

The Effect of Mechanical Vibration on Human PDL Cell Differentiation and Response to Inflammation

Megan DesRoches
Marquette University

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THE EFFECT OF MECHANICAL VIBRATION ON HUMAN PDL CELL
DIFFERENTIATION AND RESPONSE TO INFLAMMATION

by

Megan S. DesRoches, D.D.S.

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Marquette University,
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ABSTRACT
THE EFFECT OF MECHANICAL VIBRATION ON HUMAN PDL CELL
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Megan S. DesRoches, D.D.S.

Marquette University, 2016

Low magnitude mechanical vibration is a therapeutic adjunct being investigated to alter bone remodeling and inflammation in areas such as osteoporosis, bone fracture healing, and muscle soreness after exercise. In orthodontics a device named AcceleDent has been marketed that claims to increase the rate of tooth movement and decrease pain. However evidence for these claims is lacking. In this study we looked at two potential cellular mechanisms for these claims: periodontal ligament (PDL) cell differentiation and inflammation under an orthodontic model of strain (IL-1 β).

Increased PDL cell differentiation into osteogenic cells could be an avenue of increasing orthodontic tooth movement. To test for cell differentiation, cultured human PDL cells were observed for calcification by Alizarin Red staining. Also the gene expression of periostin, a cytokine with roles in bone formation, was analyzed using qPCR. To test for inflammation, the gene expression of MMP-13, an inflammatory cytokine was tested. The cells were treated under conditions of +/- IL-1 β (10 ng/ml) and +/- 0.3g/30Hz vibration.

PDL cell differentiation was decreased with addition of IL-1 β (1 ng/ml), as expected under an inflammatory environment. However vibration exhibited no observable effect. Gene expressions of periostin and MMP-13 under all conditions showed no statistically significant results. However, a general trend was noted that vibration may decrease inflammation/MMP-13 production. These findings warrant further investigation.

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CHAPTER I

INTRODUCTION

In the field of orthodontics, many people would benefit from treatment. Dentists estimate 35% of individuals as having normal occlusion, and recommend approximately 55% of people to seek orthodontic care, while 10% could benefit but have only mild malocclusion. Even the general public sees 35% of individuals as needing care. However, less than 25% of people pursue treatment (Proffit, 2007). There are many deterrents to care, including financial constraints, the extended treatment time needed for care, pain associated, and esthetics concerns of appliances. Of interest to this study is the time needed to provide orthodontic care and associated pain.

Over the last several years, there has been a focus on clinical adjuncts that can help speed orthodontic tooth movement. Surgical adjuncts including open flap procedures to remove the bony cortex with either conventional mechanical instrumentation or piezoelectrics have seen increased tooth movement, leading to shorter overall orthodontic treatment time (Wu, 2015). However the surgical nature of this adjunct makes it unappealing to patients. This has led to more conservative procedures such as cortical micro-perforations without a gingival flap, low energy laser radiation, low magnitude vibration, pulsed electromagnetic waves, and various pharmacological methods (El-Angbawi, 2015).

Of these adjuncts, a device called AcceleDent claims that the low magnitude vibration it provides speeds orthodontic tooth movement and can decrease pain. AcceleDent's relative ease of use and patient acceptance is appealing to both practitioners and patients. AcceleDent is used in the comfort of the home and patients are instructed to

gently hold the mouthpiece-shaped wafer between their teeth for 20 minutes a day while in active orthodontic treatment. It delivers a low magnitude vibration stimulus to the teeth of 0.3g/30Hz while its hands free and low maintenance style allow a patient to continue to address other aspects of their busy day. The company, OrthoAccel Technologies, claims that AcceleDent is a “Leader in Accelerated Orthodontics®, is an FDA-cleared, class II medical device clinically proven to move teeth up to 50% faster. Simple-to-use and hands-free, AcceleDent is clinically shown to reduce discomfort that may be associated with orthodontic treatment. AcceleDent is the fast, safe and gentle way to speed orthodontic treatment” (retrieved June 20, 2016 from <http://acceleddent.com/how-it-works/introducing-acceleddent>). However, literature backing these claims is of controversy. A recent Cochrane Collaboration review of non-surgical adjuncts for accelerating tooth movement concluded that “Although there have been claims that there may be a positive effect of light vibrational forces, the results of the current studies do not reach either statistical or clinical significance and are at high risk of bias” (El-Angbawi, 2015). The aims of this study are to determine if there are molecular and cellular mechanisms associated with low magnitude vibrational therapy to periodontium cells in regards to accelerated tooth movement or decreased pain. Periodontal ligament cells were chosen due to their key role in the orthodontic tooth movement process.

Chapter II

Literature Review

A. Orthodontic Tooth Movement

Pressure-Tension Theory of Orthodontic Tooth Movement

Several theories exist in regards to orthodontic tooth movement. One of the most prominent theories is the pressure-tension theory. When orthodontic forces are applied to a tooth, areas within the periodontal ligament (PDL) are compressed in the direction of tooth movement on the pressure side. Conversely on the opposite side, tension exists as orthodontic force moves the tooth away from the alveolar bone and the PDL is stretched. The stretching and compression of the periodontal ligament causes a cascade of molecular and cellular events and is dependent on the degree of force applied.

