The Effect of Mechanical Vibration on Alveolar Bone Following Experimental Periodontitis - A Micro-CT Study

Andrei Dan Taut
Marquette University

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THE EFFECT OF MECHANICAL VIBRATION ON ALVEOLAR BONE FOLLOWING EXPERIMENTAL PERIODONTITIS – A MICRO-CT STUDY

by

Andrei D. Taut, D.D.S.

A Thesis submitted to the Faculty of the Graduate School, Marquette University, in Partial Fulfillment of the Requirements for the Degree of Master of Science

Milwaukee, Wisconsin

August 2019
ABSTRACT

THE EFFECT OF MECHANICAL VIBRATION ON ALVEOLAR BONE FOLLOWING EXPERIMENTAL PERIODONTITIS – A MICRO-CT STUDY

Andrei D. Taut, D.D.S.

Marquette University, 2019

Introduction: Vibration in the form of high frequency, low magnitude acceleration has paradoxical effects on craniofacial bones – with anabolic effects under physiological conditions and catabolic effects in the presence of inflammation. The objectives of this study are to establish a murine model for periodontitis and to investigate the effects of high frequency, low magnitude mechanical vibration on alveolar bone following ligature-induced experimental periodontitis.

Materials and Methods: Ninety-five 11-week-old inbred strain C57BL/6J male mice were randomly assigned into four groups: 1) healthy control (n = 9); 2) healthy + mechanical vibration (n = 8); 3) experimental periodontitis + no treatment (n=7); and 4) experimental periodontitis + vibration (n = 9). All mice in the disease groups had ligature-induced experimental periodontitis induced for 8 days to generate localized alveolar bone loss. In mechanical vibration treatment groups, the mice received high frequency mechanical vibration (60 Hz, 0.3g) for 5min/day on the maxillary right first molar for consecutive 7 and 21 days respectively to determine the effects on alveolar bone following experimental periodontitis. Micro computed tomography (micro-CT) was used to quantify new bone formation through bone volume fraction (BVF), bone mineral density (BMD), and crestal bone heights post treatment with or without mechanical vibration. For each of the parameters, one-way ANOVA was used to determine a statistically significant difference among the 5 experimental groups, with Bonferroni comparison being used to measure significance between each 2 groups (p < 0.05 is considered significant).

Results: Experimental periodontitis resulted in significant reductions in BVF, TMD and alveolar bone height compared to healthy controls. Treatment with mechanical vibration for 21 days led to a non-significant, local anabolic effect, but also resulted in statistically significant decreases in BVF and TMD of alveolar bone adjacent to the site of application of mechanical vibration.

Conclusion: Mechanical vibration (60Hz, 0.3g, 5min/day) modestly increased bone volume and density when applied directly to the tooth, indicating a potential clinical application for improving bone quantity and quality following periodontitis.
ACKNOWLEDGEMENTS

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CHAPTER I – INTRODUCTION

To manage periodontal diseases, the combination of surgical and pharmaceutical treatments for the regeneration of alveolar bone have been suggested, but are often invasive, costly, and are limited to small regions of bone loss. Thus, a significant demand exists for an effective, non-invasive, and safe treatment for alveolar bone loss in order to address this emerging public health concern and help maintain the overall health and well-being of an aging population.

In the United States, one out of every two adults age 30 or older – 64.7 million American adults - has periodontal diseases. In adults 65 and older, the prevalence increases to 70.1 percent (Eke et al., 2012). Periodontal disease is a chronic inflammatory disease which affects gum tissue and alveolar tooth-supporting bone, which if left untreated, can lead to tooth loss and have an adverse impact on oral health in clinical dentistry, including the stability of removable prostheses and success of dental implants (Alikhani et al., 2012). Periodontitis also exerts an adverse impact on systemic health and has been associated with other chronic diseases such as diabetes and cardiovascular diseases (Eke et al., 2012).

Many surgical techniques used in treating bone loss in periodontitis, such as implantation of various types of bone graft and/or bone substitutes, among others, have been shown to be effective for treatment of alveolar bone loss, but are also expensive, invasive and associated with significant morbidity, especially in older patients. Studies have shown that dynamic loading via low intensity, high frequency vibration induce therapeutic musculoskeletal anabolic responses in human and animal studies, leading to improved bone strength, fracture healing, and even improved wound healing. The
positive anabolic effects of exercise and loading on weight-bearing bones have been demonstrated (Honda, Umemura, & Nagasawa, 2001). Furthermore, high frequency mechanical vibration also activates periodontal tissues and has anabolic effects on bone mass and architecture. Animal and human studies demonstrate that high-frequency, low-magnitude vibration improves bone volume and density by increasing bone formation and decreasing bone resorption (Alikhani et al., 2018; Alikhani et al., 2012; Teixeira et al., 2010). Furthermore, vibration promotes mesenchymal stem cell differentiation and MSC commitment to the osteogenic lineage and restricts MSC adipogenic commitment (Thompson, Yen, & Rubin, 2014). However, no knowledge is available on the effects of localized mechanical vibration on the regeneration of alveolar bone in periodontitis. The aims of this study are to 1) establish a mouse periodontitis model and to 2) investigate the effects of high frequency, low magnitude mechanical vibration on alveolar bone following ligature-induced experimental periodontitis.
CHAPTER II – LITERATURE REVIEW

PERIODONTAL BONE LOSS

Periodontal disease leads to the progressive, irreversible breakdown of essential tooth-supporting oral tissues, including the periodontal ligament (PDL), tooth root surface cementum, alveolar bone, and gingiva. The bacterial etiology of periodontal disease has been well established in both animal models and human clinical studies, with its pathogenesis being initiated and propagated by groups of periodontal pathogens – predominantly Gram-negative anaerobes in chronic lesions – found in the bacterial biofilm clinically known as dental plaque (Hasan & Palmer, 2014) – and associated virulence factors such as lipopolysaccharide (LPS) (Hasan & Palmer, 2014).

