Mechanism of Mechanical Vibration in Enhancing Orthodontic Retention

Brent A. Ito  
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MECHANISM OF MECHANICAL VIBRATION IN
ENHANCING ORTHODONTIC
RETENTION

by

Brent A. Ito, D.M.D.

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

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ABSTRACT
MECHANISM OF MECHANICAL VIBRATION IN ENHANCING ORTHODONTIC RETENTION

Brent A. Ito, D.M.D.
Marquette University, 2021

Introduction: Low-magnitude, high-frequency (LMHF) mechanical vibration is proven to be anabolic and can increase alveolar bone mass. The objectives of this study were to generate an orthodontic retention cell culture model and investigate the effects of LMHF mechanical vibration on human periodontal ligament fibroblast (HPLF) cells after orthodontic loading (retention) in vitro.

Materials and Methods: HPLF cells were seeded at a density of 4x10^5/well in 6-well Flexcell culture plates (Day 0). On day 3, the cells were mechanically stretched (15% surface extension) for 1 hour to mimic orthodontic loading/tension in vitro. On day 5, the cells were randomly assigned to 5 groups: a sham control group (0 Hz) and 4 vibration groups (30, 60, 90, and 120 Hz). Cell culture media were changed to fresh growth media (wells #1-3) and differentiation media (wells #4-6), and subsequently refreshed every 5 days until the end of the experiment. Mechanical vibration was applied for 20 minutes per day for 28 consecutive days, while static cells (0 Hz) were used as control. The cells were then stained for collagen type I and bone nodules, photographed, and quantified using a customized computer program. The experiment was repeated four times (n=4). Statistically, one-way ANOVA was used to test for significant differences in collagen type I production and bone nodule formation with Tukey post hoc comparison to find differences between the groups. P value less than 0.05 was considered statistically significant.

Results: After 28 days of retention in culture, with or without mechanical vibration, HPLF cells produced collagen type I and bone nodules in all groups. All vibrated groups (30, 60, 90 and 120 Hz) showed increased production of collagen type I compared to sham control (0 Hz). However, 120 Hz produced the highest amount of collagen type I, which was significantly higher than all other groups (p=0.028). Changes in bone nodule formation were like that of collagen type I, but with no statistically significant difference (p=0.056).

Conclusion: LMHF mechanical vibration increases the production of collagen type I and bone nodules by HPLF cells in vitro, suggesting its possibility of enhancing tooth stability during orthodontic retention.
ACKNOWLEDGMENTS

Brent A. Ito, D.M.D.

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CHAPTER I – INTRODUCTION

Orthodontic tooth movement (OTM) occurs as the alveolar bone around a tooth remodels in response to prolonged pressure from orthodontic appliances. Following OTM, after appliances are removed, periodontal tissues require time for reorganization (Proffit et al., 2013). Therefore, retention is necessary to maintain teeth in ideal functional and esthetic positions. Without adequate retention, teeth tend to move back to their previous positions, and relapse can occur (Maltha et al., 2017). Many patients struggle to wear retainers well, and compliance decreases with time. Thus, a significant demand exists for strategies to combat periodontal relapse and shorten retention time following orthodontic treatment.

Retention is a significant challenge facing all orthodontists. Although many patients may feel that orthodontic treatment is complete when appliances are removed, it is at this point that the retention phase has just begun. Most standard orthodontic retention protocols utilize fixed or removable retainers worn full or part-time to keep teeth in their corrected positions. Given that orthodontic patients already frequently complain about long duration of active treatment (1.5-2 years), many reject or cease to wear retainers full time for the additional 6-12 months needed for periodontal tissues to remodel (Zhang et al., 2014). This can lead to relapse and compromise the long-term functional and esthetic results of orthodontic treatment.

Over the past two decades, various biomedical agents, methods, and techniques have been introduced to enhance orthodontic retention, thus minimizing relapse (Swidi et al., 2018). One such method is mechanical vibration. Recent studies suggest that low-magnitude, high-frequency (LMHF) mechanical vibration can alter bone metabolism to
stimulate cell metabolism, osteoblastic gene expression and bone formation (Zhang et al., 2014). This suggests the possible application of LMHF mechanical vibration as an adjunct method to accelerate bone and periodontal remodeling following orthodontic treatment. In effect, this may promote stability, shorten retention time and increase patient satisfaction.