The cellular events that happen on the pressure side of orthodontic tooth movement differ based on the length and quantity of force applied. If heavy or light pressure is applied but is less than one second in length, the internal pressure in the PDL causes the tissue to be incompressible and any tooth movement occurs within the surrounding alveolar bone. However when a longer duration of force is applied, changes occur. A force of 1-2 seconds will cause a tooth to move within the socket and PDL fluid to be expressed from the tissue. If a force continues but is light in nature (20-25g/cm² of root surface) after 2 seconds the capillary bed located within the PDL is partially occluded and PDL fibers and cells are mechanically distorted. Within minutes blood flow is altered, oxygen levels change and inflammatory mediators such as prostaglandins and cytokines are released. Within several hours of prolonged force these chemical mediators affect cellular activity, differentiation of cells within the PDL and tooth

movement begins as alveolar bone is remodeled by osteoclasts and osteoblasts approximately 2 days after initial consistent force. However if forces are heavy the PDL capillary bed is occluded fully within seconds as seen below in figure 1 adapted from Proffit, 2007. Within hours tissue necrosis occurs as cells are cut off from oxygen and nutrients. Physical contact between the tooth and bone can occur with great forces. This necrosis results in undermining resorption or hyalinization of adjacent marrow spaces as cells are not viable within the PDL to help with remodeling of bone in a physiologic fashion. During this process, inflammatory cell mediators recruit cells such as macrophages, foreign body giant cells and osteoclasts which resorb bone and dead tissue adjacent to the PDL over a process of days. Tooth movement eventually occurs once enough undermining resorption occurs usually after 7-14 days. While light forces within orthodontics are preferable and have a better physiologic response, in clinical situations a mix of light, physiologic and heavy, hyalinized undermining resorption will likely occur in different areas of PDL (Krishnan, 2006 and Proffit, 2007).

Effect of orthodontic force on blood flow in the periodontal ligament

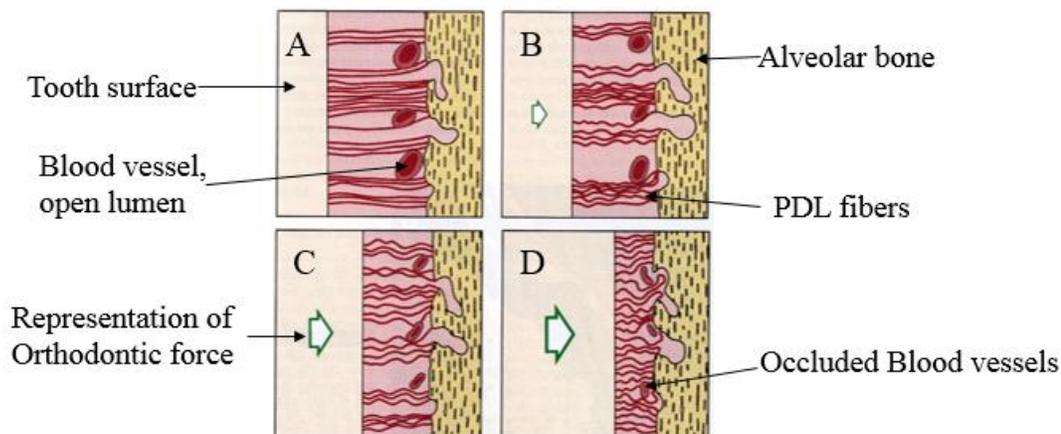


Figure 1: Representation on how increasing force occludes capillaries within the PDL. **A.** normal periodontal ligament, attached to tooth surface on right and alveolar bone, left. **B.** light orthodontic forces begin to compress PDL space. **C.** As forces increase, blood vessels are compressed. **D.** Heavy forces fully occlude blood vessels. *Adapted from Contemporary Orthodontics (p337), by W.R. Proffit, 2007, St. Louis, MO: Mosby Elsevier. Copyright 2007 by Mosby Elsevier. Reprinted with permission.*

Conversely on the tension side of orthodontic tooth movement, PDL fibers are stretched between the moving tooth and alveolar bone. This causes blood flow to be maintained or even increased. This alteration, just as in the pressure side of tooth movement, causes a change in metabolites, chemical signaling, and cellular differentiation. These changes allow new periodontal tissue, such as bone, ligament fibers and cementum to be produced (Proffit, 2007).

Orthodontic Tooth Movement Phases

In all, orthodontic tooth movement can be summarized into three phases; an initial phase, lag phase and a post-lag phase. The initial phase lasts approximately 1-2 days where initial forces displace the tooth within the alveolar socket. Here some movement

occurs and blood flow is altered leading to a cascade of cellular and tissue reactions. Following the initial phase, a lag phase occurs when areas of hyalinization arise as a result of heavy forces. No tooth movement occurs during this time and depending on the magnitude of force this phase can last 4-40 days. During this time, cells are busy removing any necrotic tissue to allow for tooth movement to continue. Cells such as macrophages, foreign body giant cells and osteoclasts are recruited, while on the tension side pre-osteoblasts migrate towards the socket wall and fibroblasts remodel PDL tissues. After enough necrotic tissue is removed, active tooth movement can occur in a post-lag period. Here osteoclasts continue to resorb bone on areas in pressure while newly differentiated osteoblasts lay down new bone on the tension side.

Although phases are a good way to understand the biologic sequence of events occurring within the PDL, tooth movement in reality is a dynamic process occurring over the entire tooth surface. There can be simultaneous areas of physiologic tooth movement in areas subjected to light pressure as well as hyalinization due to heavy force application. (Krishnan, 2006).