Periodontal pathogens cause both direct and indirect destruction of soft and hard tissues. Direct action of bacteria include damage to crevicular epithelium, secretion of leukotoxin for impairment and/or destruction of leukocytes and polymorphonuclear leukocytes (PMNs), degradation of immunoglobulins, breakdown of protein and non-protein components of the extracellular matrix of periodontal tissues, activation of complement by endotoxin (LPS), and stimulation of bone resorption via the action of endotoxin and lipoteichoic acid from both Gram-negative and Gram-positive periodontal pathogens respectively (Hasan & Palmer, 2014). A host of pro-inflammatory molecules, such as lipid-based mediators, cytokines, and chemokines, are upregulated in periodontal tissues in response to the periodontal pathogens. Higher concentrations of lipid-based mediators, including prostaglandins and leukotrienes, have been measured in gingival-crevicular fluid of patients with periodontal disease. Furthermore, human clinical trials have demonstrated decreased periodontal tissue destruction in the presence of
cyclooxygenase inhibitors, highlighting the importance of prostaglandins and leukotrienes in the early disease progression process (Graves, Li, & Cochran, 2011).

In advanced periodontal lesions, indirect damage and further disease progression is believed to develop via the modulation of host response. More specifically, activation of complement and additional inflammatory responses by periodontal pathogens subsequently results in the recruitment and activation of B cells and T cells, which in turn release their own tissue destructive enzymes (Graves et al., 2011). The actions of LPS – an example of a B-cell mitogen – prevents the adaptive immune responses from secreting specific antibodies that would focus against LPS antigens and lead to protection. Instead, LPS leads to polyclonal activation of B cells and non-specific antibody production occurs. The inflammatory cascade initiated by periodontal pathogens also results in the activation of T cells, antigen-presenting cells and macrophages, which release the cytokines IL-1β, TNF-α, and IL-6 to produce cytotoxic responses in periodontal tissues. Finally, PMNs are stimulated to secrete enzymes known as matrix metalloproteinases that destroy host tissues rather than the periodontal pathogens present in the dental plaque (Hasan & Palmer, 2014). IL-1β and TNF-α both stimulate bone resorption and possess further pro-inflammatory actions. IL-6 leads to B-cell differentiation, polyclonal antibody production, and osteoclast differentiation. The destructive potential of periodontal disease can be exacerbated by a myriad of risk factors including inherited host factors, lifestyle, and environmental exposure such as age, systemic disease, genetics, stress, and trauma (Lindhe, Lang, & Karring, 2008).

Under physiologic conditions, bone – including alveolar bone - in the human skeleton is constantly undergoing equal amounts of bone formation and bone resorption. This coupled nature of physiologic bone resorption and formation is disrupted, and the
balance shifts towards increased bone resorption, when periodontal pathogen-induced inflammatory responses are activated deep to the sub-epithelial space and in the vicinity of alveolar bone (Hasan & Palmer, 2014).

**RECONSTRUCTIVE PERIODONTAL THERAPIES**

Non-surgical periodontal therapy has generally been utilized to successfully treat and prevent future disease progression in patients with moderate periodontitis. In cases of severe periodontitis, surgical practices such as, gingivectomy, apical repositioning of raised tissue flaps, and bone contouring, must also be implemented to treat and prevent future disease recurrence (Lindhe et al., 2008). In addition, regenerative procedures can be used in combination with surgical periodontal therapy to restore or regenerate lost periodontal tissues. Regeneration of soft and hard tissues of the periodontium is the ideal treatment modality following destruction of alveolar bone and soft tissues – and serves to improve the esthetics, function and long-term prognosis of the involved teeth. The purpose(s) of reconstructive therapies fall into one or more of the following categories: (1) to regenerate the missing regions of the periodontal ligament (PDL), (2) to increase the amount of attached gingiva, and (3) to increase coverage of previously exposed roots (Floyd, Ide, & Palmer, 2014). According to the American Academy of Periodontology, regeneration is defined as the “reproduction or reconstitution of a lost or injured part in a manner similar or identical to its original form. In periodontics, refers to the formation of new bone, cementum, and a functionally-oriented periodontal ligament at a site deprived of its original attachment apparatus” (American Academy of Periodontology, 2001). Although the soft and hard tissues of the periodontium are intimately linked, the remainder of this section will focus on therapeutic strategies for regeneration of alveolar
bone – and the periodontal ligament - only, in order to provide a sufficient amount of background which is most relevant to the scope of the research study.

Alveolar bone deficiencies in the oral cavity have traditionally been treated surgically using autologous bone grafting techniques. The assumption with regenerative periodontal surgery is that the presence of newly-formed bone will not only provide structural support for the periodontally compromised tooth, but ultimately allow for the differentiation of progenitor cells within bone and the remaining periodontal ligament to form a new cementum layer and collagen fiber/periodontal ligament on the exposed tooth root surfaces (Lindhe et al., 2008).

