# VASCULAR AND BONE CHANGES AFTER A FULL MUCOPERIOSTEAL FLAP VERSUS CONTROL: A RANDOMIZED SPLIT-MOUTH EXPERIMENTAL STUDY

A Thesis

by

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# ABSTRACT

The purpose of the present study was to investigate the changes in the vascular bed structure of medullary bone after a full mucoperiosteal flap is elevated. In addition, the 3D microstructure of medullary and cortical bone was studied. Thirteen Sprague Dawley rats were used in a randomized split-mouth design experiment. A full mucoperiosteal flap was elevated on the buccal aspect above the first and second maxillary molars. The flap was then approximated, but not sutured back. The animals were allowed to heal for two weeks prior sacrifice. At the time of sacrifice, the animals were perfused with a barium sulfate based solution. Bone and vasculature were analyzed by micro-CT. Bone remodeling was analyzed through H&E and TRAP staining. There were no differences between the experimental and control sides in bone volume fraction and bone mineral density for the buccal region of interest. There also were no differences in bone mineral density, vessel volume and vessel diameter in the inter-radicular region of interest. Histological sections showed a decrease in the width of cortical buccal bone, increased osteoclastic activity in the inter-radicular region, and areas of necrotic bone in the experimental group. Elevation of a full mucoperiosteal flap increases osteoclastic activity in the trabecular inter-radicular bone, but has little or no effect on the micro-vascular bed of the inter-radicular region.

# DEDICATION

This work is dedicated to my parents, Esmeralda and Gazmend Abdi, and to my fiancé, Erik Selden, for their constant support. Thank you for your outpouring of love and encouragement.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

# CHAPTER I

# INTRODUCTION

Orthodontic treatment times range from 21-35 months.[1] Increased treatment time is problematic due to associated risks of demineralization, root resorption and dental caries.[2-6] Over the years, multiple approaches have been taken to decrease treatment time by accelerating tooth movement. While prostaglandin, vitamin D3 and osteocalcin injections have all been shown to increase rates of tooth movement, their clinical applications are limited due to pain associated with injections, the necessity to repeat the procedure periodically and possible systemic effects.[7-9] The most promising approach seems to be alveolar decortication with corticotomies. Alveolar decortication induces the Regional Acceleratory Phenomenon (RAP), which increases rates of bone remodeling and decreased bone density, leading to faster tooth movement. [10-12] Numerous studies have shown that corticotomies performed through the cortex only, along with a full-thickness mucoperiosteal flap, increase (double to quadruple) tooth movement rates, decrease the amount of bone, and decrease the density of bone through which the teeth are being moved.[10-12] However, this procedure is invasive, it affects crestal bone heights and it is associated with pain and swelling.

In order to reduce the morbidity and the invasiveness of corticotomies, flapless corticotomy procedures have been proposed. In 2009, Dibart et al[13] described a more conservative flapless approach for performing corticotomies in making small incisions with a piezo knife approximately 3 mm deep to lower morbidity and increase rates of

tooth movement. Several other studies have reported similar findings with increases in tooth movement rates ranging from 30-300% of control value. [14-19] However, other studies have not reported increases in tooth movement with flapless corticotomies. Recently Swapp et al[20] found no changes in medullary bone density and no effects on tooth movement rates when flapless corticotomies were limited to the cortical bone. Other studies support these findings making it less clear if flapless corticotomies are efficient in increasing tooth movement rates.[21-23]

Interestingly, Yaffe et al[24] were able to demonstrate that the RAP is induced by only elevating a full mucoperiosteal flap. More recently, a 30% increase in tooth movement was produced by simply elevating a full mucoperiosteal flap without performing corticotomies. The increased tooth movement was associated with an 8% decrease in bone density. It presently remains unclear why flap surgery alone decreases the amount and density of medullary bone though which teeth are being moved. Because raising a flap damages the greatest blood supply to bone, injury to vasculature may play a role.

To date, there have been no studies that have investigated the role that vascular damage has in initiating the RAP and producing changes in medullary bone, cortical bone and angiogenesis. The purpose of the present study was to investigate the changes in the vascular bed structure of medullary bone after a full mucoperiosteal flap is elevated. In addition, the 3D microstructure of medullary and cortical bone will be studied.

The results of this study should provide a better understanding of the RAP effects when there is no direct damage to medullary bone.

# CHAPTER II

# LITERATURE REVIEW

## *Tooth Movement and Bone Biology*

Movement of teeth in bone requires increased bone turnover. King et al[25] analyzed bone near teeth being moved in rats. His team found that in the orthodontically moved molars, bone resorption was found on the pressure side and that bone formation was found primarily on the tension side.

There is a correlation between bone remodeling and the rates of orthodontics tooth movement. Verna et al[26] used fifty-two Winstar rats in three groups: control, high bone turnover, and low bone turnover. The high and low turnover groups were pharmacologically induced by changes in thyroid function for four weeks prior to application of orthodontic force. Serum levels of tri-iodothyronine (TT3) and thyroxin (TT4) were measured to check the state of hyper- and hypothyroidism that is directly correlated to bone turnover. Maxillary first molars were moved through the application of a mesial force for three weeks. They were able to show that the high turnover group had higher rates of tooth movement while the low turnover group had decreased rates of tooth movement compared to the control group.

Bone density also affects the rate of tooth movement. Teeth move slower though denser bone and faster in less dense bone. Goldie et al[27] showed this in study using lactating rats and a control group. The lactating rats group had lower bone density than the controls. Teeth were moved mesially and the amount of tooth movement was

recorded. They found that the test animals had a 36% decrease in bone density and almost double the rate of tooth movement on day fourteen.

Similarly, study by Ashcraft et al,[28] was able to show that decreased bone density increases the rates of tooth movement. They used sixteen New Zealand white rabbits and divided them in a low bone density experimental group and a control group. The experimental group received cortisone acetate to induce osteoporosis and decrease the bone density. They then mesialized the maxillary first molars in both groups and recorded the amount of tooth movement. They found that the rabbits with corticosteroid-induced osteoporosis exhibited 18% decrease in bone and a three to four fold increase in the rate of tooth movement compared to controls.

Recently, Hashimoto et al[29] demonstrated a change in the rate of tooth movement in rats with which bone density was altered. They 21 Winstar rats that were allocated in three groups: ovariectomy, ovariectomy and zoledronic acid administration and sham surgery (control). Zoledronic acid was injected in the second groups two weeks after the surgery and continued every two weeks. Maxillary first molars were moved using NiTi coils in all three groups. In vivo microCT was used to analyze bone density parameters. They were able to conclude that orthodontic tooth movement was almost two times more in the ovariectomy compared to control and zolendronic acid injection groups. In addition, a reverse correlation was found between orthodontic tooth movement and bone mineral content, bone mineral density, bone volume and the ratio between bone mineral content to tissue volume. Less bone and less dense bone increase the rate of tooth movement.

In summary, there is an increase in the rate of tooth movement with increased bone turnover and decreased bone density.

*Regional Acceleratory Phenomenon*

Frost[30,31] first described the Regional Acceleratory Phenomenon in 1983. He defined the RAP effect as an increase in the normal healing process of bone. In his review article, Frost explained that the RAP begins within a few days after injury to bone. The remodeling process begins with the formation of granulation tissue. Precursor cells begin to produce new cells that differentiate to provide new vessels, intercellular materials, and supporting cells. Collectively they form a soft tissue mass between the two fracture segments. This stage lasts about two weeks.

Further cell differentiation, begins to create new chondroblasts and osteoblasts, which produce the extracellular matrices. After a week these matrices begin to mineralize and form a callus around the fracture.

In humans, it takes from four to sixteen weeks for the complete mineralization of the callus[30]. After complete mineralization, a process of reorganization and remodeling of the callus begins. The Basic Multicellular Unit is responsible for the remodeling processes that occur. These processes include replacing mineralized cartilage into woven bone, woven bone into laminar bone and removal of any callus plugs from the marrow cavity. The Basic Multicellular Unit is composed of many kinds of cells including osteoclasts and later osteoblasts, intercellular material and capillaries. The osteoclasts remove previously mineralized tissue, while the osteoblast will lay new lamellar bone.

This process will last from one to four years.