Cytokines in Orthodontic Tooth Movement

Of particular interest is the role of cytokines in orthodontic tooth movement. Cytokines are signaling molecules that are produced in connective tissue cells such as fibroblasts and osteoblasts and have roles in bone remodeling and hence orthodontic tooth movement. They work as autocrine or paracrine signaling molecules and there are numerous families and functions for each molecule, some of which are not fully understood (Meikle, 2006). Interleukin 1, interleukin 2, interleukin 3, interleukin 6,

interleukin 8, tumor necrosis factor alpha, gamma interferon, and osteoclast differentiation factor all have roles in orthodontic tooth movement, with IL-1 having the most important role as it stimulates osteoclast function (Krishnan, 2006). IL-1, with its two isoforms IL-1 α and IL-1 β , is a pro inflammatory cytokine and has roles in the regulation of feeding, sleep, temperature, and bone remodeling. However overproduction can lead to acute and chronic inflammation as well as autoimmune disorders and pain (Ren, 2009). In tooth movement, IL-1 β regulates bone resorption and bone formation by mechanical stress (Davidovitch, 1988 and Preiss, 1994). IL-1 β can stimulate other cytokines such as adhesion molecules, RANKL and matrix metalloproteinases (MMPs) (Meikle, 2006, and Nogueira, 2013). Together these chemical signaling molecules regulate the cellular changes in the PDL needed to organize clearing out necrotic tissue, and remodeling the bone, connective tissue, and vascular elements under orthodontic strain. Leukocytes are attracted to clear necrotic tissue, fibroblasts are stimulated to produce connective tissue, osteoclasts to resorb bone on the pressure areas and osteoblasts to build new bone on the tension sites (Krishnan, 2006). Together these changes allow orthodontic tooth movement to occur. However, these molecular processes are still being researched and interactions between cytokines and their intended receptors are complex. Below are figures by Meikle of hypothesized pathways for these cytokines on the pressure and tension sides of orthodontic tooth movement. The diagrams show the complexity of cytokine and cellular interactions and illustrate that molecular pathways under pressure and tension are different.

Cellular effects of PDL compression

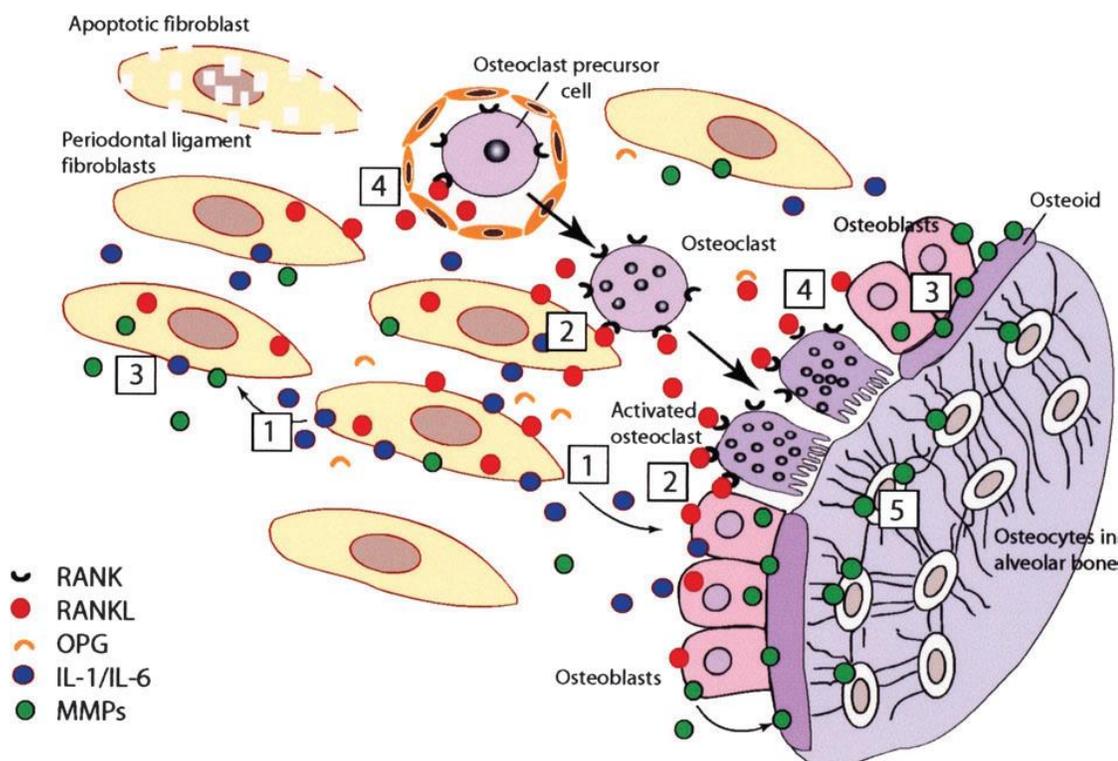


Figure 2 Hypothetical PDL model of the remodeling of the periodontium: compression side. “PDL cells under compressive strain synthesize interleukin-1 (IL-1) and IL-6 (1); IL-1 and IL-6 act in an autocrine and paracrine manner to up-regulate receptor activator of nuclear factor κ B ligand (RANKL) (2) and matrix metalloproteinase (MMP) (3) expression by PDL cells and osteoblasts. Osteoblast-derived MMPs degrade the non-mineralized surface osteoid layer of bone, while MMPs produced by PDL cells degrade their extracellular matrix; (4) RANKL stimulates the formation and function of osteoclasts from mononuclear precursor cells which access the bone surface and degrade the mineralized matrix; (5) deformation of the alveolar bone up-regulates MMPs expression by osteocytes adjacent to the bone surface.” Reprinted from “*The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt*,” by M.C. Meikle, 2006, *European Journal of Orthodontics*, 28, 221-240. Copyright 2006 by the Oxford University Press. Reprinted with permission.