Mechanical vibration has been used successfully in the field of orthopedics to promote anabolic bone metabolism. In addition, mechanical vibration has been studied in the field of dentistry to promote alveolar bone healing following tooth extraction. However, there are few studies on the effects of mechanical vibration following an active orthodontic tooth movement. Without knowing the mechanism by which LMHF mechanical vibration affects periodontal tissues following OTM, it is impossible to optimize vibration protocols to enhance orthodontic retention. The aims of this study were to 1) generate an orthodontic retention cell culture model, and 2) investigate the effects of LMHF mechanical vibration on human PDL cells after orthodontic loading (retention) in vitro.
CHAPTER II – LITERATURE REVIEW

Biology of Orthodontic Tooth Movement

Orthodontic tooth movement (OTM) is a mechanically induced modeling and remodeling process of the periodontium (gingiva, periodontal ligament, and alveolar bone). As depicted in Figure 1, prolonged force applied to a tooth creates pressure and tension areas within the periodontal ligament (PDL). Bone resorption on the compressed areas of the PDL is coupled with bone formation on the tension areas of the PDL (Melsen et al., 2006). This pressure-tension theory is the main control element that describes the mechanism by which OTM occurs. OTM is mediated by pro-inflammatory factors, including Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and various cytokines (e.g., IL-1\textbeta, IL-6, TNF-\alpha), which are differentially expressed in response to forces within the PDL (Li et al., 2018). When certain areas of the PDL experience pressure or tension, blood flow is altered, which signals the release of these chemical messengers (Proffit et al., 2013). Compression within the PDL signals the release of chemical messengers that stimulate osteoclastic activity, whereas tension within the PDL signals the release of chemical messengers that stimulate osteoblastic activity (Raisz, 1999). Since the PDL is both the medium of force transfer and controls how alveolar bone responds, PDL is the main biological mediator of OTM.
Figure 1. Tissue responses during orthodontic tooth movement (OTM). This histologic cross-section view of a tooth root under orthodontic force demonstrates areas of compression and tension within the periodontal ligament (PDL). On the side away from the direction of tooth movement, the PDL space is enlarged, and blood vessels dilate. On the compressed side of the PDL, blood flow is restricted. Bone formation occurs on the tension side, and bone resorption occurs on the compression side (Melsen et al., 2006).

In addition to the pressure-tension theory, the biologic electricity theory can also describe the control of OTM. When light pressure is applied to a tooth, biological electricity is produced that can create changes in bone metabolism. Piezoelectricity describes the flow of electric current as electrons are displaced from one part of a crystal lattice to another due to external force. This phenomenon has been described in many crystalline materials, including bone (inorganic crystals) and collagen in PDL (organic crystals). It is generally accepted that piezoelectricity plays an important role in the maintenance of the skeleton. The bone around teeth is maintained by signals generated
during normal chewing. Without these piezoelectric signals, bone mineral is lost, and atrophy results (Proffit et al., 2013). Piezoelectric signals have a quick decay rate and produce an equivalent signal in the opposite direction when the force is released. For this reason, the sustained force used to induce OTM does not produce piezoelectric signals. Thus, the pressure-tension theory remains predominant in describing the biological control of OTM. However, some studies have suggested that mechanical vibration can amplify piezoelectric signals during OTM and accelerate anabolic bone metabolism in the alveolar bone (Shapiro et al., 1979).

**Biology of Orthodontic Retention**

Following OTM, retention is the final phase of orthodontic treatment in which teeth are maintained in their corrected positions. During active tooth movement, a pro-inflammatory environment was created that facilitated bone catabolism, enlargement of PDL spaces, and disruption of collagen fiber bundles (Vinod & Davidovitch, 2006). Accordingly, the teeth must be held passively in their final positions to allow healing of the periodontal tissues. In general, reorganization of the PDL occurs over a 3-4-month period, after which the mobility of teeth disappears. Recent reports indicate that occlusal forces are an important factor in expediting periodontal recovery (Terespolsky et al., 2002). However, supracrestal fibers are particularly slow to remodel and can take over a year before they are stable. Therefore, following orthodontic treatment, full-time retention is advised for at least 3-4 months, after which part-time retention should continue until at least the 1-year mark (Proffit et al., 2013).

Orthodontic relapse can occur during the retention phase when teeth start to move back toward their original positions (Horowitz & Hixon, 1969). Relapse is thought to be
of multifactorial causes and is directly related to insufficient bone formation in the alveolus within a given time. Relapse has been related to muscular and soft tissue imbalance causing catabolic bone modeling and remodeling (Yadav et al., 2015). Pressures from the lips and tongue or growth of the jaws following treatment are common causes of relapse. Overexpansion of dental arch form has also been cited as an iatrogenic cause (Graber et al., 2017).

