expressed as means and standard deviations for each group. Means were compared using parametric analysis of variance (ANOVA). Examination of residual error normal quantile plots and the Shapiro-Wilks test for normality confirmed that the data followed the normal distribution, allowing ANOVA to be used among the groups: (1) graft/saline, (2) graft/BP/T0, (3) graft/BP/T1, and (4) graft/BP/T2 ($\alpha = 0.05$). Mean differences among subgroups under the ANOVA model were evaluated using the Fisher least significant difference criterion. Computations were carried out using JMP 12.0 (SAS Inc, Cary, NC). Adjusted values of $P < .05$ were considered statistically significant.

Results

Establishment of Intraoral Cleft Bone Graft Model in Rats

To develop an animal model for cleft bone graft that can better represent the intraoral environment, we created a circular, midpalatal CSD in rats. A defect 3 mm in diameter was created in the palatal areas reproducibly without any technical difficulties (Fig 1). In addition, oral mucosal tissues at the surgical lesions closed within 1 week and healed without any complications, including breathing problems, nasal obstruction, bleeding, and infection. Using this model, we first examined the bone-healing patterns of the CSD with or without bone graft. Micro-CT analysis of the control group confirmed that this newly developed intraoral defect was indeed nonhealing (Fig 2A, top). Conversely, when bone graft was placed, significant incorporation of bone occurred (Fig 2A, bottom). This difference in healing pattern was confirmed up to 10 weeks. Further quantification of mineralized tissues in the defect area showed a significant increase in bone volume fraction (BV/TV) in the graft group compared with the control group (Fig 2B). These results indicate the successful establishment of an intraoral cleft bone graft model in rats.

Effects of BPs at the Different Delivery Time Points in the Cleft Bone Graft Rat Model

BPs are known to reduce bone resorption leading to increased bone volume. (Russell et al., 2008) As such, we next examined the effects of administering BPs at different time points during the course of palatal bone graft healing: systemic BP injection at the time of surgery (graft/BP/T0), 1 week after surgery (graft/BP/ T1), and 3 weeks after surgery (graft/BP/T2). Systemic injection of saline at the time of surgery was used as a control group (graft/saline). As an additional control, the defect was created without bone graft placement (control). The group assignment and experimental timeline are outlined in Figure 3. Oral mucosal tissues at the surgical lesions in all groups, including BP injection groups, were completely healed and closed without any complications up to 6 weeks postsurgery.

Micro-CT 3D reconstructed images of the defect area reconfirmed the nonhealing defect in the control group and revealed integration of defect margins with existing palatal bone in the graft/BP/T1 and the graft/BP/T2 groups, indicating clinical success of the bone graft procedure (Fig 4A). Most of the graft/BP/T0 and graft/ saline animals had no union at the margins. Micro-CT analysis showed a significant increase in BV/TV in the graft/BP/T1 (45.4% ± 8.8%) and the graft/BP/T2 (46.1% ± 12.4%) group compared with the graft/saline group (31.0% ± 7.9%) (Fig 4B). There was no statistically significant difference in BV/TV between the graft/BP/T1 and graft/BP/T2 groups. Interestingly, BP administration at the time of surgery did not increase bone volume and in fact had the lowest BV/ TV (27.6% ± 5.9%) (Fig 4B). Our BMD analysis, however, did not show significant difference among groups (Fig 4c).

Similarly, H&E staining of the defect verified a clear increase in bone mass in the graft/BP/T1 and the graft/ BP/T2 groups (Fig 5). There was a significantly increased mineralized area/total area in the graft/BP/ T1 (0.72 ± 0.10) and the graft/BP/T2 (0.68 ± 0.09) groups compared with the graft/BP/T0 group (0.21 ± 0.08) and the graft/saline group (0.24 ± 0.06) (Fig 5B). In these delayed BP groups, more exuberant angiogenesis and new bone formation were 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 were visualized. In the graft/BP/ T1 and the graft/BP/T2 samples, complete fusion of defect margins with existing palatal bone was present histologically confirming 3D micro-CT images, while most of the graft/saline and graft/BP/T0 groups had no union (Fig 5A). Indeed, one of the rats in the graft/ saline group showed severe bone resorption and lacked new bone formation entirely, indicating the clinical unpredictability of oral cleft bone graft surgery (Fig 5). Interestingly, all animals in the graft/BP/T0 group showed significantly decreased bone volume with signs of acute inflammation (Fig 5A). 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 (Fig 5A).

TRAP staining showed no significant difference in quantity of osteoclasts, with 25% detached from the bone surface; however, 3.75% of TRAP+ multinucleated osteoclasts in BP-treated rats exhibited abnormal morphology, appearing rounded and detached from the bone surface (Fig 6). Taken together, our data suggest that delayed BP administration following the cleft bone graft surgical procedure enhanced the formation of bone at the grafted sites.

Discussion

In this study, we showed successful establishment of an intraoral CSD rat model that best represents the bone grafting in patients with CLP. Also, we demonstrated the use of BPs to suppress bone resorption without any adverse effects in this rat model, providing preclinical evidence that a single dose of BP can be utilized to improve cleft bone graft in patients.

Currently, there are several animal models for CLP but with multiple shortcomings. While genetic rat models for CLP exist, they die soon after birth, preventing the study of CLP

postnatally (Juriloff and Harris, 2008). Therefore, in order to study bone grafting in oral clefts, a 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 (Aghaloo et al., 2010). However it fails to accurately represent bone graft in patients with CLP as intraoral factors such as the oral bacterial flora, mastication forces, and salivary components are not present in these models. We recreated a previously published intraoral CSD design in which the rats experienced severe incisal root damage. The defect cut through the tooth, causing unnecessary pain and introducing pulpal bleeding and cells into the defect (Nguyen et al., 2009). In addition, the design had an irregular shape, compromising reproducibility of the study. Here, we developed a novel animal model that accurately simulates clinical conditions by creating a palatal defect 3 mm in diameter that circumvents problems with lethal genetic models. Therefore, our model represents more accurately the conditions associated with CLP grafting, such as oral bacterial flora, mastication forces, and salivary components. Furthermore, we demonstrated that our novel palatal defect was non–bone healing and resulted in complete closure of the overlaying soft tissues in 6 weeks with no significant complications. All rats used in this study survived without postoperative complications, demonstrating the surgical predictability and reproducibility of this procedure.

The gold standard for graft sources in patients with CLP is autologous bone (Lichte et al., 2011) with the iliac crest (Boyne and Sands, 1972), calvaria (Wolfe and Berkowitz, 1983), or tibia (Kalaaji et al., 2001) being the most common sources. This is because autografts are osteoinductive, osteoconductive, and nonimmunogenic (Dimitriou et al., 2011). In this study, instead of using autologous bone, we used Fischer F344 Inbred rats as donors for bone graft material not only to minimize any complications originating from surgery for obtaining autologous bone but also due to their genetic continuity, resulting in isografts that closely mimic autografts. Indeed, our histologic findings showed minimal inflammatory reaction against isograft materials, demonstrating successful establishment of an effective intraoral CSD-based animal model that can be applied to the study of different agents in future studies.

