PFI-2 INDUCED BONE FORMATION IN A RAT MODEL

COMPARING TWO SURGICAL TECHNIQUES

A Thesis

by

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ABSTRACT

Bone grafting materials are commonly used in dentistry for a variety of surgical procedures and new materials are regularly being developed. Evaluation of these newly developed materials has been done in animal models using a calvarial defect. This study was done to test the effectiveness of an emerging material, PFI-2, for bone grafting and to compare two surgical techniques in a rat model including the calvarium osteotomy and tooth extraction defect.

Thirty two Wistar rats were divided into two groups: the tooth extraction group and the calvarium osteotomy group. The two surgical groups were divided into a control and test group. The control group had the surgical procedure performed without any grafting material while the test group had a PFI-2 scaffold placed into the bony defects. All other surgical methods were the same between the control and test group. Fluorochrome labels were used to identify new bone formation in ground sections. Animals were sacrificed at 4 and 8 weeks for evaluation using radiographs, micro-CT, histology, and RT-PCR.

New bone formation was observed with both surgical techniques. PFI-2 induced 81.7% bone formation at eight weeks compared to 44.5% in the eight week control group. Histologic sections showed greater amounts of new bone formation in the PFI-2 groups with mesenchymal cell proliferation at primary ossification centers. RT-PCR was used to evaluate gene expression. In the extraction model, collagen 3, OCN, and ALP were significantly increased with a significant decrease in RunX2, and iBSP. In the calvarium model, collagen 1, RunX2, and ALP were significantly increased compared to corresponding controls.

PFI-2 induced new bone formation with 81.7% defect coverage compared to 44.5% in the control group. The extraction model and calvarium model produced significantly different

responses in gene expression suggesting different molecular pathways in two surgical groups. Use of PFI-2 shows promise as a bioactive material for alveolar ridge augmentation and bone regeneration.

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Contributors

This work was supervised by a thesis committee consisting of Dr. Thomas Diekwisch and Dr. Xianghog Luan of the department of periodontics and Dr. Phillip Kramer of the department of biomedical sciences.

The data for the RT-PCR was analyzed by Dr. Mirali Pandya. The histologic sections were processed and embedded by Connie Tillberg.

All other work conducted for the thesis was completed by the student independently.

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1. INTRODUCTION

Bone grafting materials are commonly used in dentistry for alveolar ridge preservation, guided tissue regeneration, guided bone regeneration, and maxillary sinus grafting. New materials are constantly being developed to aid wound healing and regeneration of lost alveolar bone. Testing of these new materials will begin in animal models before it can be translated into human use. The aim of this study is to evaluate the effectiveness of PFI-2 as a bone grafting material and to compare two surgical techniques in a rat model including the tooth extraction technique and the calvarium osteotomy technique.

Rats are frequently used as an animal model for evaluating new materials for a variety of reasons. They are chosen due to their ease of handling, reproductive capacity, completed genome mapping, low cost, and genetic similarity to humans (Sharma 2013). Rats are more suitable than mice for periodontal research due to their larger jaw size which allows for manageable extraction of teeth and better manipulation of the oral cavity.

The most common surgical technique that is used in the rat model is the calvarium osteotomy as it has been described in the literature (Cooper 2010, Guskuma 2010, Patrick 2012, Schmitz 1986). The calvarium of the rat is accessed surgically so that a section of bone may be removed for test material application. Typically a "critical size defect" is created. Critical sized defects are defined as, "the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal." (Schmitz 1986). In rats the critical size defect is described as eight millimeters (Schmitz 1986). Even though the generally accepted eight millimeter defect is used, smaller defects may be used to allow two defects per animal, allowing for fewer animals to be required for a given study (Patrick 2012). The concept of using a smaller sized critical defect in rats has been examined by Cooper et al. 2010. He found that

small defects of 2.3 mm in the rat calvarium only showed approximately 35% healing after 6 weeks (Cooper 2010). Short term studies of 12 weeks or less may incorporate defects that are smaller than the generally accepted eight millimeter defect to compare different grafting materials effectively while using a smaller total number of animal subjects. In this study five millimeter defects were used to allow space to create two adjacent defects in the rat calvarium to reduce the total number of animals needed to minimize animal pain and distress (Guskuma 2010).

The tooth extraction defect has also been used in the rat model to evaluate new grafting materials. No critical sized defects have been described for extraction sockets in rats. Healing in these extraction sockets in rats was first described by Hubsch 1952. Most previous publications involve extraction of the right or left maxillary incisor without any additional manipulation of the underlying bone (Talwar 2006, Willett 2017). The graft materials that are placed into these defects may be left exposed to the oral cavity (Talwar 2006), contained with suture material alone (Ehrnford 1980), or may be contained with cyanoacrylate (Willett 2017). In this study the maxillary first molars were extracted and a standardized defect created for effective comparison between the groups.

PFI-2 is a potent and selective inhibitor of SETD7 methyltransferase activity in cells that was first described in 2014 (Dalia 2014). The precise function of SETD7 in biology is unknown, however, it is involved in multiple molecular pathways including cancer, metabolism, and inflammation (Dalia 2014). Even in SETD7 knockout mice there are no apparent developmental deficiencies and cancer does not develop any sooner than non-knockout mice (Lehnertz 2011). SETD7 functions by monomethylation of lysine 4 on histone H3. More recent studies show that SETD7 is also capable of monomethylation of non-histone proteins including p53 and Forkhead

box O3 (Wu 2019). PFI-2 and SETD7 are undergoing current research to better understand its role and function in biological systems.

We hypothesized that by inhibiting the inflammatory activity of SETD7 by application of PFI-2 we would be able to improve healing and promote bone growth. In smooth muscle cells it has been shown that activation of SETD7 promotes TNF-α proliferation by enhancing NF-κB/CD38 signaling (Li 2008). TNF-α is a cell signaling cytokine involved in systemic inflammation. Increase in pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 increase expression of RANKL. In turn RANKL expression activates its receptor RANK to stimulate osteoclastogenesis and osteoclast activation (Boyce 2007). NF-κB signaling in osteoclasts causes secretion of protons, chloride ions, and collagenases to the ruffled border which interacts with the mineral portion of the bone. Hydrochloric acid is formed at the osteoclast/bone border causing dissolution of the mineralized bone matrix (Brendan 2015). Use of PFI-2 to inhibit SETD7 may be used to potentially inhibit osteoclast maturation and activation.

2. MATERIALS AND METHODS

2.1 Animal Groups

Thirty two male Wistar rats were chosen for use during this study. All procedures for animal testing were performed in accordance with federal regulations and was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC). All procedures were performed in the same animal facility in Texas A&M University College of Dentistry. The study was conducted from April 2019 through March 2020.