The limitations of autologous bone grafting techniques are significant and include limited availability of autograft, donor site complications, and prolonged surgical procedures (Silva, Cortez, Moreira, & Mazzonetto, 2006). More recently, bone tissue engineering approaches have also been investigated in order to circumvent the limitations of autologous bone grafting procedures. Tissue engineering treatment approaches involve the utilization of a triad of components - scaffold materials, growth factors/gene delivery, and progenitor cells, as evident in Figure 1 (Lindhe, Lang, Berglundh, Giannobile, & Sanz; Pilipchuk et al., 2015).
The purpose of a scaffold in tissue engineering and periodontal regeneration is to act as a compartment that promotes cell attachment, migration, proliferation and three-dimensional structural organization of cells. Several characteristics of the scaffold must be carefully controlled for optimal results. Scaffolds used for periodontal regeneration and tissue engineering must be biocompatible, biodegradable, have suitable mechanical
properties and ideal architecture, and have the potential to induce, or at least sustain, the cells required for ongoing periodontal regeneration (Dellatore, Garcia, & Miller, 2008).

The availability of the necessary cell types and the presence or absence of signals required to recruit and stimulate these cells are both essential for periodontal tissue regeneration to occur, and are the distinguishing factors between wound healing via scar tissue and true tissue regeneration (Lindhe et al., 2008). In advanced tissue engineering techniques, the desired cell types and/or the signaling molecules can be seeded onto the scaffold prior to delivery. Customized, three-dimensionally printed, hybrid scaffolds seeded with human PDL cells have been shown to result in the regeneration of both alveolar bone and PDL in a rodent fenestration model, but the results of this and other preclinical studies have not been predictable clinically (Park et al., 2014). Autologous bone marrow stromal cells, periodontal ligament cells, periodontal ligament stem cells, and dental follicle cells have shown potential in regenerating alveolar bone and connective tissue in vivo, but significant challenges remain (Hasegawa et al., 2006; H. Li, Yan, Lei, Li, & Xiao, 2009; Liu et al., 2019; Rios, Lin, Oh, Park, & Giannobile, 2011). Some of the limitations in the success of current tissue engineering strategies seem to be related to the large size of bone defects and will require further collaboration between biomaterial scientists and clinical personnel.

**MURINE MODEL FOR PERIODONTITIS**

Experimental animal models are essential for studying the mechanisms underlying periodontal pathology and new strategies for treatment of the disease (Abe & Hajishengallis, 2013). Every animal model has its advantages and disadvantages, and although no perfect simulation of the pathophysiology of human periodontal disease
exists, mouse periodontitis models offer a cost effective, easily standardized method for testing novel therapeutic strategies for periodontal regeneration (Abe & Hajishengallis, 2013; Graves, Fine, Teng, Van Dyke, & Hajishengallis, 2008; Gyurko et al., 2006). More specifically, the ligature-induced model offers the important advantage of being able to initiate disease at a known time with rapid progression resulting in alveolar bone loss within a few days in mice and rats. Studies have demonstrated that the placement of ligatures – silk or cotton – in the gingival sulcus interproximally, or around the molar teeth, disrupts the gingival epithelium and increases biofilm accumulation to elicit an inflammatory response that enhances osteoclast formation and ultimately results in significant horizontal bone loss (Bezerra et al., 2000; C. H. Li & Amar, 2007; Marchesan et al., 2018; Oz & Puleo, 2011; Taut et al., 2013). However, not all rodents respond similarly to the physical and inflammatory insult of ligature placement. The inherent susceptibility to periodontal bone loss and the regenerative potential vary significantly with the age, sex, and strain of animal.

The C57BL/67 strain of mouse has been demonstrated to be highly susceptible to LPS-induced periodontal bone loss, despite its low incidence of naturally occurring periodontal disease (Hiyari et al., 2015; Saadi-Thiers et al., 2013). Similarities have also been shown between the local and systemic inflammatory responses of humans and experimental periodontitis mouse models. C57BL/67 mice with ligature-induced periodontitis displayed elevated serum levels of IL-6 during the early, acute phase of periodontal disease, as well as elevated serum IL-1β that correlated to both increased osteoclastic activity and bone loss as has been shown in humans. Finally, expression patterns of proteases MMP-9 and Cathepsin B within the gingival inflammatory response also closely matched patterns evident in human periodontitis (Saadi-Thiers et al., 2013).
In contrast to human subjects, female C57BL/67 mice have been shown to be more susceptible to ligature-induced periodontal bone loss compared to males, with significantly greater linear bone loss, pro-inflammatory cytokine production, and presence of oral bacteria (Duan et al., 2016). Regardless, male C57BL/67 mice are still utilized to avoid the confounding effects of estrogen (Saadi-Thiers et al., 2013; Wehrle et al., 2015).