The traditional view of orthodontic stability put forward by Dr. Edward Angle was that relapse would not occur if teeth are put in ideal occlusion. However, it is commonly observed that adults with little growth remaining may still experience lower incisor crowding following orthodontic treatment. The current view of orthodontic stability is that changes to alignment and occlusion of teeth are part of the normal aging process. Therefore, long-term stability may only be achieved by long-term retention (Nanda & Nanda, 1992).

**Traditional Methods of Orthodontic Retention**

There are two main types of retainers traditionally utilized by orthodontists to maintain tooth positions following treatment: fixed retainers and removable retainers (shown in Figure 2). A fixed (bonded) retainer is a segment of wire bonded to the surfaces of teeth. While fixed retainers may be used routinely by some clinicians, they are particularly indicated when intra-arch instability is anticipated and prolonged retention is planned (Zachrisson, 2007). Fixed retainers may be used for maintenance of lower incisor position during late growth, diastema maintenance, and maintenance of posterior tooth position in adults (Proffit et al., 2013). Patients may prefer fixed retainers over removable ones as they do not need to be inserted by the patient themselves and are not visible
during smiling or talking (Axelsson & Zachrisson, 1992). However, the fixed retainer may make maintaining oral hygiene more difficult and require more frequent visits to the orthodontist if they come loose.

Figure 2. Examples of orthodontic retainers. From left to right: Hawley retainer, Clear (vacuum-formed) retainer, and Fixed (bonded) retainer (Images from Dolphin Aquarium software).

The main types of removable retainers include Hawley retainers and clear (vacuum-formed) retainers. Hawley retainers contain clasps on molar teeth, an outer bow with adjustment loops (usually from canine to canine), and the acrylic covering the palate. These features provide excellent control of incisors and a potential bite plane for control of overbite. Hawley retainers also allow posterior teeth to settle after appliance removal. Clear retainers are made with heat-softened plastic that is sucked down tightly over teeth with a vacuum-forming device. These invisible retainers are often more esthetic to patients and can maintain incisor alignment as well as fixed retainers. However, they do not allow for settling of posterior occlusion like Hawley retainers and need to be replaced more frequently due to cracking or discoloration (Proffit et al., 2013).

New Adjunct Therapies for Orthodontic Retention

Over the past two decades, many new methods and techniques have been introduced to enhance orthodontic retention. These include surgical techniques,
biomedical molecules, lasers, and vibrational therapy (Swidi et al., 2018). The main surgical procedure is the circumferential supracrestal fibrotomy (CSF), in which supracrestal fibers are sectioned and allowed to heal while teeth are held in position. While this procedure will decrease relapse caused by gingival elasticity, it comes at the cost of a surgical procedure that may reduce the height of the gingival attachment (Proffit et al., 2013). The pharmacological agents that have been investigated recently typically target factors that control bone metabolism. The most commonly studied agents in orthodontics include osteoprotegerin, bisphosphonates, bone morphogenic proteins, and relaxin. In general, these agents inhibit tooth movement by either inhibiting bone resorption or stimulating bone and PDL formation. RANKL inhibitor agents, particularly denosumab, appear to hold the greatest potential for use in orthodontic retention in the future. However, most pharmacological agents exhibit systemic rather than localized effects, causing unwanted side effects (Yadav et al., 2016). Low-level laser therapy (LLLT) has a stimulatory effect on the submucosal subcellular environment and can increase cell metabolism. LLLT has been associated with faster PDL maturation, which may shorten the time required for orthodontic retention (Jahanbin et al., 2014). However, more clinical studies are needed. Mechanical vibration has a proven osteogenic effect on bone, however, its effects on orthodontic relapse prevention remain unclear now.

**Effect of Mechanical Vibration on Bone**

During normal daily activities, bones are subjected to a variety of mechanical loads resulting in continuous adaptive changes in bone mass and architecture. Osteocytes regulate these changes by sensing mechanical loads (including vibrations) and responding by altering the balance of bone formation and resorption (Cowin et al., 1991; Lau et al.,
Low-magnitude, high-frequency (LMHF, acceleration < 1g where g = 9.81m/s$^2$ and 20-90 Hz) vibration has been shown to inhibit the formation of osteoclasts \textit{in vitro} (Wu et al., 2012). In animal studies, LMHF vibration stimulated an anabolic response in weight-bearing and non-weight-bearing bones (Rubin et al., 2002; Garman et al., 2007). Therefore, whole-body vibration therapy has recently been investigated for many applications as a non-invasive therapy to improve bone quantity and quality. Vibration is believed to mimic the effects of dynamic bone and muscle loading that occurs because of small persistent postural muscle contractions (Thompson et al., 2014). This type of muscle activity is likely more critical in preserving bone mass and facilitating repair than high-impact activity (Edwards & Reilly, 2015).