Cellular effects of PDL tension

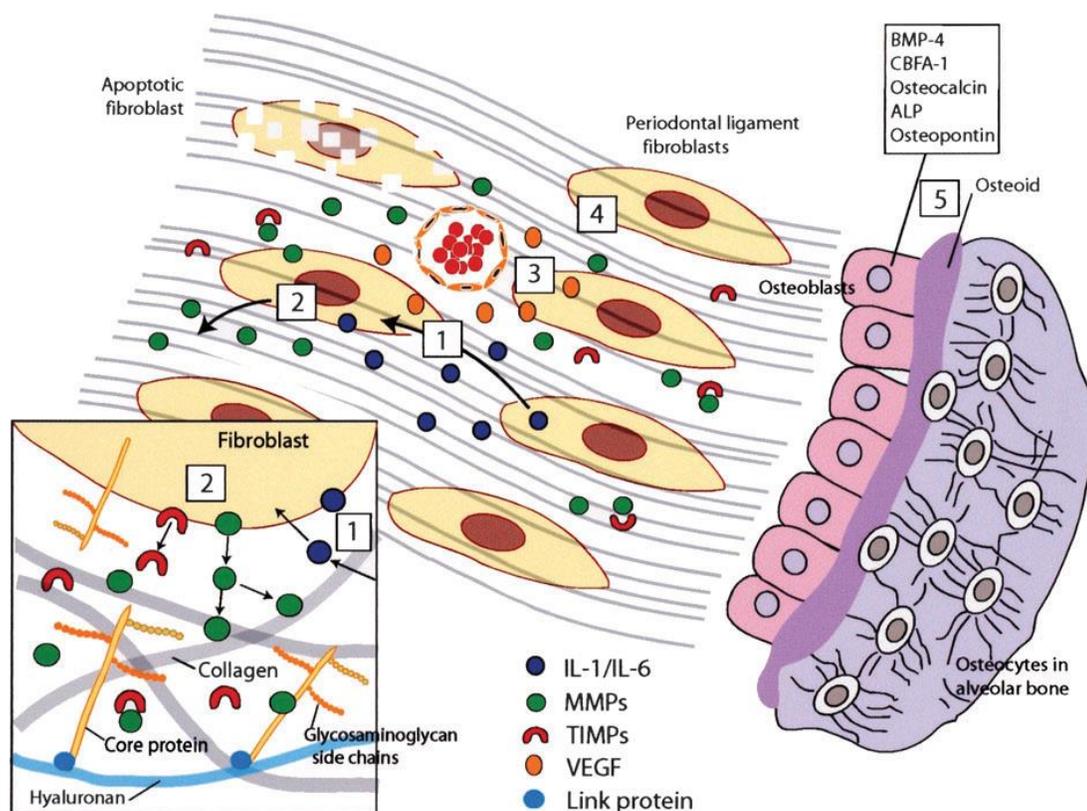


Figure 3 Hypothetical PDL model of the remodeling of the periodontium: tension side. “PDL fibroblasts under tensile strain synthesize cytokines such as interleukin-1 (IL-1) and IL-6 (1); IL-1 and IL-6 in turn stimulate matrix metalloproteinase (MMP) and inhibit tissue inhibitor of metalloproteinase (TIMP) synthesis by PDL cells via autocrine and paracrine mechanisms (2); vascular endothelial growth factor (VEGF) produced by mechanically activated fibroblasts promotes angiogenesis (3). Degradation of the extracellular matrix by MMPs facilitates cell proliferation and capillary growth; PDL cells (4), osteoblasts, and bone-lining cells (5) enter a biosynthetic phase with the synthesis of structural and other matrix molecules.” *Reprinted from “The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt,” by M.C. Meikle, 2006, European Journal of Orthodontics, 28, 221-240. Copyright 2006 by the Oxford University Press. Reprinted with permission.*

B. Negative side effects of OTM

While the goal of orthodontic tooth movement is to align teeth, create better occlusion, esthetics and health, there are negative side effects. Inflammation plays a key role within orthodontic tooth movement as cellular necrosis and release of various

inflammatory molecules such as IL-1 β and prostaglandins occur. Although helpful in recruitment and differentiation of cells to move teeth, inflammation can also lead to unwanted consequences such as pain, root resorption, and periodontal breakdown.