**CLINICAL APPLICATIONS OF VIBRATION THERAPY**

Vibration therapy has been investigated as a potential non-invasive, non-pharmacological therapy for improving bone quantity and quality in susceptible individuals, as well as for preserving bone health in non-pathological conditions. High frequency, low magnitude vibration therapy (frequency >30 Hz, magnitude/intensity <1 g where g = acceleration of 9.81 m/s²) is believed to mimic the anabolic effects of dynamic loading on bone and muscle tissue that occurs as a result of small persistent postural muscle contractions (high-frequency, low-magnitude signals) throughout the day (Thompson et al., 2014). Evidence exists that this type of muscle activity may be more critical than high impact activity in preserving bone mass and facilitating repair (Edwards & Reilly, 2015; Rubin, Judex, & Qin, 2006). In contrast, dynamic high-intensity vibration therapy and static loads can result in tissue disruption and even induce bone resorption, respectively. It is also well understood that the prolonged absence of functional mechanical loading results in deleterious effects on bone, as well as the rest of the neuromuscular system.

In the absence of inflammation or pathology, high frequency mechanical vibration (HFMV) in the form of whole-body vibration therapy has demonstrated success in the treatment of preclinical models for osteoporosis, osteogenesis imperfecta, and fracture
healing by improving bone structure and augmenting healing processes (Alikhani et al., 2018; Edwards & Reilly, 2015). At a cellular level, HFMV seems to directly activate bone formation pathways, while down-regulating bone resorption, especially in the absence of inflammation (Zhou et al., 2015). On a molecular level, HFMV has been shown to stimulate osteogenic intracellular mechanotransduction pathway signaling to regulate gene expression, downstream protein synthesis and growth factor release in several mature cell types, including osteoblasts, osteocytes, and periodontal ligament fibroblasts (Alikhani et al., 2016; Benjakul, Leethanakul, & Jitpukdeebodintra, 2019; Edwards & Reilly, 2015; Moustafa et al., 2012). HFMV has also been shown to enhance vascularization and accelerate soft and hard tissue wound healing by stimulating an increase in the pro-angiogenic growth factors IGF-1 and VEGF (Matsumoto & Goto, 2017; Weinheimer-Haus, Judex, Ennis, & Koh, 2014). In addition to its favorable effects on mature tissue cells, HFMV also modulates stem cell proliferation and differentiation in progenitor cell lineages, including in mesenchymal and periodontal ligament stem cells (Edwards & Reilly, 2015; Zhang et al., 2015). Mesenchymal stem cells (MSCs) are multipotent stem cells capable of differentiating into a number of mature cell types – osteoblasts, chondrocytes, adipocytes, and myocytes. In a diet-induced obesity mouse model, HFMV applied at 90 Hz with 0.2g for 15 minutes per day/5 days per week for 6 weeks not only resulted in increased MSC numbers, but also increased MSC commitment to the osteogenic lineage, while reducing adipogenic differentiation compared to controls (Luu et al., 2009).
In the craniofacial region, HFMV has been most widely studied as a potential therapy for accelerating orthodontic tooth movement. The findings have been controversial, primarily because early research has demonstrated that accelerated tooth movement may occur due to an increase in bone breakdown when HFMV is applied concurrently with orthodontic tooth movement. Furthermore, other studies demonstrate no effect on the rate of orthodontic tooth movement and even enhanced bone formation and bone density in alveolar bone (Alikhani et al., 2012; Kalajzic et al., 2014; Yadav et al., 2015). The paradoxical effects of mechanical vibration on craniofacial alveolar bone, have been explained by several key differences between the circumstances and application of whole-body vibration on long bone and localized HFMV on alveolar bone. First, the endochondral embryologic origin of long bones permits increased weight-bearing capacity and adaptation under heavy dynamic loads, while tooth-supporting alveolar bone, of intramembranous origin, is considered non-weight bearing and is infrequently exposed to the same heavy dynamic loads (Alikhani, Sangsuwon, Alansari, Nervina, & Teixeira, 2017). Despite these differences in origin and structure, anabolic responses have been observed in both healthy alveolar bone and alveolar bone of osteoporotic rats with localized HFMV (Alikhani et al., 2019; Alikhani et al., 2012; Alikhani et al., 2016).

Second, the physiologic conditions under which a bone anabolic response is demonstrated, differ from conditions related to orthodontic tooth movement – the first scenario being characterized by a subdued pro-inflammatory environment, while a significant increase in pro-inflammatory mediators is usually associated with orthodontic tooth movement (Alikhani et al., 2018). Recent in vitro and in vivo studies have
demonstrated that mechanical vibration and compressive forces act synergistically on the expression of pro-inflammatory molecules Prostaglandin E2 (PGE$_2$), IL-6, and RANKL in human periodontal ligament cells and RANKL in osteocytes (indirectly) to enhance alveolar bone resorption (Benjakul et al., 2019; Phusuntornsakul, Jitpukdeebodintra, Pivasant, & Leethanakul, 2018; Sakamoto et al., 2019). Furthermore, vibration applied during experimental tooth movement also increases RANKL expression in vivo on the compression side only, compared to orthodontic tooth movement alone (Sakamoto et al., 2019). Together, the studies allow us to hypothesize that the application of force with HFMV leads to bone breakdown in the presence of active, elevated inflammation, by worsening inflammatory responses, delaying healing, and enhancing bone resorption (Alikhani et al., 2018). Table 1 provides a summary of the conditions under which HFMV elicits anabolic and catabolic responses in alveolar bone.