Rats were divided into two groups depending on the surgical technique. Half were assigned to the tooth extraction group and the other half into the calvarium defect group. Within the two surgical groups the rats were divided equally into the control and test groups. The test group had the surgery performed with a collagen sponge soaked in a 10 μM solution of PFI-2. The control group had the same surgery performed only without any graft material or collagen sponge. After the surgical procedure the rats were sacrificed at 4 and 8 weeks. Fluorochrome labels were used to identify new bone formation and calcium incorporation (Milch 1957, Sun 1992).

2.2 Tooth Extraction Procedure

Wistar rats were sedated under general anesthesia with a mixture of ketamine (50mg/Kg) and xylazine (7mg/Kg). Anesthesia was administered as intraperitoneal injection. Depth of anesthesia was assessed from lack of reflex to toe pinch. Extraction of the maxillary right and left first molars was accomplished with a curette applying a lateral force to luxate the tooth. Following tooth removal, a standardized 2.1 mm defect was created with a #703 dental bur extending apically 2 mm beyond the crest of the alveolar ridge. In the test group a collagen sponge soaked in a 10μM solution of PFI-2 was placed into the standardized defect. In the control group the standardized

defect was left open. In both groups a resorbable collagen membrane (Geistlich BioGide) was trimmed to approximately 1 x 2 mm and placed over the defect to contain the graft material. The resorbable collagen membrane was secured in place with cyanoacrylate (PeriAcryl). Immediately following surgery animals were given an injection of tetracycline 50mg/Kg as a fluorochrome label. At 4 weeks animals were then injected with another fluorochrome label: alizarin red.

Animals were held in a warmed incubator for observation until signs of purposeful movement was noted. At completion of observation they were transferred back into normal husbandry cages and administered post-operative doses of nalbuphene (5mg/Kg) at 12, 24, and 36 hours for post-operative pain management. Animals were sacrificed at 4 and 8 weeks following surgery by CO_2 asphyxiation and decapitation. Samples that were used for RT-PCR were immediately frozen at -80°C. All other samples were placed in 70% ethanol for processing.

2.3 Calvarium Osteotomy Procedure

The Wistar rats in the calvarium osteotomy group were sedated under general anesthesia using the same methods described in the tooth extraction procedure. The rats were shaved with an electric razor from the bridge of the snout to the caudal end of the calvarium. The surgical site was disinfected with an iodine solution and followed by 70% ethanol. A single midline sagittal incision was made through the skin and through the periosteum. Skin and periosteum was the moved laterally to access the underlying bone of the calvarium. Two 5 mm bony defects were created in the right and left parietal bones using a trephine bur at 1,200 rpm with saline solution irrigation to prevent overheating of the calvarial bone. Once a near full thickness corticotomy was made in the calvarium, an elevator was then used to apply gentle pressure around the defect margin to free the bone from the underlying dura mater. The defect was irrigated with copious saline solution and sites were evaluated to ensure no damage was done to the dura mater. In the control groups, no

graft material was placed in the defect. In the test group, a collagen sponge soaked in a 10μM solution of PFI-2 was placed into the defect. In both groups, a resorbable collagen membrane (Geistlich BioGide) was trimmed into a 6 mm circle approximately. The resorbable collagen membrane extending beyond the defect margins by 1 mm circumferentially. The membrane was secured in place with cyanoacrylate (PeriAcryl) as was done in the tooth extraction group. The skin and periosteum was sutured with primary closure using a 4-0 monofilament resorbable suture material (Glycolon). Immediately following surgery animals were given an injection of tetracycline 50mg/Kg as a fluorochrome label. At 4 weeks animals were then injected with another fluorochrome label: alizarin red.

Animals were held in a warmed incubator for observation until signs of purposeful movement was noted. At completion of observation they were transferred back into normal husbandry cages and administered post-operative doses of nalbuphine (5mg/Kg) at 12, 24, and 36 hours for post-operative pain management. Animals were sacrificed at 4 and 8 weeks following surgery by CO_2 asphyxiation and decapitation. Samples that were used for RT-PCR were immediately frozen at -80°C. All other samples were placed in 70% ethanol for processing.

2.4 Evaluation methods

All evaluation methods were performed for selected samples in both surgical technique groups. Evaluation methods included conventional radiographs, micro-CT, histologic analysis (H&E and ground section), and RT-PCR.

All radiographs were taken with the same settings for milliamps, kilovolts, and exposure time. All samples were positioned at the same orientation and distance from the x-ray tube head. ImageJ software was used to analyze the bony defects of the calvarium osteotomy group. Micro-CT images of the samples were taken using standardized settings. Three-dimensional orientation

of the micro-CT images was completed to view the cranial samples from a coronal dimension to allow defect visualization. Tooth extraction defects were oriented to observe the samples from a lateral view. Sagittal sections were taken to allow visualization of the standardized bony defects.

Samples were prepared for histologic sectioning using paraffin for H&E staining and in plastic for ground sectioning. Specimens in the cranial group were oriented and sectioned perpendicular to the bony defect for visualization of the complete 5 mm osteotomy. Specimens in the tooth extraction group were oriented and sectioned from a sagittal view to allow defect visualization. Cell types and fluorescence of the samples were analyzed by a single examiner using light microscope and digital camera software.

RT-PCR was conducted on selected samples to evaluate and compare gene expression between the tooth extraction group and the calvarium osteotomy group. The following genes were evaluated in both groups: Col1, Col2, Col3, SETD7, OCN (osteocalcin), iBSP (bone sialoprotein), RunX2, and ALP (alkaline phosphatase).

3. RESULTS

3.1 Radiographs and Micro-CT

Representative radiographs of the cranial osteotomy group showing radiographic bone fill at 4 and 8 weeks for the control and test groups are shown in Figure 1. Histograms were generated from ImageJ software to analyze the radiopacity of the 5 mm defects. The histograms show the isolated cranial defect as a grayscale as seen in Figure 2. A value is assigned for the number of pixels for each of the 256 shades of gray that are found within the defect. A value of 0 corresponds to a completely black pixel and a value of 255 corresponds to a completely white pixel. The higher numbers correspond to greater amounts of radiopacity and mineralization within the defects. Overall, the PFI-2 groups show more radiopacity than the control groups. The 4 week PFI-2 group has an average value of 80.7 while the 4 week control has a value of 57.4 on the grayscale. The 8 week PFI-2 group has an average value of 117.3 compared to the 62.9 in the 8 week control group. The percentage of pixels greater than 60 shades of gray on the grayscale was calculated as a measure of overall mineralization of the cranial defects. The values for overall mineralization with > 60 shades of gray were as follows: Control 4 weeks 36.9%, Control 8 weeks 44.5%, PFI-2 4 weeks 66.5%, and PFI-2 8 weeks 81.7%.