Several studies have been able to establish that corticotomies initiate the RAP by increasing remodeling rates, reduce mineral density and bone density.[32-34] Lee et al[35] used thirty rats distributed to five groups; a control group where teeth were moved without any adjunctive procedures and four experimental groups where either corticotomies or osteotomies were performed either alone or in conjunction with tooth movement. The surgeries were performed in one side of the maxilla while the contralateral side served as control. Micro-CT analyses were performed on the animals at three time points: immediately after surgery, 21 days after surgery, and 2 months after surgery. Bone demineralization was observed throughout the sections in the corticotomy group, while the osteotomy-assisted group showed demineralization only on the distal cuts. These results indicate that corticotomies can generate the regional acceleratory phenomenon.

*Corticotomies*

Cho et al[10] performed corticotomies around the maxillary and mandibular third premolars of two beagle dogs and then closed second premolar extraction spaces. Two weeks after surgery, the corticotomy side showed more tooth movement than the control side. By eight weeks the experimental teeth had moved 4 times as much as the control teeth in the maxilla, and 2 times as much in the mandible.

Iino et al[11] performed a similar study after extracting the mandibular second premolars. Corticotomies were performed on the experimental side in 12 beagle dogs.

The third premolars on both groups were then moved mesially using continuous forces. They found that tooth movement rates were significantly increased on the corticotomy side compared to controls during the first two weeks. By the fourth week, they showed that teeth on corticotomy side had moved approximately twice as far as the control teeth.

Sanjideh et al[12] also studied orthodontic space closure in five foxhound dogs after extraction of mandibular third premolars and maxillary second premolars in a split mouth design. The dogs were randomly assigned to either tooth movement alone or tooth movement in conjunction with corticotomies. In addition, a second corticotomy procedure was performed in the experimental group in the maxillary 28 days after the first surgery. A coil spring was used to provide constant mesial force. After eight weeks, there was 2.4 mm of tooth movement on the corticotomy side and 1.3 mm on the control side in the mandible. In addition, the maxillary teeth in the group that had two corticotomies moved only 15% farther than the group that had only one corticotomy procedure.

Fischer et al[36] performed a split mouth design experiment in 6 consecutively treated patients with bilaterally impacted canines. The impacted canines were randomly assigned to one of two groups: conventional exposure group, which served as control and corticotomy-assisted exposure. In the corticotomy-assisted exposure, the canine was exposed and corticotomy cuts were made to the cortical bone mesial and distal to the exposed canine. Their results indicate that there is a decrease of 28-33% of treatment time for the corticotomy-facilitated procedure.

Aboul-Ela et al[37] used a sample consisting of thirteen consecutively treated

patients with maxillary first premolar extraction. A split-mouth design was used where corticotomies were randomly assigned to one side of the maxillary arch while the other side served as control. The corticotomy cuts were made at the canine region. Miniscrew implants, placed between the maxillary first molars and maxillary second premolars, were used to retract canines using closed nickel-titanium coil springs on both experimental and control sides. They were able to show that the average daily rate of canine retraction was two times greater on the corticotomy side two months after surgery. During the third month of retraction the rate of tooth movement on the experimental side was only 15% higher than control. At the end of the fourth month, no differences in tooth movement were observed between the corticotomy and control side. The canines on the experimental side had been retracted more than the contralateral control canines at the end of the forth month, but this difference is mostly attributed to increased tooth movement rate during the first two months after surgery.

The corticotomy studies, all of which were performed with flaps, agree that the procedures approximately doubles tooth movements. They also show that the increased tooth movements are limited in terms of their duration, with peak differences occurring after 2-3 weeks and no differences in tooth movement evident after 6-8 weeks.

*Flapless Corticotomies*

Dibart et al[13] first described piezocision in a case report published in 2009. Small incisions apical to the interdental papilla were performed on the buccal aspect of each jaw. A piezo knife was then used to create the cortical incision approximately 3 mm

deep. A tunnel technique was used in the areas that needed bone grafting. In this case report, they were able to complete orthodontic treatment of a mildly crowded adult female in 17 weeks. No swelling, bruising or severe discomfort was reported.

Keser et al[15] published another case report where piezocision was performed sequentially in a patient. A 25-year-old female with Class III malocclusion, severe crowding, maxillary transverse deficiency and anterior crossbite was treated using piezocision to facilitate her treatment. Six flapless cortical incisions were made in the maxilla to a depth of 3 mm. The same incisions were made in the mandible 2 weeks after the lower arch was bonded. The authors reported an increase in the maxillary transverse dimension facilitated by piezocision. The orthodontic treatment was completed in 8 months.

There is also an increase in buccal tooth movement and tipping using piezocision compared to controls. Ruso et al[14] investigated the amount of buccal tooth movement and tipping in a randomized split-mouth study. The sample consisted of six female adult foxhound-mix dogs. Mesial, distal and inter-radicular incisions were made without elevating a flap. The corticotomy cuts were made to a depth of 5 mm using a Piezo knife. The maxillary second premolars were then actively moved for nine weeks and stabilized for another two weeks before sacrifice. The authors reported a 35% increase in tooth movement and 105% increase in tipping. In addition, their histological analyses showed that there was less bone, less dense done and less mature bone in the apico-buccal and cervico-lingual regions surrounding the teeth on the piezocision side.

There are increases in tooth movement because there are also increases in bone remodeling with piezocision-facilitated tooth movement. In a study by Dibart at al,[16] 94 young adult rats were divided into four groups: control, tooth movement alone, piezocision alone and tooth movement plus piezocision. Seven time points were studied: 1, 3, 7, 14, 28, 42 and 56 days. Palatal mesial and distal incisions were made around the maxillary first molar. Corticotomy cuts to a depth of 0.5 mm were made mesially and distally using a piezo-knife. At day 7, there were significant decreases in the amount of alveolar bone for the piezocision plus tooth movement group compared to the tooth movement only group. In addition, piezocision led to an increase in osteoclastic activity. The increases were most significant in the piezocision plus tooth movement group, indicating that the increase in osteoclastic activity can be partially contributed to the tooth movement, even though the tooth movement group alone did not show a difference. They reported a 2-fold increase in tooth movement at day 28 for the piezocision plus tooth movement group, compared to the tooth movement only group. Interestingly, this study reported that the extent of demineralization induced by piezocision was greatest at day 7 extended to a distance of 2.5 mm from the corticotomy site.

In another type of flapless corticotomy surgery, a reinforced scalpel separates the interproximal cortices transmucosally without reflecting a flap and yet still achieves accelerated tooth movement[18,19]. Kim et al[17] described another flapless corticotomy procedure utilizing an ultrasonic piezotome tip to induce cortex damage to a depth of 3 mm. The 6-week study was conducted with 10 beagles grouped as follows: an

orthodontic only group (control) and orthodontics plus piezopuncture. Tooth movements were significantly higher in the piezopuncture group; 3.26 fold and 2.45 fold for the maxillary and mandibular experimental groups respectively as compared to the controls.

Swapp et al[20], however, failed to report a difference in medullary bone density and tooth movement for flapless corticotomies. They used a split-mouth design on seven foxhounds to evaluate the protraction of mandibular third premolar. Sixty buccal and lingual corticotomies were performed 2-3 mm into the cortical bone without elevating a flap. Their results indicated that there was a decrease in bone density and increase in bone modeling in cortical bone following corticotomies. However, microCT and histological analysis indicated that there were no differences in bone density and bone remodeling in medullary bone. This explains why there was no difference in the rate of tooth movement for the experimental and control side.

The same results as the Swapp et al were previously described by Safavi et al[21]. Five adult German Sheppard dogs were used in a split-mouth study where the second premolars were immediately loaded for mesialization into first premolar extraction spaces. The corticotomies were produced with a surgical bur drilled 2-mm deep through the buccal cortex mesial, buccal and distal to the second premolar. The corticotomies were performed at extraction, at 1 month, and at 2 months. The study lasted 3 months. Results showed the experimental side moved 1.6 fold faster than the control over the first month, however the second and third month showed no statistically significant difference in tooth movement velocities compared to the control.