<table>
<thead>
<tr>
<th>Anabolic effect</th>
<th>Catabolic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method of application</td>
<td></td>
</tr>
<tr>
<td>Directly on teeth in the target area, or</td>
<td>Directly on tooth or teeth that are moving</td>
</tr>
<tr>
<td>Indirectly on adjacent teeth close to the target area</td>
<td></td>
</tr>
<tr>
<td>Initial state of tissue</td>
<td></td>
</tr>
<tr>
<td>Physiologic condition</td>
<td>Inflammatory condition</td>
</tr>
<tr>
<td>Target tissue</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>Periodontal ligament</td>
</tr>
<tr>
<td>Responding cells</td>
<td></td>
</tr>
<tr>
<td>Osteocytes</td>
<td>Osteoclasts</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td></td>
</tr>
<tr>
<td>Resulting effect</td>
<td></td>
</tr>
<tr>
<td>Bone formation</td>
<td>Bone resorption</td>
</tr>
<tr>
<td>Load-independent</td>
<td>Load-dependent</td>
</tr>
<tr>
<td>Extension of effect</td>
<td></td>
</tr>
<tr>
<td>Gradient effect with highest response on bone surrounding target tooth and extending to adjacent bone</td>
<td>No gradient effect, effective only on target tooth exposed to orthodontic forces with no effect on adjacent teeth</td>
</tr>
<tr>
<td>Potential clinical uses</td>
<td></td>
</tr>
<tr>
<td>Preservation of alveolar bone after extractions</td>
<td>Accelerated tooth movement</td>
</tr>
<tr>
<td>Bone regeneration after periodontal disease</td>
<td>Increase in magnitude of movement (distance)</td>
</tr>
<tr>
<td>Enhance Implant and graft integration</td>
<td>Differential anchorage</td>
</tr>
<tr>
<td>Increased bone formation after Orthopedic treatment</td>
<td>Increase in magnitude of Orthopedic correction</td>
</tr>
<tr>
<td>Improved retention after Orthodontic treatment</td>
<td>Reduced bone density around target tooth to facilitate different types of tooth movement</td>
</tr>
<tr>
<td>Increased bone formation after Orthognathiic Surgery</td>
<td>Reduced necrotic (hyalinized) area in response to static Orthodontic forces</td>
</tr>
<tr>
<td></td>
<td>Possible increased frontal resorption</td>
</tr>
</tbody>
</table>

**Table 1. Summary of the anabolic and catabolic effect of HFA in orthodontic treatment.**

“Vibration paradox in orthodontics: Anabolic and catabolic effects.” By Alikhani et al. PLOS ONE 2018 May 7;13(5); permission conveyed by and licensed under CC BY 2.0.
STUDY AIMS

To establish a mouse periodontitis model and investigate the effects of mechanical vibration on alveolar bone following experimental periodontitis in mice.
CHAPTER III – MATERIALS AND METHODS

ANIMAL MODEL AND STUDY PROTOCOL

Adult male C57BL/6J (n = 95, average weight 21–26 g, 11 weeks old) were housed and treated according to a protocol conforming to ARRIVE (Animal Research Reporting of the In Vivo Experiments) guidelines and approved by the Marquette University Institutional Animal Care and Use Committee (IACUC). The timeline of the experiment is detailed in Figure 2. Two weeks following arrival at Marquette University, animals were divided into four groups: 1) Healthy Group that served as the control with no intervention, 2) Healthy + Vib group that received HFMV for 7 and 21 days (frequency = 60 Hz, acceleration = 0.3 g where ‘g’ represents the acceleration of gravity (1 g = 9.81 m/s²)) for 5 minutes per day, 3) Perio Group which had sterilized silk sutures/ligatures placed according to protocol below and received no intervention; and 4) Perio + Vib Group that received silk ligatures and HFMV for 7 and 21 days (frequency = 60 Hz, acceleration = 0.3 g where ‘g’ represents the acceleration of gravity (1 g = 9.81 m/s²)) for 5 minutes per day. The 5-minute daily duration of HFMV was chosen to remain consistent with previous internal and external experimental designs (Alikhani et al., 2019; Alikhani et al., 2018).

![Figure 2. Study Timeline](image)

**Figure 2. Study Timeline.** Study timeline began with 8 days of experimental periodontitis, followed by ligature removal and 7 and 21 days of HFMV treatment (HFMV treatment initiated 24 hours following removal of ligatures).
In accordance with the Recommended Best Practices for Mouse Anesthesia designed by the Marquette University’s Office of Research and Compliance (https://www.marquette.edu/orc/animal-care-use/documents/AnestheticsandAnalgesicsRodent2017.pdf), animals were anesthetized using isoflurane inhalation (Charles River Laboratories International, Inc.). Using the Simplified Ligature Model Materials – custom 3-D printed mouse dental bed and 3-D printed U-tipped ligature holder (Marchesan Lab, University of North Carolina Adams School of Dentistry, Chapel Hill, NC, USA) seen in Figure 3, silk sutures (5/0) were placed unilaterally into the interproximal gingival sulci of the right maxillary first and second molar teeth according to previously described protocol (Marchesan et al., 2018) to induce experimental periodontitis. Sutures were checked every other day to ensure their presence and were replaced as necessary. Experimental periodontitis was induced for a period of 8 days. Intact controls (Healthy) were not ligated and served as controls. Ligatures were removed at the end of the experimental periodontitis phase before HFMV treatment. HFMV treatment was initiated 24 hours following ligature removal to allow for the inflammatory response to subside.
Figure 3. “Tools and technical procedures required to set up the simplified ligature model in mice. a–h, The tools required. a, Mouse dental bed. b represents high magnification of a. c, U-tipped ligature holder (U-shaped for holding silk). d, Assembled U-tipped ligature holder. e represents high magnification of U-tipped ligature holder. f, The U-tipped holder with 5-0 silk suture. g, High-magnification view of the U-tipped holder, showing two knots in the inside of the forceps tips (~2.5-mm distance between knots). h, Experimental setup immediately before anesthetizing the mouse with isoflurane. i–p, The stages required to insert the ligature are shown as photos (i,k,m,o) and diagrammatically (j,l,n,p). i,j, The left hand is used to hold the dental explorer while the tip of the dental explorer and the 2.5-mm silk between the knots are carefully located in the gap between the first and second molars, using the U-tipped ligature holder held in the right hand. k,l, The suture is then pushed through the interdentium between the first and second molars. m,n, The silk is cut, and the U-tipped forceps are removed. o,p, Finally, the silk is trimmed at the end of the knot. Appropriate institutional regulatory board permission was obtained to carry out the experimental procedure on the mouse shown here.” Republished with permission of Springer Nature from “An Experimental Murine Model to Study Periodontitis,” by Marchesan J.T., et al, 2018, October, Nature Protocols, 13(10):2247-2267; permission conveyed through Copyright Clearance Center, Inc.
MECHANICAL VIBRATION APPLICATION AND FLUORESCENT BONE LABELING