The cranial micro-CT images are shown in Figure 3. The quality of the mineralization in the bony defects is visible in the micro-CT images. The control images show islands of mineralization with less density. The 4 week PFI-2 image shows more radio-dense material than either the 4 or 8 week control with an immature appearance. The 8 week PFI-2 image shows near-complete defect coverage with new bone that is similar in visual quality to the surrounding native bone.

Representative radiographs of the tooth extraction group are shown in Figure 4. Extensive new bone formation is visible in the 8 week PFI-2 group compared to the incomplete fill in the control group at the same time point. The 8 week PFI-2 radiographs show alveolar bone levels that are higher compared to the 8 week control with more homogenous radiopacity.

3.2 Histology

Histologic sections of H&E staining and fluorescent ground sections for both the tooth extraction group and the calvarium osteotomy group are shown in Figures 5, 6, 7, and 8.

The H&E staining for both the tooth extraction group and the calvarium osteotomy group show new bone formation in the standardized defects. New bone with osteocytes were visualized in the PFI-2 at four and eight weeks post-surgery. In the tooth extraction group PFI-2 application resulted in mesenchymal cell proliferation and megakaryocyte formation at primary ossification centers as seen in Figure 5D.

Ground sections of the calvarium osteotomy group resulted in near-complete coverage of the standardized defect with a homogenous tetracycline-fluorescent fibrous tissue after 8 weeks of PFI-2 application indicative of calcium incorporation, see Figure 8. Ground sections of the tooth extraction group show higher amounts of trabeculation compared to the calvarium group. Tetracycline and Alizarin Red fluoresce are visible in Figure 7 showing new trabeculae in the bony defects. Calcium incorporation is present in greater amounts in the PFI-2 groups compared to the controls.

3.3 RT-PCR

The data for RT-PCR in both surgical technique groups is visible in Figures 9, 10, 11, and 12. Statistically significant differences are noted with an asterisk.

In the tooth extraction model, there was a statistically significant difference between control and PFI at 8 weeks for Collagen 3 with greater amounts of Collagen 3 in the PFI-2 group. SETD7 was significantly reduced at 8 weeks in the PFI-2 group compared to the control. The PFI-2 group showed significantly greater levels of Osteocalcin and ALP at 8 weeks. Surprisingly, there was a significant decrease in the mineralization genes RunX2, and iBSP at 4 and 8 weeks in the PFI-2 group compared to controls.

In the calvarium osteotomy model, there was a significant increase in collagen 1 at 8 weeks and collagen 3 at 4 weeks in the PFI-2 group compared to controls. There were no statistically significant differences in SETD7 levels between the test and control groups at either time point. The mineralization gene expression for iBSP, RunX2, and ALP were doubled compared to the controls at the same time points for the PFI-2 groups compared to the controls. These results were statistically significant for the RunX2 and ALP at 4 and 8 weeks following surgery.

4. DISCUSSION

Dentists involved in alveolar ridge preservation and bone regeneration procedures have long been interested in effective materials for use during surgical procedures (Sun 2013). Research continues to find new and more effective materials to improve the volume of bone regenerated and to improve the healing time. This study shows the potential for the use of PFI-2 as a bioactive material to aid bone regeneration for a variety of surgeries in dentistry and possibly for other applications in regeneration in general medicine.

These findings further outline that the exact function of PFI-2 in its inhibition of SETD7 are still unknown. In the calvarium osteotomy model there was no significant differences in the levels of SETD7 between the control and PFI-2 groups for either time point. In the extraction model there was only a significant decrease in SETD7 activity at 8 weeks without any significant difference at the 4 week time point. This would suggest that the effect of PFI-2 in inhibition of SETD7 may occur for only a short amount of time before returning to normal at 4 and 8 weeks. Another explanation could be that PFI-2 is using a different biological pathway to produce the significant amount of bone regeneration as compared to the control samples.

The biological pathways between the calvarium osteotomy and tooth extraction appeared to be different between the groups. Levels of iBSP and RunX2 were significantly elevated in the calvarium model while they were significantly decreased in the extraction model. This suggests that PFI-2 may follow a different biological pathway depending on its location in the body. The calvarium osteotomy defect differs from the tooth extraction defect in a number of ways. The calvarium defect presents with a cortical bilayer separated by a very thin trabecular layer while the extraction defect in the alveolar bone presents with significantly more trabecular bone. The base of the extraction defect is a bony floor of trabecular bone while the calvarium defect has no bony

floor. Instead, the base of the defect is the dura mater of the brain. The different types of cells that are exposed to the PFI-2 may influence the molecular pathways that are activated as evidenced by the difference in gene expression between the two surgical groups.

Further research will be necessary to better understand the role of PFI-2 in bone formation. With continued study PFI-2 may be translated into human studies where is shows great promise in improve the amount of bone formation and to increase the speed with which wounds heal.

5. CONCLUSIONS

The following conclusions can be drawn from the study: 1) PFI-2 induced new bone formation with 81.7% defect coverage at 8 weeks compared to 44.5% in the control group in the calvarial model. 2) In the extraction model, new bone formation was accompanied by a significant increase in collagen III, osteocalcin, and alkaline phosphatase, while there was a remarkable decrease in RunX2 and iBSP. 3) In the calvarial defect model, new mineralized tissue formation was facilitated by increased expression of iBSP, RunX2, ALP, and Collagen I. 4) Use of PFI-2 shows promise as a bioactive material for alveolar ridge augmentation and bone regeneration.