A previous study showed that delaying BP administration may allow the endogenous anabolic and catabolic responses to establish themselves before administering the drug, resulting in a higher rate of bone turnover (Little et al., 2005). In a femoral osteotomy model, BP injections delayed by 2 weeks had increased callus volume and density (Bosemark et al., 2013, Mathavan et al., 2013). Indeed, upon examining the effect of timing of BP delivery on bone graft success, we observed a significant increase in BV/TV following delayed BP administration at 1 and 3 weeks postoperatively. Furthermore, these results were confirmed through histologic analysis, which found a significant increase in mineralization following delayed BP administration. These results suggest that when BPs are administered at the time of surgery, osteoclast inhibition results in an inability to resorb the dead bone that now occupies the space. Consequently, proper bone remodeling is also inhibited. Moreover, the results suggest that BPs, which have greater affinity for bone undergoing active remodeling, bind at a greater rate to the grafted region after first allowing time for the anabolic and catabolic responses to establish themselves, thereby resulting in an increased anticatabolic response (Little et al., 2005).

Additionally, the bone remodeling rate in the maxillary bone, which is more relevant for CLP studies, is higher than bone remodeling in other anatomic regions (Huja and Beck, 2008). In a study of young dogs, a threefold to sixfold difference in bone turnover rate between the maxilla and femur was observed (Huja et al., 2006, Huja and Beck, 2008). Accordingly, this high bone remodeling rate in the maxilla may allow for even greater binding of BP (Bertoldo et al., 2007), thereby inducing a higher anticatabolic effect in cleft bone grafting and shifting the balance between osteoclastic and osteoblastic activity toward increased bone formation (Wermelin et al., 2007, Aspenberg, 2009).

Interestingly, BP administration at the time of the surgery resulted in a lowest BV/TV (Fig 4). Studies have found that BP accumulation in bone can be directly toxic to the oral epithelium and can impair epithelial homeostasis and repair by inhibiting re-epithelialization, thereby delaying soft tissue healing and increasing the likelihood of secondary infections (Reid et al., 2007; Donetti et al., 2013; Donetti et al., 2014). However, there was complete primary soft tissue healing by 1 week after the bone graft surgery, suggesting that soft tissue toxicity of BP is less likely to be the reason for this differences in BV/TV.

On the other hand, BP is known to induce inflammation as clinically evident by occurrence of acute phase responses in 30% to 40% of intravenous BP users (Adami et al., 1987). Indeed, animals in the graft/BP/T0 group exhibited signs of extensive acute inflammation upon histologic examination in addition to the lower BV/TV (Figure 4). BP can induce expression of proinflammatory cytokines, such as interleukin-6 and tumor necrosis factor–α, both *in vitro* and *in vivo* (Schweitzer et al., 1995, Thiebaud et al., 1997). As such, it is possible that administration of BP immediately after the surgery exacerbates and prolongs proinflammatory signals in the grafted areas, preventing proper healing process and resulting in lower BV/TV.

BP prevents bone resorption by targeting osteoclasts and suppressing their functions (Russell et al., 2008). When we examined the presence of osteoclasts using TRAP staining, we observed no significant difference in the number of matured TRAP+ osteoclasts between different treatment groups. However, it is noteworthy that 3.75% of TRAP+ multinucleated osteoclasts in BP-treated rats exhibited abnormal morphology, appearing rounded and detached from the bone surface (Fig 5). In a study utilizing transgenic mice that overexpressed TRAP, no significant difference in osteoclast number was observed despite a significant decrease in trabecular bone (Angel et al., 2000), suggesting that TRAP expression is not directly associated with osteoclast number. Other studies examining high-dosage BP treatment in animals also found no difference in TRAP+ cells (Kang et al., 2013, Park et al., 2013, Williams et al., 2014), but found that they had abnormal morphology in BP-treated groups (Kang et al., 2013). This morphologic aberration may indicate a disruption of osteoclast function. Alternatively, another explanation of our results may be that at the time of euthanasia (6 weeks), most of the dysfunctional osteoclasts affected by BP had already undergone apoptosis, while the newly generated osteoclasts showed normal resorptive activity. Studies have shown that the osteoclast life span ranges from just a few days (Miller and Bowman, 2007) to 6 weeks (Marks and Seifert, 1985). Future studies in

which animals are euthanized soon after BP administration with multiple shorter intervals of sacrifice could demonstrate osteoclast activity levels as a function of time.

Although we provide compelling evidence that proves antiresorptive effects of BP in cleft bone graft at the preclinical level, pleotropic effects of BP should be carefully considered before translating our findings onto the clinical level. First, BP is known to inhibit or delay permanent tooth eruption, as demonstrated by other studies showing that daily injections of BP at high concentrations for up to 1 month resulted in delayed tooth eruption or no eruption in animals (Grier and Wise, 1998; Bradaschia-Correa et al., 2007). However, close examination revealed that these previous studies administered much higher BP dosages, ranging from 1.25 to 2.5 mg/kg daily injections, compared with the 0.1 mg/kg single injection used in our study based on the commonly used therapeutic low dose given to humans for treatment of osteoporosis and osteogenesis imperfect (OI) (Schmitz and Hollinger, 1986, Ward et al., 2007). Second, there are concerns regarding the long-term effects on bone remodeling and maturation when BP is used in growing patients. However, it should be noted that BP therapy for OI in children has become a safe and established protocol to increase BMD and reduce fracture incidence (Glorieux et al., 1998, Seikaly et al., 2005, DiMeglio and Peacock, 2006). The literature conclusively shows that BP use in growing patients is justified, but it should be used carefully in regard to transient but possibly reversible changes at bony growth plates. Third, long-term use of BP is associated with the risk of developing BP-related osteonecrosis of the jaw (BRONJ), particularly with intravenous administration (Aghaloo et al., 2015). The incidence of BRONJ ranges from 0.8% to 12% when used intravenously to treat hypercalcemia and bone metastases (Ruggiero et al., 2009). However, a large study examining 64 children treated with BP for OI found 0% incidence of BRONJ (Malmgren et al., 2008). In line with this finding, cleft bone grafting is done in the maxilla, where BRONJ is half as likely to occur compared with the mandible (Weeda, 2009). Based on these previous clinical studies, we anticipate the concentration of BP used in our palatal defect animal model poses a minimal risk for developing BRONJ.

In this study, the effect of systemic BP on cleft bone grafting was examined in female rats. Although a previous study reported that drug uptake by the bone between male and female rats presents similarly (Lin et al., 1992), this current study can be further expanded by examining BP effects on males. In addition, further histologic analysis using calcein binding will be beneficial as this allows direct quantification of extracellular matrix mineral content in monolayer cultures of bone-forming cells such as primary osteoblasts (Hale et al., 2000) to further evaluate the effect of BPs on cleft grafting.