In accordance with the Recommended Best Practices for Mouse Anesthesia designed by the Marquette University’s Office of Research and Compliance (https://www.marquette.edu/orc/animal-care-use/documents/AnestheticsandAnalgesicsRodent2017.pdf), animals were anesthetized using isoflurane inhalation (Charles River Laboratories International, Inc.) and unilateral mechanical vibration was conducted through an electromechanical actuator held in place by a custom apparatus as demonstrated in the diagrammatic representation in Figure 4. LabView Custom software (National Instruments, Austin, TX) was designed to communicate with the electromechanical actuator to produce the specific vibration frequencies. Vibration was conducted at 0.3g (acceleration), 20 micrometers of micro-vertical displacement, and 60 Hz frequency, for 5min/day for 7 days and 21 days. The 7-day experimental period for early assessment of the effects of HFMV on inflammation, as well as on osteogenic and bone resorptive signaling cascades. Previous studies demonstrated a statistically significant increase of alveolar bone starting at 14 days after initiation of HFMV and up to 56 days of HFMV, thus the 21-day experimental period was selected as the practical way to assess the long term effects of HFMV on alveolar bone following experimental periodontitis (Alikhani et al., 2016). During the 21-day experimental period, mice were given two fluorescent markers—Calcein (50 mg/kg body weight) and Alizarin Red (50 mg/kg body weight) – at days 7 and 14, by subcutaneous injection (total of 2 injections).
Figure 4. Diagram of Mechanical Vibration Set-Up. Republished with minor edits with permission of Elsevier Inc./International Bone and Mineral Society, from Mechanical Vibration Inhibits Osteoclast Formation by Reducing DC-STAMP Receptor Expression in Osteoclast Precursor Cells; Kulkarni RN, Voglewede PA, and Liu D., Volume 57, Issue 2, 2013; permission conveyed through Copyright Clearance Center, Inc.

MICRO-COMPUTED TOMOGRAPHY SCANNING AND BONE ANALYSIS

Maxillary bone tissue – the first, second, and third molars – were collected at the designated end points, placed in 10% neutral-buffered formalin for 2 days, and transferred to 70% ethanol (EtOH) for microCT scanning. Formalin-fixed maxillae were subjected to micro-computed tomography (CT) image analysis. The specimens were scanned in all three spatial planes at a resolution of 8 x 8 x 8 μm (μCT40, Scanco Medical, Brüttisellen, Switzerland) as previously described (Park et al., 2007). Peak
voltage was set to 55 kVp. To assess alveolar bone loss, the distance between the cementoenamel junction (CEJ) and alveolar bone crest (ABC) was measured at two sites for the first molars (disto-palatal and disto-buccal) and two sites for the second molars (mesio-palatal and mesio-buccal) in three-dimensional images viewed from the buccal and palatal sides as described (Park et al., 2007) and detailed in Figure 5A. Using MicroView 2.5.0-rc25 software (Parallax Innovations Inc., Ilderton, ON, Canada), each reconstructed image was rotated into a standardized orientation, and a region of interest (ROI) for each specimen was created as shown in Figure 5B. Briefly, for volumetric analysis of the maxillary tooth-supporting alveolar bone, the inter-radicular alveolar ridge crests, inter-radicular surfaces of the roots of the maxillary first and second molars, cemento-enamel junction, and root apex of the mesio-buccal root of the first maxillary molar and disto-buccal root of the second maxillary molar were used as landmarks for quantifying alveolar bone loss and regeneration within a reproducible region Figure 5B. Using the average Grayscale threshold value for all of the samples, the the alveolar bone interproximally between the first and second maxillary molars, and the inter-radicular bone area of the maxillary first and second molars, including bone volume fraction (BVF) and bone mineral density in mg/cc (BMD) were quantified.
Figure 5. Linear and Three-Dimensional Regions of Interest. Linear alveolar bone loss (ABC-CEJ), or the linear distance (orange line) between the cementoenamel junction (CEJ; maroon-dashed line) and alveolar bone crest (purple line), was measured along two roots for M1 and two roots of M2. (D) Anatomical landmarks of M1 and M2 were used to create a three-dimensional ROI encompassing the inter-radicular bone (yellow triangles) and interproximal bone (blue parallelogram).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software. Data were pooled by experimental group, and the mean, standard deviation, and standard error were calculated. One-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests were performed for measuring statistically significant differences between groups.
for volumetric and linear bone levels. Mean and standard error was plotted in bar graphs. 