LMHF mechanical vibration has been used in preclinical models as a therapy to aid in treating conditions including osteoporosis, osteogenesis imperfecta, and bone fracture (Alikhani et al., 2018). Mechanical vibration is thought to inhibit bone turnover by stimulating osteocytes, which act as mechanosensors and regulate the bone remodeling process by releasing soluble factors that affect both osteoclasts and osteoblasts. High-frequency mechanical vibration causes osteocytes to release factors that inhibit osteoclast formation. In one study, RANKL levels were reduced by 50% with 60 Hz mechanical vibration, and PGE$_2$ (which stimulates osteoclast differentiation) decreased by 60% (Lau et al., 2010). Additional studies suggest that LMHF vibration stimulates osteogenic intracellular mechanotransduction pathways to regulate gene expression, downstream protein synthesis and growth factor release in several mature cell types, including osteoblasts, osteocytes, and PDL fibroblasts (Alikhani et al., 2016; Benjakul et al., 2019; Edwards & Reilly, 2015; Moustafa et al., 2012).
Mechanical Vibration in Dentistry and Orthodontics

In dentistry, physiological vibration (mastication) can maintain alveolar bone mass (Lai & Liu, 2009). Similarly, mechanical vibration has been shown to rehabilitate resorbed alveolar bone in rats and maintain alveolar bone mass after tooth extraction in mice (Mavropoulos et al., 2010; Alikhani et al., 2017). In orthodontics, LMHF mechanical vibration has been most widely studied as a therapy to accelerate orthodontic tooth movement. In one study, mechanical vibration at 60 Hz was shown to increase rates of tooth movement, increase RANKL expression, and increase osteoclast numbers (Nishimura et al., 2008). However, in another study, mechanical vibration at 30 Hz had a significant inhibitory effect on tooth movement (Kalajzic et al., 2014). Despite studies with varying and sometimes controversial results, several companies (Propel Orthodontics and OrthoAccel Technologies) have made mechanical vibration devices (shown in Figure 3) marketed to accelerate orthodontic tooth movement. A recent study compared the effects of the vibratory devices VPro5 (Propel Orthodontics) and AcceleDent (OrthoAccel Technologies) on PDL fibroblasts and osteoclasts in vitro. Both devices caused increased cell proliferation and gene expression in osteoblasts and fibroblasts, but the response to VPro5 treatment was greater than for AcceleDent. In contrast, the ability to increase osteoclast activity was device-independent. VPro5, which had a higher vibration frequency (120 Hz vs. 30 Hz) and larger acceleration (70% greater), was superior in stimulating osteoblast and fibroblast cell proliferation/gene expression, although the duration of each treatment bout was 75% shorter than for the AcceleDent (Judex & Pongkitwitoon, 2018).
Figure 3. Orthodontic vibration devices. AcceleDent by OrthoAccel Technologies (left) provides mechanical vibration at a frequency of 30 Hz, used 20 min/day. VPro5 by Propel Orthodontics (right) provides mechanical vibration at a frequency of 120 Hz, used 5 min/day. Peak accelerations generated by the VPro5 were 70% greater than those generated by the AcceleDent (Judex & Pongkitwitoon, 2018).

The seemingly paradoxical effects of mechanical vibration on bone have been explained by several factors. First, long bones are of endochondral origin and intended to permit weight-bearing capacity. This permits the adaptation of long bones under heavy dynamic loads. In contrast, alveolar bone is of intramembranous origin and is not exposed to the same heavy dynamic loads (Alikhani et al., 2017). Second, the physiologic conditions under which mechanical vibration causes an anabolic response in long bone differ from what is present during OTM. Specifically, alveolar bone during OTM is characterized by a pro-inflammatory environment (Alikhani et al., 2018). Recent studies using human PDL cells suggest that when compressive forces like what is present in OTM are combined with mechanical vibration, there is a synergistic effect on the expression of pro-inflammatory molecules PGE₂, IL-6, and RANKL (Benjakul et al., 2019; Sakamoto et al., 2019). It appears that the application of force combined with mechanical vibration enhances the inflammatory response causing enhanced bone
resorption. This is contrasted to normal physiologic conditions during which mechanical vibration will have a bone-forming effect. **Table 1** provides a summary of the conditions under which LMHF mechanical vibration elicits an anabolic and catabolic response in alveolar bone and the PDL.