With the development of a novel palatal defect animal model, which can be applied to the study of different agents in regulation of endogenous metabolic activities during bone graft incorporation, we demonstrated that a single, delayed systemic injection of ZA significantly increased percent bone volume 6 weeks after bone graft surgery. This innovative approach resulted in the clinical success of bone grafts, with clear evidence of angiogenesis, osteocytes, and bony integration. In addition to establishing an effective model for studying the effects of various agents on bone grafting, this study demonstrates that the anticatabolic

properties of BP could offer promising new clinical applications towards effectively managing patients with CLP.

Acknowledgments

This work was funded by the Cleft Palate Foundation and American Association of Orthodontists.

References

Adami S, Bhalla AK, Dorizzi R, Montesanti F, Rosini S, Salvagno G, Lo Cascio V. The acute-phase response after bisphosphonate administration. Calcif Tissue Int. 1987; 41:326–331. [PubMed: 3124942]

Aghaloo T, Cowan CM, Zhang X, Freymiller E, Soo C, Wu B, Ting K, Zhang X. The effect of NELL1 and bone morphogenetic protein-2 on calvarial bone regeneration. J Oral Maxillofac Surg. 2010; 68:300–308. [PubMed: 20116699]

Aghaloo T, Hazboun R, Tetradis S. Pathophysiology of osteonecrosis of the Jaws. Oral Maxillofac Surg Clin North Am. 2015; 27:489–496. [PubMed: 26412796]

Alonso N, Tanikawa DY, da Freitas R, Canan L Jr, Ozawa TO, Rocha DL. Evaluation of maxillary alveolar reconstruction using a resorbable collagen sponge with recombinant human bone morphogenetic protein-2 in cleft lip and palate patients. Tissue Eng Part C Methods. 2010; 16:1183–1189. [PubMed: 20163243]

Angel NZ, Walsh N, Forwood MR, Ostrowski MC, Cassady AI, Hume DA. Transgenic mice overexpressing tartrate-resistant acid phosphatase exhibit an increased rate of bone turnover. J Bone Miner Res. 2000; 15:103–110. [PubMed: 10646119]

Argintar E, Edwards S, Delahay J. Bone morphogenetic proteins in orthopaedic trauma surgery. Injury. 2011; 42:730–734. [PubMed: 21145058]

Aspenberg P. Bisphosphonates and implants: an overview. Acta Orthop. 2009; 80:119–123. [PubMed: 19234893]

Aspenberg P, Astrand J. Bone allografts pretreated with a bisphosphonate are not resorbed. Acta Orthop Scand. 2002; 73:20–23. [PubMed: 11928906]

Ayoub A, Roshan CP, Gillgrass T, Naudi K, Ray A. The clinical application of rhBMP-7 for the reconstruction of alveolar cleft. J Plast Reconstr Aesthet Surg. 2016; 69:101–107. [PubMed: 26507862]

Bergland O, Semb G, Abyholm FE. Elimination of the residual alveolar cleft by secondary bone grafting and subsequent orthodontic treatment. Cleft Palate J. 1986; 23:175–205. [PubMed: 3524905]

Bertoldo F, Santini D, Lo Cascio V. Bisphosphonates and osteomyelitis of the jaw: a pathogenic puzzle. Nat Clin Pract Oncol. 2007; 4:711–721. [PubMed: 18037875]

Bosemark P, Isaksson H, McDonald MM, Little DG, Tagil M. Augmentation of autologous bone graft by a combination of bone morphogenic protein and bisphosphonate increased both callus volume and strength. Acta Orthop. 2013; 84:106–111. [PubMed: 23409846]

Boyne PJ, Sands NR. Secondary bone grafting of residual alveolar and palatal clefts. J Oral Surg. 1972; 30:87–92. [PubMed: 4550446]

Bradaschia-Correa V, Massa LF, Arana-Chavez VE. Effects of alendronate on tooth eruption and molar root formation in young growing rats. Cell Tissue Res. 2007; 330:475–485. [PubMed: 17901984]

Burchardt H, Enneking WF. Transplantation of bone. Surg Clin North Am. 1978; 58:403–427. [PubMed: 349741]

DiMeglio LA, Peacock M. Two-year clinical trial of oral alendronate versus intravenous pamidronate in children with osteogenesis imperfecta. J Bone Miner Res. 2006; 21:132–140. [PubMed: 16355282]

Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC Med. 2011; 9:66. [PubMed: 21627784]

Dixo MJ, Marazita ML, Beaty TH, Murray JC. Cleft lip and palate: understanding genetic and environmental influences. Nat Rev Genet. 2011; 12:167–178. [PubMed: 21331089]

DonettI E, Gualerzi A, Sardella A, Lodi G, Carrassi A, Sforza c. Alendronate impairs epithelial adhesion, differentiation and proliferation in human oral mucosa. Oral Dis. 2013 XX:XX-XXX.

Donetti E, Gualerzi A, Sardella A, Lodi L, Carrassi Sforza c. Alendronate impairs epithelial adhesion, differentiation and proliferation in human oral mucosa. Oral Dis. 2014; 20:466–172. [PubMed: 23837876]

Enemark H, Krantz-Simonsen E, Schramm JE. Secondary bone-grafting in unilateral cleft lip palate patients: indications and treatment procedure. Int J Oral Surg. 1985; 14:2–10. [PubMed: 3921475]

Feichtinger M, Mossbock R, Karcher H. Evaluation of bone volume following bone grafting in patients with unilateral clefts of lip, alveolus and palate using a CT-guided three-dimensional navigation system. J Craniomaxillofac Surg. 2006; 34:144–149. [PubMed: 16537109]

Frost HM. A 2003 update of bone physiology and Wolff's Law for clinicians. Angle Orthod. 2004; 74:3–15. [PubMed: 15038485]

Glorieux FH, Bishop NJ, Plotkin H, Chabot G, Lanoue G, Travers R. Cyclic administration of pamidronate in children with severe osteogenesis imperfecta. N Engl J Med. 1998; 339:947–952. [PubMed: 9753709]

Grier RLT, Wise GE. Inhibition of tooth eruption in the rat by a bisphosphonate. J Dent Res. 1998; 77:8–15. [PubMed: 9437395]

Hale LV, Ma YF, Santerre RF. Semi-quantitative fluorescence analysis of calcein binding as a measurement of in vitro mineralization. Calcif Tissue Int. 2000; 67:80–84. [PubMed: 10908418]

Huja SS, Beck FM. Bone remodeling in maxilla, mandible, and femur of young dogs. Anat Rec (Hoboken). 2008; 291:1–5. [PubMed: 18085627]

Huja SS, Fernandez SA, Hill KJ, Li Y. Remodeling dynamics in the alveolar process in skeletally mature dogs. Anat Rec A Discov Mol Cell Evol Biol. 2006; 288:1243–1249. [PubMed: 17075846]

Hupp, JR., Ellis, E., Tucker, M. Contemporary Oral and Maxillofacial Surgery. City, state: Publisher's name; 2008.