*Flap Elevation and Rate of Tooth Movement*

Yaffe et al[24] were able to demonstrate that the RAP is induced by only elevating a full mucoperiosteal flap. 60 Winstar rats were divided in three groups. Group A served as control, in Group B a mucoperiosteal flap was reflected on the buccal surface of the mandible and in Group C flaps were elevated on buccal and lingual surfaces simultaneously. The rats were sacrificed after 3, 7, 10, 14, 21 and 120 days after surgery. After sacrifice, microCT studies, histological studies and calcium retention studies were performed on the samples. They were able to observe the regional acceleratory phenomenon ten days after surgery in the experimental group. The resorption was more prominent when the flaps were elevated on both buccal and lingual surfaces. The alveolar bone had almost recovered at day 120.

Similarly, Owen recently demonstrated that raising a mucoperiosteal flap increases the rate of tooth movement by 30%. She used seven beagle dogs in which tooth movement was measured. Using a randomized split-mouth design, a full mucoperiosteal flap was raised in one side of the mouth. Tooth movement, bone density and histological sections were studied. Their results indicate that there is a 30% increase in tooth movement and an 8% decrease in bone density on the flap side. It presently remains unclear why the amount and density of medullary bone decreased without corticotomies.

Since raising a flap disturbs the normal blood flow in the bone, it can be hypothesized that vascularization and bone remodeling are somehow related to each other. The following literature will describe bone vascularization and the mechanisms used to influence bone remodeling.

*Vascularization of Bone*

The periosteum is the primary supplier of blood to the cortical bone[38-40]. Chanavaz et al[39] were able to demonstrate that in long bones the periosteum is responsible for up to 70-80% of the arterial supply to cortical bone, as well as 100% of venous return. Additionally, it has been shown that in cases of damaged periosteum, cortical bone relies more heavily on the centromedullary arterial supply, receiving up to 30-40% of its arterial supply from the centromedullary blood system in those cases

Triffit et al[38] used 36 adult rabbits divided into three osteotomy groups; osteotomy alone, osteotomy with exclusion of the periosteal blood supply and osteotomy with exclusion of the marrow blood supply. Blood flow was measured one and two weeks after the osteotomy procedures. In the osteotomy alone group, cortical flow increased in the proximal segment one week after surgery while it increased in the distal segment two weeks after surgery. In the osteotomy group with periosteum exclusion, there were no differences in cortical blood flow. In the osteotomy group with marrow exclusion, cortical flow was not significantly reduced. This indicates that the increase in blood flow during healing of an osteotomy of the diaphysis comes from the periosteum.

Saka et al[40] studied the mandibles of twelve minipigs and mandibles of four human cavaders. The mandibles of the minipigs were perfused with a methyl-metacrylate resin and the anatomy of the vessels was studied. Their results showed that the cortex of the body of the mandible was supplied mainly by the periosteum, the ramus of the mandible was supplied by both periosteal and endosteal routes while the condyle was supplied mainly by endosteal arteries. Therefore it could be anticipated that

damages to the periosteum in the body of the mandible would directly affect the cortical bone, which houses teeth.

*Coupling of Vascularization and Osteogenesis*

Bone vascularization may play a significant role in coordinating osteoprogenitor cell activities, which in turn can influence the vessels themselves.[41-43] In 1994, Collin-Osdoby et al[44] first described the potential molecular linkage between osteogenesis and vascularization.

Laminin-1, a major basement membrane component of blood vessels, recruits in vitro osteoprogenitors in early rat calvaria cell populations. In 1999, Roche et al[45] used fetal rat calvaria cells plated on wells coated with various proteins including tenascin, laminin, osteopontin etc. Osteoprogenitor attachment was assessed through counting bone nodules formed in the different protein coated mediums. Laminin-1 showed a constant increase in the number of bone nodule forms. This indicates that osteoprogenitor cells selectively attach to laminin-1. In another study, Roche et al[46] were able to conclude that osteoprogenitors are recruited by laminin-1. In vitro assays of rat calvaria cells showed the number of new bone nodule formation was higher for the laminin-1 group even though the total number of rat calvaria cell attachment was overall lower. The number of bone nodule formation was directly correlated to the number of osteoprogenitor cells attached to laminin-1. In contrast, laminin-5, which is a truncated version of laminin-1, had an increase in rat calvaria cell attachment but not an increase in the number of bone nodule formation. This indicates that osteoprogenitor cells have a

specific affinity to laminin-1, specifically to parts of it that are missing from laminin-5.

In addition, bone sialoprotein, a protein synthesized by osteoblasts and osteoclasts, facilitates human endothelial cell attachment in vitro. Bellahcene at al[47] incubated human umbilical vein endothelial cells in wells pre-coated with either bone sialoprotein or vitronectin. Their results showed that bone sialoprotein was chemotactic for endothelial cells. In addition, they were able to show that bone sialoprotein had angiogenic properties by monitoring a chicken chorioallantoic assay. The assays in which bone sialoprotein was applied to, showed a greater vascular index which suggests that new blood vessels were formed in those assays.

*Vascularization and Bone Density*

There is a reciprocal link between bone remodeling and blood flow. The earliest studies that tried to correlate bone perfusion to bone remodeling date in 1970. Sim et al[48] used 29 dogs divided into one control group and four experimental groups that represented states of high to low bone remodeling. The level of bone remodeling was determined by adjusting the thyroid and parathyroid hormone levels. Blood entering and leaving the tibia was evaluated by analyzing $^{85}$Sr clearance. $^{85}$Sr clearance, which is a measure of blood flow volume, was decreased in the low bone remodeling groups and increased in high bone remodeling groups compared to control. This indicates that in a state of high bone remodeling, an increase in blood flow in necessary to sustain the bone turnover.

A recent study, by Sammarco et al[49] used mice to evaluate the role of oxygen in

bone regeneration. They amputated the second and forth digits of the hind limbs of the mice while using the third digit as a control. The regenerated digits were then harvested at different time points. The oxygen level of the remodeling bone of the regenerated digits was measured using Hypoxyprobe-1 Plus and FBXL5 which signal when oxygen levels are below 1.3% and above 6% respectively. In addition micro-CT and histological analysis were used to determine the level of bone regeneration at different time points. They concluded that the initial stages of bone regeneration, which include bone degradation, are characterized by hypoxic events. New bone started to form 10 days after amputation, however the hypoxic signal did not decrease until after 21 days after amputation. Moreover, an increase in oxygen tension was associated with both an increase and decrease in bone mineralization, and was strictly dependent on the stage of bone remodeling in which hyperbaric oxygen was applied. These results indicate that oxygen levels could be part of a signaling mechanism during bone healing.

In cases of disease, bone mineral density and vascular perfusion of the surrounding bone are correlated. Studies from Shih et al[50], Griffith at al[51] and Wang et al[52] were able to correlate a decrease in bone perfusion with decreased mineral density in osteoporotic patients. However, they were not able to deduce a causal relationship.

Yang et al[53] were able to deduce a causal relationship between decreased microcirculation and decreased bone mineral density. Their study subjects included 186 people randomly enrolled by stratified sampling divided in 8 groups depending on their age. Both computed tomographic perfusion and bone mineral density of the third and fourth lumbar vertebral marrow were measured. They evaluated age-associated changes

of perfusion, bone mineral density and disc degeneration. Their results showed that decreased microcirculatory perfusion, which is associated with aging, preceded decreased bone mineral density and disk degeneration. This suggests that changes in vascular perfusion could possible cause bone loss and deterioration of intervertebral disks.

A decrease in microcirculation induces hypoxia in the surrounding tissues. Hypoxia is directly correlated with an increase in osteoclasic activity and bone resorption.[54-59] Arnett et al[60] used mouse marrow cells cultures to study the effect of different oxygen tensions on osteoclasts. They found that reducing oxygen tension from the atmospheric level of 20% caused increases in the number of multinucleated osteoclasts and resorption pits. This effect was the strongest at 2% oxygen where resorption was stimulated up to 21-fold. Furthermore, osteoclast stimulation and resorption pits were observed even in severe hypoxia with 0.2% oxygen tension.

On the other hand, hypoxia inhibits the growth and proliferation of osteoblasts. Utting et al[61] used primary rat osteoblastic cell cultures on Melanex discs. The effect of oxygen tension was studied on stable osteoblastic cultures and on differentiating ones. Reduction of oxygen pressure from 20% to 5% decreased formation of mineralized bone nodules 1.7 times, while reduction to 2% decreased bone formation 11 times. A further reduction of oxygen pressure to 0.2%, almost completely eliminated formation of bone nodules. In this study, they were able to show a decrease in osteoblast alkaline phosphatase activity and expression of mRNAs for ALP and osteocalcin in hypoxic environments. This suggests an inhibition of osteoblast differentiation and proliferation.

