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Effects of Externally Applied, Cyclical, Low Magnitude Forces on Orthodontic Tooth Movement in Rats

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TABLE OF CONTENTS

List of Figures

Figure 11: Graph of inter-molar distances for tooth movement in rats with CLMF (100 and 200 Hz, 0.4N) and the control (tooth movement only). Individual non-parametric, unpaired t-tests determined there was no significant difference in the dose frequency for CLMF.

Figure 12: Graph of the quantification of ostoclasts in controls and CLMF (30 Hz and 60Hz, 0.4N, 4 weeks). Individual non-parametric, unpaired t-tests determined there was no significant difference in the number of osteoclasts.

Figure 13: TRAP staining showing no significant difference in the number of osteoclasts comparing controls vs. CLMF (30 Hz, 0.4N, at 4 weeks).

Figure 14: Graph of the proliferation of PDL cells in rats with CLMF only (30 Hz and 60Hz, 0.4N, 4 weeks). BrdU was used to compare the control (right side, non-CLMF) vs. CLMF (left side). Individual non-parametric, unpaired t-tests determined there was no significant difference in the number of proliferating PDL cells between the two sides.

ABSTRACT

Orthodontic tooth movement requires external orthodontic forces to be converted to cellular signals that result in the coordinated removal of bone on one side of the tooth (compression side) by osteoclasts, and the formation of new bone by osteoblasts on the other side (tension side). The length of orthodontic treatment can take several years, leading to problems of caries, periodontal disease, root resorption, and patient dissatisfaction. It appears that the velocity of tooth movement is largely dependent on the rate of alveolar bone remodeling. Pharmacological approaches to increase the rate of tooth movement are limited due to patient discomfort, severe root resorption, and drug-induced side effects. Recently, externally applied, cyclical, low magnitude forces (CLMF) have been shown to cause an increase in the bone mineral density of long bones, and in the growth of craniofacial structures in a variety of animal models. In addition, CLMF is well tolerated by the patient and produces no known adverse effects. However, its application in orthodontic tooth movement has not been specifically determined. Since factors that increase alveolar bone remodeling enhance the rate of orthodontic tooth movement, we hypothesized that externally applied, cyclical, low magnitude forces (CLMF) will increase the rate of orthodontic tooth movement. In order to test this hypothesis we used an in vivo rat orthodontic tooth movement model. Our specific aims were:

Specific Aim 1: ***To develop an in vivo rat model for tooth movement.***

We developed a tooth movement model based upon two established rodent models (Ren and Yoshimatsu *et al,* See Figure 1.). The amount of variation of tooth movement in rats exposed to 25-60 g of mesial force activated

from the first molar to the incisor for 4 weeks was calculated.

Specific Aim 2: ***To determine the frequency dose response of externally applied, cyclical, low magnitude forces (CLMF) for maximal tooth movement and osteoclast numbers.***

Our working hypothesis for this aim was that the amount of tooth movement would be dose dependent on the frequency of application of the CLMF. In order to test this working hypothesis, we varied the frequency of the CLMF from 30, 60, 100, and 200 Hz, 0.4N, two times per week, for 10 minutes for 4 weeks, and measured the amount of tooth movement. We also looked at the number of osteoclasts for the different frequencies; we hypothesized an increase in osteoclasts for the dose respnse of different frequencies.

Specific Aim 3: ***To determine the effects of externally applied, cyclical, low magnitude forces (CLMF) on PDL proliferation.***

Our working hypothesis for this aim was that PDL proliferation would increase with CLMF. In order to test this hypothesis we compared CLMF (30 Hz, 0.4N, two times per week, for 10 minutes for 4 weeks) performed on the left side (experimental side), to the non-CLMF side, on the right (control side).

This was an experimental study with 24 rats in total. The experimental group contained fifteen (15) rats in total, and they all received a spring plus a different frequency of CLMF. Three (3) received a spring and CLMF at 30 Hz, 0.4N for 10 minutes. Six (6) received a spring and CLMF at 60 Hz, 0.4N for 10 minutes. Three (3) received a spring and CLMF at 100 Hz, 0.4N for 10 minutes. Three (3) received a spring and CLMF at 200 Hz, 0.4N for 10 minutes. The control group contained six (6) rats, and received only a spring. An additional

three (3) rats received CLMF (30 Hz, 0.4N, two times per week, for 10 minutes for 4 weeks) only, with no spring, and were used only for histological purposes.

Rats were subjected to the application of orthodontic force from their maxillary left first molar to their left central incisor. In addition some of the rats received externally applied, cyclical, low magnitude force (CLMF) on their maxillary left first molar. micro-CT was used to measure the amount of orthodontic tooth movement. The distance between the maxillary first and second molars, at the most mesial point of the second molar and the most distal point of the first molar (1M-2M distance) were used to evaluate the distance of tooth movement. Immunohistochemistry was performed with TRAP staining and BrdU quantification.

Externally applied, cyclical, low magnitude forces (CLMF) do appear to have an effect on the rate, while not significant, of orthodontic tooth movement in rats. It appears that lower CLMF decreases the rate of tooth movement, while higher CLMF increases the rate of tooth movement. Future studies with larger sample sizes are needed to clarify this issue. CLMF does not appear to affect the proliferation in PDL cells, and has no effect on the number of osteoclasts.

Chapter I: Introduction

A. Background

i. Anatomy, Biological Responses, and Orthodontic Tooth Movement

From a clinical perspective, one premise still remains indisputable- precisely executed mechanics are still subject to the dominance of the underlying cellular responses. It appears that the speed of tooth movement greatly depends on the speed of alveolar bone remodeling [1]. The length of orthodontic treatment can take several years, leading to problems of caries, periodontal disease, root resorption, and disgruntlement of the patient. Efforts to shorten the time of orthodontic treatment and accelerate the alveolar bone response would be beneficial to both the patient and the profession. A global perspective of these biological processes may be suitable for the clinician, but specificities in cellular and molecular pathways are paramount for the advancement of the field of orthodontics [2].

The supportive structures of the teeth consist of cementum, the alveolar bone, and the periodontal ligament (PDL). Cementum is the hard, bonelike tissue covering the roots of teeth. The alveolar bone is the thin covering of compact bone that surrounds the teeth; when viewed radiographically, it is called the lamina dura. From the lamina dura extend the collagenous fibers of the periodontal ligament. These fibers are embedded in alveolar bundle bone on one side, extend across the 0.5mm ligament space, and attach to the cementum layer of the tooth root on the other side. Additionally, the PDL space contains a network of capillaries and nerve fibers, fibroblasts, as well as an amorphous

ground substance consisting of connective tissue polysaccharides, salts and water [3].

The orientation of the collagenous fiber bundles of the PDL varies with the functional demands of the dentition. The majority of fibers, the oblique fibers, extend from the cementum in a coronal direction obliquely to the bone. This arrangement of fibers functions as a “shock absorber”, enabling teeth to withstand the forces of normal function. When forces are applied to the teeth, the underlying PDL fibers, cells, interstitial fluid and alveolar bone flex to dissipate the stress [4]. Although the alveolar bone is constantly remodeling in response to the intermittent, masticatory forces, these forces are inadequate to produce tooth movement.

According to the pressure-tension theory, it is the light, continuous compression and tension within the PDL space that stimulate a sequence of events that initiates remodeling of the surrounding alveolar bone. These stresses alter the local fluid pressure and vary the blood flow in the PDL. This change in pressure and blood flow leads to the release of chemical mediators. These chemical mediators initiate a cascade of signals that lead to the activation of osteoclasts and osteoblasts, the primary bone remodeling cells [1].

An osteoblast is a mononucleate cell responsible for bone formation, in areas of tension; an osteoclast is a multinucleate cell responsible for bone resorption, in areas of compression. The osteoclasts create space in the alveolar bone for the tooth to move, while the osteoblasts form new bone in the areas vacated by the moving tooth. The formation of mature bone-resorbing osteoclasts from hematopoietic precursors requires cell–cell interaction with cells

from the osteoblastic lineage [5]. Osteoblastic cells are, therefore, said to be necessary to "support" osteoclastogenesis. The molecule mediating this interaction is called receptor activator of NF-kappa B (RANK) ligand, or RANKL [6]. Osteoblastic cells express RANKL as a membrane-associated factor, and expression of RANKL is induced by multiple stimulators of resorption, including PGE2 [7]. Osteoclast precursors express, RANK, the receptor for RANKL.

RANKL is also a ligand for osteoprotegerin (OPG) [8]. OPG, which is produced by osteoblastic cells, and acts as a decoy receptor for RANKL, thereby preventing RANKL-RANK binding. Increased OPG expression can, therefore, suppress osteoclast formation [9].

ii. Phamacological regulation on bone remodeling

Not surprisingly, recent in vivo experiments have shown that exogenously, pharmacologically, added OPG decreases the rate of orthodontic tooth movement [10,11] and exogenously added RANKL [12] increases the rate of orthodontic tooth movement. The expression of RANKL and OPG in the PDL seems to be dependent on the type of mechanical loading (i.e. compression vs. tension). Compressive forces on PDL cells, cause the induction of RANKL expression [13,14] with little changes in OPG expression [15]. In contrast, tensile forces on PDL cells cause the up regulation of both OPG [16] and RANKL expression [17]. These differences may explain why the compression side of orthodontic tooth movement is associated with an increase in bone resorption.

Factors that increase the rate of bone remodeling have also been shown to increase the rate of tooth movement. Orthodontic movement of teeth

stimulates prostaglandin production, and endogenous or exogenous prostaglandins enhance the rate of tooth movement. This enhancement is presumably the result, at least in part, of prostaglandin-stimulated bone remodeling. In a number of studies it has been shown that prostaglandins are involved in the bone removal component of orthodontic tooth movement. Prostaglandin levels have been shown to increase on the compression side of the tooth during orthodontic tooth movement [18]. In addition, inhibitors to prostaglandin production, (cyclooxygenase (COX) inhibitors) have been shown to decrease both the total amount of orthodontic tooth movement, and the number of osteoclasts on the compression surface [19-23]. In other studies, it has been shown that locally administered PGE_1 caused an increase in orthodontic bone resorption [24] and tooth movement [25-27].

Parathyroid hormone (PTH) is a potent bone-remodeling factor. Continuous infusion of PTH has been shown to cause a 2 fold increase in the rate of orthodontic tooth movement in rats, and a corresponding 2-3 fold increase in the number of osteoclasts on the compression side of the periodontal ligament during orthodontic tooth movement [28]. Similar findings of increased tooth movement in a rat model were reported with the local injection of PTH in a slow-release formulation [29].

The active form of Vitamin D3, [1,25 $(OH)_2D_3$], is known to be a potent stimulator of osteoclastic bone resorption. In 1988, Collins et al. using a cat model, showed that after 21 days of canine retraction with a light-wire retraction spring, and weekly intraligamentous injections of a solution of 1,25 $(OH)_2D_3$ in dimethylsulfoxide (DMSO), the teeth had moved 60% further than matched

control teeth. At the histologic level, increased numbers of mononuclear osteoclasts precursors were recruited and activated, resulting in greater amounts of alveolar bone resorption on the pressure side of the periodontal ligament [30]. Similar findings were reported in 1992 by Takano-Yamamoto et al., who injected rats with 1,25 $(OH)_2D_3$, along with placing an elastic band separator between the maxillary first and second molars. 1,25 $(OH)_2D_3$ was synergistic with mechanical stimuli (the elastic separator), enhancing the numbers of osteoclasts induced, compared to the elastic separator alone [31].

iii. Mechanical regulation of bone formation

Although many pharmacological approaches have been shown to increase tooth movement, many side effects, such as local pain, severe root resorption [32], and drug-induced side effects [33] have been reported. This turned the trend to finding a physical approach to accelerate tooth movement. One approach such is low-energy laser irradiation, known to have anabolic effects, such as the acceleration of bone formation. In 2000, Kawasaki *et al*, examined the effects in rats. In the laser irradiation group, the amount of tooth movement was significantly greater (1.3-fold) than that of the nonirradiation group at the end of the experiment. The amount of bone formation and rate of cellular proliferation on the tension side and the number of osteoclasts on the pressure side were all significantly increased in the irradiation group when compared with the nonirradiation group [34].