Jia YL, Fu MK, Ma L. Long-term outcome of secondary alveolar bone grafting in patients with various types of cleft. Br J Oral Maxillofac Surg. 2006; 44:308–312. [PubMed: 16107298]

Johanson B, Ohlsson A, Friede H, Ahlgren J. A follow-up study of cleft lip and palate patients treated with orthodontics, secondary bone grafting, and prosthetic rehabilitation. Scand J Plast Reconstr Surg. 1974; 8:121–135. [PubMed: 4615365]

Juriloff DM, Harris MJ. Mouse genetic models of cleft lip with or without cleft palate. Birth Defects Res A Clin Mol Teratol. 2008; 82:63–77. [PubMed: 18181213]

Kalaaji A, Lilja J, Elander A, Friede H. Tibia as donor site for alveolar bone grafting in patients with cleft lip and palate: long-term experience. Scand J Plast Reconstr Surg Hand Surg. 2001; 35:35–42. [PubMed: 11291348]

Kang B, Cheong S, Chaichanasakul T, Bezouglaia O, Atti E, Dry SM, Pirih FQ, Aghaloo Tl, Tetradis S. Periapical disease and bisphosphonates induce osteonecrosis of the jaws in mice. J Bone Miner Res. 2013; 28:1631–1640. [PubMed: 23426919]

Kesteris U, Aspenberg P. Rinsing morcellised bone grafts with bisphosphonate solution prevents their resorption. A prospective randomised double-blinded study. J Bone Joint Surg Br. 2006; 88:993–996. [PubMed: 16877594]

Kortebein MJ, Nelson CL, Sadove AM. Retrospective analysis of 135 secondary alveolar cleft grafts using iliac or calvarial bone. J Oral Maxillofac Surg. 1991; 49:493–498. [PubMed: 2019895]

Lewis BR, Stern MR, Willmot DR. Maxillary anterior tooth size and arch dimensions in unilateral cleft lip and palate. Cleft Palate Craniofac J. 2008; 45:639–646. [PubMed: 18956935]

Lichte P, Pape HC, Pufe T, Kobbe P, Fischer H. Scaffolds for bone healing: concepts, materials and evidence. Injury. 2011; 42:569–573. [PubMed: 21489531]

Lidral, AC., Vig, WL. Role of Orthodontists in the Management of Patients with Cleft Lip and Palate. City, state/country: Oxford University Press; 2002. p. 381-396.

Lilja J, Moller M, Friede H, Lauritzen C, Petterson LE, Johanson B. Bone grafting at the stage of mixed dentition in cleft lip and palate patients. Scand J Plast Reconstr Surg Hand Surg. 1987; 21:73–79. [PubMed: 3296143]

Lin JH, Chen IW, Duggan DE. Effects of dose, sex, and age on the disposition of alendronate, a potent antiosteolytic bisphosphonate, in rats. Drug Metab Dispos. 1992; 20:473–478. [PubMed: 1356720]

Little DG, McDonald M, Bransford R, Godfrey CB, Amanat N. Manipulation of the anabolic and catabolic responses with OP-1 and zoledronic acid in a rat critical defect model. J Bone Miner Res. 2005; 20:2044–2052. [PubMed: 16234978]

Long RE, Semb G, Shaw WC. Orthodontic treatment of the patient with complete clefts of lip, alveolus, and palate: lessons of the past 60 years. Cleft Palate Craniofac J. 2000; 37:533–533.

Malmgren B, Astrom E, Soderhall S. No osteonecrosis in jaws of young patients with osteogenesis imperfecta treated with bisphosphonates. J Oral Pathol Med. 2008; 37:196–200. [PubMed: 18321345]

Marks SC Jr, Seifert MF. The lifespan of osteoclasts: experimental studies using the giant granule cytoplasmic marker characteristic of beige mice. Bone. 1985; 6:451–455. [PubMed: 3006733]

Marukawa E, Oshina H, lino G, Morita K, Omurav K. Reduction of bone resorption by the application of platelet-rich plasma (PRP) in bone grafting of the alveolar cleft. J Craniomaxillofac Surg. 2011; 39:278–283. [PubMed: 20542707]

Mathavan N, Bosemark P, Isaksson H, Tagil M. Investigating the synergistic efficacy of BMP-7 and zoledronate on bone allografts using an open rat osteotomy model. Bone. 2013; 56:440–448. [PubMed: 23845325]

Miller SC, Bowman BM. Rapid inactivation and apoptosis of osteoclasts in the maternal skeleton during the bone remodeling reversal at the end of lactation. Anat Rec (Hoboken). 2007; 290:65–73. [PubMed: 17441199]

Nguyen PD, Lin CD, Allori AC, Ricci JL, Saadeh PB, Warren SM. Establishment of a critical-sized alveolar defect in the rat: a model for human gingivoperiosteoplasty. Plast Reconstr Surg. 2009; 123:817–825. [PubMed: 19319044]

Nguyen PD, Lin CD, Allori AC, Schachar JS, Ricci JL, Saadeh PB, Warren SM. Scaffold-based rhBMP-2 therapy in a rat alveolar defect model: implications for human gingivoperiosteoplasty. Plast Reconstr Surg. 2009; 124:1829–1839. [PubMed: 19952639]

Nicholson PT, Plint DA. A long-term study of rapid maxillary expansion and bone grafting in cleft lip and palate patients. Eur J Orthod. 1989; 11:186–192. [PubMed: 2670591]

Park R, Kim JH, Choi H, Park YB, Jung HS, Moon HS. Effect of alendronate on bone remodeling around implant in the rat. J Adv Prosthodont. 2013; 5:374–381. [PubMed: 24353873]

Reid IR, Bolland MJ, Grey AB. Is bisphosphonate-associated osteonecrosis of the jaw caused by soft tissue toxicity? Bone. 2007; 41:318–320. [PubMed: 17572168]

Rossell-Perry P. Flap necrosis after palatoplasty in patients with cleft palate. BioMed Res Intl. 2015; 14 xxx-xxx.

Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws—2009 update. J Oral Maxillofac Surg. 2009; 67:2–12.