In addition, an increase in osteoblast apoptosis was not observed. Collagen produced by osteoblasts cultured in 20% oxygen was more abundant that collagen deposited by osteoblasts cultured in 2% oxygen. Furthermore, the collagen deposited by hypoxic osteoblasts exhibited an increased sensitivity to pepsin degradation. The results of this study indicate that there is an oxygen requirement for osteoblast formation and differentiation and for effective bone formation. It also stresses the importance of vasculature for bone health.

An increase in oxygen tension suppressed osteoclast activity and bone resorption while increasing osteoblastic activity. Hadi et al[62] used RAW 264.7 monocytic cells to study the effect of varying levels of oxygen. For hypoxia (2% $O_2$) and normoxia (21% $O_2$) treatments cells were incubated in airtight chambers. In addition, some cells were exposed to hyperbaric oxygen, pressure and hyperoxia in stainless steel hyperbaric chambers. RT-PCR and Western Blotting was performed to study osteoclast differentiation. Osteoclast formation and bone resorption were significantly reduced by administration of hyperbaric oxygen or hyperoxia in 21% oxygen culture conditions.

Hyperbaric oxygen has the opposite effect on osteoblasts: it stimulates them towards an osteogenic phenotype. Wu et al[63] used human osteoblasts to evaluate the effect of hyperbaric oxygen on osteoblasts. Cell cultures were used to study the proliferation, differentiation and the cell membrane integrity. Hyperbaric oxygen stimulated the proliferation and differentiation of osteoblasts cultured in 10% fetal calf serum up to 8 days of hyperbaric oxygen application. After day 8, there were no differences between control and experimental cultures. In addition, calcium deposition

was observed in the experimental cultures at day 3, while it could only be observed in control cultures after day 6. These results indicate that there is a direct correlation between exposure to hyperbaric oxygen and bone healing.

Similar results were observed by Gokce et al[64] in study where 24 Sprague Dawley were used to study the effects of hyperbaric oxygen (HBO) on bone healing during orthodontic tooth movement. The rats were divided in two groups: one group in which HBO was administered twice daily and a control group. Coil springs to move the mandibular first molar were placed in both groups. After sacrifice, the histomorphology of samples was studied. They were able to conclude that HBO enhanced bone formation during orthodontic tooth movement as measured by an increase in bone volume/tissue volume ratio and trabecular bone number and a decrease in trabecular separation.

The literature presented indicates that changes in vasculature and therefore of the oxygen supply to bone will shift the bone remodeling processes. In addition, corticotomy studies indicate that damage caused to periosteum and cortical bone will induce the RAP in medullary bone and increase tooth movement rates. To date, there have been no studies that have investigated the role that vascular damage has in initiating the RAP and producing changes in medullary bone, cortical bone and angiogenesis in the two bone compartments.

# CHAPTER III

# MATERIALS AND METHODS

Thirteen Sprague Dawley male rats, weighing 400 to 450 g, were acquired from Envigo. All animals were healthy. This model was used because the barium sulfate perfusion limited the size of the animal that could be used. The Institutional Animal Care and Use Committee of Texas A&M College of Dentistry approved the experimental protocol and animal housing and care (IACUC # 2016-0083-CD). Two pilot animals were used to develop the barium sulfate perfusion protocol and to standardize the surgical procedures.

## *Surgical Protocol*

The animals were acclimated for at least two days. After acclimation, they were anesthetized by injecting 0.3-0.4 mg of ketamine/xylazine (40-80 mg/kg: 5-10mg/kg) intraperitoneally. A split-mouth design was used. The side of the surgical insult was randomly allocated before the surgeries using Excel's random number generator. After the animals were fully anesthetized, a cotton swab dipped in Peridex was applied in the oral cavity. A periosteal elevator was used to prop the rat's mouth open and laminated 3x magnification loupes were used for visualization. A full mucoperiosteal flap, approximately 6 mm x 3 mm, was elevated on the buccal aspect above the first and second maxillary molars using the sharp end of a 28G needle (Figure 1). The flap was

immediately re-approximated, but not sutured. All animals were fed food pellets during the experimental period; food and water intake were monitored daily.

*Barium Sulfate Perfusion and Euthanasia*

The rats were allowed to heal for two weeks because the greatest osteoclastic activity has been reported two weeks after injury.[16] One hour before sacrifice, the animals were injected with 0.5 ml of papaverine solution (0.4 g/L) and 1 ml of heparin (1000IU) intraperitoneally. They were anesthetized by being placed in an isoflourane (4% isoflourane) chamber for at least 10 minutes. The chest cavity was cut open using surgical scissors and the left ventricle was located. A 20G needle was inserted and maintained in the left ventricle (Figure 2A). Bilateral saphenous veins and the tail vein were severed to allow the blood and perfusion solution to escape the cardiovascular system. The heart of the animal was beating most of the time during perfusion. Approximately 200-250 ml of heparinized solution (1 g/L) maintained at $60^{o}$ C was perfused until the drained blood and the iris of the rats appeared clear.

The BriteVu™ (Scarlet Imaging, Murray, UT) perfusion solution was prepared by mixing 2 packets of BriteVu Rat with 200 ml of water that had been previously heated to $45^{0}$C. The mixture was heated to $80^{0}$C for 10 minutes and then allowed to cool to $60^{0}$C while stirring the entire time. Approximately 0.5 ml of dish soap (Dawn, USA) and a drop of green dye were added to the solution. The perfusion solution, which had been kept heated and agitated, was drawn from the beaker with a 10 ml syringe while avoiding any bubbles. The syringe was then connected to the needle inserted to the

animal's left ventricle. Gentle hand pressure was applied to the syringe to perfuse the solution. Care was taken to not apply too much pressure in order to not burst any vessels. Approximately 100-150 ml of BriteVu perfusion solution was perfused per animal. The eyes, the heart, and the skin of the rats were monitored for color changes to green (Figure 2B).

After the BriteVu perfusion, the rats were placed in plastic bags and refrigerated. Twenty-four hours after perfusion, the heads were separated from the body and placed in 4% PFA. After 3-4 days, the right and left maxillary first and second molars were sectioned and placed in 4% PFA.

*MicroCT Analysis*

After sacrifice, all specimens were stripped of soft tissues and scanned using the SkyScan1172 scanner (Bruker, Kontich, Belgium). The specimens were imaged at 3μm resolution, 50 kV and 167μA. Scan time was 130 minutes per specimen. 3D and 2D morphometric parameters were calculated for the inter-radicular and buccal regions of interest (ROI). The buccal ROI was 2 mm long and 1 mm deep extending from the edge of the buccal cortical bone to the PDL space. It was of varying heights depending on the alveolar heights. The inter-radicular ROI was defined as the volume of bone starting cervically from the root furcation of the maxillary first molar and extending apically to the tip of the longest root of the same tooth. The mesio-distal depth of the inter-radicular ROI was limited laterally by the PDL space of the teeth. Greyscale threshold values (30-255) were applied for the inter-radicular and buccal bone ROI. For barium

sulfate vessel isolation and quantification, grey threshold values of 90-255 were used.

3D reconstructions of the μCt scans were done using Skyscan NRecon software with histogram limits 0.0-0.45. After reconstruction, the barium sulfate filled vessels were separated from the bone volumes. They were then analyzed.

*Histologic Analysis*

The samples were fixed in 4% PFA and then decalcified for two weeks in EDTA. Radiographs were used to monitor decalcification. They were then dehydrated in graded alcohol, cleared with xylene and embedded in paraffin blocks. The blocks were cooled and then sectioned to be 6 μm thick with a microtome oriented in a coronal direction. Every $10^{th}$ and $11^{th}$ slice was mounted on glass slides and stained for H&E and TRAP, respectively. The H&E and TRAP slides were visualized by a blinded investigator under a Zeiss Axioplan microscope (Carl Zeiss Microimaging, Germany) and photographed using SPOT 5.0 software (SPOT Imaging Solutions, Sterling Heights, MI). The slides were photographed at 2.5X magnification to capture the entire tooth and at 10X to capture the buccal, inter-radicular and palatal sections of bone.