Another physical approach is mechanical loading of bone, which is essential for maintaining bone mass and integrity. Conceptually, bone adapts to

natural (weight bearing, muscle pull) and therapeutic (orthodontic) mechanical strains to achieve a better balance between mechanical stress and the load bearing capacity of the bone tissue [35, 36]. For example, increased loading of the arms of tennis players results in increased bone formation [37]. In contrast, loss of loading, during immobilization [38] or spaceflight [39], can decrease bone formation and increase bone resorption. This is not a new concept; Wolff was the first to make this association in 1892. Wolff's Law of Bone Remodeling stated that "every change in the form and function of bones, or of their function alone, is followed by certain definite changes in their internal architecture, and equally definite alterations in their external conformation" [40].

Frost hypothesized that mechanically induced bone remodeling was dependent on the strain, not the stress, or more specifically on a minimum effective strain (MES) [41]. Experimental evidence has suggested that the MES range is about 0.0008-0.002 units bone surface strain, and that strains below this MES do not cause bone remodeling [41]. In 1971, Liskova showed that dynamic, not static, strains caused increased bone formation in rabbits [42]. This has been supported by a number of studies [43-46]. In fact, it has been shown that static loading may actually suppress both appositional and longitudinal bone formation [47].

Increased duration of loading does not cause increased bone formation. In fact, as loading duration is increased, the bone formation response tends to saturate. In one study, the effects of jump training on bone morphological and mechanical properties were investigated in immature rat bone. The rats were divided into a control group or groups of 5-, 10-, 20-, 40-, and 100-jumps per day.

It was found that the 5 jumps a day group generated the same amount of new bone formation as compared to the higher jump groups [48]. In another study, the effects of the number of load cycles per day on new bone formation were investigated in an isolated avian-bone preparation to which external loads could be applied *in vivo* [49]. It was found that neither the extent, nor the character, of the mechanically induced bone changes were affected by additional increases in the number of load cycles from 36 to 1800. These observations have led to the hypothesis that bone cells are able to sense and respond to mechanical forces, but that the mechanosensitivity of bone declines soon after the application of the force. Therefore, under continued stimulation, bone is desensitized to mechanical stimuli. In support of this hypothesis, it has been shown that if bone is given a sufficient recovery period between loading regimens (8 h), it is able to regain its mechanosensitivity. [50].

A current hypothesis is that the adaptive response of bone is not the result of the numerous cycles of "small" strain magnitudes during routine activity, but rather of the far fewer cycles of relatively "large" strain magnitudes produced during unusual loading situations [51]. A number of studies have shown that large strain magnitudes applied to bone at low loading frequencies cause more bone formation than smaller strain magnitudes at higher loading frequencies [45,52]. It has also been shown that girls who have a larger number of large strain occurrences by being active in impact loading sports (gymnastics and volleyball) have a higher bone mineral density than girls who are active in non-impact sports such as swimming [53-55].

This is not to say that small strain magnitudes have no influence on bone. Muscle contractions from activities such as standing and talking create very small strains on the relevant bones. These strain magnitudes occur thousands of times a day. The role of these strain magnitudes in the maintenance of the skeletal structure has recently been shown. In one study it was found that if very low magnitude strains at high frequency-vibrations were applied for only 20 minutes a day to sheep, it caused a 34% increase in trabecular femur bone density as compared to control sheep [56]. It is important to note in this study that the strain (5 με) the animals received via the high frequency vibration was 20-fold higher than that which normally occurs in the sheep at the same frequency from activities such as standing. Therefore, even though the stimulus was for only 20 minutes, it still represents an order of magnitude increase in the total strain energy induced at that frequency from routine activities over a 12 h period [40].

To determine whether oscillatory forces stimulated sutural growth, static and cyclic oscillatory forces were applied with the same peak magnitude of 5N to sutures in the maxilla of growing rabbits. Application of repetitive 5N cyclic and static forces *in vivo* for 10 minutes/day over 12 days resulted in cyclic loading inducing significantly greater sutural widths than sham control and static loading. Fluorescent labeling of newly formed sutural bone demonstrated more osteogenesis on cyclic loading in comparison with sham control and static loading [57]. Similarly, cyclic loading applied to the growth plate of neonatal rabbit explants at 200 mN and 1 Hz for 60 minutes revealed that cyclic loading induced significantly more proliferating chondrocytes than unloaded controls, as

well as significantly higher growth plate height than the unloaded controls [58]. Taken together, these data suggest that brief doses of cyclic, intermittent forces activate cellular and molecular responses.

a. Tooth Movement Models

Cyclical, ossicilatory force application in orthodontic tooth movement has not been specifically determined. In order to test this, an animal model needs to be established. Previously, large numbers of animal models, such as rats, dogs, cats, and monkeys have been used to obtain insight on tooth movement. The biggest limitations related to these animal models are their similarity and applicative value to humans. Of the literature from 1981-2002, 57% of the orthodontic tooth movement models were rats, making the rat the investigative workhorse for unraveling the processes of mechanotransduction and alveolar bone remodeling in orthodontic tooth movement [59].

The use of the rat has several advantages: they are relatively inexpensive, which allows large samples; they can be housed for long periods of time; histological preparation of the rat is easier than other models; greater availability of antibodies required for cellular and molecular biological techniques; and they are larger than mice, which makes it easier to place orthodontic appliances. The rat does have its own limitations: denser alveolar bone as compared to humans; the lack of osteons and less abundant osteoid tissue; structural dissimilarities in the arrangement of PDL fibers and the supporting structures; and tissue development during root formation and tissue changes incident to orthodontic treatment appear to be faster in rats than in humans, although their principle mechanisms are the same [59].

In review of the 153 (57% of the total tooth movement models) studies done on rats from 1981-2002, only 3 met Ren's inclusion criteria for a good model [59]. Ren's inclusion criteria were: a force magnitude of less than 20cN; mesial movement of molars; an experimental duration greater than 2 weeks; and no extra experimental conditions, such as drug intervention. Most of the studies failed to take into account the physiology of the rat (ie. natural distal drift of the molars and the continual eruption of the incisors), or the orthodontic appliance design was faulty. The distal drift of the molars underestimates the amount of mesial movement of the molars; continual eruption of the incisors can lead to a deficient control of force direction. The appliance design can be considered poor when it does not take into account the 50-fold decreased rat molar root surface area compared to humans, or it lacks a constant and continual force [59].

In 2000, Pavlin *et al.* experimented with the loading conditions that would produce an optimal biological response of paradental tissues. They used an elastomeric "o-ring" tied between maxillary incisors and the first molar, and a red elgiloy (alloy of nickel and cobalt) open coil spring (0.0056 x 0.022 inches, Rocky Mountain Orthodontics, Denver, CO) tied and bonded to the same teeth, respectively. In the study, they found that the coil spring has considerable advantages over the "o-ring." First, the spring has a lower force/deflection rate (F/Δ). This allows for a more precise and reproducible application of a low level force, which also remains more constant compared with that delivered by an elastomeric "o-ring." Second, bonding of a coil spring to the molar and the incisors eliminates contact of the appliance with gingival tissues, minimizing the risk of tissue irritation [2]. This correlates with the criticisms of Charles Waldo,

whom in 1954, was among the first pioneers responsible for the advent of the rat model. His method, known as the Waldo method, utilized an orthodontic intermaxillary elastic, which was stretched taut and inserted into the interproximal space just cervical to the contact area between the molars of rats [60]. This method has been criticized due to the unknown force decay of the elastic. Springs have proven to be more reliable, and to deliver a reproducible force of 10 +-2CN over a range of 3-15mm of activation [59].

In the early 1990's, King, Keeling, and Nixon produced the only 3 articles that met all of Ren's criteria for an ideal rat model [59]. Forces of 20, 40, and 60cN were used in all 3 articles. They are criticized for having an initial constant force, but not reactivating it, and forces of 40 and 60cN being too high. The appliance consisted of a 9 mm length of closed coil spring (0.006 inch Hi T; arbor diameter: 0.022 inch, Unitek, Monrovia, Calif.) suspended between a cleat bonded to the occlusal surface of the maxillary first molars and the lateral surface of the maxillary incisors. Initial force values were determined by suspending known weights from the anterior end of these coils before fixation to the incisors. Tooth movement measurements were based on enlarged cephalograms, and were measured from the position of a reproducible landmark on the molar cleat, with respect to either zygomatic amalgam implants, or a barbed broach placed submucosally on the palate. Palatally placed barbed broaches represented a more reliable, less traumatic, and more easily executed superpositional landmark than zygomatic amalgams. They only had a 79% appliance success rate, the animals lost weight, and they extracted mandibular first and second molars. All of these factors contributed to poor overall animal care [59,61,62,63].

In 2004, Ren's model was fabricated due to the shortcomings of the rat models used from 1981-2002, and used a spilt-mouth design. This design compensated for the physiological distal drift of the molars, growth of the snout and concomitant forward movement of the incisors, and the continuous eruption and possible distal tipping of the incisors. Stainless steel ligature wires with a diameter of 0.2 mm were bent to enclose all three maxillary molars as one unit. To this ligature wire a Sentalloy® closed coil spring (Ni Ti, 10 cN, wire diameter 0.22 mm, eyelet diameter 0.56 mm, GAC, New York, USA) was attached to deliver a reproducible force of 10 ± 2 cN over a range of 3-15 mm activation. A transverse hole was drilled through the alveolar bone and both maxillary incisors at the mid-root level using a drilling bur (D0205, Dentsply). A stainless steel ligature wire (diameter 0.3 mm, Dentaurum) was inserted through the hole. Bonding was applied until the buccal and palatal wires were completely embedded in the bonding material, then it was light cured. It was activated and subsequently attached to the ligature wire through the snout and the incisors [59].

Most recently, in 2006, Yoshimatsu *et al.* used a variation of the Ren model using NiTi closed coil springs. Their mouse model included a NiTi closed coil spring, with the wire diameter of 0.15mm, and the coil diameter 0.9mm. The appliance was inserted between the maxillary incisor and the first molar on the left side. It was fixed with a 0.1mm wire around each tooth using a dental adhesive agent (Superbond; Sunmedical Shiga, Japan). To prevent detachment of the maxillary incisors during the experiment, a shallow groove, 0.5mm from the gingiva, was made on the maxillary incisor every 4 days, and the wire was

reattached at the new groove. According to the manufacturer's database, the force level of the coil spring after activation was approximately 10g. The maxillary left molar was used as the experimental side, and the right as the control, taking into account the distal molar drift that would naturally occur [64].

In this study, we used a modified collaboration of both Ren and Yoshimatsu's tooth movement models. We utilized Ren's mesial movement of molars, and an experimental duration greater than 2 weeks. We added to the model, Yoshimatsu's method of fixing a 0.1mm diameter wire around both the incisor and first molar only. We did not use all three molars as a unit, because we measured tooth movement as the distance between the first and second molars. We also utilized the incisor notching to stabilize the anterior portion of the spring, and to deliver control over the direction of the force. In addition, we used the maxillary left molar as the experimental side, and the right as the control, taking into account the distal molar drift that would naturally occur.

b. Application of CLMF to Tooth Movement

Studies have used a variety of methods to deliver low magnitude, high frequency forces to accelerate the rate of orthodontic tooth movement. In 1987, Stark *et al*. applied a pulsed electromagnetic field (PEMF) to increase both the rate and amount of orthodontic tooth movement observed in guinea pigs, to evaluate the electromagnetic field's effects on bony physiology and metabolism, and to search for possible systemic side effects [65]. In 2007, vibration induced by PEMF was studied in rats. Neodymium-Iron-Boron (Nd-Fe-B) magnets and Sentalloy closed coil springs were placed between maxillary or mandibular first

molars and incisors to activate tooth movement. The animals of experimental subgroups were exposed to the vibration induced by PEMF, while the control subgroups were under normal atmosphere. The changes in the space between the molar and incisor were measured to indicate the amount of tooth movement. Under PEMF, the coil spring had a significantly greater amount of tooth movement than that of the coil-magnet combination, as did the magnets compared to sham magnets. Under a non-PEMF scenario, there was no significant difference in tooth movement between coil spring and coil-magnets combination, nor was there difference between magnets and sham magnets [66]. In 2000, as to be expected, Tengku showed that a static magnetic field had no effect on orthodontic tooth movement [67].