Russell RG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int. 2008; 19:733–759. [PubMed: 18214569]

Schmitz JP, Hollinger JO. The critical size defect as an experimental model for craniomandibulofacial nonunions. Clin Orthop Relat Res. 1986:299–308. xx. [PubMed: 3084153]

Schweitzer DH, Oostendorp-van de Ruit M, Van der Pluijm G, Lowik CW, Papapoulos SE. Interleukin-6 and the acute phase response during treatment of patients with Paget's disease with the nitrogen-containing bisphosphonate dimethylaminohydroxypropylidene bisphosphonate. J Bone Miner Res. 1995; 10:956–962. [PubMed: 7572320]

Segura-Castillo JL, Aguirre-Camacho H, Gonzalez-Ojeda A, Michel-Perez J. Reduction of bone resorption by the application of fibrin glue in the reconstruction of the alveolar cleft. J Craniofac Surg. 2005; 16:105–112. [PubMed: 15699654]

Seikaly MG, Kopanati S, Salhab N, Waber P, Patterson D, Browne R, Herring JA. Impact of alendronate on quality of life in children with osteogenesis imperfecta. J Pediatr Orthop. 2005; 25:786–791. [PubMed: 16294137]

Smith DM, Cooper GM, Mooney MP, Marra KG, Losee JE. Bone morphogenetic protein 2 therapy for craniofacial surgery. J Craniofac Surg. 2008; 19:1244–1259. [PubMed: 18812847]

Springer IN, Acil Y, Kuchenbecker S, Bolte H, Warnke PH, Abboud M, Wiltfang J, Terheyden H. Bone graft versus BMP-7 in a critical size defect—cranioplasty in a growing infant model. Bone. 2005; 37:563–9. [PubMed: 16046207]

Tagil M, Aspenberg P, Astrand J. Systemic zoledronate precoating of a bone graft reduces bone resorption during remodeling. Acta Orthop. 2006; 77:23–26. [PubMed: 16534698]

Thiebaud D, Sauty A, Burckhardt P, Leuenberger P, Sitzler L, Green JR, Kandra A, Zieschang J, Ibarra de Palacios P. An in vitro and in vivo study of cytokines in the acute-phase response associated with bisphosphonates. Calcif Tissue Int. 1997; 61:386–392. [PubMed: 9351880]

Tindlund RS. Skeletal response to maxillary protraction in patients with cleft lip and palate before age 10 years. Cleft Palate Craniofac J. 1994; 31:295–308. [PubMed: 7918525]

Ward L, Tricco AC, Phuong P, Cranney A, Barrowman N, Gaboury I, Rauch F, Tugwell P, Moher D. Bisphosphonate therapy for children and adolescents with secondary osteoporosis. Cochrane Database Syst Rev. 2007 Cd005324.

Weeda L Jr. Bisphosphonate related osteonecrosis of the jaws: a review and update. J Tenn Dent Assoc. 2009; 89:16–19. quiz 20-11. [PubMed: 19630246]

Wermelin K, Tengvall P, Aspenberg P. Surface-bound bisphosphonates enhance screw fixation in rats—increasing effect up to 8 weeks after insertion. Acta Orthop. 2007; 78:385–392. [PubMed: 17611854]

Williams DW, Lee C, Kim T, Yagita H, Wu H, Park S, Yang P, Liu H, Shi S, Shin KH, et al. Impaired bone resorption and woven bone formation are associated with development of osteonecrosis of the jaw-like lesions by bisphosphonate and anti-receptor activator of NF-kappaB ligand antibody in mice. Am J Pathol. 2014; 184:3084–3093. [PubMed: 25173134]

Wolfe SA, Berkowitz S. The use of cranial bone grafts in the closure of alveolar and anterior palatal clefts. Plast Reconstr Surg. 1983; 72:659–671. [PubMed: 6622573]

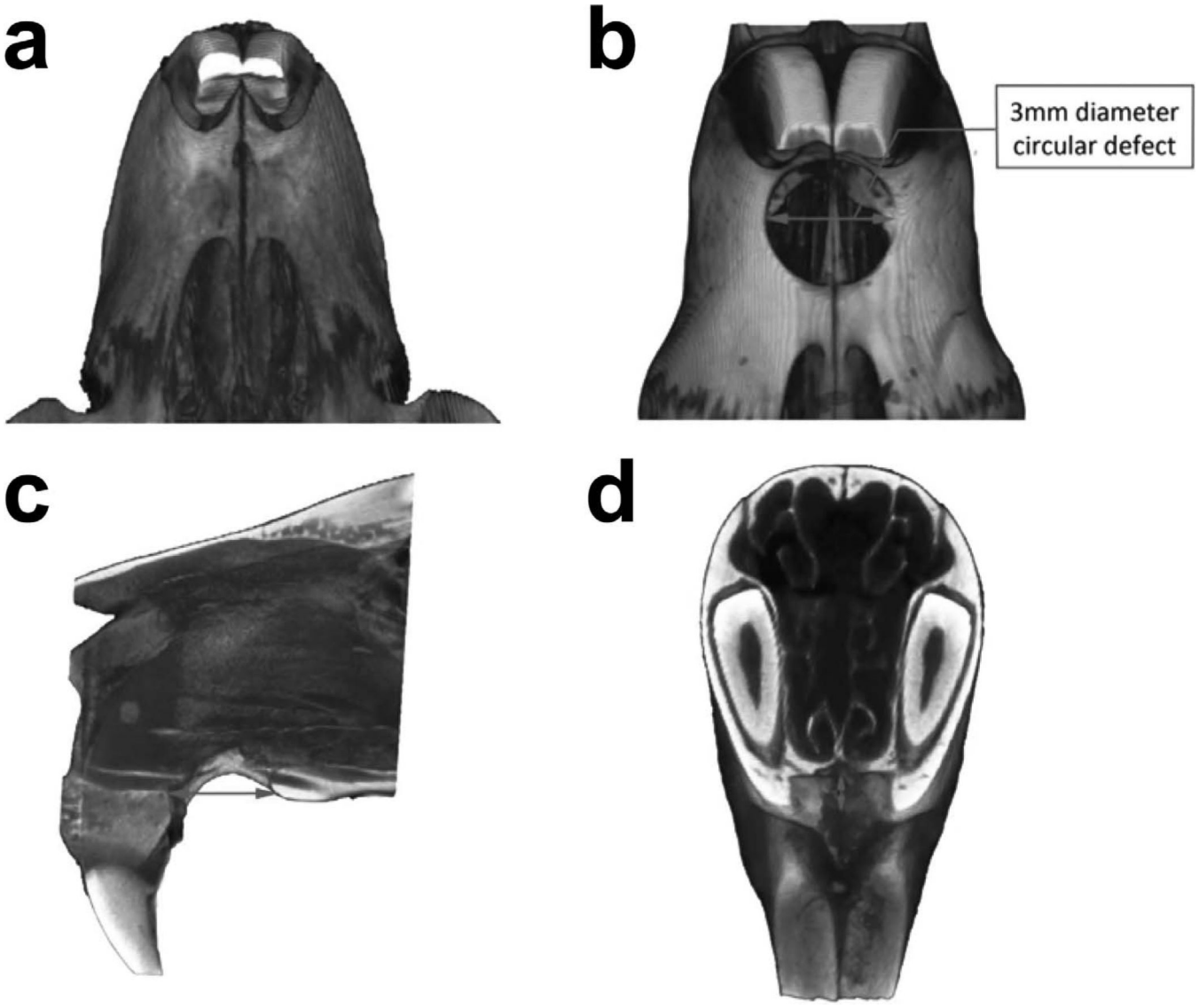

FIGURE 1.