*Statistical Analysis and Determination of Significance*

Statistical tests showed that the data were normally distributed. Paired t-tests were used to compare the control and experimental sides. A single blinded operator performed all statistical analysis using SPSS.

# CHAPTER IV

# RESULTS

Following surgery, healing of the flap site progressed normally, with little to no swelling and no infections in any of the animals. The perfusion was not successful in one animal (#5) that was not included in the analysis.

## *MicroCT*

There were no statistically significant side difference in buccal bone volume fraction, or bone mineral density, between control and experimental sides. A statistically significant greater trabecular separation was found for the experimental group compared to control in the buccal ROIs (Table 1, Figures 3, 4). In addition, there was no difference in bone mineral density between control and experimental sides for the inter-radicular trabecular bone (Figure 5).

There were no statistically significant differences in inter-radicular vessel volume, vessel diameter or vessel volume/surface ratio between control and experimental groups in the inter-radicular ROIs. (Table 2, Figure 6-7)

## *H&E Staining*

The H&E sections showed a decrease in the bucco-palatal width of the buccal cortical bone (Figures 8, 9) and an increase in the size of the osteocyte lacunae on the experimental side. In four animals (#2, #4, #7 and #13) the increase in the osteocyte

lacunae size was so pronounced as to give the buccal bone a "shredded" appearance. In some animals, the increase in lacunae size was accompanied by cell death. Acellular regions were present in most sections on the experimental side, but not on the control side. The sizes and number of acellular regions on the buccal aspect decreased moving lingually.

New bone formation was observed on the experimental side in some animals (Figure 9). The new bone was added buccal to the cortical layer on the flap side. The newly added bone appeared woven with areas of bone remodeling. Lines of bone forming osteoblasts were present throughout the new woven bone.

Small blood vessels were observed in the trabecular bone on the both control and experimental sides. No differences were observed in the number and/or size of the bone vessels present on the buccal slices (Figures 8, 9).

The inter-radicular slices showed woven bone on both the experimental and control sides (Figure 10). Resorption lacunae and/or lines were abundant on both sides. Multiple small diameter vessels were observed on the control and experimental slices. The barium sulfate solution was present in all vessels. Vessels of large diameter were visible only in some animals. No consistent differences were observed histologically in the number and/or size of the vessel between the experimental and control sides.

*TRAP Staining*

TRAP staining showed minimal TRAP activity in the buccal sections on both experimental and control sides. Most of the TRAP activity in the buccal sections was

observed in the PDL space, around the roots of teeth. Some TRAP activity was observed in the newly formed buccal bone of six animals (#3, #6, #7, #9, #12 and #13) on the flap side (Figures 11,12).

For the interadicular sections, significantly more TRAP activity was observed on the experimental side compared to the control side. There were no apparent side differences in the numbers of the osteoclasts. However, the experimental flap side showed more low-grade TRAP activity than the control side around the blood vessels (Figures 13).

# CHAPTER V

# DISCUSSION

Buccal cortical bone thickness decreases after flap surgery. In the present study, there was a decrease in the bucco-palatal width of buccal cortical bone on the experiment side. Yaffe et al[24] reported similar findings in rats. Radiographically, they observed a decrease in the buccal-lingual width of the buccal bone ten days post-surgery after flap surgery. They also showed bone resorption on the periodontal side of the buccal cortical bone, with the greatest resorption occurring three weeks after surgery. The decrease in buccal bone width was associated with increased osteoclastic activity. However, the present study did not show an increased osteoclastic activity. As such, the decrease in cortical bone width could be due to osteocitic osteolysis. It has been established that a decrease in oxygen levels will induce osteocyte death.[65,66] In this study, we can expect to have osteocyte necrosis because studies have shown that apoptosis of osteocytes is followed by a secondary necrosis due to osteocytes being "trapped" in their lacunae.[67-70] During necrosis, the cell membrane integrity is compromised and the contents of the cytosol are released in the osteocyte lacunae.[71] It is hypothesized that the release of cell contents in the osteocyte lacunae could be implicated with the increase in size of the lacunae. These findings support the notion that osteocytes have the ability to resorb bone within their own lacunae.[72-76] In addition to cell death, osteocyte lacunae in the buccal cortical bone increased in size with flap surgery. In the present study, areas of acellularity accompanied the increase in osteocyte

lacunae size. Empty lacunae have been previously observed following ischemic necrosis.[77,78] In addition, flap surgery has been previously associated with areas of sequestered bone.[79] The specimens that showed the greatest acellularity were those that showed the greatest size increases of the osteocyte lacunae. The increase in size of the osteocyte lacunae could be an early indicator of distressed osteocytes.

Flap surgery has little or no effect on mineral density or bone volume fraction two weeks after surgery. In the present study, no statistically significant differences were found for bone mineral density of the buccal sections between the flap and the experimental group. In contrast, Owen et al[80] reported decreased mineral density on the flap side, while Yaffe et al[24] found decreased $^{45}Ca$ retention two weeks after flap surgery on the experimental side. Although the differences were not statistically significant, the present study did show a decrease in bone mineral density in the buccal sections on the experiment side. It is possible that due to small sample size the present study did not have sufficient power to establish the difference.

Flap surgery does not increase osteoclastic activity in the buccal bone of rats two weeks after surgery. The TRAP analyses did not show any differences in osteoclast activity in the buccal sections between experimental and control sides. Previous studies have reported greater osteoclastic activity on the flap side compared to the control side.[24,80-84] Hypoxia is associated with an increase in osteoclastic activity and bone resorption. [54-60] However, most of the studies demonstrating this relationship have either been performed in vitro or studied on bone that was highly vascularized. The buccal bone of the rat is comprised mostly of cortical bone, vascularized by the periosteum,

with minimal vessels/osteons present in the bone proper. Without the appropriate vasculature support it would be impossible to have osteoclasts present in the site of injury.

Osteoclastic activity in the trabecular inter-radicular bone increases after flap surgery. In the present study, the flap group showed consistently more of a low-grade TRAP activity than the control group around the blood vessels. As previously noted, osteocyte death plays a major role in osteoclast recruitment through different mechanisms.[85-87] Hypoxia is one of the mechanisms that induces osteocytes to stimulate osteoclast differentiation by up-regulating factors like GDF15[88,89] and osteopontin.[90] By raising a full mucoperiosteal flap, the periosteum was removed from the buccal cortical bone, rapidly decreases oxygen levels for the osteocytes close to the periosteum. This is important because the periosteum provides most of the blood supply to the bone.[39] Any damage to the periosteum will decrease the oxygen levels of the cells it supplies. The cells closer to the periosteum (i.e. osteocytes of the buccal cortex) might be expected to be more susceptible to hypoxia due to limited peripheral vascularization of the buccal bone. However, the effects of hypoxia could be transmitted to distant osteocytes through osteocyte "communication". It has been shown that osteocyte processes could form up to 12.7 terminal connections with other osteocyte processes, indicating that signals from one osteocyte could be relayed to by others at far distances.[91] This could explain the increased osteoclastic activity in the inter-radicular bone without direct injury to the trabecular bone. Yaffe et al[24] found an increase in osteoclasts in his experimental groups, but Owen et al[80] did not. However, Owen and co-workers sacrificed their experimental

animals at 8 weeks during which the osteoclastic activity could have subsided. They did find a decrease in bone density in the inter-radicular region on the experimental side that indicates a previous increase in osteoclastic activity. The lack of statistically significant differences in bone mineral density in the present study could again be due to small sample size.

Raising a flap will potentially cause new bone to be added to the buccal cortical bone. Approximately half of the samples showed new buccal bone formed on the experimental side. Periosteum is a highly vascularized tissue that stores progenitor cells and local growth factors.[92,93] This is why autologous periosteum grafts are used as membranes in bone repair procedures.[94,95] The amount of the newly formed bone could be related to the time and extent that the periosteum might have attached to the buccal bone. The samples with new bone formation probably had blood clots that formed between the periosteum and the buccal bone. Some fibrous tissue is present in all sections of new bone. This indicates that the new bone was formed through soft callus mineralization. In this case the periosteum would've helped in the new bone formation by providing osteoprogenitors and the vascular supply needed to support new bone formation.