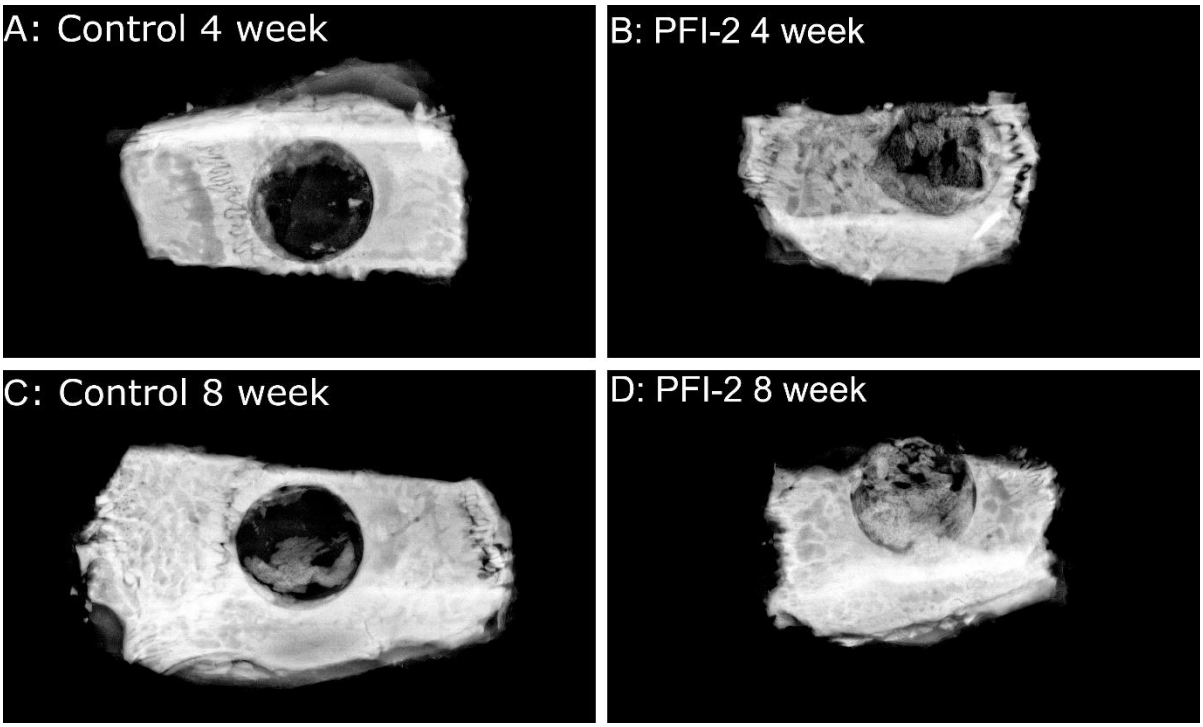

Figure 1. Calvarium Radiographs

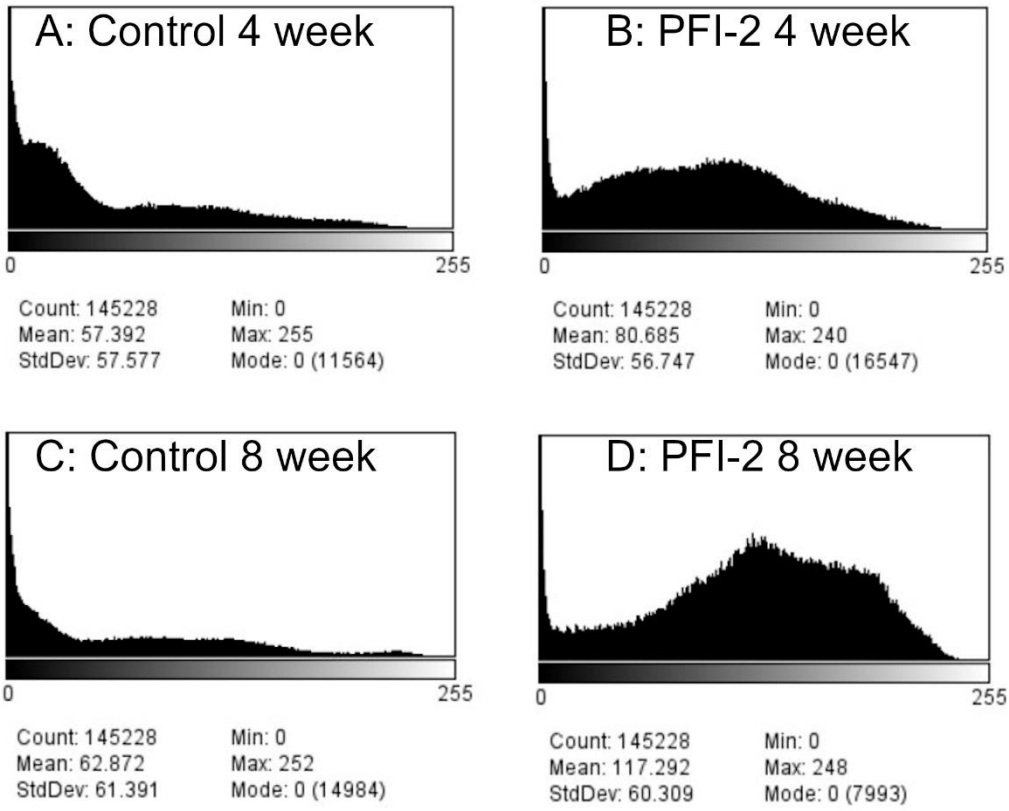

Figure 2. ImageJ Histograms of Calvarium Radiographs

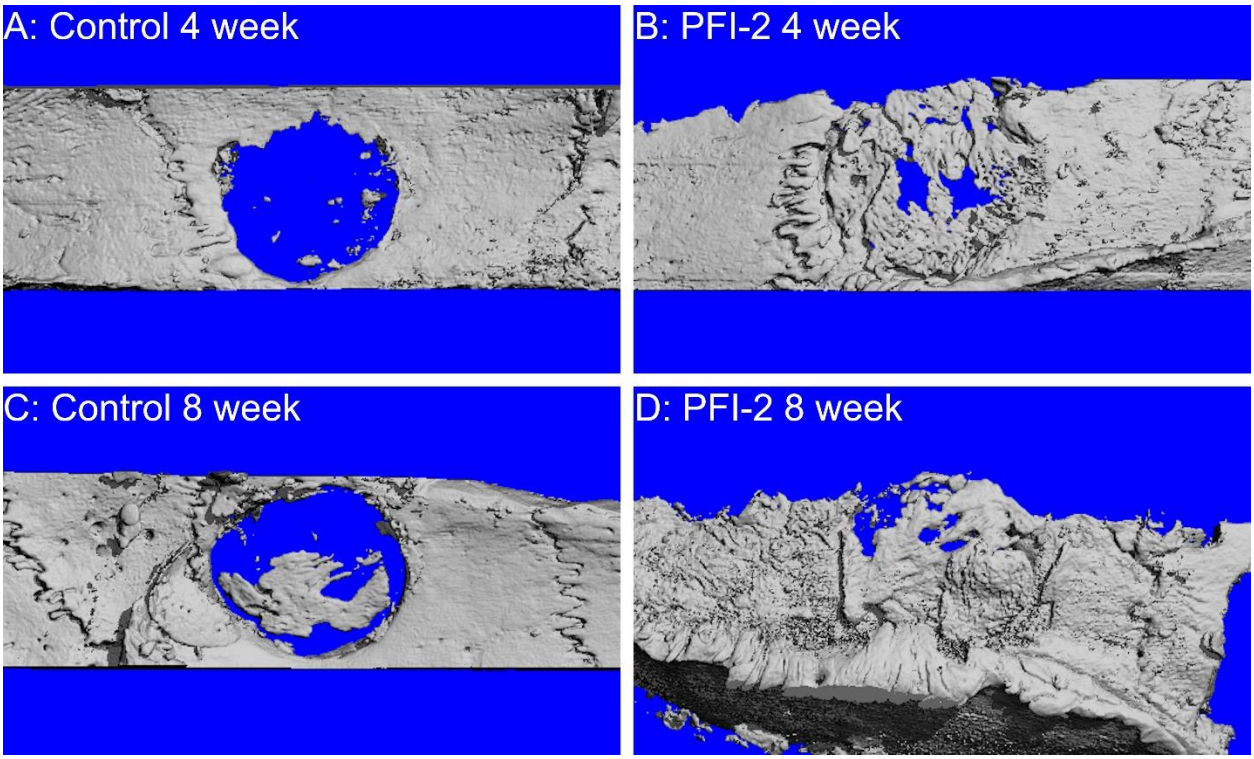