In 1986, Shimizu studied the movement of the lateral incisor in *Macaca fusca* loaded with a vibrating force. The vibration was done for 1.5 hours per day over 3 weeks. The results showed 1.3-1.4 times greater tooth movement than loading a static force. The duration of vibration can arguably cause mental and physical stresses on the animal [68]. In 2008, Nishimura along with Shimizu again investigated the effects of stimulation by resonance vibration on the speed of tooth movement in rats. The maxillary first molars were moved to the buccal with an expansion spring for 21 days. The experimental group consisted of adding a vibrational stimulant (60 Hz, 1.0 m/s^2) to the maxillary first molars for 8 minutes on days 0, 7, and 14. Tooth movement in the experimental group was observed to be significantly greater by 15% than the control group. Enhanced RANKL expression was observed in fibroblasts and osteoclasts in the periodontal ligament of the experimental group on day 3. The number of osteoclasts in the

experimental group was significantly increased over the control on day 8. This gave promise that the application of resonance vibration might accelerate tooth movement, and gave insight into the response to the activation of the RANK-RANKL pathway from the resonance vibration. It was also concluded that a force of 12.8g was the optimal force level to move rat molars [69]. Limitations of this experimental design are that an unknown force value for vibration was used, and the appliance consisted of an expansion spring. The use of the expansion spring can lead to possible skeletal effects, and can overestimate the actual amount of dental tooth movement. In addition, this appliance design does not correlate with Ren's criteria of mesial movement of the molars, and the use of a coil spring to decrease the force/deflection rate (F/Δ).

B. Rationale

Recently, externally applied, cyclical, low magnitude forces have been shown to cause an increase in the bone mineral density of long bones, and in the growth of craniofacial structures in a variety of animal models. In 2008, it was shown by Nishimura *et al.* to increase the rate of orthodontic tooth movement; however, a frequency dose response was not performed, and the appliance design consisted of an expansion device. Therefore the goals of this study were to develop a coil spring, rat tooth movement model, which delivered a constant force, and mesial movement of the maxillary molars. A second goal was to quantify the frequency dose response, which caused maximal tooth movement, and osteoclast numbers.

Chapter II: Hypotheses and Aims

A. Hypotheses and General Objectives

1. **Hypothesis 1:** We hypothesized that there would be greater orthodontic tooth movement in the CLMF experimental group, than for the control group. We hypothesized that there would be an increase in the number of osteoclasts, and an increase in PDL proliferation.
2. **Hypothesis 2:** We hypothesized that there would be a frequency dependent dose response increase of orthodontic tooth movement with CLMF.

Null Hypotheses:

1. **Null Hypothesis 1**: There would be no difference in the amount of tooth movement in the CLMF experimental group versus the control group. There would be no difference in the number of osteoclasts, and no difference in PDL proliferation.
2. **Null Hypothesis 2**: There would be no difference in the amount of tooth movement given different frequency doses of CLMF.

B. Specific Aims/Objectives

Aim 1: To develop an *in-vivo* coil spring, rat tooth movement model, which delivered a constant force, and mesial movement of the maxillary molars.

We developed a tooth movement model based upon two established rodent models (Ren and Yoshimatsu *et al,* See Figure 1.). The amount of variation of tooth movement in rats exposed to 25-60 g of mesial force activated from the first molar to the incisor for 4 weeks was calculated.

Aim 2: To determine the frequency dose response of externally applied, cyclical, low magnitude forces (CLMF) for maximal tooth movement and osteoclast numbers.

Micro-CT was used to measure the amount of tooth movement. Tartrate-resistant acid phosphatase (TRAP) staining was used to assess the number of osteoclasts in the periodontal ligament of the maxillary first molar after CLMF.

Aim 3: To determine the effects of externally applied, cyclical, low magnitude forces (CLMF) on PDL proliferation.

BrdU immunostaining was used for assessment of cell proliferation in the periodontal ligament of the maxillary first molar after CLMF.

Chapter III: Materials and Methods

All experiments were performed under an institutionally approved protocol for the use of animals in research (University of Connecticut Health Center #2007-341).

This was an experimental study with 24 rats in total.

The experimental group contained fifteen (15) rats in total, and they all received a spring plus a different frequency of CLMF. Three (3) received a spring and CLMF at 30 Hz, 0.4N for 10 minutes. Six (6) received a spring and CLMF at 60 Hz, 0.4N for 10 minutes. Three (3) received a spring and CLMF at 100 Hz, 0.4N for 10 minutes. Three (3) received a spring and CLMF at 200 Hz, 0.4N for 10 minutes. The control group contained six (6) rats, and received only a spring. An additional three (3) rats received CLMF (30 Hz, 0.4N, two times per week, for 10 minutes for 4 weeks) only, with no spring, and were used only for histological BrdU staining only.

Control Group	Experimental Group
(6) rats, spring only	(3) spring and CLMF at 30Hz, 0.4 N
	(6) spring and CLMF at 60 HZ, 0.4N
	(3) spring and CLMF at 100 Hz, 0.4N
	(3) spring and CLMF at 200 Hz, 0.4N

Histology Group
(3) CLMF at 30 Hz, 0.4N only

Rats were subjected to the application of orthodontic force from their maxillary left first molar to their left central incisor. In addition some of the rats received externally applied, cyclical, low magnitude force on their maxillary left first molar. In order to ensure that the rats were eating customarily, we weighed the rats every week. Any rat that lost more than 20% of their weight in one week, or who had weight loss in two consecutive weeks, was sacrificed and excluded from the study.

Upon completion of the research study, the rats were euthanized by $CO_{2,}$ followed by cervical dislocation. Animals showed no signs of apparent pain or distress. All animal experimental procedures were in compliance with the guidelines in the Care and Use of Animals in the *American Journal of Physiology* and the University of Connecticut Health Center.

A. Rat Tooth Movement Model

Young, female, Sprague Dawley rats (6 weeks, body weight 150-250g) were used for the experiment. The animals were acclimatized for at least 1 week before the experiment started. The animals were housed under normal laboratory conditions, and powdered, crushed food provided by the UCHC Animal Care Facility and water *ad libitum*. The food was checked and changed everyday. A standard 12 hour light and dark cycle was maintained.

B. Method for orthodontic force application

Animals were first placed under general anesthesia with isoflurane and ketamine (87 mg/kg) for initial appliance placement. A 9mm nickel-titanium, closed coil spring (.010 x .030mm, Rocky Mountain Orthodontics. Denver, CO) was used for the application of orthodontic force. The force/deflection rate (F/Δ) for the spring was determined in order to calibrate the amount of force produced by activation of the spring.

Prior to appliance delivery a 0.014 mm SS ligature was threaded through the contact between the first and second left maxillary molars. Self-etching primer (Transbond Plus self etching primer, 3M Unitek) was applied to the lingual surface of the first molar, and the ligature was bonded with light-cured dental adhesive resin cement (Transbond 3M Unitek), and cured with commercial unit (LEDemetron 1, Dentsply). The spring was then attached to the 0.014 mm SS ligature around the first molar and activated to the incisor. A second 0.014 mm SS ligature was placed around the incisor, activating the spring, and reinforced with the same bonding procedure as the molar. In addition, grooves 0.5mm from the gingiva were prepared on the facial, lingual, and distal surfaces of the maxillary central incisors to prevent the ligatures from dislodging from the incisor due to their lingual curvature and eruption pattern. After the ligatures were tied and cut, composite resin (Transbond XT Light Cure Adhesive Paste, 3M Unitek, Monrovia, CA) was placed over the wire to prevent slipping and gingival irritation, as well as pulpal irritation due to exposed dentin. See Figure 1 and 2A.

The entire procedure of orthodontic appliance application took 30-45 minutes, which could be completed once adequate anesthesia was obtained. Subsequent to the procedures the rats were allowed to recover in the presence

of an incandescent light for warmth and the animals were returned to their cages once full ambulation and self-cleansing had returned. The appliance was checked twice weekly, and more bonding material was added when necessary. The incisal grooves 0.5mm from the gingiva were done once per week to compensate for the continual incisal eruption.

Only the left side of the maxilla was treated; the contralateral side (non-treated) served as the control side for histological purposes, and was used to evaluate the physiological distal drift of the molars.

C. micro-CT Analysis

Micro-CT analysis was performed by the micro-CT facility at the University of Connecticut Health Center headed by Dr. Douglas J. Adams. Three (3) different time points (0, 2, and 4 weeks) were used to measure tooth movement. The distance between the maxillary first and second molars, at the most mesial point of the second molar and the most distal point of the first molar (1M-2M distance) were used to evaluate the distance of tooth movement. See Figure 3 for an example of micro-CT after 4 weeks of tooth movement.

The measurements were made on the 2D chosen slice from the micro-CT scan. See Figure 3C. The slice that showed the most root structure was determined to be the correct slice for the midpoint of the two molars. The slice before and after was also measured, and the three were averaged to result in the measurement of the distance used in this study.

Scanning was performed at 55 kV and 145 mA, collecting 1,000 projections per rotation at 300 millisecond integration time. Three-dimensional images were constructed using standard convolution and back projection

algorithms with Shepp and Logan filtering and rendered within a 12.3 mm field of view at a discrete density of 578,704 voxels/mm3 (isometric 12 mm voxels).

D. Application of CLMF

Anesthesia was induced, and a 0.036 SS fabricated mouthprop, placed between the maxillary and mandibular incisors, was used to hold the rat's mouth open. A feedback loop, controlled, electromechanical actuator was used to apply unilateral CLMF to the left first maxillary molar of the rat, in similar fashion to current mouse mandible CLMF performed in *ex vivo* culture conditions (Model 3230, Bose/EnduraTec, Minnetonka,MN). See Figure 4. Loading protocols for individual animals consisted of 10 minutes of CLMF, at a force magnitude of 0.4 Newtons, applied at a frequency of 30, 60, 100, or 200 Hertz (cycles/second), two times per week for 4 weeks. The magnitude, frequency, and duration of these ranges were chosen based upon our ongoing in-vitro studies.

At 4 weeks, the rats undergoing CLMF were injected intraperitoneally with 0.1 mg Bromodeoxyuridine (BrdU) per gram body weight 2 hours prior to CLMF, and were euthanized 6 hours after CLMF. This was chosen based upon our ongoing *in-vitro* studies being 6 hours in culture. The rats serving as controls were injected intraperitoneally with 0.1 mg Bromodeoxyuridine (BrdU) per gram body weight, and euthanized 3 hours after injection. This allowed maximal BrdU incorporation. Immunohistological analyses were performed on all rats.

E. Wellness monitoring and Euthanasia

Rats were subjected to the application of orthodontic force from their molars to their central incisors. In addition some of the rats received externally applied, cyclical, low magnitude force (CLMF). In order to ensure that the rats

were eating customarily, we weighed the rats every week. Any rat that lost more than 20% of their weight in one week or who had weight loss in two consecutive weeks was sacrificed and excluded from the studies.

Upon completion of the research study, the rats were euthanized by CO_2 followed by cervical dislocation. Animals showed no signs of apparent pain or distress. All animal experimental procedures were in compliance with the guidelines in the Care and Use of animals in the *American Journal of Physiology* and the University of Connecticut Health Center.

F. Dissection and Tissue Preparation

After decapitation, the mandibles were removed. See Figure 2B. The maxilla was then hemisected, and cleansed of soft tissues and muscles. The hemisected maxilla subsequently was placed in 10% Formalin for five days at 4°C with constant agitation. The maxilla was then washed in PBS two times for 30 minutes, and placed in 30% sucrose overnight.

G. Frozen Embedding

Prior to embedding, a 200ml beaker containing 2-methylbutane was pre-chilled over dry ice under a hood. Disposable base molds (Thermo Shandon) were filled with frozen embedding medium (Thermo Shandon), and care was taken to avoid the introduction of bubbles. The maxilla was immersed in individual molds containing the embedding medium. The embedding media was flash frozen by holding the mold with forceps in a solution of 2-methylbutane, while keeping the embedding mold on a horizontal level. Once the medium is frozen, the mold was allowed to sink to the bottom of the beaker until it was completely frozen. The molds were removed from the methyl butane solution

and wrapped in a square of aluminum foil, placed in a plastic container, and stored at -20°C.