Acute inflammation and pain

Acute inflammation occurs during the initial phase of orthodontic tooth movement and results in plasma and leukocytes leaving capillaries that are under strain. Molecularly, the gingival crevicular fluid expressed from the periodontal ligament contains high concentrations of inflammatory mediators such as cytokines and prostaglandins. One of the hallmark symptoms from this acute period is pain and this discomfort occurs shortly after orthodontic appliances are activated. (Proffit, 2007 and Krishnan, 2006). Pain itself is a very individual experience and depends on such things as age, gender, individual pain threshold, magnitude of force applied, present emotional state and stress, cultural differences and previous pain experiences (Krishnan, 2007). Patient surveys have shown that pain is a strong deterrent of treatment, and one survey by O'Connor showed that pain was the greatest dislike of treatment and rated fourth for fear prior to start of treatment (O'Connor, 2000). Pain during orthodontic treatment causes a decrease in quality of life and can influence several aspects in a patient's life including sleep and diet, causing patients to take analgesic medication (Krishnan, 2007). Pain generally starts as early as 4 hours after activation of an orthodontic appliance and peaks at 24 hours (Jones, 1984). The most intense period of orthodontic pain typically lasts 2-4 days and in most patients will cease within 7 days (Jones, 1992). This pain can interfere with treatment progress as patients may have decreased compliance with treatment

recommendations, such as wearing elastics or headgear due to experienced pain (Egolf, 1990). This can lead to compromised orthodontic treatment or an increase in total treatment time.

The mechanism for orthodontic pain, however, is not fully understood. A linkage with initial inflammatory mediators released and a resultant rise in hyperalgesia of the PDL are the best known pathways for pain. Changes in blood flow in the pulp release many molecules including substance P, IL-1 β , histamine, enkephalin, dopamine, serotonin, glycine, glutamate gamma-amino butyric acid, prostaglandins, leukotrienes and cytokines. An increase in these mediators is associated with hyperalgesia (Krishnan, 2007 and Ren, 2009). Orthodontic patients are recommended to take anti-inflammatory medications to offset pain, with short-term Ibuprofen showing the most efficacy. This leads support to orthodontic pain as a primarily inflammatory response (Ngan, 1994).

Chronic Inflammation, Root Resorption, and Periodontal Breakdown

Within a couple of days after orthodontic activation a chronic inflammatory response takes over where leukocytes, fibroblasts, endothelial cells, osteoblasts and alveolar bone marrow cells differentiate and proliferate. They migrate into the surrounding periodontal tissues as the remodeling process begins (Krishnan, 2006). This allows for the desirable effect of tooth movement to occur as bone is resorbed and new bone constructed. However, deleterious side effects of chronic inflammation can also occur including root resorption, and periodontal breakdown.

Like the orthodontic tooth movement process, remodeling of the root surface also occurs. However, unlike the bony resorption of the alveolar bone in orthodontic tooth

movement, root resorption is an unwanted side effect. Root resorption has been theorized to be induced by inflammation (Brezniak, 2002) and has the tendency to occur with increased force levels (Proffit, 2007). Thus having a physiologic level of force and inflammation while avoiding high force and unhealthy levels of inflammation is important.

Similarly, periodontal breakdown can be worsened by orthodontic tooth movement if a patient has periodontal disease. Periodontal disease is caused by infective bacteria and the body's inflammatory reaction towards these bacterium. It is important for clinicians to treat any pre-existing periodontal disease and ensure that a patient is stable prior to start of orthodontics. If active periodontal disease is in progress, then the inflammatory nature of orthodontics can increase the amount of gingival and periodontal inflammation (Proffit, 2007). This in turn can worsen alveolar bone loss (Cardaropoli, 2014). Since root resorption and periodontal breakdown can be worsened by increased levels of inflammation, keeping inflammation low while still allowing orthodontic tooth movement to occur, is also imperative.

C. Low magnitude Vibration Therapy

Vibration therapy within medicine

Application of low magnitude vibration to human tissue is a novel idea and research is ongoing into potential therapeutic uses. In medicine, full body vibration helps in bone formation, which could be useful to treat osteoporosis or fractures (Thompson, 2014). Two biologic mechanisms for increased bone formation have been proposed. First, mechanical loading by low magnitude vibration lowers osteoclast formation by

preventing differentiation of osteoclast precursor cells (Lau, 2010, and Kulkarni, 2013). Second, dynamic loads to bone have shown to increase bone formation. This is illustrated by the fact that static loads, such as external force from orthodontic appliances or growth from tumors, cause resorption of bone. However, dynamic loads such as muscles pulling on the skeleton during exercise increase bone formation, with skeletons of more active individuals having increased density of bone. Similarly, vibration acts as a dynamic load, stimulating the bone formative processes of the skeleton (Thompson, 2014). Thus, low magnitude vibration promotes bone formation by its abilities to decrease osteoclast formation and resultant bone resorption and by increasing bone formation in stimulating anabolic capacity (Thompson, 2014).

Vibration is also being investigated as it relates to pain perception, especially within the field of exercise science. After intensive exercise, muscular soreness occurs with pain, stiffness, reduced range of motion and decreased muscle force being key symptoms. This muscular soreness is a result of microscopic muscle tears (Veqar, 2014). Several studies have found that low magnitude vibration applied post exercise reduce post exercise soreness, increasing the blood flow under the skin and increasing a patient's range of motion (Veqar, 2014). This has led to the suggestion that whole body low magnitude vibration can be used as a post exercise recovery therapy and a way to decrease pain (Wheeler, 2013).

Vibration therapy within orthodontics

Recently in the field of orthodontics, various adjuncts to treatment have been introduced with aims to reduce the treatment time of orthodontic tooth movement. One

adjunct is AcceleDent, a device that provides a low magnitude vibrational force of 0.3g/30Hz to the occlusal surfaces of teeth. Patients in orthodontic treatment are directed to gently bite on the AcceleDent mouthguard for 20 minutes a day. The product claims two major benefits, decreased treatment time of orthodontics (faster orthodontic tooth movement) and decreased discomfort or associated orthodontic pain. However studies of these claims are mixed (El-Aghbawi, 2015).