$P$ value <0.05 was considered to be statistically significant.
CHAPTER IV – RESULTS

Figure 6. Linear Bone Height (ABC-CEJ) at Day 0 and Day 21. Day 0 ABC-CEJ values for all four roots were pooled into one graph due to the limited variability. Linear bone height (ABC-CEJ) significantly reduced after 8 days of ligature placement at all four sites. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

Figure 7. First maxillary molar inter-radicular bone volume (BVF) and density (BMD) at Day 0. Alveolar bone volume (BVF) and density (BMD) significantly reduced at adjacent site – inter-radicular region of M1. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.
Figure 8. Interproximal bone volume (BVF) and density (BMD) at Day 0.
Alveolar bone volume (BVF) and density (BMD) significantly reduced interproximally at site of ligature placement between M1 and M2. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

Figure 9. Second maxillary molar inter-radicular bone volume (BVF) and density (BMD) at Day 0. Alveolar bone volume (BVF) and density (BMD) significantly reduced at adjacent site – inter-radicular region of M2. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.
Figure 10. Alveolar Bone Loss Following Experimental Periodontitis. Representative micro-CT images of maxillary alveolar bone surrounding the first (M1) and second (M2) molars at Day 0 for healthy and experimental periodontitis groups. Representative coronal slices (2D) as well as 3D images of maxillary specimens showcase the visual differences between the amount of bone resorption following 8 days of ligature-induced periodontitis.

Figure 11. First maxillary molar inter-radicular bone volume (BVF) and density (BMD) at Day 21. Non-significant increase in BVF (~5%) and BMD (~5%) in inter-radicular bone of M1 following application of HFMV for 21 days compared to healthy controls and compared to perio + no treatment group. Marked wound healing response/rebound effect also evident in perio + no treatment group back to healthy alveolar bone levels. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.
Figure 12. Interproximal bone volume (BVF) and density (BMD) at Day 21.
Statistically significant impaired bone healing (BVF & TMD) in interproximal alveolar bone with application of HFMV for 21 days following experimental periodontitis ($p < 0.05$) compared to healthy controls and compared to healthy + vibration group. No bone anabolic effects on healthy interproximal bone with application of HFMV for 21 days. * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.

Figure 13. Second maxillary molar inter-radicular bone volume (BVF) and density (BMD) at Day 21. Non-statistically significant impaired bone healing (BVF & TMD) in inter-radicular bone of M2 with application of HFMV for 21 days following experimental periodontitis compared to healthy controls and compared to healthy + vibration group. Slight increase in BVF and BMD in healthy alveolar bone (inter-radicular of M2) following application of HFMV to healthy bone for 21 days * indicates statistical significance of $p < 0.05$ using one-way analysis of variance (ANOVA) with Bonferroni post hoc tests.
Figure 14. Alveolar Bone Following 21 Days of HFMV Treatment. Representative micro-CT images of maxillary alveolar bone surrounding the first (M1) and second (M2) molars at Day 21 for healthy, experimental periodontitis, and HFMV-treated groups. Representative coronal slices (2D) as well as 3D images of maxillary specimens showcase the relatively minor differences between the tooth supporting alveolar bone in the control and treatment groups.
CHAPTER V – DISCUSSION

The goal of the study was to establish a mouse periodontitis model and investigate the effects of mechanical vibration on alveolar bone following experimental periodontitis. The ligature-induced periodontitis model has been widely utilized in both large and small animal studies (Kantarci, Hasturk, & Van Dyke, 2015) to investigate disease pathogenesis and as a translational research model for novel therapeutic strategies. Mice are the smallest widely used species for this animal model and due to the small body size and dimensions of their dentition, ligature placement presents with significant technical challenges. Several studies have demonstrated successful ligature placement and induction of periodontitis (Jiao et al., 2013; C. H. Li & Amar, 2007; Marchesan et al., 2018), our study is one of the few to utilize the model to measure the regenerative potential of a novel therapy for periodontal bone loss (Abe et al., 2012). The advantages of the ligature-induced periodontitis model is that periodontitis can be initiated at a known time with significant, predictable inflammatory responses involving periodontal pathogens and bone loss – approximately ten times more bone loss than the oral gavage model – occurring in a defined location within a relatively short duration (Abe & Hajishengallis, 2013; de Molon et al., 2016; Marchesan et al., 2018). The mouse model offers several significant advantages in comparison to the use of larger animal models. Mice are extremely cost-effective and are the most widely used species for knockout studies. Furthermore, the mouse genome has been sequenced and the roles of specific genes in the pathogenesis, wound healing, and regeneration are well understood. Last, antibodies against mouse antigens are as widely available as for human use (Kantarci et al., 2015).
Both linear and volumetric alveolar bone loss were achieved with ligature placement for 8 days in our experiment. Alveolar bone height, bone volume, and bone density were reduced by approximately 33 to 50% interproximally between the first and second maxillary molars at the site of ligature placement, as demonstrated in previous studies (Abe & Hajishengallis, 2013; Marchesan et al., 2018). A significant reduction in bone volume and density was also measured extending beyond the immediate location of the ligature and into the inter-radicular bone/furcation region of the first and second maxillary molars, indicating that a robust, established inflammatory response likely contributed to the bone resorption. Ligature placement does not elicit a robust inflammatory response and alveolar bone loss in GF mice, indicating that induction of periodontal bone loss in the ligature model is dependent on bacterial accumulation at the site of ligature placement (Marchesan et al., 2018; Xiao et al., 2017).