G. Frozen Sectioning

Frozen sectioning was performed on a Leica CM1900 Cryostat (D-69226; Leica, Inc., Nussloch, Germany). Frozen sectioning is designed to capture a frozen section of undecalcified tissue, cut by a tungsten knife (TC-65; Leica, Inc. Nussloch, Germany), adhered on special cold, adhesive, Cryofilm tape (Cyrofilm type II (C); Section Lab Co. Ltd, Japan) to assist transferring the sagittal section to a cold glass microscope slide. Once the Cryofilm transfer tape is removed from the slide, it leaves the frozen section behind on the microscope slide.

The block containing the maxilla was oriented in the block holder to obtain a 5-μm sagittal section, allowing analyses of the mesial-buccal, and mesial-distal roots of the maxillary first molars. The slides were air-dried and kept in a dark slide box at 4°C before histological stainings.

I. Immunohistochemistry

Immunohistochemistry staining was carried out using the Zymed® BrdU Staining Kit (Invitrogen©, SKU #93-3943, Carlsbad, CA) following the procedure recommended by the manufacturer. The negative control consisted of substitution of the monoclonal anti-BrdU antibody with the blocking solution.

To quantify BrdU staining, a rectangular box of fixed area was superimposed on 10x images of each section and a labeling index (number of BrdU positive cells/ total number of cells) was calculated in the PDL area of the

maxillary first molar. Quantitative analysis was calculated as the number of positive cells in all cells observed according to the following formulae:
Ratio of BrdU positive cells = (Number of BrdU positive cells/number of all cells) ×100

Tartrate-resistant acid phosphatase **(**TRAP) staining was performed using the acid phosphatase leukocyte kit (Sigma Chemical, St Louis, MO), to identify the osteoclasts. TRAP was carried out after rinsing the sections in PBS for 5 minutes, three times. The sections were then washed in the detection buffer. The detection buffer contains 112mM sodium acetate anhydrous, 76mM tartrate, and 11mM sodium nitrite. Next, the sections were incubated for 5 minutes at room temperature with ELF 97 substrate (Molecular Probes. Inc. E-6601) diluted 20-fold in the same detection buffer for 5 minutes. The slides were monitored under the microscope. The reaction was stopped by submerging the slides in three changes of wash buffer for 15 minutes with gentle agitation. The wash buffer contained 25mM EDTA, 5mM levamisole, and PBS. The slides were mounted with 50% glycerin in PBS. The slides could then be visualized with fluorescent microscopy. A red staining was used for quantification (AEC (RED) Subsrate Kit; Symed Laboratories Inc. Invierogen, CA).

Four sections were taken around the mesial root from each rat, and the TRAP positive cells were counted, and averaged to obtain one number per frequency.

J. Statistics

The data collected were not normally distributed. Therefore, the analyses used were non-parametric tests. Statistical significance of differences among means was determined using non-parametric, unpaired t-tests. Significance was accepted when $P<0.05$. Statistical analyses were carried out using GraphPad Prism. (GraphPad Software, Inc., La Jolla, CA).

Chapter IV. Results

Spring design for the *in-vivo* coil spring, rat tooth movement model

To determine the type of spring design for the tooth movement model, two springs were used: 25 gram and 60 gram springs. The amount of tooth movement was measured after 4 weeks. The measurement was made from the distal of the maxillary left first molar to the mesial of the maxillary second molar with micro-CT (Figure 5). A non-parametric t-test was used to compare the two groups. There was no significant difference between 25g and 60g force springs for the amount of tooth movement achieved after 4 weeks (P= 0.4674). See Figure 6.

Table 1

Non-parametric t-tests for comparison of orthodontic tooth movement (M1-M2) after 4 weeks with 25 grams vs. 60 grams springs

Spring Force	M1-M2 (mm)
25 grams	
Rat 1	1.626
Rat 2	0.707
60 grams	
Rat 3	0.716
Rat 4	0.796
P= 0.4674	

Table 2

Measurements of orthodontic tooth movement (M1-M2) after 2 and 4 weeks: control (tooth movement only), CLMF 30 Hz, 60 Hz, 100 Hz, and 200 Hz (0.4N, two times per week, for 10 minutes)

Frequencies	M1-M2 (mm)	
	2 weeks	4 weeks
Control (0 Hz)		
Rat 1	0.676	0.716
Rat 2	0.596	0.796
Rat 3	0.287	0.245
Rat 4	0.373	0.454
Rat 5	0.087	0.208
Rat 6	0.429	0.212
CLMF (30 Hz, 0.4N)		
Rat 7	0.146	0.255
Rat 8	0.106	0.183
Rat 9	0.443	0.226
CLMF (60 Hz, 0.4N)		
Rat 10	0.066	1.028
Rat 11	0.455	0.670
Rat 12	0.759	0.701
Rat 13	0.481	0.258
Rat 14	0.212	0.309
Rat 15	0.630	0.780
CLMF (100 Hz, 0.4N)		
Rat 16	0.533	0.709
Rat 17	0.464	0.245
Rat 18	0.374	0.625
CLMF (200 Hz, 0.4N)		
Rat 19	0.255	0.180
Rat 20	0.700	0.744
Rat 21	0.517	0.505

Control vs. CLMF at 30 Hz, 0.4N, two times per week, for 10 minutes for 2 and 4 weeks

To determine if CLMF at 30 Hz, 0.4N, two times per week, for 10 minutes for 2 and 4 weeks had an effect on tooth movement, two groups were used: 3 control (tooth movement only) rats and 3 CLMF (30 Hz, 0.4N, two times per week, for 10 minutes). The measurement was made from the distal of the maxillary left first molar to the mesial of the maxillary second molar with micro CT

(Figures 7 and 8). A non-parametric unpaired t-test was used to compare the two groups. There was no significant difference in the amount of tooth movement for the CLMF group (30 Hz, 0.4N, two times per week, for 10 minutes for 2 weeks) as compared to the control group (P= 0.1448). Likewise, there was no significant difference in the amount of tooth movement for the CLMF group (30 Hz, 0.4N, two times per week, for 10 minutes for 4 weeks) as compared to the control group (P= 0.0957). The mean of the control group at 4 weeks was 0.5857, and the mean of the experimental group at 4 weeks is 0.2112, with a difference of the means of 0.3745. This suggests that CLMF at 30Hz, 0.4N after 4 weeks decreases tooth movement, compared to the controls, by 63.94% (Figure 9).

Table 3

Non-parametric t-tests for comparison of orthodontic tooth movement (M1-M2) after 2 and 4 weeks: control (tooth movement only) vs. CLMF (30 Hz, 0.4N, two times per week, for 10 minutes).

Groups	2 weeks M1-M2 (mm)
Control	
Rat 1	0.676
Rat 2	0.596
Rat 3	0.287
CLMF (30 Hz, 0.4N)	
Rat 7	0.146
Rat 8	0.106
Rat 9	0.443
P= 0.1448	

Groups	4 weeks M1-M2 (mm)
Control	
Rat 1	0.716
Rat 2	0.796
Rat 3	0.245
CLMF (30 Hz, 0.4N)	
Rat 7	0.255
Rat 8	0.183
Rat 9	0.226
P= 0.0957	

Table 4

Comparison of the means of orthodontic tooth movement (M1-M2) after 4 weeks: control (tooth movement only) vs. CLMF (30 Hz, 0.4N, two times per week, for 10 minutes for 4 weeks).

Groups	Mean M1-M2 (mm)
Control	0.5857 +_0.1719
CLMF (30 Hz, 0.4N)	0.2112+_0.01410

Difference between means 0.3745+_0.1725

Control vs. CLMF at 60 Hz, 0.4N, two times per week, for 10 minutes for 2 and 4 weeks

To determine if CLMF at 60 Hz, 0.4N, two times per week, for 10 minutes for 2 and 4 weeks had an effect on tooth movement, two groups were used: 3 control (tooth movement only) rats and 6 CLMF (60 Hz, 0.4N, two times per week, for 10 minutes). The measurement was made from the distal of the maxillary left first molar to the mesial of the maxillary second molar with micro CT. A non-parametric unpaired t-test was used to compare the two groups. There was no significant difference in the amount of tooth movement for the CLMF group (60 Hz, 0.4N, two times per week, for 10 minutes for 2 weeks) as compared to the control group (P= 0.4420). Likewise, there was no significant difference in the amount of tooth movement for the CLMF group (60 Hz, 0.4N, two times per week, for 10 minutes for 4 weeks) as compared to the control group (P= 0.1116). The mean of the control group after 4 weeks was 0.2913, and the mean of the experimental group after 4 weeks is 0.6243, with a difference of the means of -0.3330. This suggests that CLMF at 60Hz, 0.4N after

4 weeks increases tooth movement, compared to the controls, by 114.31% (Figure 9).

Table 5

Non-parametric t-tests for comparison of orthodontic tooth movement (M1-M2) after 2 and 4 weeks: control (tooth movement only) vs. CLMF 60 Hz, 0.4N, two times per week, for 10 minutes).

Groups	2 weeks M1-M2 (mm)
Control	
Rat 4	0.373
Rat 5	0.087
Rat 6	0.429
CLMF (60 Hz, 0.4N)	
Rat 10	0.066
Rat 11	0.455
Rat 12	0.759
Rat 13	0.481
Rat 14	0.212
Rat 15	0.630
P= 0.4420	

Groups	4 weeks M1-M2 (mm)
Control	
Rat 4	0.454
Rat 5	0.208
Rat 6	0.212
CLMF (60 Hz, 0.4N)	
Rat 10	1.028
Rat 11	0.670
Rat 12	0.701
Rat 13	0.258
Rat 14	0.309
Rat 15	0.780
P= 0.1116	

Table 6

Comparison of the means of orthodontic tooth movement (M1-M2) after 4 weeks: control (tooth movement only) vs. CLMF (60 Hz, 0.4N, two times per week, for 10 minutes for 4 weeks).

Groups	Mean M1-M2 (mm)
Control	0.2913 +_0.0813
CLMF (30 Hz, 0.4N)	0.6243+_0.1196
Difference between means	-0.3330+_0.1830

Control vs. 30 Hz vs. 60 Hz (0.4N, two times per week, for 10 minutes for 4 weeks)

Comparison of the controls to both 30 Hz and 60 Hz CLMF after 4 weeks was not significant (P value of 0.0957, and 0.1116, respectively). However, comparison of 30 Hz to 60 Hz CLMF after 4 weeks was significant (P= 0.05). See Figure 9. Interestingly, comparison of 30 Hz to 60 Hz after 2 weeks was not significant (P= 0.2701).

Frequency dose response for CLMF at 100 Hz and 200 Hz (0.4N, two times per week, for 10 minutes for 2 and 4 weeks)

To determine the frequency dose response for CLMF at 100 Hz and 200 Hz, (0.4N, two times per week, for 10 minutes for 2 and 4 weeks) 3 groups were used: Group 1 (control tooth movement only); Group 2 (100 Hz); Group 3 (200 Hz). The measurement was made from the distal of the maxillary left first molar to the mesial of the maxillary second molar with micro-CT (Figure 10). Individual t-tests to compare each frequency (100 and 200 Hz) versus the control group were performed. There was no significance in the intermolar distance for CLMF at all frequencies. See Figure 11.

Table 7

Individual t-tests comparison of the means of orthodontic tooth movement (M1-M2) after 2 and 4 weeks: control (tooth movement only) vs. CLMF 100 Hz, and 200 Hz (0.4N, two times per week, for 10 minutes).