Figure 3. Calvarium Micro-CT

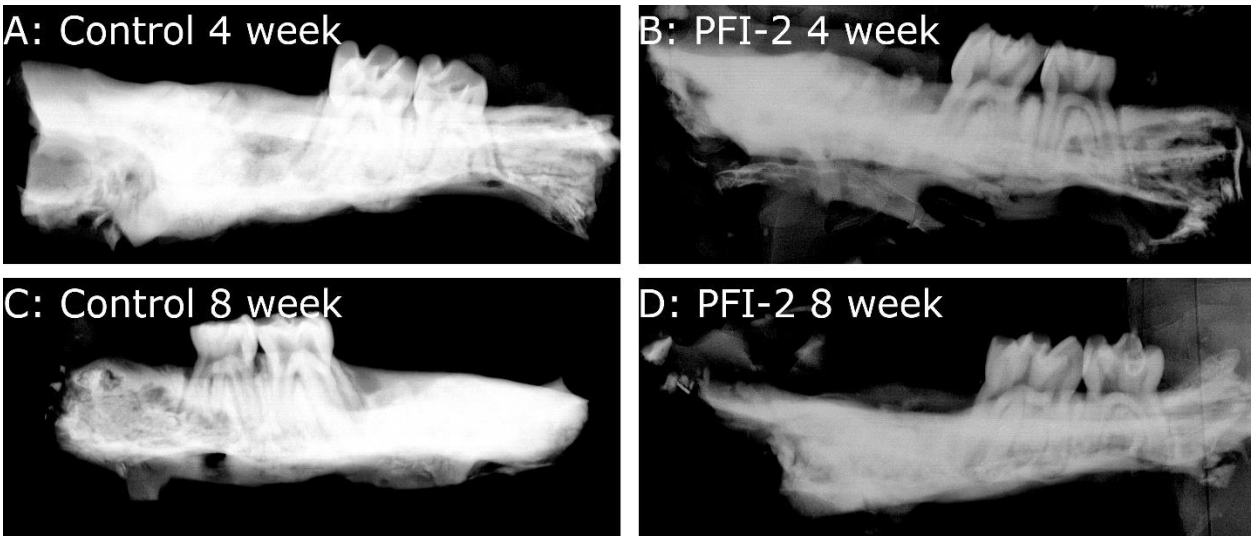

Figure 4. Extraction Radiographs

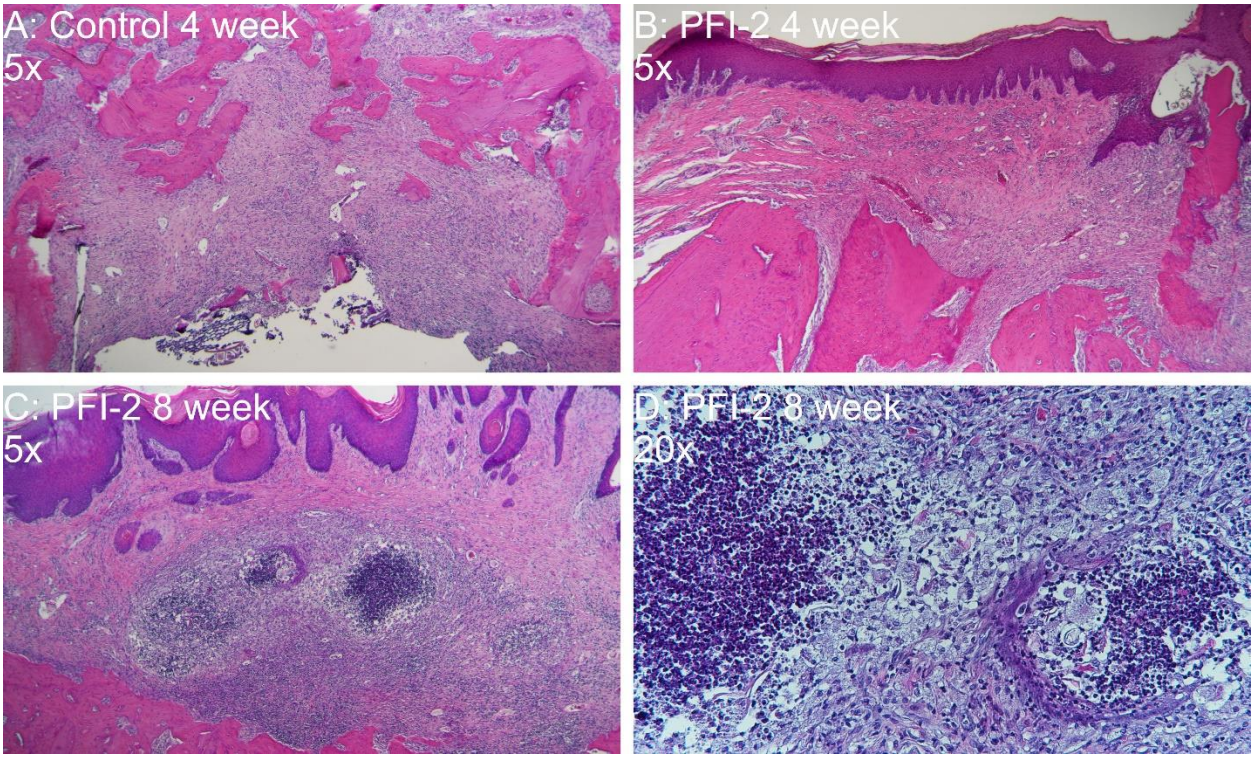

Figure 5. Extraction H&E Histology

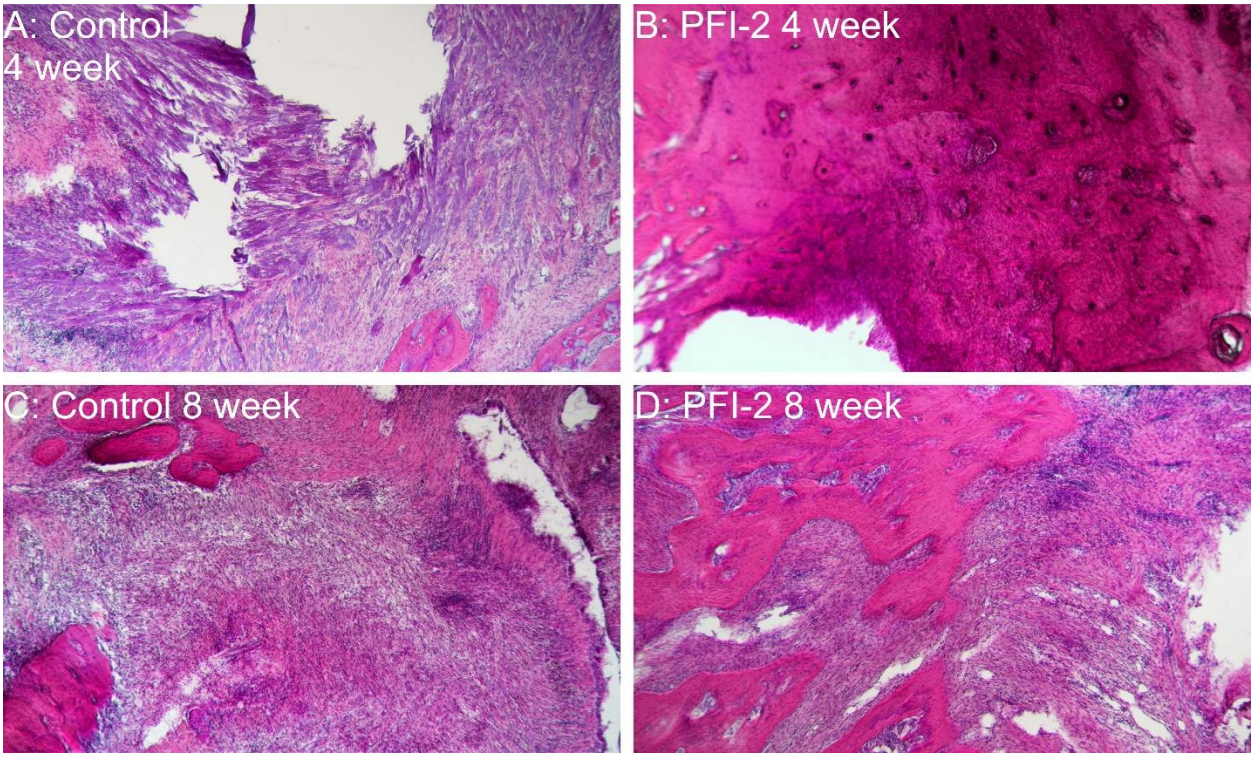