Localized high frequency, low magnitude mechanical vibration showed a trend towards increased bone volume and bone density at the site of application in both control and experimental periodontitis groups. Compared to the healthy controls without any intervention, HFMV (60 Hz, 0.3 g) of healthy mouse alveolar bone over 21 days led to approximately 5% increase in bone volume and bone density in tooth-supporting, inter-radicular bone in the furcation of the first maxillary molar. Similar results regarding the anabolic effect of HFMV on alveolar bone have been recorded in previous studies. When HFMV (60 Hz, 0.3 g) was applied to the maxillary first molar of rats for 5 minutes/day for 28 days, an increase in bone volume of approximately 20% was recorded in comparison to static controls (Alikhani et al., 2012). Although the osteogenic effect of HFMV was not as pronounced in our study, strict comparisons should not be made due to the different animal species and total length of the study.
The osteogenic effect of localized HFMV application did not extend beyond the bone around site of application, the maxillary first molar with healthy, intact alveolar bone. In this study, no increase was recorded in bone volume, bone density, and linear bone height of interproximal bone or inter-radicular bone of the second maxillary molar. This is in contrast to previous studies in rats, which have shown that the osteogenic effect is not limited to the area of application but has a gradient response which is greatest at the point of application. An increase in bone volume of approximately 18% was measured in the region of the second maxillary molar and an 11% increase in bone volume around the maxillary third molar when HFMV was applied locally on the maxillary first molar (Alikhani et al., 2012).

In animals with ligature-induced periodontitis, a modest, non-significant increase in bone volume and bone density – compared to the experimental periodontitis group receiving no treatment – was recorded with HFMV for 21 days that was also limited to the immediate site of application. After ligature removal, natural healing of the alveolar bone resulted in a rebound effect of bone volume and bone density at all disease sites back to healthy levels, which may have masked some of the osteogenic effects of mechanical vibration therapy. Although samples were collected 7 days after HFMV therapy, analysis of the results was outside the scope of this study. Relative to the area of mechanical vibration application, distant sites of periodontitis located interproximal between the first and second maxillary molars and in the inter-radicular space of the second maxillary molar were negatively affected by HFMV applied to the first maxillary molar, with a statistically significant decrease in both bone volume and density compared to healthy controls was measured in the interproximal bone for the periodontitis + mechanical vibration group. In the murine ligature-induced model of periodontitis, the
pattern of the inflammatory response infiltration, predominated by neutrophils, peaks at 9 days following ligature placement (Marchesan et al., 2018) and the resorptive effect of HFMV on bone in the presence of an elevated inflammatory response has been well documented (Alikhani et al., 2018). Although HFMV was not initiated until 24 hours after ligature removal, the elevated inflammatory response may have persisted when the HFMV treatment began. It is also possible that the application of vibrational force to the first molar could have simply resulted in additional damage to the already weakened interproximal periodontal support structures, which include the periodontal ligament and alveolar bone (Kalajzic et al., 2014). Further studies are needed to characterize the pattern of inflammation, bone resorption, and wound healing in the murine periodontal ligature model for its optimization of its use in preclinical regenerative studies.

LIMITATIONS OF THE STUDY

Several limitations could have affected the outcomes of our study. First, the peak strain translated to the alveolar bone was not measured as in previous studies. Although the device for mechanical stimulation was calibrated, alveolar bone is exposed to indirect loading via the teeth and periodontal ligament, which can produce a complex pattern of strain distribution that is further complicated by the compromised periodontal ligament and reduced bone density present in periodontitis (Alikhani et al., 2017). Thus, the HFMV regimen may need to be adjusted from the optimal regimen routinely used in teeth with intact periodontal ligament. Second, the ligature-induced mouse model for periodontitis may not be well suited for measurement of the regenerative potential of lengthier doses of treatment. In a rat tooth extraction model study, the most significant increases in bone volume occurred after 28 days of daily application of HFMV (Alikhani
et al., 2016). However, as evidenced in this study, the natural healing response of the C57BL/67 strain of mice in alveolar bone is robust by 21 days, which may lead to challenges assessing the true regenerative potential of HFMV unless a potent osteogenic response occurs much earlier in the treatment regimen with HFMV. One possible solution for this limitation could involve induction of ligature-induced periodontitis for longer than 8 days, as previous research has shown that longer ligature placement may lead to the induction of more of a chronic inflammatory response, compared to an acute response short term.

**CONCLUSION AND CLINICAL IMPLICATIONS**

Mechanical vibration (60Hz, 0.3g, 5min/day) modestly increases bone volume and density when applied directly to the tooth, indicating a potential clinical application for improving bone quantity and quality following periodontitis. Its potential clinical application as an adjunct for improving the success of surgical therapy involving scaffolds, growth factors, and autologous cells should also be investigated.
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