Groups	2 weeks M1-M2 (mm)
Control	
Rat 1	0.676
Rat 2	0.596
Rat 3	0.287
Rat 4	0.373
Rat 5	0.087
Rat 6	0.429
CLMF (100 Hz, 0.4N)	
Rat 16	0.533
Rat 17	0.464
Rat 18	0.374
P= 0.7189	

Groups	4 weeks M1-M2 (mm)
Control	
Rat 1	0.716
Rat 2	0.796
Rat 3	0.245
Rat 4	0.454
Rat 5	0.208
Rat 6	0.212
CLMF (100 Hz, 0.4N)	
Rat 16	0.709
Rat 17	0.245
Rat 18	0.625
P= 0.6460	

Groups	2 weeks M1-M2 (mm)
Control	
Rat 1	0.676
Rat 2	0.596
Rat 3	0.287
Rat 4	0.373
Rat 5	0.087
Rat 6	0.429
CLMF (200 Hz, 0.4N)	
Rat 16	0.255
Rat 17	0.700
Rat 18	0.517
P= 0.6051	

Groups	4 weeks M1-M2 (mm)
Control	
Rat 1	0.716
Rat 2	0.796
Rat 3	0.245
Rat 4	0.454
Rat 5	0.208
Rat 6	0.212
CLMF (200 Hz, 0.4N)	
Rat 16	0.180
Rat 17	0.744
Rat 18	0.505
P= 0.8481	

Quantification of the osteoclasts during orthodontic tooth movement with CLMF

To determine the number of osteoclasts for the controls, CLMF at 30 Hz and 60 Hz, (0.4N, two times per week, for 10 minutes for 4 weeks) 3 groups were used: Group 1 (control tooth movement only); Group 2 (30 Hz); Group 3 (60 Hz). Four sections were taken around the mesial root from each rat, and the TRAP

positive cells were counted, and averaged to obtain one number per frequency. Individual nonparamtric, unpaired t-tests to compare each frequency (30 and 60 Hz) versus the control group were performed. There was no significant difference in the quantification of osteoclasts for controls and CLMF at 30 Hz and 60 Hz (P= 0.8229 for 30 Hz, and P=0.3993, for 60 Hz). See Figures 12 and 13.

Groups	4 weeks TRAP positive cells
Control	
Rat 1	99.25
Rat 2	159.50
Rat 3	59.66
CLMF (30 Hz, 0.4N)	
Rat 7	116.25
Rat 8	70.26
Rat 9	159.75
P= 0.8229	

Groups	4 weeks TRAP positive cells
Control	
Rat 1	99.25
Rat 2	159.50
Rat 3	59.66
CLMF (60 Hz, 0.4N)	
Rat 10	5.75
Rat 11	73.50
Rat 12	116.50
P= 0.3993	

Quantification of PDL cell proliferation during CLMF

To determine PDL proliferation, BrdU quantification for 3 rats was performed. CLMF (30 Hz, 0.4N, two times per week, for 10 minutes for 4 weeks) was performed on the left side (experimental side), and no-CLMF was done on the right side (control side). The BrdU positive cells were quantified, and right vs. left sides of the rat were compared with nonparametric, unpaired independent t-tests. The results showed no significant difference between control vs. CMLF sides, showing no significant difference in proliferation of cells in the PDL. See Figure 14.

Rat 22	4 weeks BrdU positive cells
Control (right side)	
Section 1	0.00461
Section 2	0.00826
Section 3	0.00663
CLMF (left side, 30 Hz, 0.4N)	
Section 1	0.004620
Section 2	0.006690
Section 3	0.006870
Section 4	0.006016
P= 0.6909	

Rat 23	4 weeks BrdU positive cells
Control (right side)	
Section 1	0.02450
Section 2	0.02860
Section 3	0.02330
Section 4	0.01288
CLMF (left side, 30 Hz, 0.4N)	
Section 1	0.01029
Section 2	0.03802
Section 3	0.04984
Section 4	0.04217
P= 0.2167	

Rat 24	4 weeks BrdU positive cells
Control (right side)	
Section 1	0.03000
Section 2	0.01144
Section 3	0.00874
Section 4	0.01192
CLMF (left side, 30 Hz, 0.4N)	
Section 1	0.01530
Section 2	0.01300
Section 3	0.01056
Section 4	0.01064
P= 0.5522	

Chapter V. Discussion

In this study, we used externally applied, cyclical, low magnitude forces (CLMF) on the rats' maxillary first molars to examine the effects of orthodontic tooth movement. All of the rats' mean body weights increased linearly, and there was no significant difference between the body weights of the experimental group and the controls. The health of the rats was not affected by the anesthesia, orthodontic appliance, or the CLMF.

Two different springs were used in a pilot study to determine if there was any difference in the rate of tooth movement at 4 weeks. A light (25g) force spring, and a high (60g) force spring were used. There was found to be no significant difference in the amount of tooth movement with either spring ($P = 0.4674$). Therefore, the high force (60g) spring was used for the rat tooth movement model in this study.

Unique to this study was the use of micro-CT for the measurement of the distance between the first and second molars. The rats were scanned at time points 0, 2, and 4 weeks. Previous studies measured the distance with stone models, after taking an impression with PVS of the rat's mouth. micro-CT eliminates the need for impressions, as the measurements can be made on the 2D chosen slice from the micro-CT scan. The slice that showed the most root structure was determined to be the correct slice for the midpoint of the two molars. The slices before and after were also measured, and the three were averaged to result in the measurement of the distance used in this study.

The velocity of tooth movement is largely dependent on the rate of alveolar bone remodeling. An increase in orthodontic tooth movement should result if there is a significant increase in the amount of bone formation, the rate of cellular proliferation on the tension side, and the number of osteoclasts on the pressure side. Looking at the concept of mechanical loading of bone, increased loading results in increased bone formation [37]. Low magnitude strains at high frequency-vibrations applied for only 20 minutes a day to sheep caused a 34% increase in trabecular femur bone density as compared to control sheep [56]. Similarly, cyclic loading applied to the growth plate of neonatal rabbit explants at 200 mN and 1 Hz for 60 minutes revealed that cyclic loading induced significantly more proliferating chondrocytes than unloaded controls, as well as significantly higher growth plate height than the unloaded controls [58]. Taken together, these data suggest that brief doses of cyclic, intermittent forces activate cellular and molecular responses. However, for orthodontic tooth movement to occur these mechanical loading forces need to effect both the proliferation of the osteoblasts, and the number of osteoclasts.

In 1986, Shimizu studied the movement of the lateral incisor in *Macaca fusca* loaded with a vibrating force. The vibration was done for 1.5 hours per day over 3 weeks. The results showed 1.3-1.4 times greater tooth movement than loading a static force. The duration of vibration can arguably cause mental and physical stresses on the animal [68]. In 2008, Nishimura along with Shimizu again investigated the effects of stimulation by resonance vibration on the speed of tooth movement in rats. The experimental group consisted of adding a vibrational stimulant (60 Hz, 1.0 m/s^2) to the maxillary first molars for 8 minutes

on days 0, 7, and 14. The appliance design consisted of an expansion device. Their results showed a 15% significant increase in the rate of tooth movement [69].

In this study, we applied the CLMF to the maxillary first molars with frequencies of (30, 60, 100, and 200 Hz). Nishimura did not describe a force of the vibration in their study; we used a force of 0.4N. The CLMF lasted 10 minutes, and was performed twice a week for 4 weeks. We found no significant increase in the rate of tooth movement at all frequency doses. In 2008, it was shown by Nishimura *et al.* that the number of osteoclasts was significantly higher in the experimental group with vibration, than the control group at day 8. They also found that in the control group, the number of osteoclasts increased gradually, whereas numerous osteoclasts were found on day 8 and persisted until day 21 in the experimental group [69]. The changes in osteoclast numbers early on day 8, but not later in the experimental group makes tooth movement difficult to explain, while in our study we looked at osteoclast numbers only at one time point, after 4 weeks. We did not find a difference in the PDL cell proliferation, as well as no significant change in the number of osteoclasts. These histological findings support the result of the lack of increase in orthodontic tooth movement.

The differences in results as compared to Nishimura in 2008, may further be explained by the appliance design. In Nishimura's study they used an expansion device, which may lead to false positive tooth movement, due to any skeletal effects of the spring along the midpalatal suture. In this study, a coil spring, rat tooth movement model, which delivers a constant force, and mesial

movement of the maxillary first molar was used. This design meets the criteria for an ideal rat tooth movement model described by Ren [59].

A goal of this study was to quantify the frequency dose response, which causes maximal tooth movement and osteoclast numbers. 30, 60, 100, and 200 Hz, with 0.4 N of force were applied to the maxillary first molar of the experimental rats. While there was no significant difference in orthodontic tooth movement for any of the frequencies, comparison of the means suggests that CLMF at 30Hz, 0.4N decreases tooth movement, compared to the controls, by 37.45%, and CLMF at 60Hz, 0.4N increases tooth movement, compared to the controls, by 33.3% (Figure 9). Comparison of the controls to both 30 Hz and 60 Hz CLMF was not significant (P value of 0.0957, and 0.1116, respectively). However, comparison of 30 Hz to 60 Hz CLMF was significant (P= 0.05). See Figure 9. Future studies with larger sample sizes are needed to clarify this issue. In 2008, Nishimura had an experimental group of n=6, and they found a 15% significant change in tooth movement at day 21, while we found greater mean differences at 60 Hz, but we also had more variation making the results not significant.

We found a large variation in the amount of tooth movement within the groups. This can be compared to what we see clinically in humans. There is a large amount of clinical tooth movement in the first 4 weeks of treatment in humans. Recently, in December of 2009 in the AJODO, Karras et al. studied the effects of alendronate on orthodontic tooth movement in rats. They used a similar coil spring model, and found a large amount of variation within the groups, especially as the distance between the molars increased in size. They analyzed

the logarithm of the distance, instead of the actual measured distance. Because some of the distances were 0mm, they used the logarithm plus 0.23mm, with 0.23mm as the 2.5th percentile of the positive distance measurement [70]. Further studies with larger sample sizes could result in decreased variation.

Measurements with micro-CT were taken at the 0, 2, and 4 week timepoints. All rats at timepoint 0 had 0mm molar distances, as their molars were touching. At the 2 week timepoint the majority of tooth movement had been accomplished, as compared to the 4 week timepoints. Interesting to note, is that some measurements even decreased from the measurements taken at 2 weeks to 4 weeks. This lead to the dilemma of the questionable accuracy of the 4 week measurement, if the 2 week measurement was higher. These data suggest that future tooth movement studies do not need to exceed 2 weeks. Studies after 2 weeks can lead to complications with bond failure, and spring activation. Additionally, future studies can incorporate faxitron radiography to determine the spring's activation during the experiment.

Future studies examining the effects of CLMF will need larger sample sizes to decrease the variation within the groups. Besides the frequency of CLMF, the magnitude of force, and the duration also need to be examined for effects on the rate of orthodontic tooth movement. Results from this study do show an inhibitory trend with low CLMF that could be further examined for usage of anchorage and retention in orthodontics.

Chapter VI. Summary and Conclusions

Externally applied, cyclical, low magnitude forces (CLMF) do appear to have an effect on the rate, while not significant, of orthodontic tooth movement in rats. It appears that lower CLMF decreases the rate of tooth movement, while higher CLMF increases the rate of tooth movement. Future studies with larger sample sizes are needed to clarify this issue. CLMF does not appear to effect the proliferation in PDL cells, and has no effect on the number of osteoclasts. Besides the frequency of CLMF, the magnitude of force, and the duration also need to be examined for effects on the rate of orthodontic tooth movement.

Chapter VII: Figures

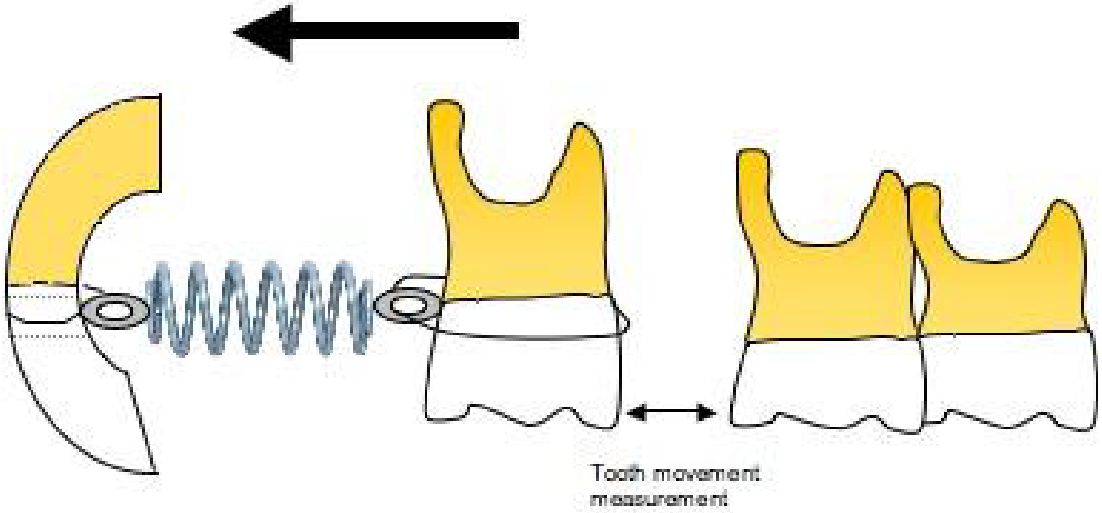

Figure 1: Cartoon schematic of the modified collaboration of both Ren and Yoshimatsu's tooth movement models used in this study.