Figure 6. Calvarium H&E Histology

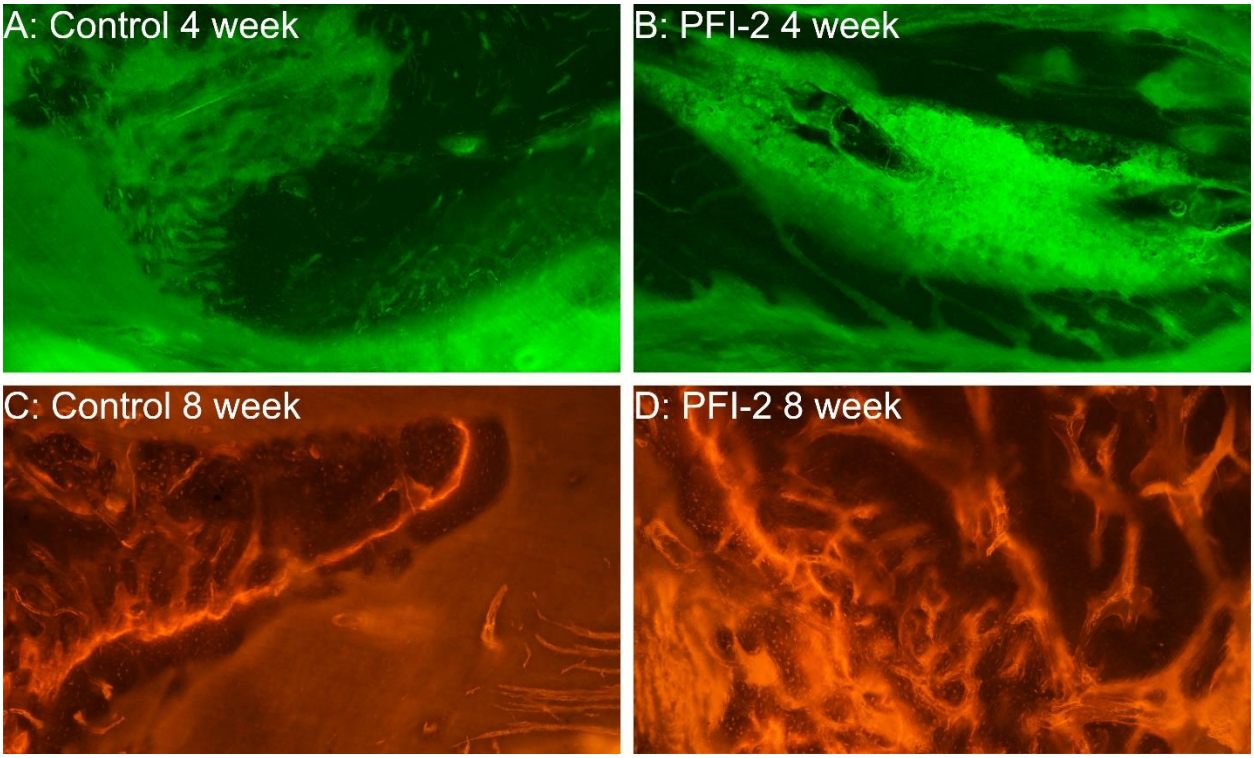

Figure 7. Extraction Ground Section

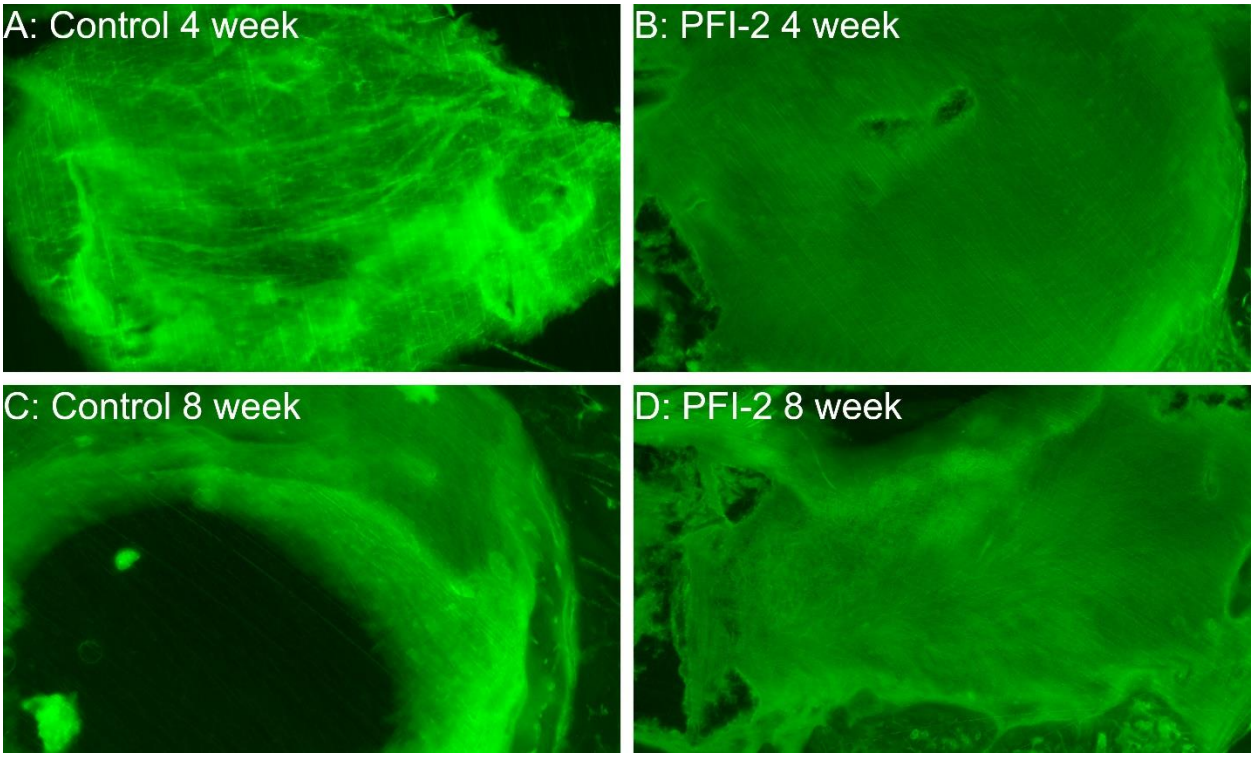

Figure 8. Calvarium Ground Section

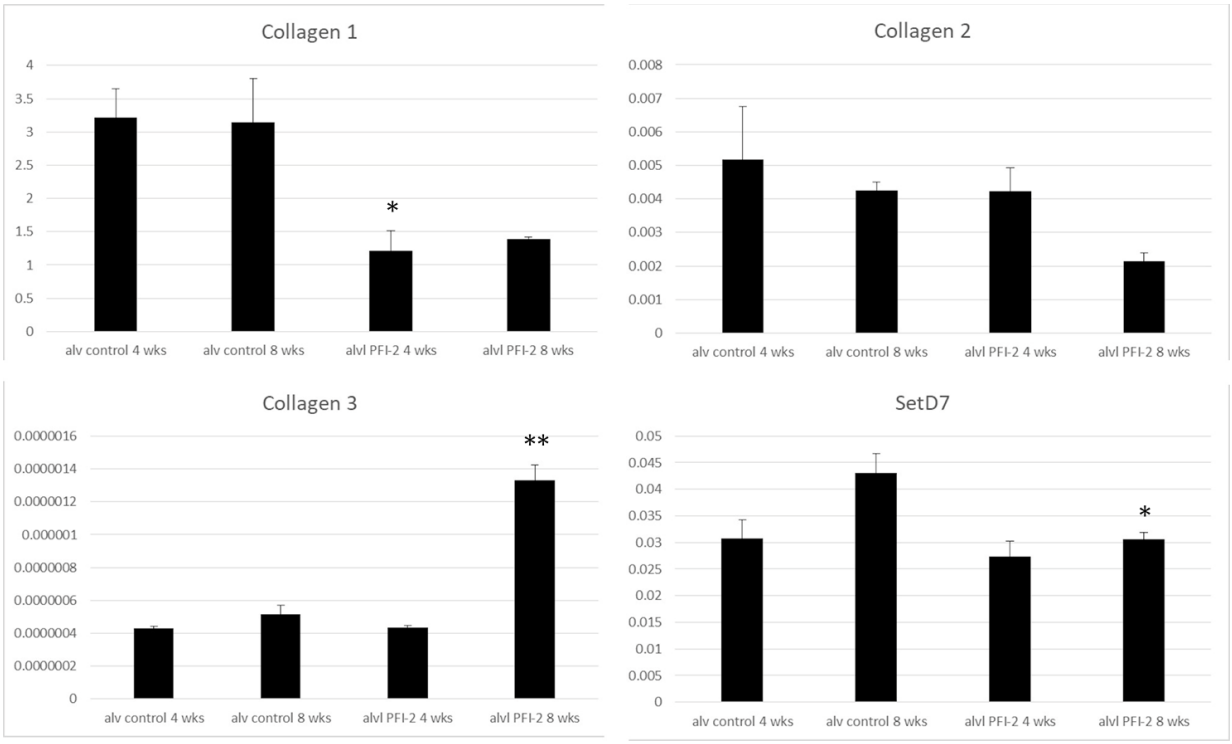