Flap surgery does not affect the microvasculature of the inter-radicular bone. In the present study, there was no statistical difference in the amount, size and shape of blood vessels between the control and experimental groups. It has been shown that an increase in blood flow is necessary to sustain bone turnover. Sim et al[48] studied the blood flow of 29 adult dogs with varying bone turnover, which was induced

experimentally over a two-week period. They concluded that an increase in blood flow follows an increase in bone turnover. It has also been shown that then the periosteum is damaged cortical bone relies more heavily on the centro-medullary arterial suppl.[39] In the present study, increased blood flow in the inter-radicular region was expected to compensate for the damage to periosteum. The damage caused by raising flap might not have been significant to require an increase in flood flow in the inter-radicular region. Even though there was an increase in osteoclastic activity in the inter-radicular region, it is possible that more pre-osteoclasts were differentiated into osteoclasts from the existing blood vessels. Albeit not statistically significant, the present study showed increases in the diameter of blood vessels, but not in their volume. Small sample size is again implicated.

# CHAPTER VI

# CONCLUSIONS

From the present study, it can be concluded the following:

1. Buccal cortical bone thickness decreases after flap surgery.
2. Flap surgery will not affect bone mineral density or bone volume fraction in rats two weeks after flap surgery.
3. Flap surgery does not increase osteoclastic activity in the buccal bone of rats two weeks after surgery.
4. Osteoclastic activity in the trabecular inter-radicular bone increases after flap surgery.
5. Raising a flap has the potential of adding new bone to the buccal cortical bone.
6. Flap surgery does not affect the microvasculature of the inter-radicular bone.

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APPENDIX A

FIGURES

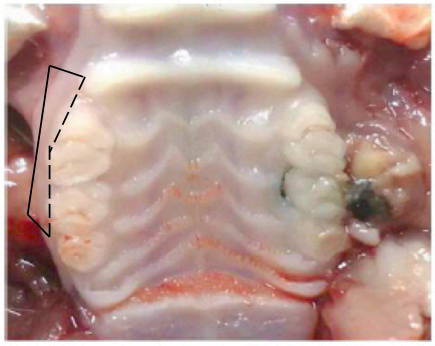

**Figure 1.** Flap surgery site. A full mucoperiosteal flap approximately 6 mm x 3 mm was elevated on the buccal aspect above the first and second maxillary molars

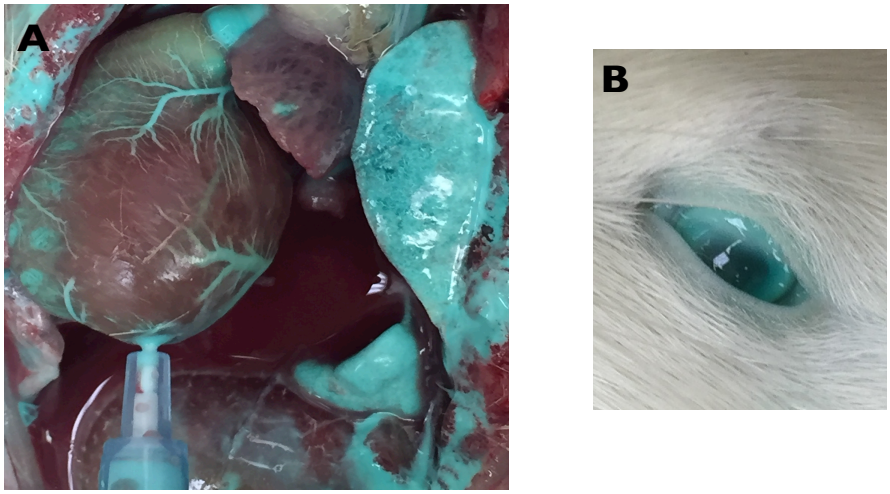


**Figure 2.** Barium sulfate perfusion A) Cannulation of the left ventricle and perfused coronary vessels, B) Green iris coloration after successful perfusion

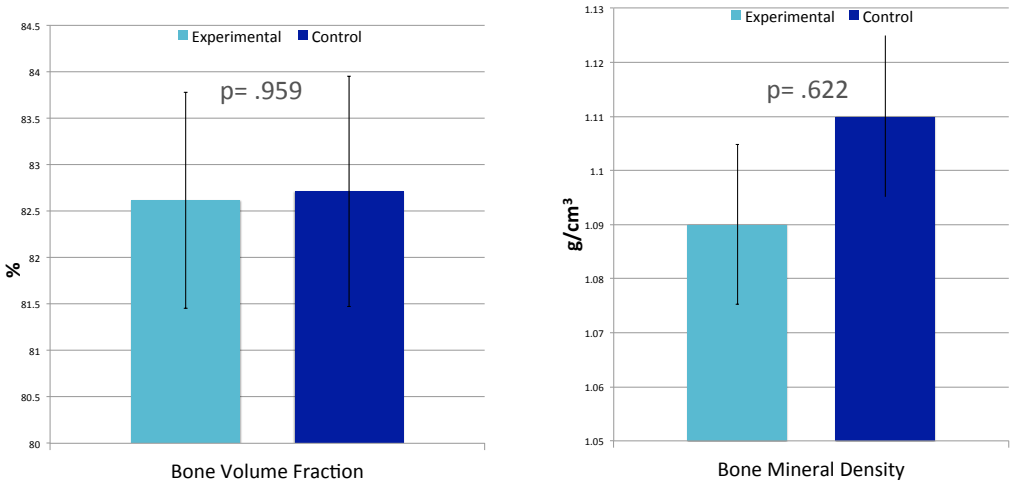


**Figure 3.** Differences between flap side and control in bone volume fraction and bone mineral density of the buccal bone ROI

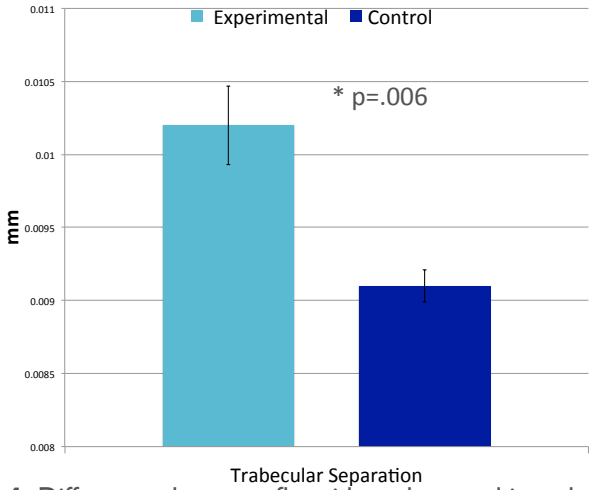


**Figure 4.** Differences between flap side and control in trabecular separation of the buccal bone ROI

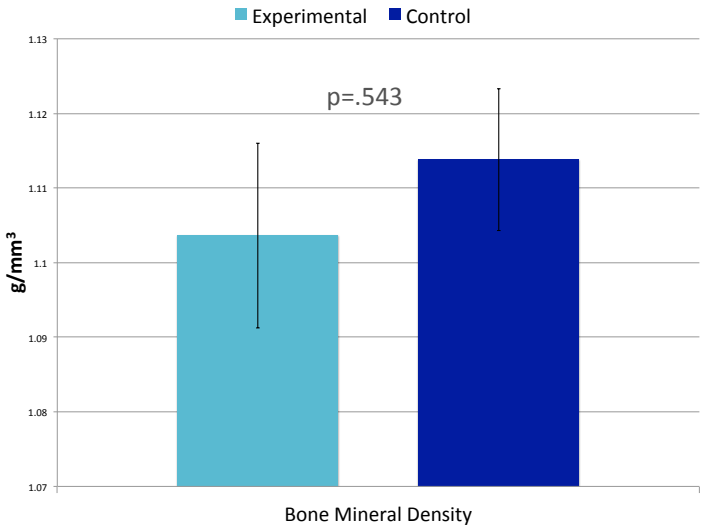


**Figure 5.** Differences between flap side and control in bone mineral density in the inter-radicular region of interest