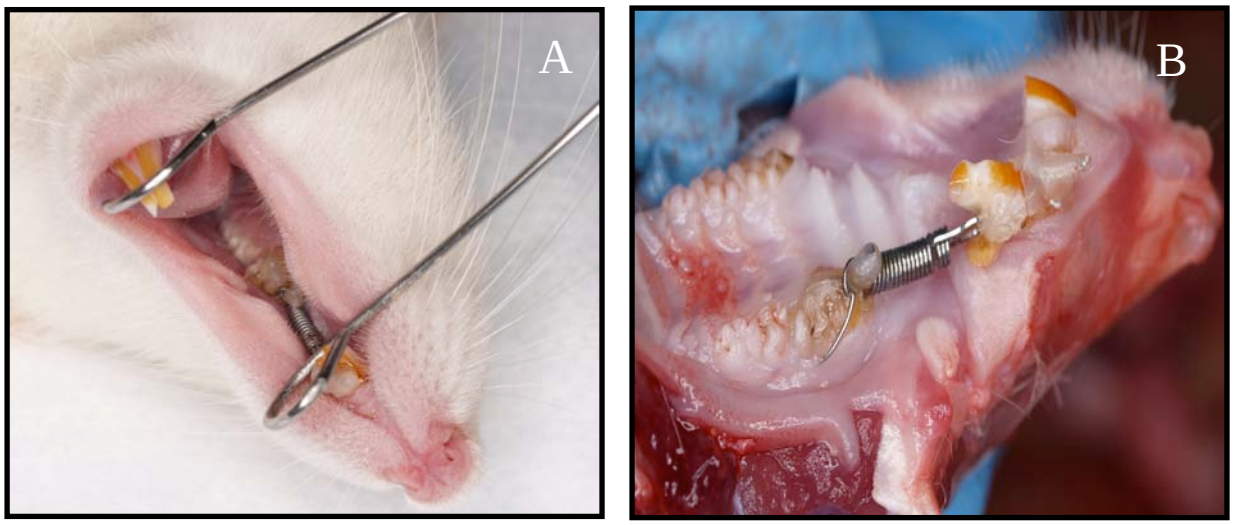

Figure 2. A. *In vivo* rat tooth movement model, with a spring being activated from the maxillary left first molar to the incisor. **B.** Dissected rat maxilla, with a spring being activated from the maxillary left first molar to the incisor.

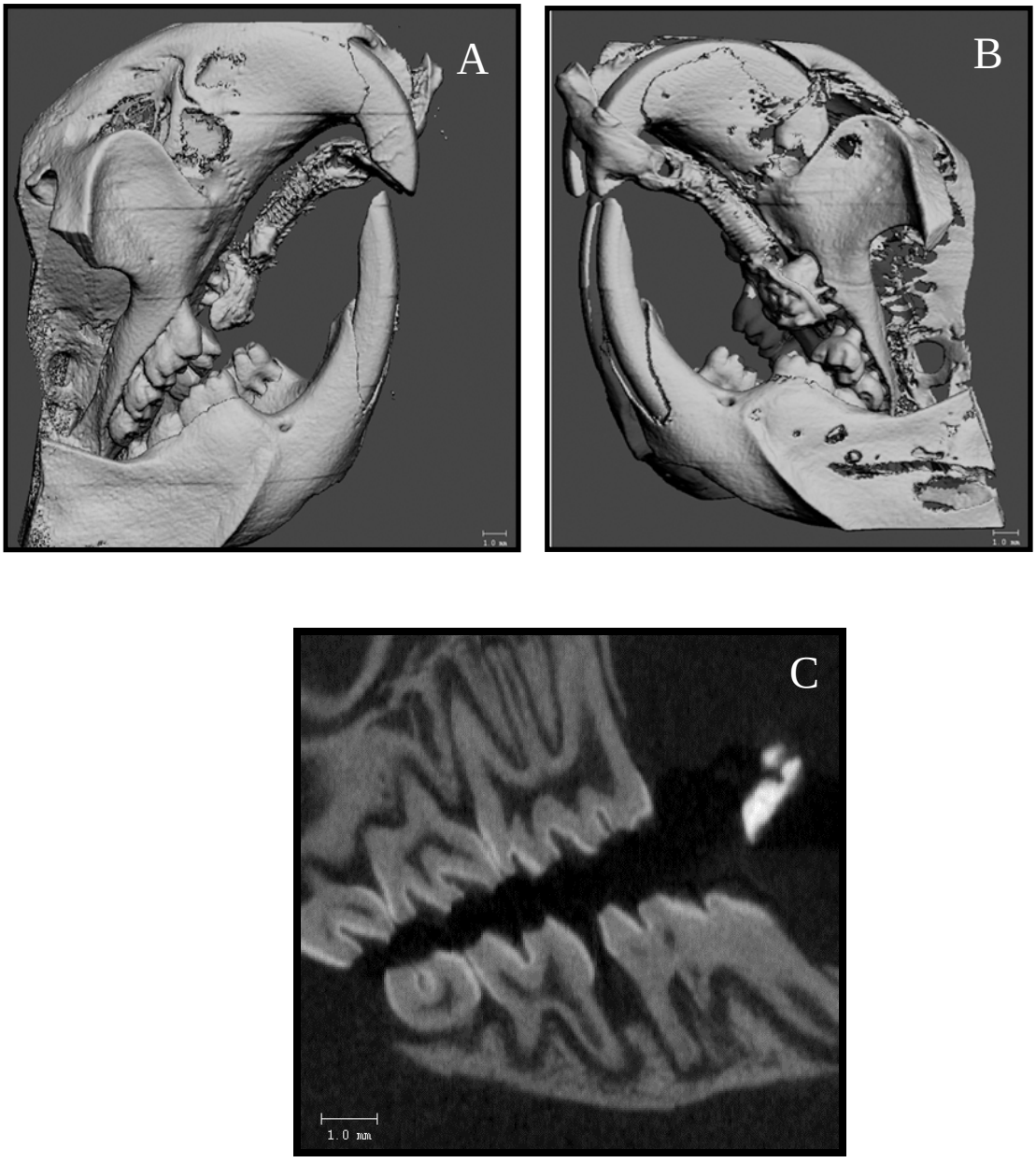

Figure 3: An example of micro-CT images of an *in-vivo* rat after 4 weeks of tooth movment. **A.** 3D reconstructed image of the control, unloaded side. **B.** 3D reconstructed image of the experimental, loaded side. **C.** 2D section used for intermolar measurements.

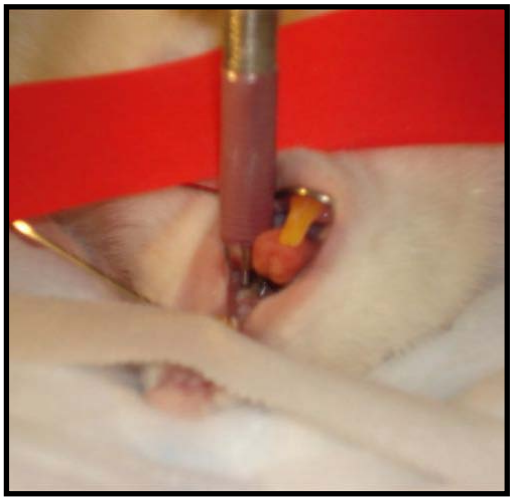

Figure 4: *In vivo* rat undergoing CLMF: A feedback loop, controlled, electromechanical actuator is used to apply unilateral CLMF to the maxillary left first molar of the rat.

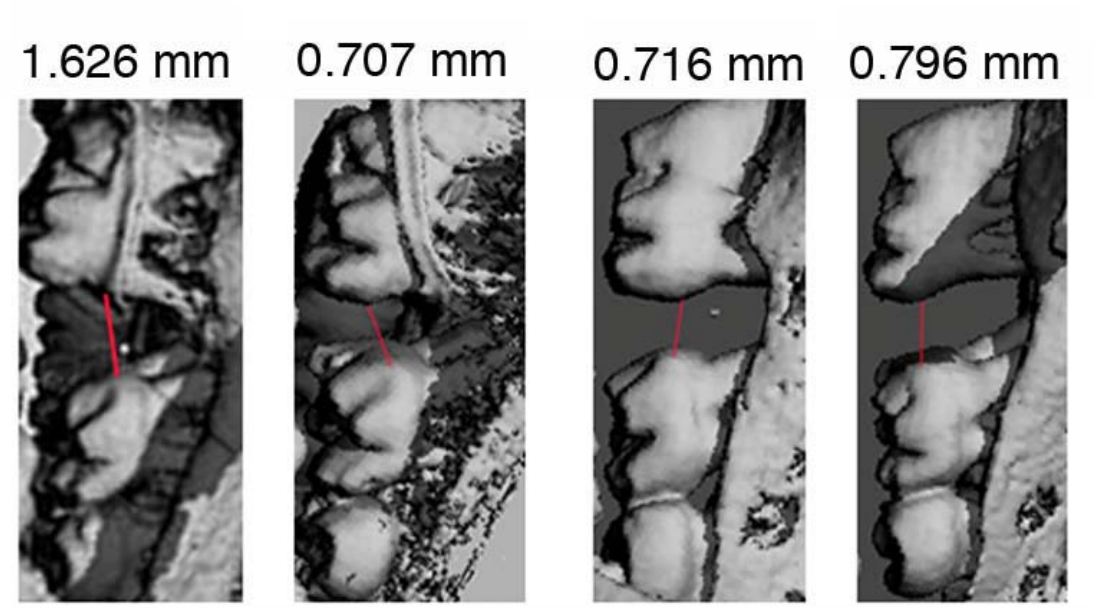

Figure 5: micro-CT with different spring force values and distance between 1st and 2nd molars after 4 weeks of orthodontic tooth movement.

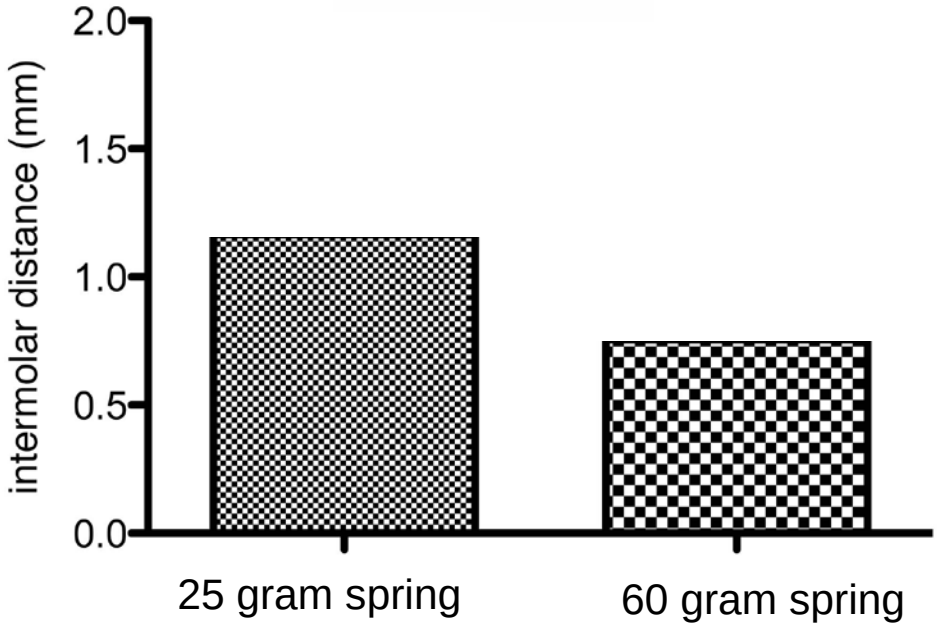

Figure 6: Graph with different spring force values and distance between 1st and 2nd molars after 4 weeks of orthodontic tooth movement. Spring force was not significant for the amount of tooth movement achieved after 4 weeks (P= 0.4674).