**Table 1. Vibration paradox in orthodontics: Anabolic and catabolic effects.** LMHF mechanical vibration will elicit different effects in target tissues dependent on if the initial state of the tissue is a physiologic condition or inflammatory condition (Alikhani et al., 2018).

<table>
<thead>
<tr>
<th></th>
<th>Anabolic effect</th>
<th>Catabolic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Method of application</strong></td>
<td>• Directly on teeth in the target area, or</td>
<td>• Directly on tooth or teeth that are moving</td>
</tr>
<tr>
<td></td>
<td>• Indirectly on adjacent teeth close to the target area</td>
<td></td>
</tr>
<tr>
<td><strong>Initial state of tissue</strong></td>
<td>• Physiologic condition</td>
<td>• Inflammatory condition</td>
</tr>
<tr>
<td><strong>Target tissue</strong></td>
<td>• Bone</td>
<td>• Periodontal ligament</td>
</tr>
<tr>
<td><strong>Responding cells</strong></td>
<td>• Osteocytes</td>
<td>• Osteoclasts</td>
</tr>
<tr>
<td></td>
<td>• Osteoblasts</td>
<td></td>
</tr>
<tr>
<td><strong>Resulting effect</strong></td>
<td>• Bone formation</td>
<td>• Bone resorption</td>
</tr>
<tr>
<td></td>
<td>• Load-independent</td>
<td>• Load-dependent</td>
</tr>
<tr>
<td><strong>Extension of effect</strong></td>
<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><strong>Potential clinical uses</strong></td>
<td>• Preservation of alveolar bone after extractions</td>
<td>• Accelerated tooth movement</td>
</tr>
<tr>
<td></td>
<td>• Bone regeneration after periodontal disease</td>
<td>• Increase in magnitude of movement (distance)</td>
</tr>
<tr>
<td></td>
<td>• Enhance Implant and graft integration</td>
<td>• Differential anchorage</td>
</tr>
<tr>
<td></td>
<td>• Increased bone formation after Orthopedic treatment</td>
<td>• Increase in magnitude of Orthopedic correction</td>
</tr>
<tr>
<td></td>
<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></td>
<td>• Increased bone formation after Orthognathic Surgery</td>
<td>• Reduced necrotic (hyalinized) area in response to static Orthodontic forces</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Possible increased frontal resorption</td>
</tr>
</tbody>
</table>

https://doi.org/10.1371/journal.pone.0196540.t001

Very few studies have evaluated the effect of mechanical vibration during the retention stage of orthodontic treatment. One study found that low-frequency mechanical vibration at 30 Hz had a tendency to decrease relapse after orthodontic tooth movement in mice, but not to a statistically significant level (Yadav et al., 2015). There was, however, a noticeable improvement in bone volume fraction (BVF), and thickness and integrity of collagen fibers within the PDL were improved. Furthermore, there was a
reduction in osteoclast numbers and sclerostin expression. These findings are consistent with the present understanding of how mechanical vibration affects bone. After termination of active orthodontic treatment, the pro-inflammatory environment should be returning to normal physiological conditions, suggesting that mechanical vibration will enhance bone formation. However, the experimental results to prove this have yet to be completed.
CHAPTER III – MATERIALS AND METHODS

Cell Culture

Human periodontal ligament fibroblasts (HPLF) from ScienCell Research Laboratories (Catalog #2630) were used for this experiment. HPLF cells were cultured to near-confluency in T75 cm$^2$ culture flasks using growth media consisting of α-MEM supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin, at 37°C and 5% CO$_2$ in air.

Orthodontic Loading of Cells

The timeline of the experiment is detailed in Figure 4. HPLF cells were seeded at a density of 4x10$^5$/well in 6-well Flexcell culture plates (Day 0). On day 3, the cells were mechanically loaded to mimic OTM. As shown in Figure 5, the cells in the Flexcell culture plates were mechanically stretched for 1 hour to mimic orthodontic tension (15% surface extension), which was consistent with previous studies (Zhang, 2017). After two days of incubation, the cells are in the post-stretching retention stage.

Figure 4. Study timeline. Mechanical loading (stretching) was executed on day 3 after cell seeding to mimic OTM. Mechanical vibration was started 2 days post-loading during the retention stage. The vibration was applied for 20 minutes per day for 28 consecutive days.
Figure 5. HPLF cells under mechanical loading (stretching) to mimic OTM *in vitro*. Flexcell culture plates contain a silicon rubber membrane bottom that is flexible. After HPLF cells form a monolayer (green) within each well, the Flexcell plate is positioned on a baseplate with posts (yellow) that stretch the surface of the silicon rubber membrane by 15%. This strain on the monolayer of HPLF cells mimics orthodontic tension.