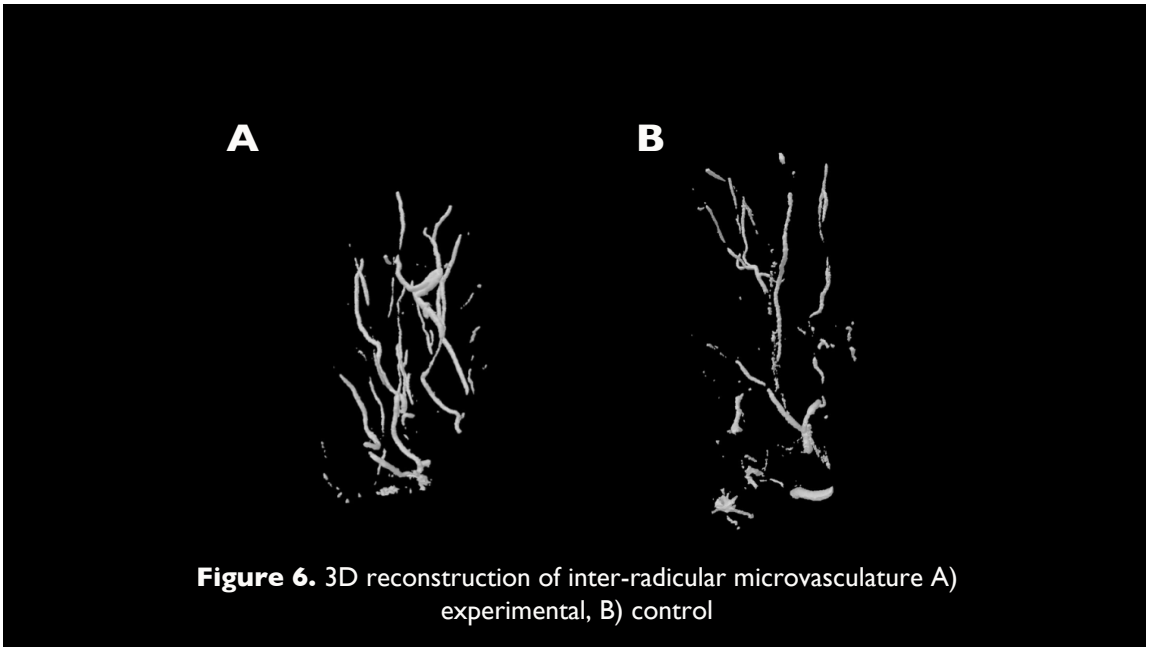


**Figure 6.** 3D reconstruction of inter-radicular microvasculature A) experimental, B) control

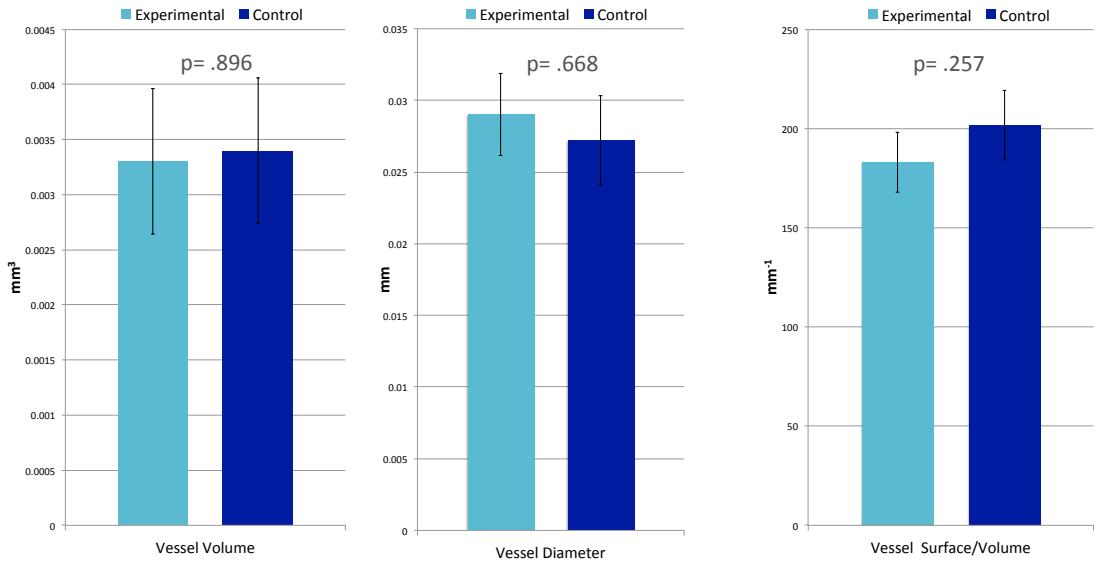


**Figure 7.** Differences in blood vessel volume, vessel diameter and vessel surface/volume of the inter-radicular bone

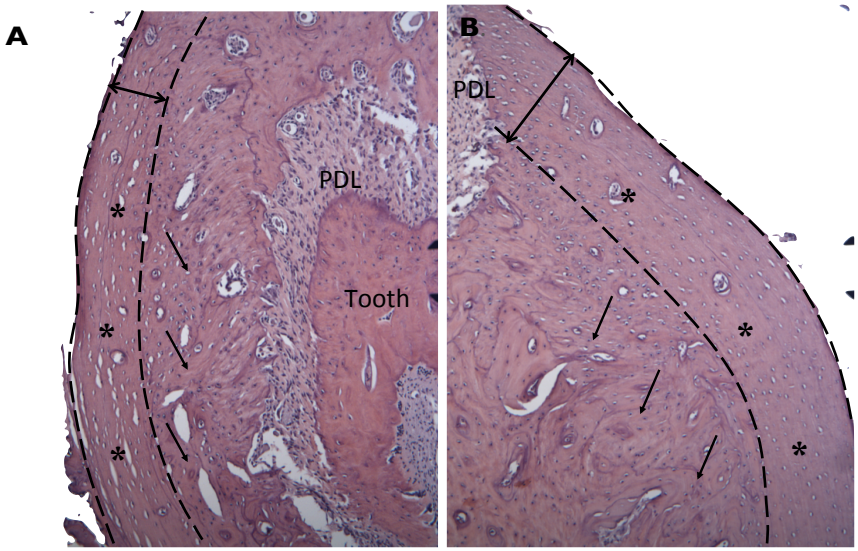


**Figure 8.** Buccal bone sections; A) experimental, B) control, cortical bone (--- ), osteocyte lacunae (*), trabecular bone (arrow)

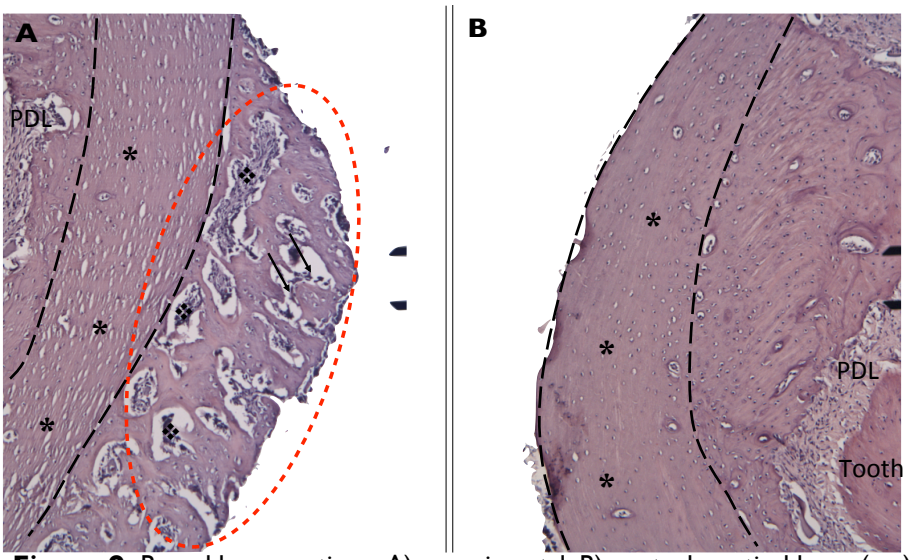


**Figure 9.** Buccal bone sections; A) experimental, B) control, cortical bone (--- ), osteocyte lacunae in cortical bone (*), new bone (---), osteoblasts (arrow), fibrous callus (❖)