Figure 9. RT-PCR Data for Extraction Model: Collagen and SETD7

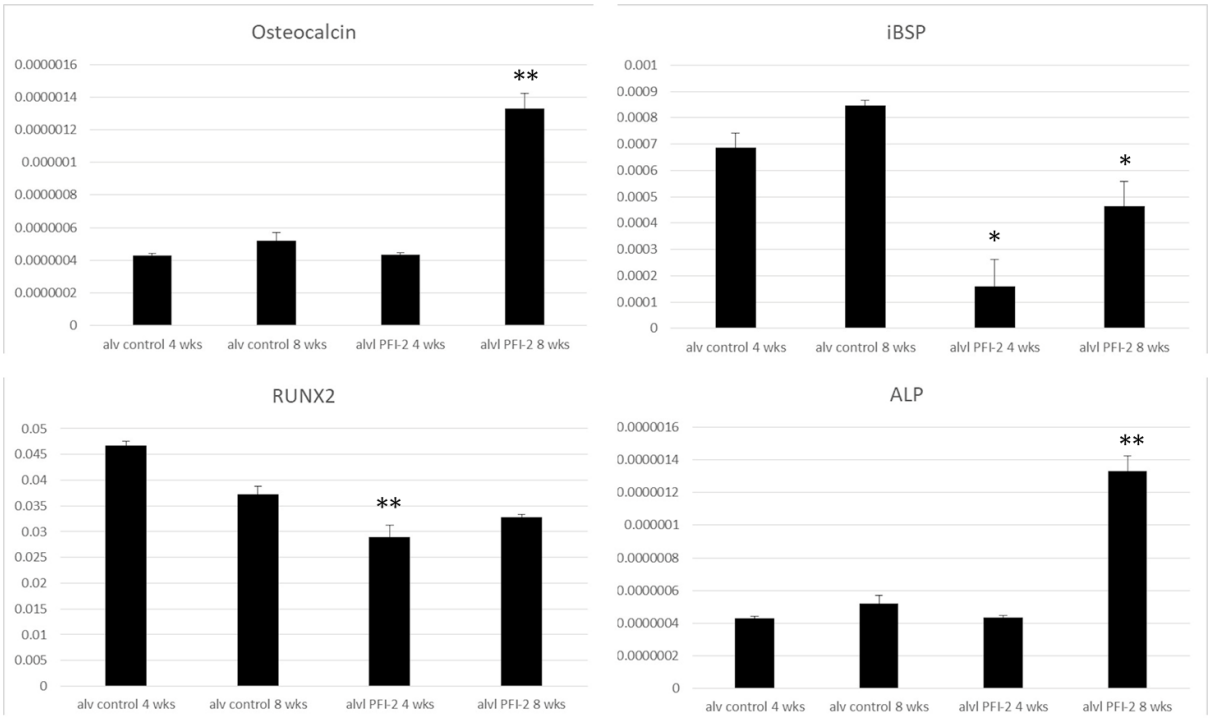

Figure 10. RT-PCR Data for Extraction Model: Mineralization Genes

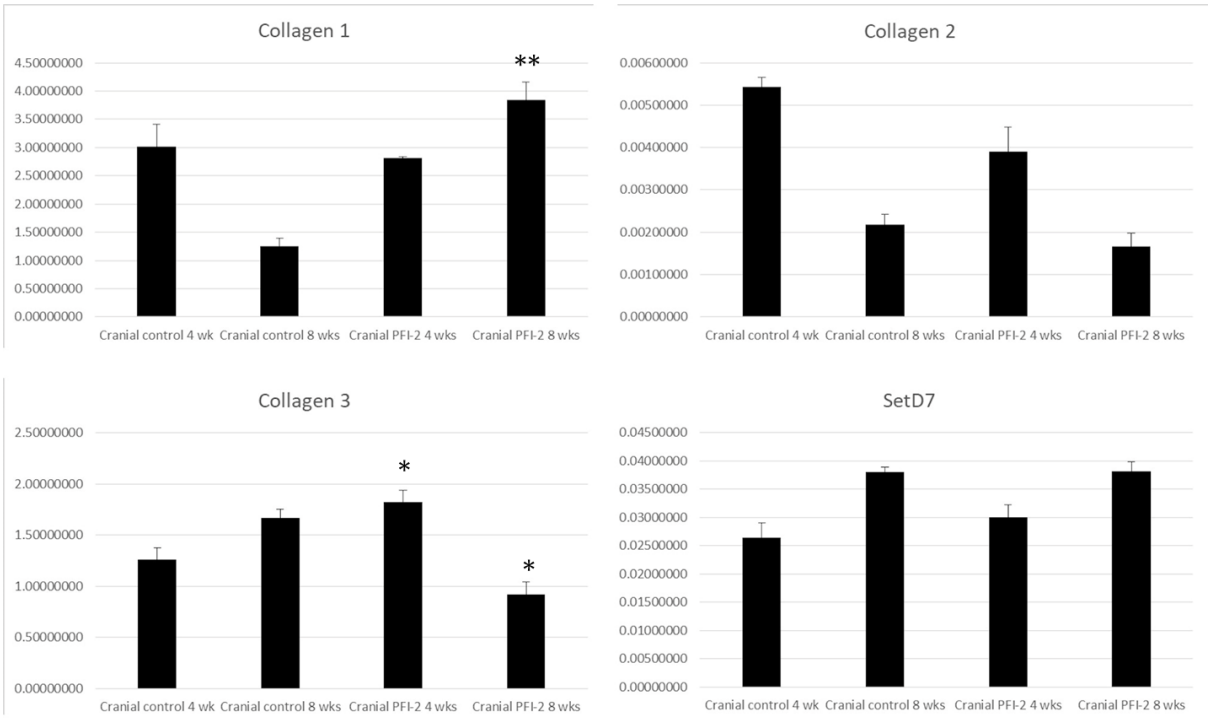