**Mechanical Vibration of Cells**

Two days post-loading (day 5), the cells were randomly assigned to 5 groups: a sham control group (0 Hz) and 4 vibration groups (30, 60, 90, and 120 Hz). The cell culture media were changed to fresh growth media (wells #1-3) and differentiation media (wells #4-6), which were refreshed respectively every 5 days until the end of the experiment. Differentiation media (α-MEM supplemented with 10% FBS, 1% Penicillin/Streptomycin, 10mM β-glycerophosphate, 0.1µM Dexamethasone and 50mg/L ascorbic acid) allowed for bone nodule formation. The mechanical vibration setup is shown in **Figure 6**. Immediately prior to vibration, each plate was sealed with Parafilm to prevent air exchange and maintain consistent pH within the media. Flexcell plates were secured to the rigid platform using four rubber bands. The 0 Hz group was placed on the
platform inside the thermal box at 37°C, but vibration was not turned on. Vibration was applied for 20 minutes per day for 28 consecutive days. The day after completing all vibrations, cells were stained for analysis as described below.

Figure 6. Mechanical vibration setup. A rigid platform was custom-made to fit Flexcell culture plates. Mechanical vibration was generated by an electrodynamic mini shaker and controlled by a piezo amplifier connected to a computer to alter vibration frequency and acceleration. An accelerometer was attached to the culture plate to confirm the vibration received by the cells. The entire vibration system was housed in a thermal box at 37°C.
Measurement of Collagen Type I Production and Bone Nodule Formation

After vibration for 28 consecutive days, the cells were stained for collagen type I/bone nodules, photographed, and quantified. The Sirius Red dye assay was used to measure collagen production by HPLF cells (Tullberg-Reinert & Jundt, 1999). Sirius Red F3BA was purchased from Chroma (Stuttgart, Germany). The dye was dissolved in saturated aqueous picric acid at a concentration of 100 mg/100 ml. Bouin’s fluid (for cell fixation) was prepared by mixing 15 ml saturated aqueous picric acid with 5 ml 35% formaldehyde and 1 ml glacial acetic acid. Cell layers were extensively washed with 1 X PBS before they were fixed with 1 ml Bouin’s fluid for 1 hour. The fixation fluid was removed, and the culture plates were washed by immersion in running tap water for 15 min. The culture plates were completely air dried before adding 1 ml Sirius Red dye. The cells were stained for 1 hour under mild shaking. The dye solution was removed, and the stained cell layers were extensively washed with 0.01 N hydrochloric acid to remove all non-bound dye. Cell morphology, type I collagen production, and bone nodule formation were then photo-documented.

For quantification, all plates with stained cells were scanned using an Epson Expression 1680 flatbed scanner. Plates were covered with a dark cloth to eliminate light distortion during the scan. Scans were acquired at 150 dpi greyscale and saved as jpg files. Image processing software, “GIMP”, was used to isolate and standardize each well from the saved jpg files. A transparent template layer was created with a fill color of black. A circle shape was deleted from the transparent template; the size of the circle was slightly smaller than the round stained well to exclude reflection and shadows from the saved jpg images. For each sample to be measured, the saved jpg image was placed in a
layer behind the template layer. The saved jpg layer was moved so that only the stained portion of the well was visible through the template layer. The saved jpg layer was positioned so that shadows and reflections from the edge of the well were not visible. A sample jpg was created using the “export as” feature. Measurement was accomplished through Matlab software using two statements for each sample. The sample image was imported into Matlab with “sample = imread (sample image filename)”. The average greyscale was computed with “imagemean = mean (sample, ‘all’)”.

**Statistical Analysis**

In this study, the experiment was repeated four times (n=4) on passages #6-10 of HPLF cells. Data were presented as mean ± SD in graphs. Differences between the means were statistically analyzed using one-way ANOVA with Tukey post hoc comparison, and P values less than 0.05 were considered statistically significant.
CHAPTER IV – RESULTS

Effect of Mechanical Vibration on Collagen Type I Production

The mean and standard deviation (SD) of collagen type I production in HPLF cells treated with different vibration frequencies are presented in Table 2. The average grayscale of the stained wells (#1-3) at each frequency was calculated using a customized computer program. A higher grayscale indicates a more intense stain and greater amount of collagen type I present. Mean and SD of the average grayscale at each frequency for all 4 trials is presented. The same data are also presented graphically in Figure 7. All vibration groups produced more collagen type I than the sham control (0 Hz), with 120 Hz having the greatest increase. Overall, for collagen type I production, there was a statistical difference (p=0.033). Tukey post hoc comparison showed that 120 Hz is statistically different from 0 Hz (p=0.028).