Palatal CSD in rats. A: Anatomy of rat anterior palate. B: Circular defect 3 mm in diameter. C: Sagittal slice indicating 3 mm in diameter. D: Axial slice indicating 0.95 mm depth of the defect without damaging the incisal roots.

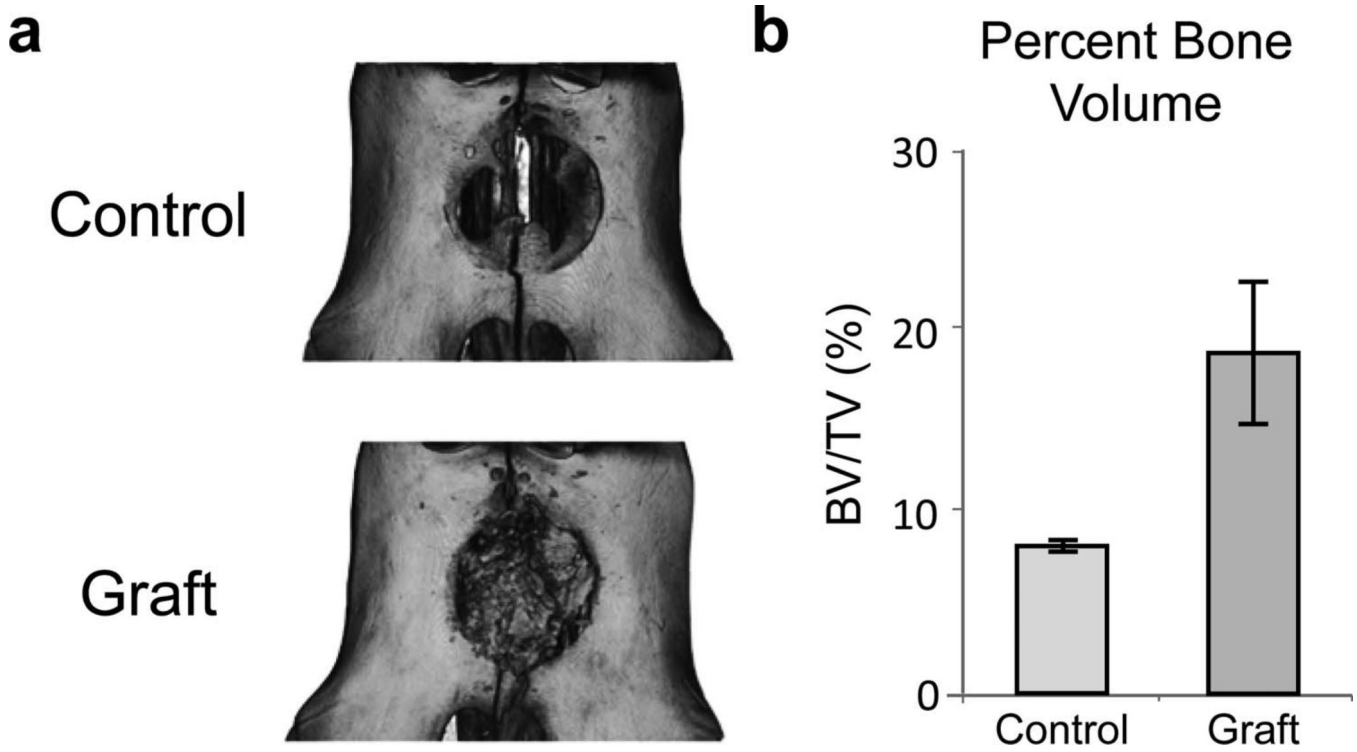

FIGURE 2.

Bone grafting in palatal defect. A: 3D micro-CT images of the control group showed that the newly developed intraoral defect was nonhealing after 10 weeks, while the graft group demonstrated a successful bone grafting at 6 weeks postoperatively. B: Quantification of bone volume by micro-CT analysis showed a statistically significant increase in bone volume in the graft group ($P < .05$). A Student's *t* test was used to calculate differences. Error bars show standard error.

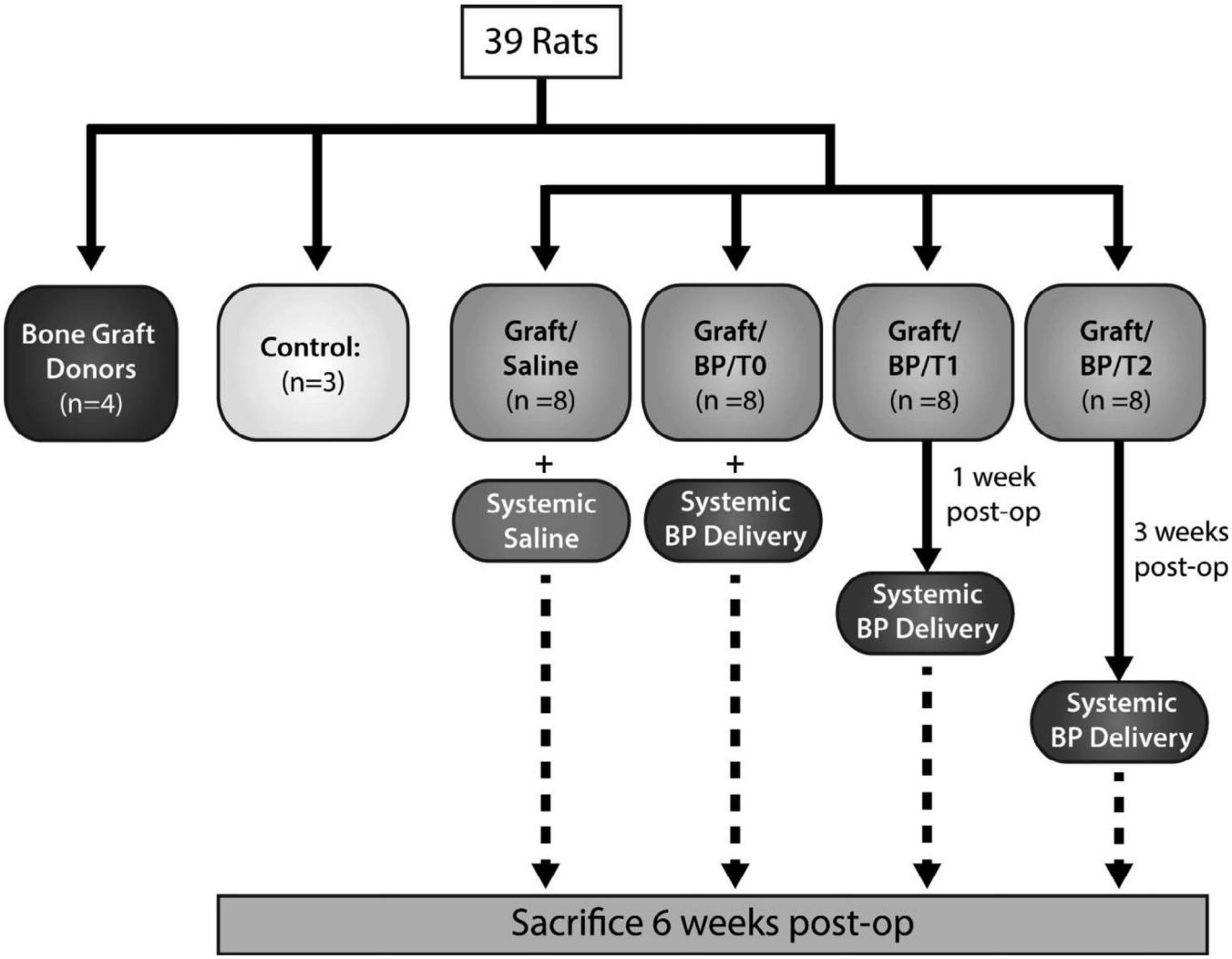

FIGURE 3.