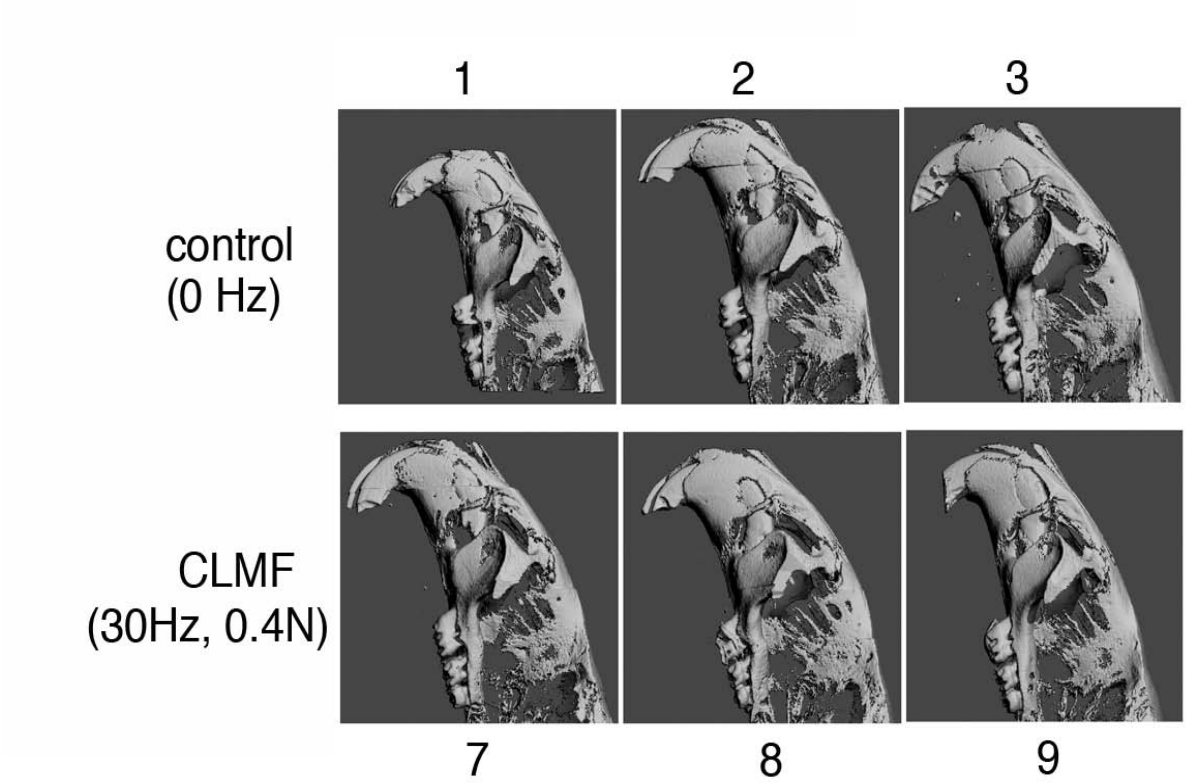

Figure 7: micro-CT comparing control rats, that received an orthodontic appliance only, and experimental rats, that received the orthodontic appliance and CLMF at 30 Hz, 0.4N, two times per week, for 10 minutes.

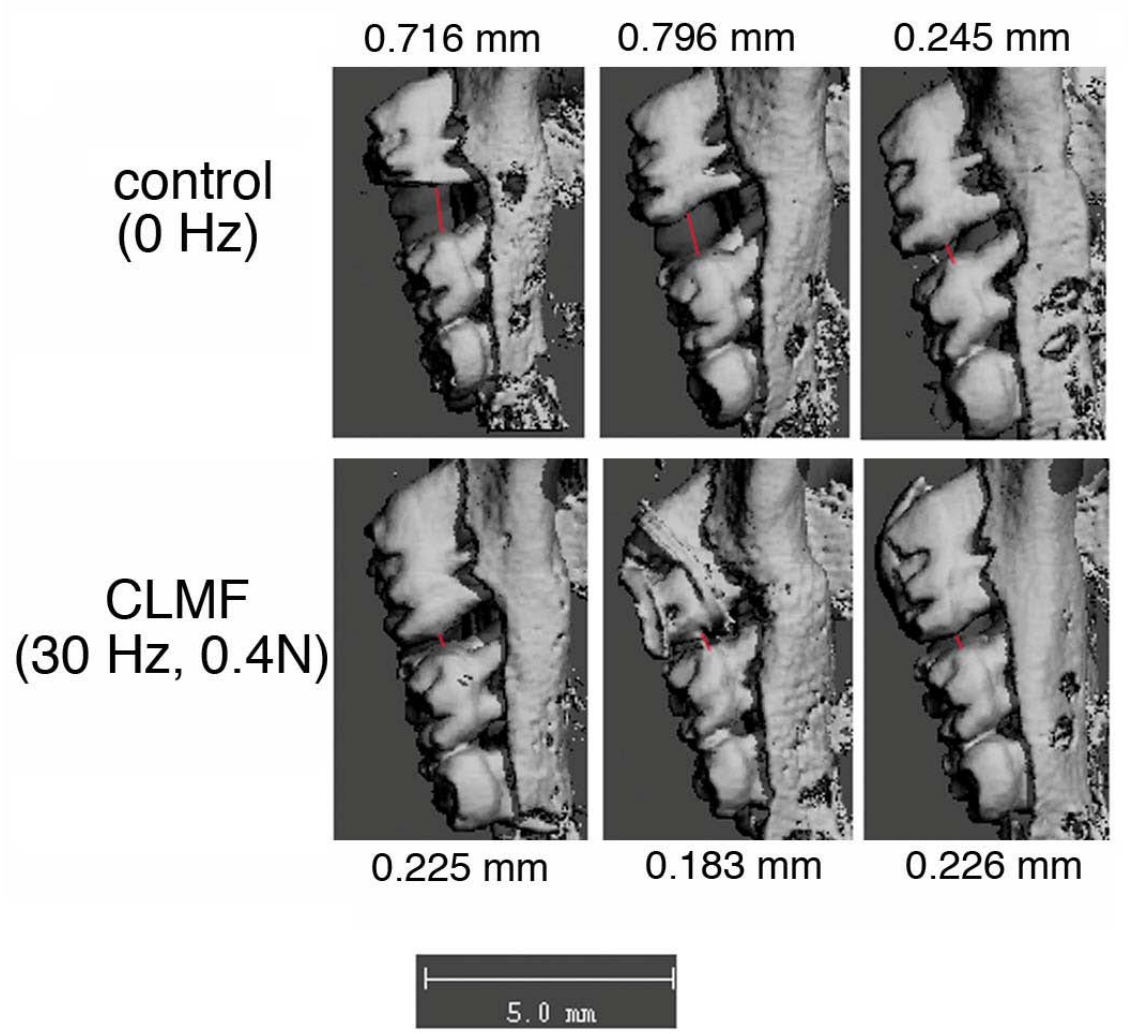

Figure 8: Intermolar measurements (M1-M2) comparing control with orthodontic appliance versus orthodontic appliance and CLMF at 30 Hz, 0.4N, two times per week, for 10 minutes.

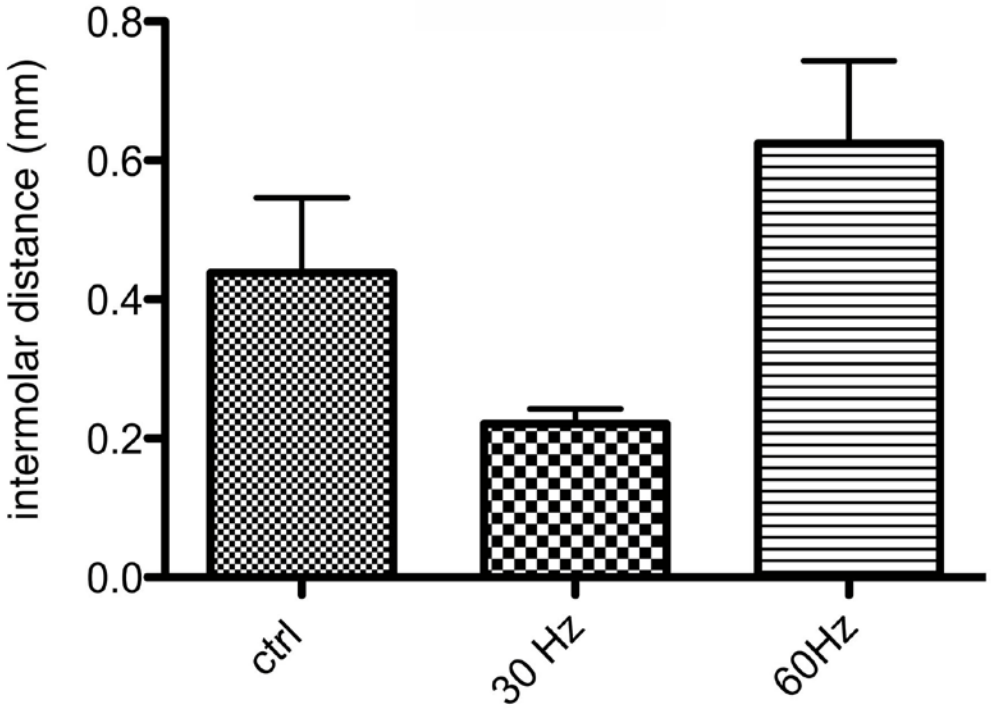

Figure 9: Graph demonstrating that CLMF (30 Hz, 0.4N, two times per week, for 10 minutes) decreases orthodontic tooth movement in rats by 37.45%, while CLMF (60 Hz, 0.4N, two times per week, for 10 minutes) increases orthodontic tooth movement in rats by 33.3%. Comparison of the controls to both 30 Hz and 60 Hz CLMF was not significant (P value of 0.0957, and 0.1116, respectively). However, comparison of 30 Hz to 60 Hz CLMF was significant (P= 0.05).

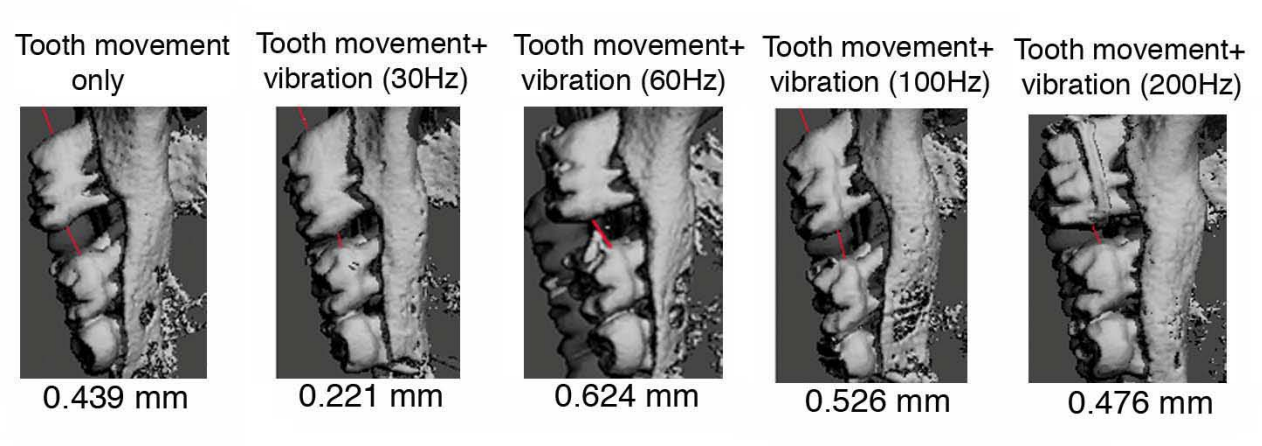

Figure 10: Average intermolar measurements (M1-M2) comparing control with orthodontic appliance only versus orthodontic appliance and dose frequencies of CLMF at 30 Hz, 60 Hz, 100 Hz, and 200 HZ (0.4N, two times per week, for 10 minutes for 4 weeks).