The idea that vibration could be utilized in orthodontics to increase bone turnover, thus increasing the frequency of orthodontic tooth movement is an attractive one. Patients continually wish to have decreased treatment times and being able to speed tooth movement could lead to several positive effects. First, orthodontic treatment could be more convenient within a patient's everyday life. Second, negative effects such as root resorption, decreased patient compliance and white spot, decalcified lesions all increase with extended treatment times. Preserving the health of tissues during treatment by shortening treatment time is a desirable goal. Pavlin et al showed a 48% greater tooth movement in clinical human based experiment, similar to Kau et al who showed 2-3mm of tooth movement per month with AcceleDent versus an average of 1mm in control populations (Kau, 2010, and Pavlin 2015). These studies are very exciting regarding the advantages of AcceleDent, however upon closer review the scientific merit of these studies needs to be questioned as neither is published into a peer reviewed journal. In fact more support can be found against vibration's effect on orthodontic tooth movement. Two animal based studies showed no or decreased tooth movement (Kalajzic, 2014, and Yadav, 2015), and Woodhouse et al found in a clinical prospective randomized clinical trial no evidence that supplemental vibration force could significantly increase the rate of

initial tooth movement or reduce treatment times (Woodhouse, 2015). Together current research led to a 2015 Cochrane review on the subject that concluded there is limited nonbiased published research available to support the claim that vibration increases the rate orthodontic tooth movement (El-Angbawi, 2015).

Similarly, the theory that vibration can decrease pain in orthodontic treatment is also alluring. Even prior to AcceleDent, cyclical forces on teeth were discussed as an option to alleviate orthodontic tooth movement associated pain. Proffit suggests that pain associated with orthodontic activation occurs as a result of blood flow being cut off in the PDL. To relieve the pain, he recommended patients engage in repetitive chewing of gum, on a plastic wafer or any other material during the first 8 hours after orthodontic activation. This is to allow the tooth to be engaged and moved enough to allow some blood through the compressed areas, keeping cytokines and other inflammatory mediators from building up, while allowing nutrients access to compressed areas (Proffit, 2007). Also prior to AcceleDent, a study from 1982 looked at vibration on the pain threshold of human teeth. They found that vibration of 100Hz and 10Hz could increase the pain threshold 1.2-1.8 times the control, respectively. Interestingly, while a smaller frequency took longer to change the pain threshold, surprisingly a larger increase in pain threshold occurred. However, the rise in pain threshold was short lived and only lasted 0-20 min after vibration ceased (Ekblom, 1982). Since the development of adjunctive vibration aids into the field of orthodontics has occurred, more recent studies have looked at AcceleDent or a similar device the Tooth Masseur in their abilities to decrease orthodontic pain. Lobre et al showed significant reductions of pain with AcceleDent in both overall pain and pain associated with biting (Lobre, 2015). However, studies

completed by Miles et al with the Tooth Masseur and Woodhouse et al with AcceleDent showed no statistically significant advantage to these devices for pain control (Miles, 2012 and Woodhouse, 2015).

D. Study Aims

AcceleDent, a low magnitude vibration therapeutic adjunct, claims to increase the rate of orthodontic tooth movement as well as decrease pain. It is important to understand the molecular and cellular mechanisms behind these claims. If vibration can indeed alter these aspects of orthodontic tooth movement there could be several benefits. First, if vibration can increase the rate of orthodontic tooth movement, more people may be willing to seek treatment as lowered time commitment would be needed. For those already seeking active treatment, lowered treatment times could decrease the risk for treatment length-dependent risks such as root resorption and caries. Looking on a broader field, if vibration positively effects bone remodeling this could have implications for osteoporosis and fracture healing. Second if vibration could decrease pain, a key deterrent and negative side effect for orthodontic treatment could be managed. Also if pain relief from vibration could be translated to other conditions, patients suffering from other acute or chronic pain conditions could benefit from a better quality of life and less morbidity. While there are many potential cellular mechanisms by which vibration could affect orthodontic tooth movement and pain, this study will focus on two possible mechanisms: 1) periodontal ligament cell differentiation and 2) inflammation.

PDL Differentiation

Within the orthodontic tooth movement process, the PDL is at the front lines. PDL cells are instantly compressed by the orthodontic force placed on teeth, with blood supply compromised. As orthodontic tooth movement occurs, PDL cellular differentiation plays an important role. Differentiation of PDL cells into osteogenic cells capable of mineralization occurs when activated by chemical messengers (Wolf, 2013). Vibration has already been shown to alter this differentiation process. A previous study by Zhang et al, showed that human PDL stem cells had increased markers for osteogenesis when vibration of 40-120Hz was applied, with 50Hz being the peak differentiation frequency (Zhang, 2012). However, we are interested in finding if increased osteogenic differentiation of PDL cells also occurs in orthodontically stressed cells and at the current commercially available frequency. Thus, we will study, in vitro, the osteogenic differentiation of PDL cells when vibration of 0.3g/30Hz is applied with the addition of IL-1 β as a model of orthodontic inflammation or strain. Our study will focus on two ways to measure differentiation. First, Alzarin red stain will determine if any calcification occurs in PDL cells signaling osteogenesis. The second will be to look at the gene expression of periostin.