Figure 11. RT-PCR Data for Calvarium Model: Collagen and SETD7

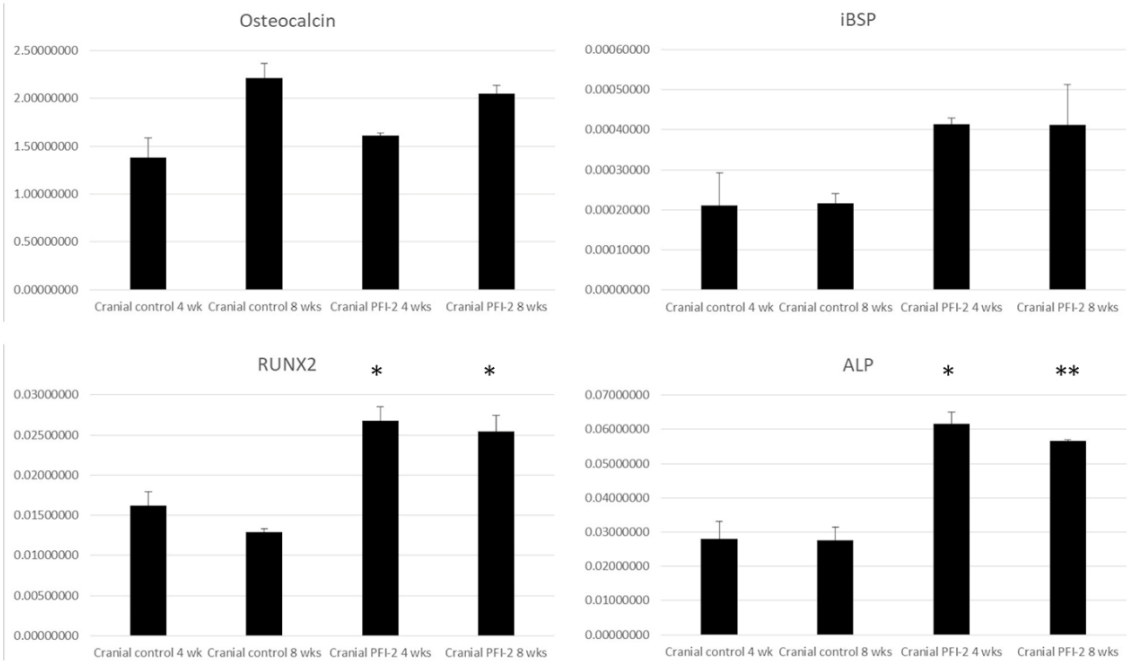

Figure 12. RT-PCR Data for Calvarium Model: Mineralization Genes

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