Experimental Design. Thirty-nine 15-week-old Fischer F344 Inbred rats were divided into five groups: (1) Control: The defect was created but no bone graft was placed (n = 3); (2) Graft/saline: Bone graft with systemic saline injection (n = 8); (3) Graft/BP/T0: Systemic BP injection at the time of surgery (n = 8); (4) Graft/BP/T1: Systemic ZA injection 1 week postoperative (n = 8); (5) Graft/BP/T2: Systemic ZA injection 3 weeks postoperative (n = 8). Four F344 Inbred rats were used as bone isograft donors. All rats were euthanized for analysis at 6 weeks postoperatively.

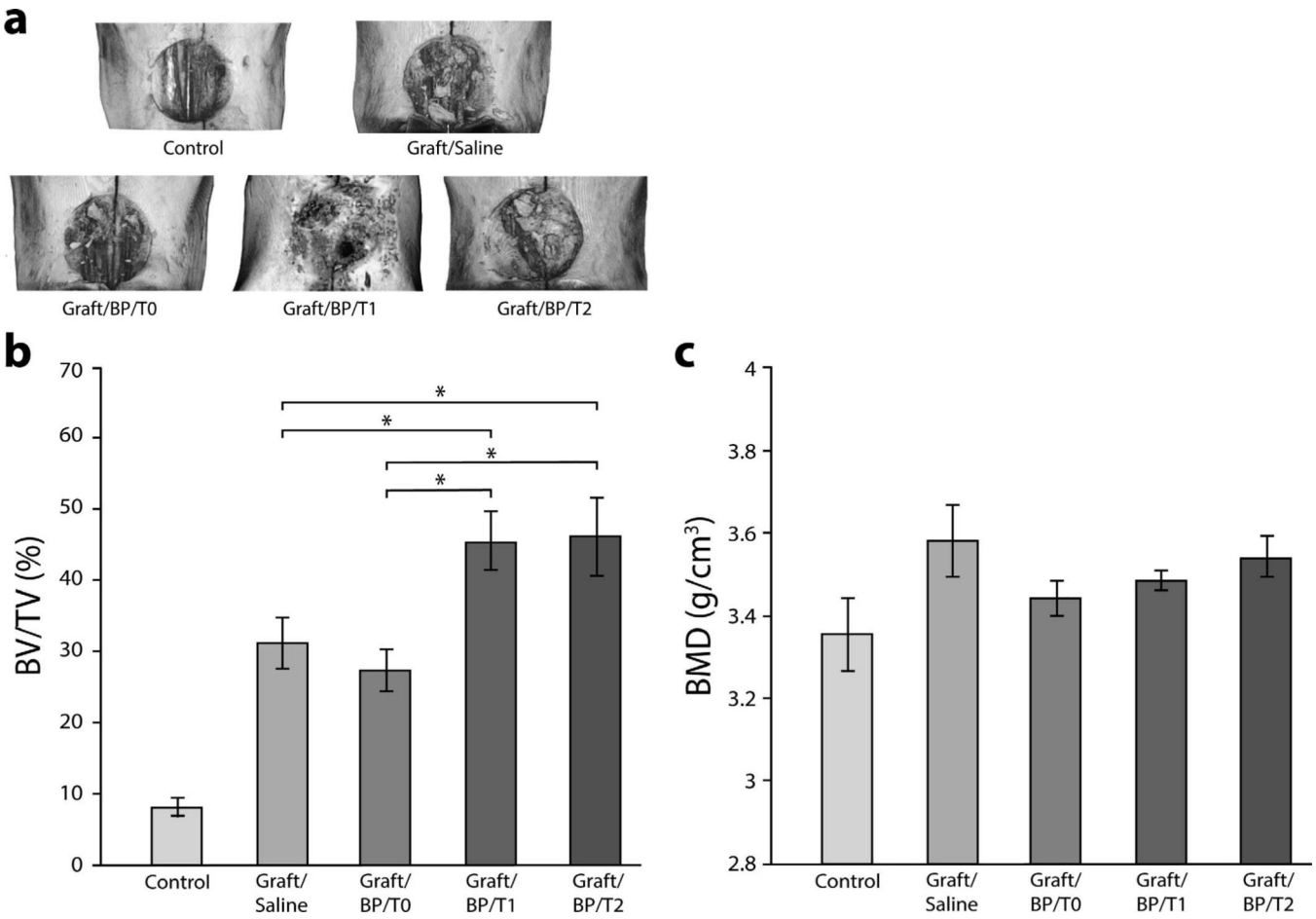

FIGURE 4.

Micro-CT images and analysis. 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 five groups demonstrated a clear increase in bone volume and bone integration in the graft/BP/T1 and graft/BP/ T2 groups. B: Quantification of bone volume by micro-CT analysis showed statistically significant increase in bone volume in graft/BP/T1 and graft/BP/ T2 groups. The graft/BP/T0 group had similar percent bone volume to the graft/ saline group. ANOVA with post hoc *t* tests was used to calculate differences. * Statistically significant, $P < .05$. Error bars show standard error. C: Quantification of bone mineral density by micro-CT analysis did not show a statistically significant difference between groups.