Periostin is part of the fascilin-1 family of proteins which are known to function in wound repair, bone and tooth morphogenesis and remodeling (Cobo, 2016). Periostin is sensitive to mechanical force (Conway, 2013), however seems to have different expression based on cell type and degree of mechanical stress (Cobo, 2016). Periostin is highly expressed in PDL cells and has a role in maintaining the integrity of periodontal tissues (Rangiani, 2015). It is involved in tissue remodeling; increasing the proliferation

and migration rates of fibroblasts (Cobo, 2016). Higher levels of periostin increase osteoblast proliferation and differentiation (Zhu, 2009). However, periostin is complex and has many regulatory roles. Even within orthodontic tooth movement, periostin has differing roles as Wilde found greater expression in compression sites and decreased in tension areas compared to control sites in rats (Wilde, 2003). However periostin was chosen in this study as it responds to mechanical stress, is found during the bone formative period within the PDL and can signal increased osteoblast differentiation. In short, periostin is important for maintaining healthy alveolar bone, preserving bone mass and promoting bone remodeling (Conway, 2013) and in this study will help us gain insight into the orthodontic tooth movement process.

Inflammation

In the second part of this study we will look at inflammation and PDL cells, where the cells will be subjected to vibration and IL-1 β as our model of orthodontic strain. Pain is intimately connected to inflammation and with AcceleDent's claim of reducing pain we expect to see a similar decrease in the expression of inflammation markers. The gene expression of MMP-13, an inflammatory cytokine will be measured.

MMP-13 is from a family of proteins known as matrix metalloproteinases which are zinc-ion-dependent proteolytic enzymes that cells produce in a variety of situations. They have functions in development, inflammation, degenerative articular diseases, tumor invasion and wound healing (Takahashi, 2003). MMPs are also key inflammatory mediators in the orthodontic tooth movement process (Capell, 2011). There are various subgroups, which MMP-13 is a collagenase. MMP-13 is regulated by inflammatory

cytokines, such as prostaglandins and interleukins as well as mechanical strain. MMP-13 is known to be expressed in PDL cells during tooth movement and plays a role in the remodeling of periodontal tissues (Takahashi, 2003). MMP-13 is integrally involved in inflammation pathways. From this study we hope to determine if vibration can alter inflammation and provide a potential molecular reason that vibration could decrease pain perception.