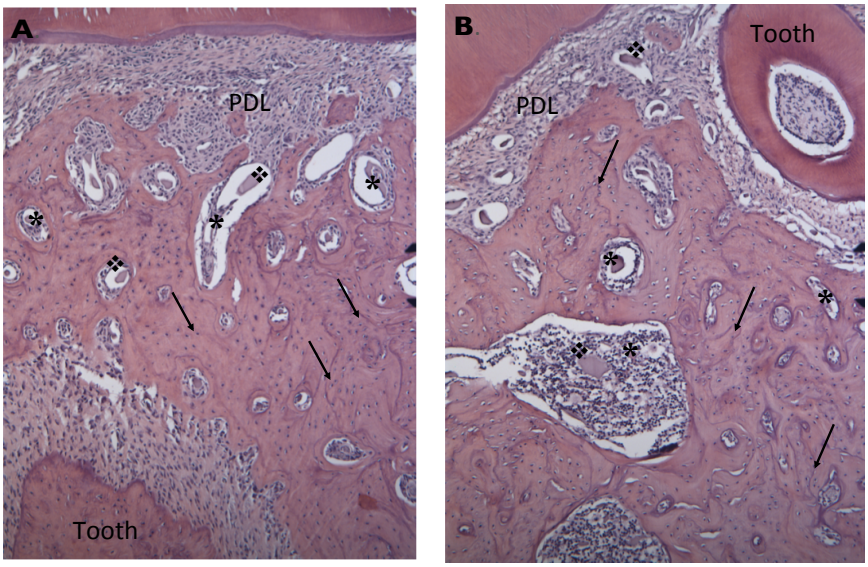


**Figure 10.** Inter-radicular bone sections; A) experimental, B) control, blood vessels(*), remodeling lacunae (arrow), barium sulfate solution (❖)

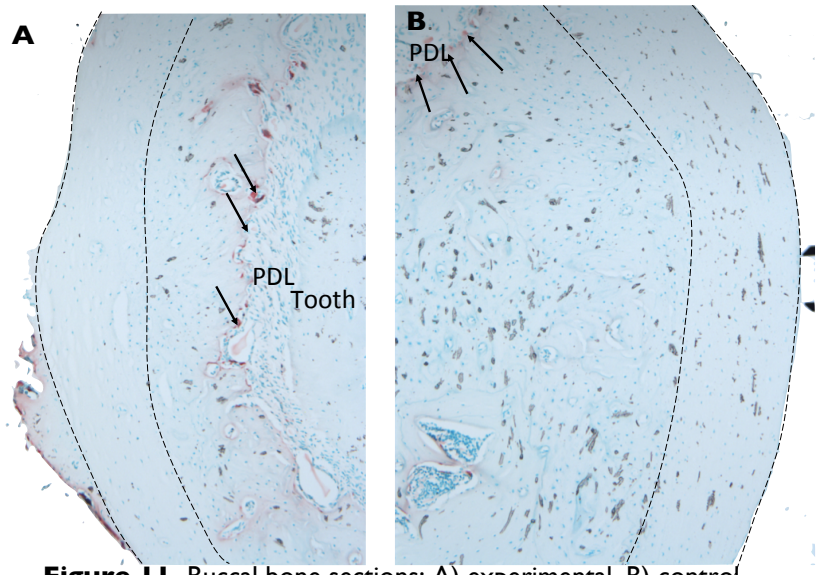


**Figure 11.** Buccal bone sections; A) experimental, B) control, cortical buccal bone (---), TRAP +(arrow)

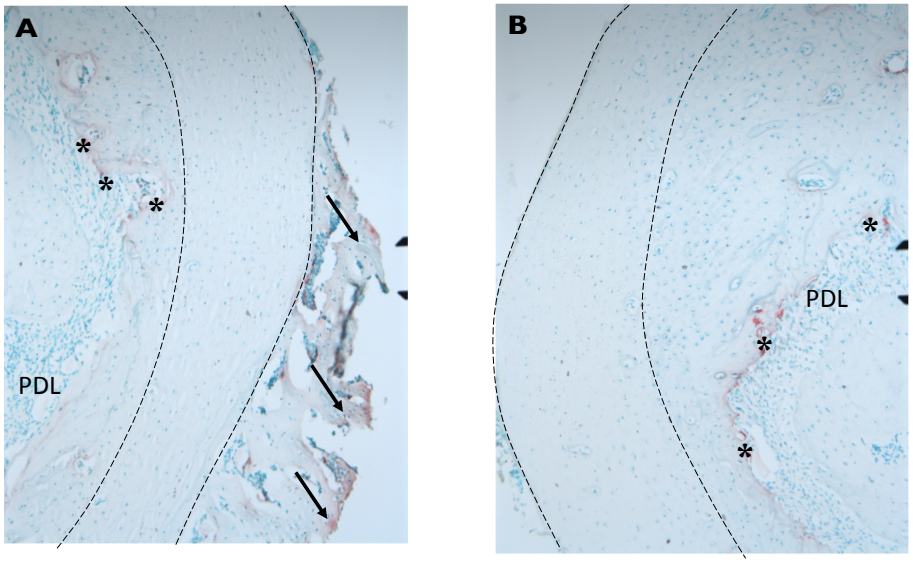


**Figure 12.** Buccal bone sections; A) experimental, B) control, cortical buccal bone (---), TRAP + PDL(*), TRAP + in new bone (arrow)

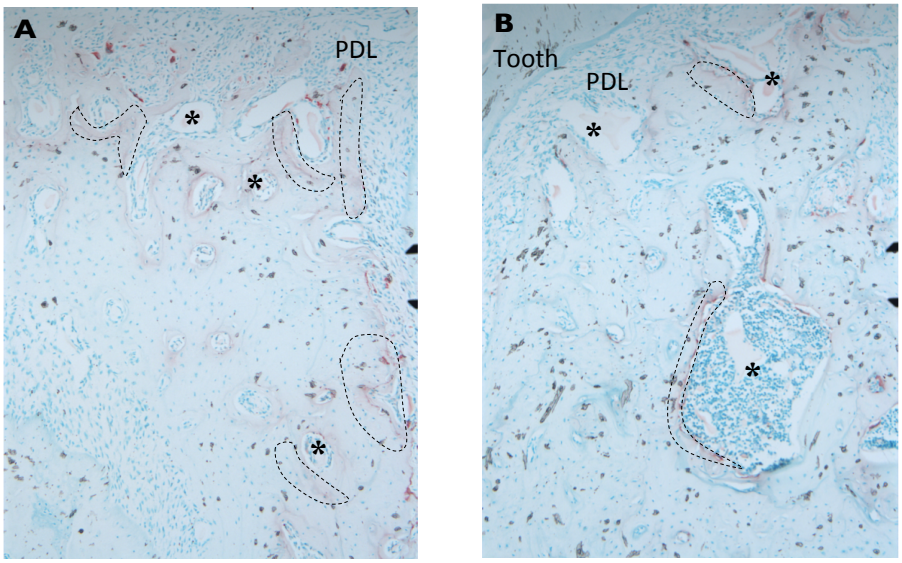


**Figure 13.** Inter-radicular bone sections; A) experimental, B) control, blood vessels(*), TRAP+ activity (---)

# APPENDIX B

## TABLES

**Table 1.** Bone changes for buccal ROI

| Measure | Experimental | | Control | | Probability |
|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | |
| Bone Volume Ratio (%) | 82.62 | 4.03 | 82.71 | 4.29 | .959 |
| Trabecular Separation (mm) | .01 | .0009 | .009 | .00038 | .006 |
| BMD ($g/cm^3$) | 1.09 | .05 | 1.11 | .05 | .622 |

**Table 2.** Bone and vascular changes for the inter-radicular ROI

| Measure | Experimental | | Control | | Probability |
|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | |
| Vessel Volume ($mm^3$) | .0033 | .00227 | .0034 | .0023 | .896 |
| Vessel Surface/Volume ($mm^{-1}$) | 183.06 | 52.57 | 201.68 | 60.86 | .257 |
| Vessel diameter (mm) | .29 | .01 | .027 | .01 | .668 |
| BMD ($g/cm^3$) | 1.10 | .04 | 1.11 | .03 | .543 |