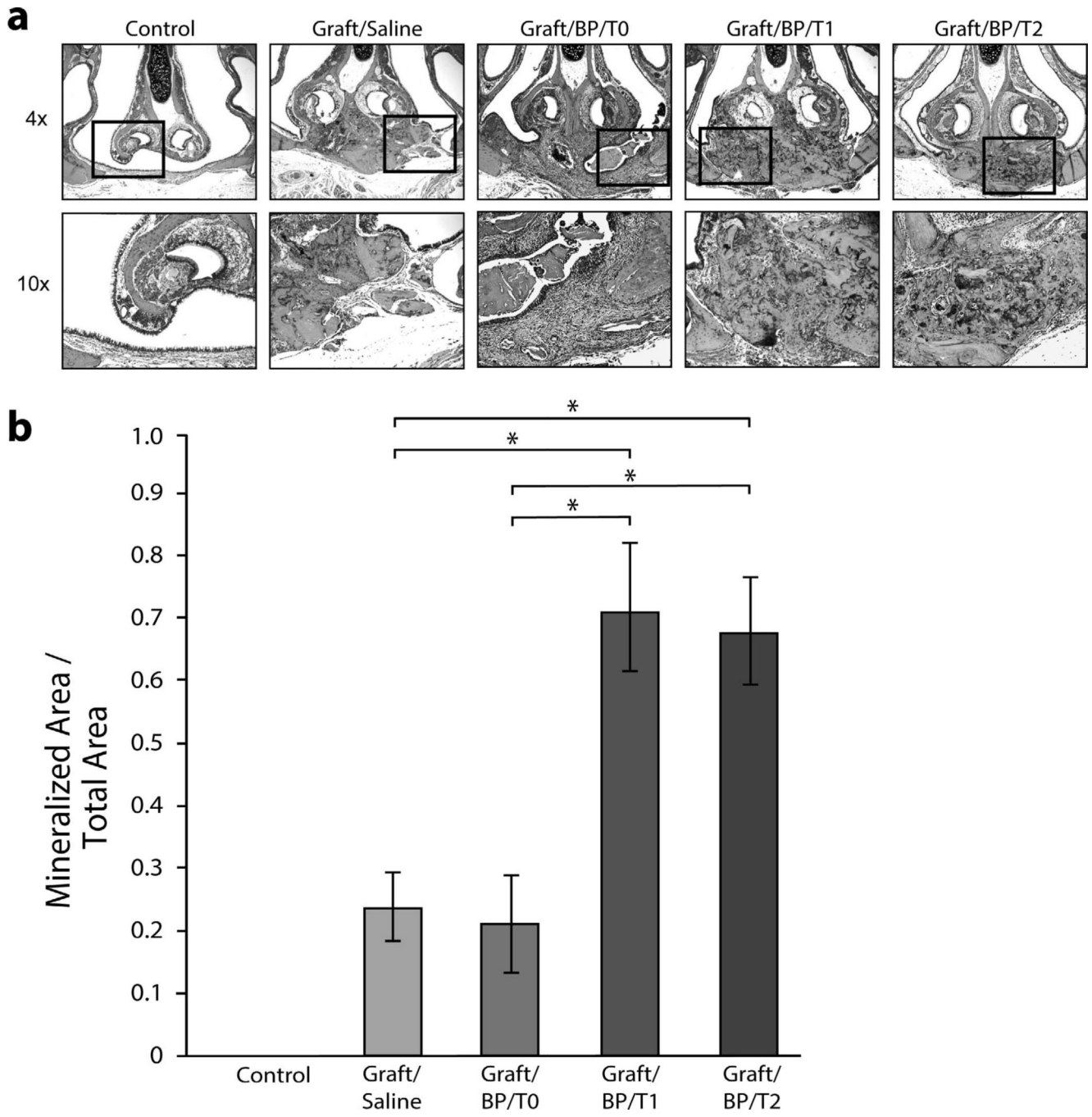

FIGURE 5.

H&E staining images. A: H&E stained coronal sections taken at 4× and 10× magnification confirmed increased bone volume in the graft/ BP/T1 and graft/BP/T2 groups, with evident amplification of osteocytes, integration of new bone with bone graft, and complete fusion of defect margins with existing palatal bone. The graft/BP/T0 group showed significantly decreased bone volume with signs of acute inflammation. B: Quantification of the ratio of mineralized area to total area for each group using Advanced SPOT 4.6 software showed significantly increased mineralization in the graft/BP/ T1 and graft/BP/T2 groups.

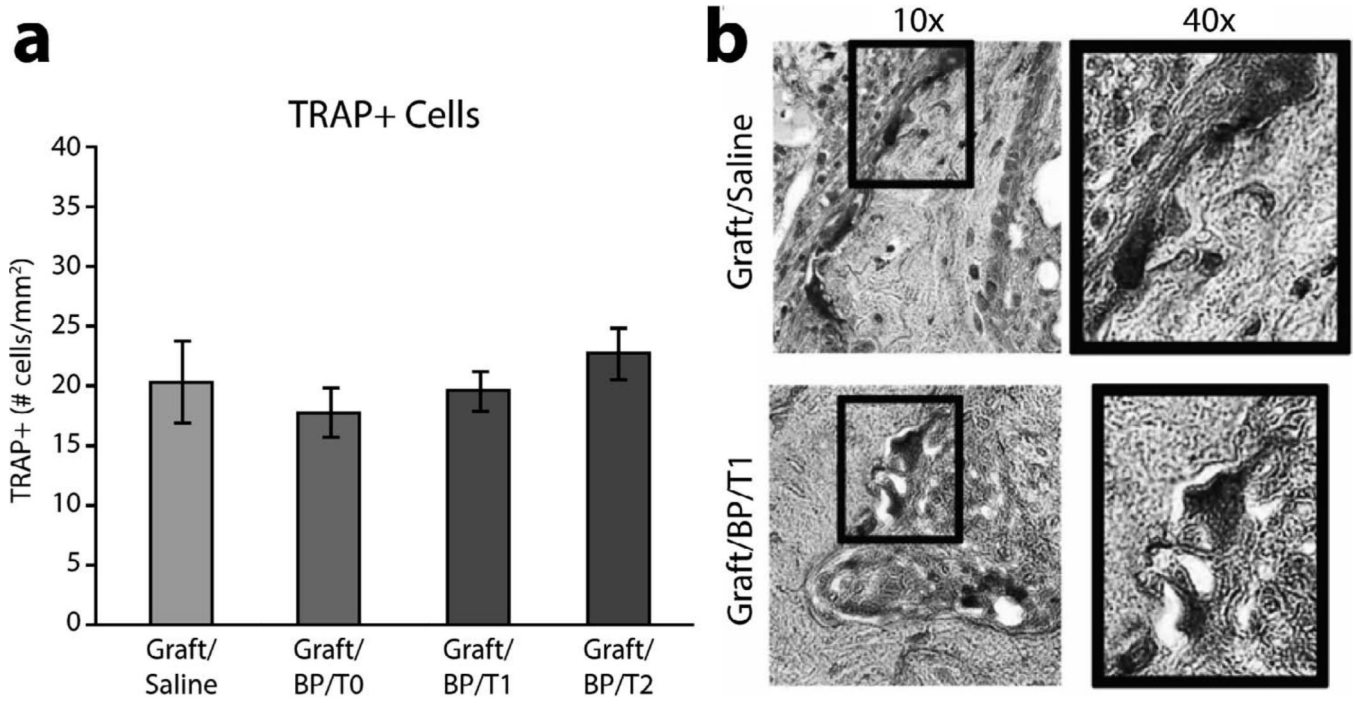

FIGURE 6.

TRAP staining images. A: There was no significant difference in the number of TRAP+ cells between groups. ANOVA with post hoc *t* tests was used to calculate differences. Error bars show standard error. B: The morphology of 3.75% of the TRAP+ cells in the graft/BP/T1 group was disrupted as compared with the graft/saline group.