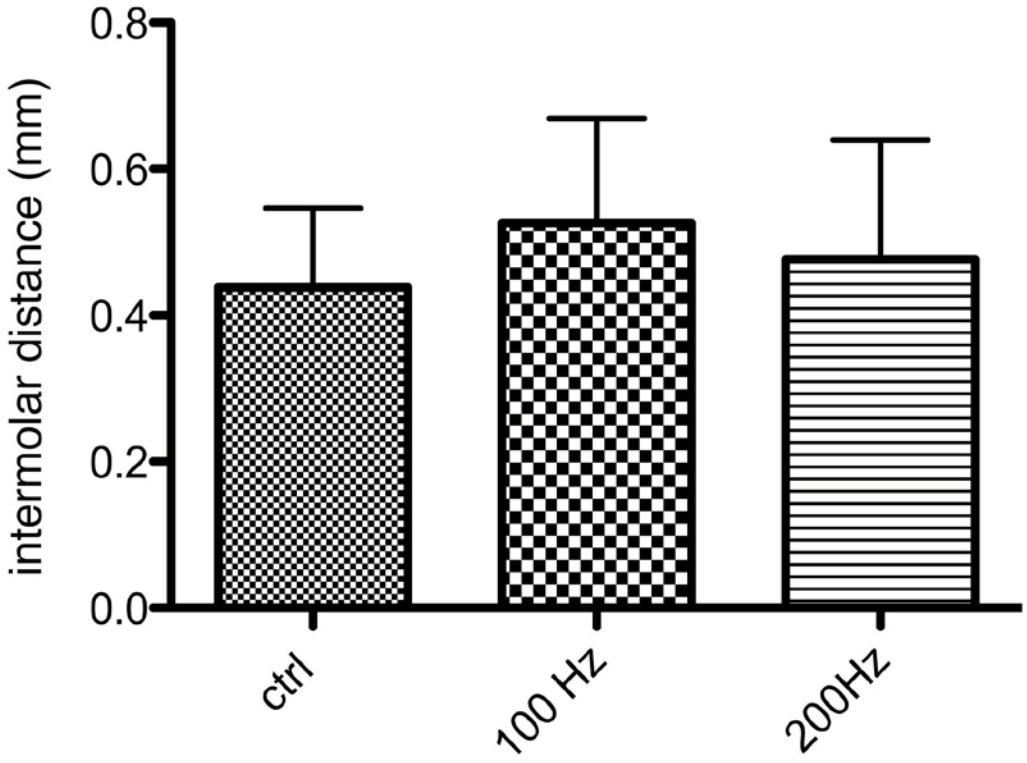

Figure 11: Graph of inter-molar distances for tooth movement in rats with CLMF (100 and 200 Hz, 0.4N) and the control (tooth movement only). Individual non-parametric, unpaired t-tests determined there was no significant difference in the dose frequency for CLMF.

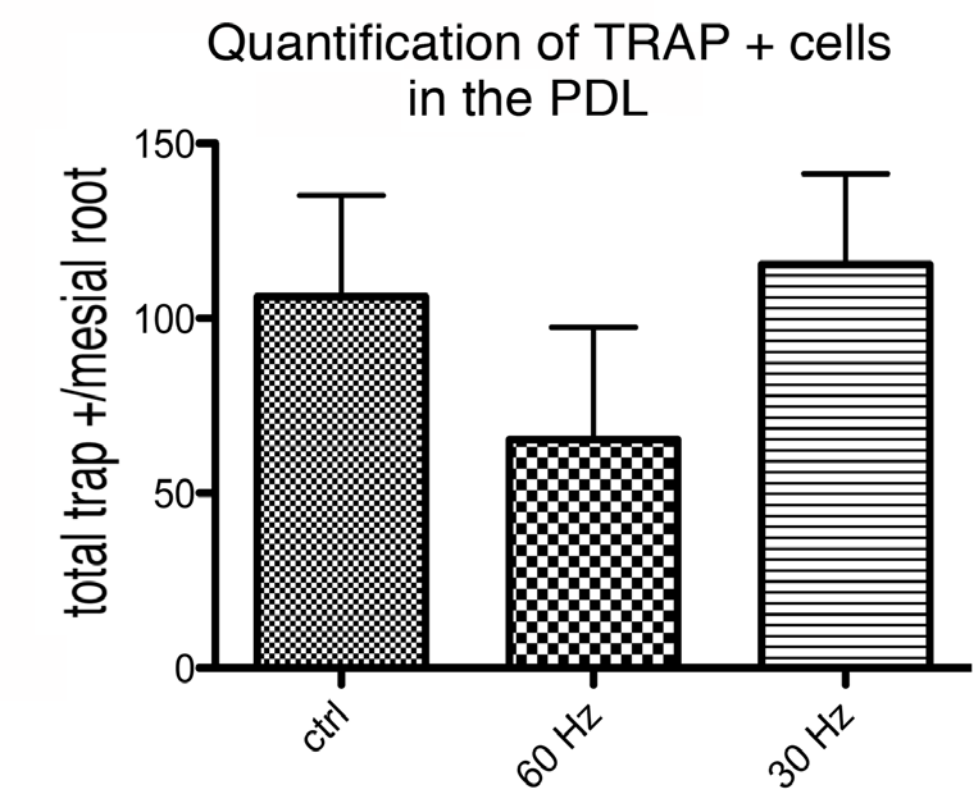

Figure 12: Graph of the quantification of ostoclasts in controls and CLMF (30 Hz and 60Hz, 0.4N, 4 weeks). Individual non-parametric, unpaired t-tests determined there was no significant difference in the number of osteoclasts.

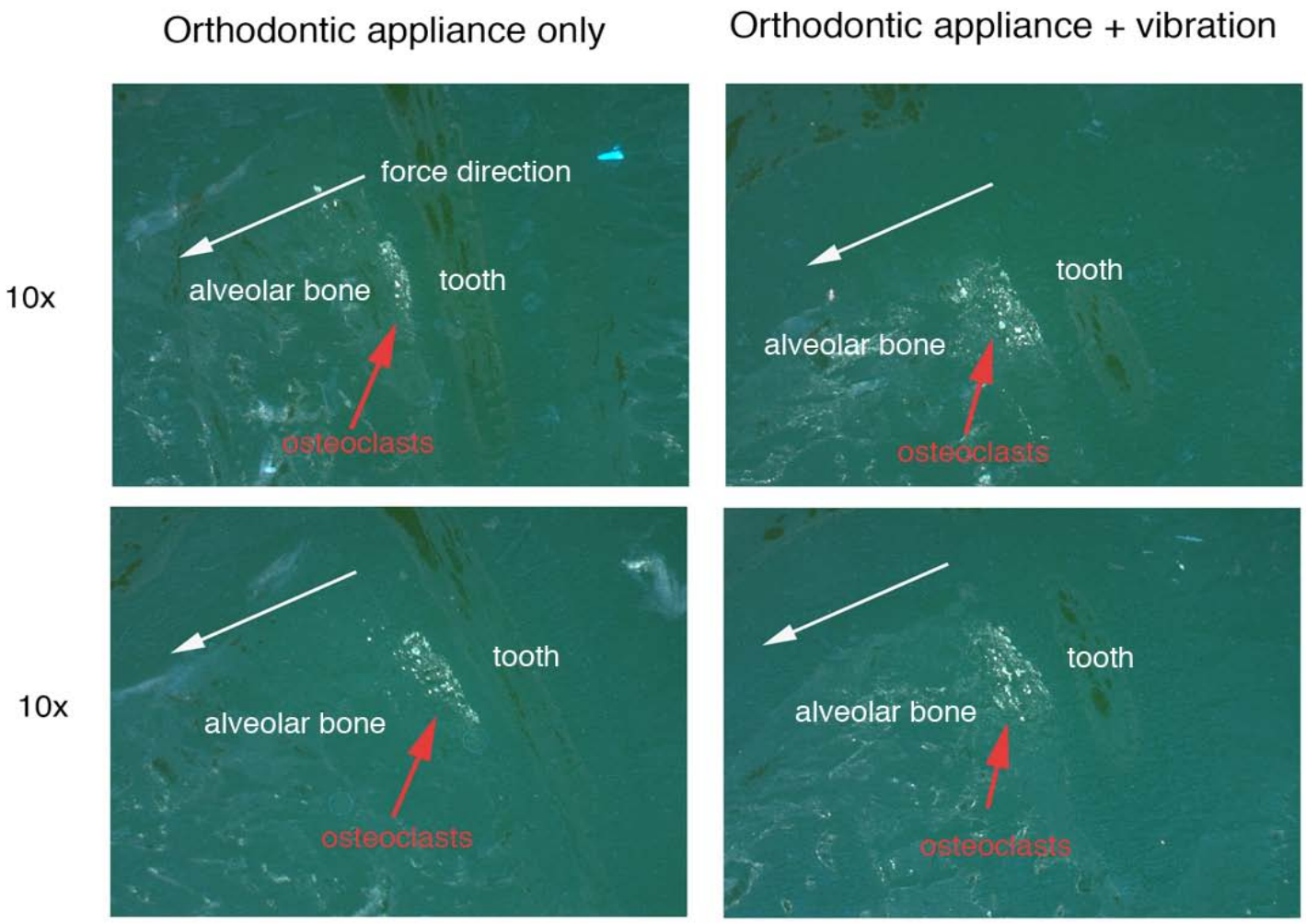

Figure 13: TRAP staining showing no significant difference in the number of osteoclasts comparing controls vs. CLMF (30 Hz, 0.4N, at 4 weeks).

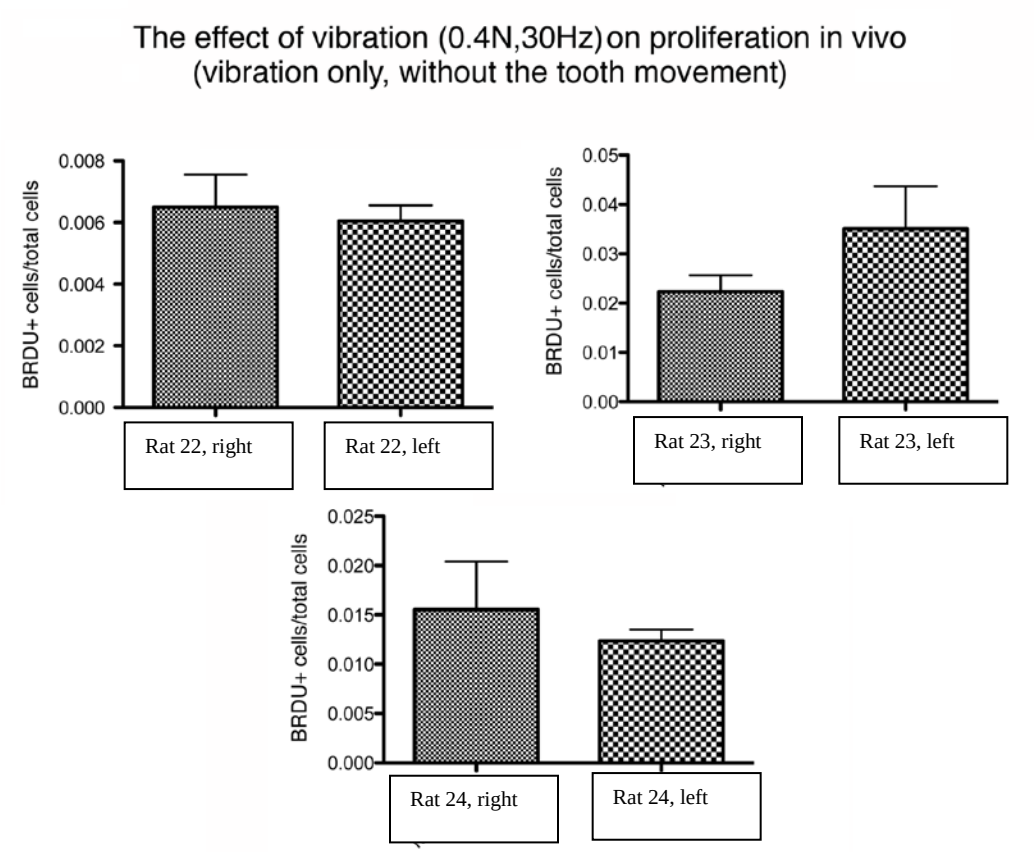

Figure 14: Graph of the proliferation of PDL cells in rats with CLMF only (30 Hz and 60Hz, 0.4N, 4 weeks). BrdU was used to compare the control (right side, non-CLMF) vs. CLMF (left side). Individual non-parametric, unpaired t-tests determined there was no significant difference in the number of proliferating PDL cells between the two sides.

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