Table 2. Mean and SD of the collagen type I production in human PDL cells.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Hz</td>
<td>8.785833</td>
<td>0.502529</td>
</tr>
<tr>
<td>30 Hz</td>
<td>8.876667</td>
<td>0.626089</td>
</tr>
<tr>
<td>60 Hz</td>
<td>9.020833</td>
<td>0.528625</td>
</tr>
<tr>
<td>90 Hz</td>
<td>9.018333</td>
<td>0.820896</td>
</tr>
<tr>
<td>120 Hz</td>
<td>9.618333</td>
<td>0.808633</td>
</tr>
</tbody>
</table>
Figure 7. Collagen type I production in human PDL cells in response to mechanical vibration at various frequencies. 120 Hz group was significantly higher than the 0 Hz sham control group (*p<0.05).

Figure 8 shows representative examples of collagen staining in the 0 Hz sham control group (A) and 120 Hz vibration group (B). As can be seen, after 28 days of culture, multiple layers of cells were formed and interlaced. Collagen type I stained in pink/purple color existed both inside the fibroblasts and extracellularly. The void spots are cell nuclei with no stain. Visually, the 120 Hz group appears more intensely stained for collagen type I than the 0 Hz group.
Figure 8. Microscopic photographs of collagen type I production and bone nodule formation in human PDL cells in response to mechanical vibrations. A) Collagen production in sham control (0 Hz); B) Collagen production in vibration group (120 Hz); C) Bone nodule formation in sham control (0 Hz); D) Bone nodule formation in vibration group (120 Hz). (Bar = 40 µm)

Effect of Mechanical Vibration on Bone Nodule Formation

The mean and SD of bone nodule formation in HPLF cells treated with different vibration frequencies are presented in Table 3. The average greyscale of the stained wells (#4-6) at each frequency was calculated using a customized computer program. A higher greyscale indicates a more intense stain and greater amount of bone nodules present. Mean and SD of the average greyscale at each frequency for all 4 trials deviation is presented. The same data are also presented graphically in Figure 9. All vibration groups formed more bone nodules than the sham control (0 Hz), with 120 Hz having the greatest increase. Although showing a similar tendency to collagen type I production, however,
bone nodule formation did not reach an overall statistical difference (p=0.056). Figure 8 shows representative examples of bone nodule formation in the 0 Hz sham control group (C) and 120 Hz vibration group (D). As can be seen, after 28 days of culture, bone nodules (dark and black) are spotted on the background of fibroblasts. Visually, the 120 Hz group appears more intensely stained for bone nodules than the 0 Hz group.

Table 3. Mean and SD of the bone nodule formation in human PDL cells.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Hz</td>
<td>9.000833</td>
<td>0.369999</td>
</tr>
<tr>
<td>30 Hz</td>
<td>9.2575</td>
<td>0.361741</td>
</tr>
<tr>
<td>60 Hz</td>
<td>9.270833</td>
<td>0.765927</td>
</tr>
<tr>
<td>90 Hz</td>
<td>9.399167</td>
<td>0.565001</td>
</tr>
<tr>
<td>120 Hz</td>
<td>9.876667</td>
<td>1.16631</td>
</tr>
</tbody>
</table>

Figure 9. Bone nodule formation in human PDL cells in response to mechanical vibration during retention in vitro.

Figure 9. Bone nodule formation in human PDL cells in response to mechanical vibration at various frequencies. Overall, there is no statistically significant difference (p=0.056).
CHAPTER V – DISCUSSION

The goals of this study were to 1) generate an orthodontic retention cell culture model, and 2) investigate the effects of low-magnitude, high-frequency (LMHF) mechanical vibration on human periodontal ligament fibroblast (HPLF) cells after orthodontic loading (retention) in vitro. Previous studies have utilized cell culture models to study the effects of mechanical vibration on various cell types (Kulkarni et al., 2013; Judex & Pongkitwitoon, 2018). Our study appears to be the first to establish an orthodontic retention cell culture model to determine the effects of mechanical vibration. We clearly demonstrated that HPLF cells actively produce collagen type I and bone nodules in long-term culture following cell stretching to simulate orthodontic tension.

We elected to test vibration frequencies of 30, 60, 90, and 120 Hz based on what is utilized by two currently available orthodontic vibration devices: VPro5 (Propel Orthodontics) and AcceleDent (OrthoAccel Technologies). A recent study verified the manufacturers’ specification of a 30 Hz vibration frequency in the AcceleDent and a 120 Hz vibration in the VPro5. The AcceleDent produced peak accelerations on the order of 0.15 g, while peak accelerations generated by the VPro5 were 70% greater than those generated by AcceleDent (Judex & Pongkitwitoon, 2018). In our study, the frequency was altered while keeping the peak acceleration constant at 0.3g.

Relapse after orthodontic tooth movement is of multifactorial causes, but is directly related to inadequate PDL remodeling and bone formation within a given time. Our study showed that LMHF mechanical vibration increased collagen type I production by HPLF cells in a frequency-dependent manner. 120 Hz produced the most significant increase in collagen type I production. Our results are consistent with previous findings.
that greater vibration frequency coincides with greater cell proliferation/gene expression in HPLF cells (Judex & Pongkitwitoon, 2018). For bone nodule formation, the null hypothesis that there would be no difference in the amount formed between experimental groups was accepted. However, there was a tendency for increased bone nodule formation when comparing the sham control (0 Hz) to 120 Hz. Our results are consistent with other in vivo studies that found that 30 Hz cyclical forces inhibit orthodontic tooth movement (Kalajzic et al., 2014) and 30 Hz of LMHF mechanical vibration increases tissue density and BVF (Yadav et al. 2016). On examination of microscopic photographs, our study showed that in vitro, HPLF cells produce collagen type I intracellularly and extracellularly, as well bone nodules within 28 days. Based on visual comparison of the microscopic photos, it is apparent that there was the greatest amount of collagen type I production in the 120 Hz vibration group. This is consistent with previous studies that found that LMHF vibration resulted in more thickened and organized PDL fibers when compared to control (Yadav et al., 2015).

When considering vibration therapy for clinical use, there are several factors that can be altered when creating devices and optimizing protocols. Vibration frequency, peak acceleration and displacement magnitude can be changed to create a different wave with possibly different biological effects. Our findings suggest that increasing vibration frequency alone will increase the anabolic activity in PDL cells. Other studies found that a greater acceleration magnitude will also increase cellular activity in PDL cells, but a greater displacement magnitude will not (Judex & Pongkitwitoon, 2018). This is of clinical importance, as a greater displacement magnitude will likely cause more patient discomfort. In addition, recent studies suggest that cells may sense vibrations
preferentially in a horizontal direction (Pongkitwitoon et al., 2016). While not addressed in this study, it may be beneficial to study the effects of altering vibration direction.

A main goal of this study was to discover more about the mechanism of LMHF mechanical vibration in enhancing orthodontic retention. Our results suggest that there is a direct effect on PDL cells. It appears that HPLF cells can sense high-frequency oscillatory accelerations directly to cause alteration in cellular activity. An alternative theory not explored here is based on stress/strain, which suggests that stresses generated in the alveolar bone by LMHF mechanical vibration result in alterations of cellular activity in surrounding cells (Judex & Pongkitwitoon, 2018).

Some limitations could have affected the outcomes of this study. First, it is important to be cautious when attempting to extrapolate data from in vitro cell culture studies to the orthodontic environment in vivo. The in vitro environment we have created does not allow cell-cell interactions that may be present in the PDL, as osteoblasts, osteocytes, and osteoclasts will be present in addition to fibroblasts. In vivo studies are necessary to confirm what we have observed in vitro. In addition, our protocol only measured collagen type I and bone nodule formation at the 28-day mark. In some other study protocols, mechanical vibration had its effect by the 7-day mark (Yadav et al., 2016). It is possible that there was a greater effect of mechanical vibration by 7 days, while the difference was minimal by 28 days. It is also possible that a more significant effect would be realized after applying vibration for much longer than 28 days. The small sample size in this experiment could have also affected the result. A larger sample size could provide more consistent results, and possibly yield results with more statistical significance.
In conclusion, LMHF mechanical vibration appears to modestly increase the production of type I collagen and bone nodules (although not statistically significant) in 
\textit{vitro} in a frequency-dependent manner, with 120 Hz causing the greatest increase. This supports the possibility of using LMHF mechanical vibration as an adjunct therapy during orthodontic retention to promote healing of the periodontium and enhance stability of the teeth. The potential clinical applications of mechanical vibration, however, still need further investigation to optimize protocols (frequency, acceleration, magnitude, timing, etc.). In addition, more information regarding specific cellular/molecular effects of mechanical vibration during orthodontic retention is indicated.
BIBLIOGRAPHY


mice are more closely associated with the subsequent osteogenic response than the peak strains engendered. *Osteoporos Int*, 23(4), 1225-1234.