Study Design

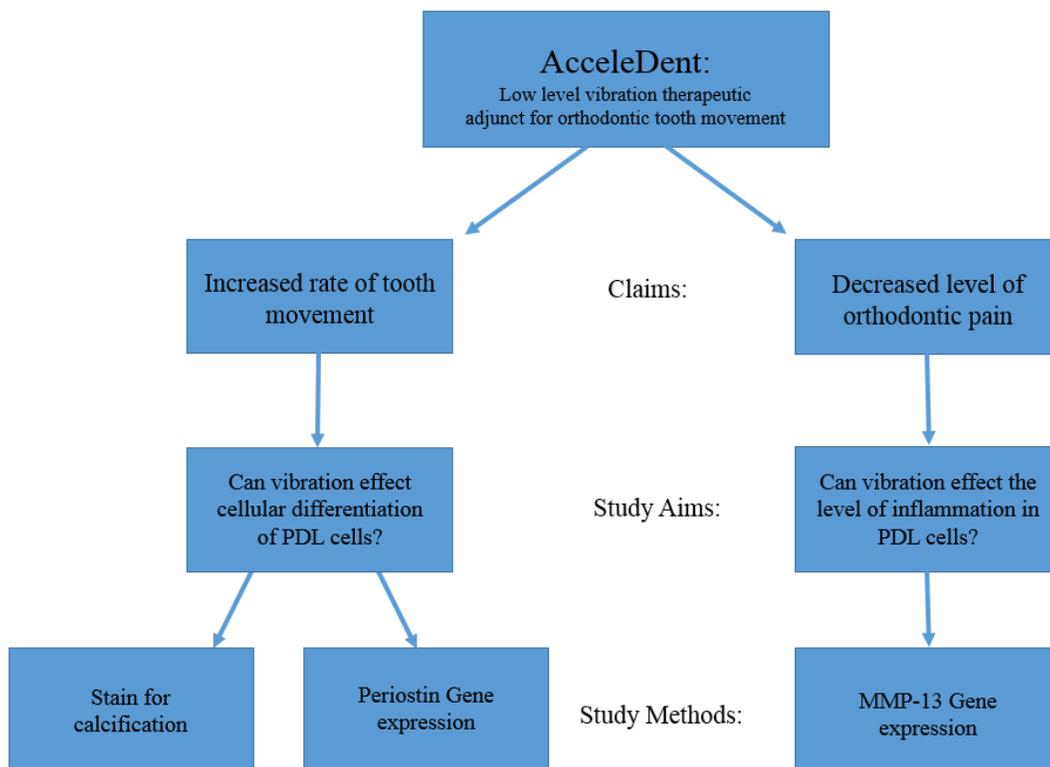


Figure 4: Study design, aims and methods

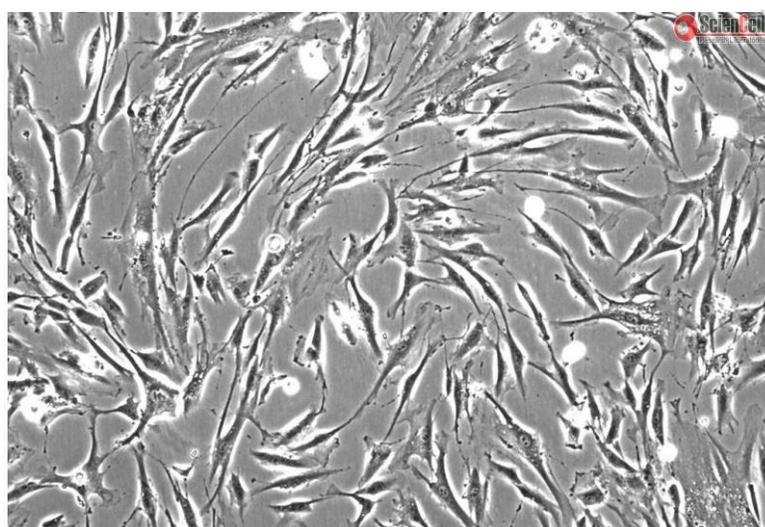
CHAPTER III

MATERIALS AND METHODS

Human Periodontal Ligament Fibroblasts

Human periodontal ligament cells (#2630) from ScienCell Research Laboratories were chosen for this study. The periodontal ligament is important in the maintenance and regeneration of periodontal tissue, and plays a key role in orthodontic tooth movement. The cells of the PDL are primarily fibroblasts but can be multipotent or composed of heterogeneous cell populations that differ in function. Below is a picture of human periodontal ligament fibroblasts #2630 from ScienCell Research Laboratories.

Human Periodontal Ligament Cells



Human Periodontal Ligament Fibroblasts (HPLF) - Phase contrast, 200x.

Figure 5: Human periodontal ligament fibroblast cells from ScienCell Research Laboratories seen under 200x magnification. *Reprinted from ScienCell Research Laboratories. (<http://www.sciencellonline.com/human-periodontal-ligament-fibroblasts.html>)*

Mechanical vibration setup

Plated cells were placed on a rigid platform. A base capable of delivering measured amounts of vertical vibration was generated by a modular piezoelectric device. The vibration frequency of 30Hz was controlled by a function generator (Instek: Model FG 8015G). A current amplifier (Advanced Motion controls, Camarillo CA, Model Brush Type PWM Servo Amplifier) delivered 0.3 g of acceleration to the vibration plate. To verify the amount of vibration cells received an accelerometer was connected to the cell plates (Endevco). The mechanical setup was housed in a thermos box of 37°C. A similar setup, used by Kulkarni et al, 2013 is shown in the figure below.

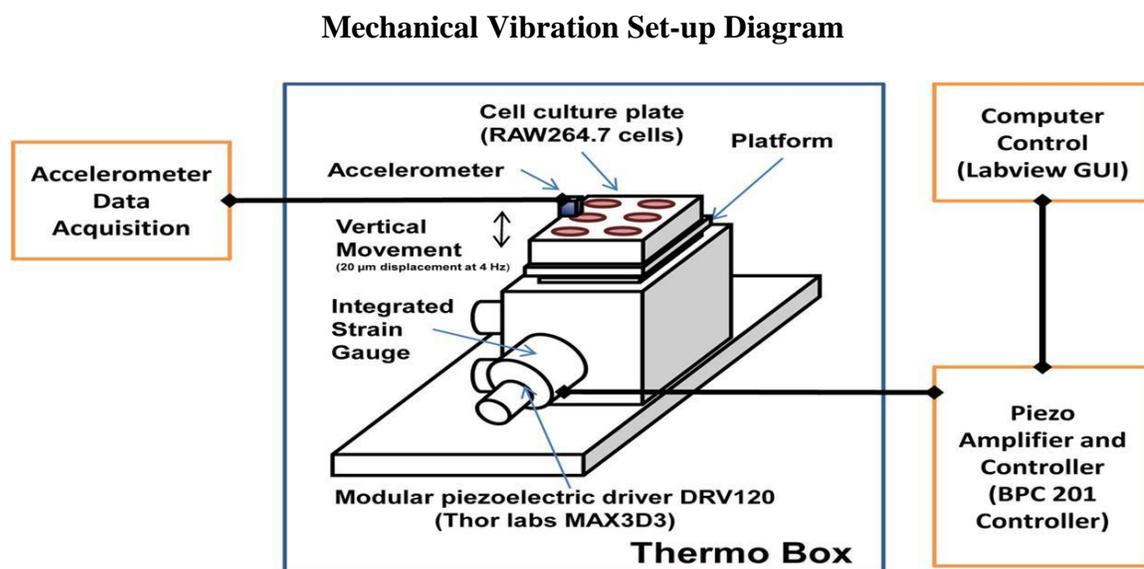


Figure 6: Mechanical vibration system composed of an enclosed 37°C heated box, vibration generator and platform for plated cells. Vibration is controlled by a piezo amplifier and controller. An accelerometer measures output. *Reprinted from “Mechanical vibration inhibits osteoclast formation by reducing DC-STAMP receptor expression in osteoclast precursor cells,” by R.N. Kulkarni, P.A Voglewede and D. Liu, 2013, Bone, 57, 493-498. Copyright 2013 by Elsevier. Reprinted with permission.*

Mechanical Vibration Set-up

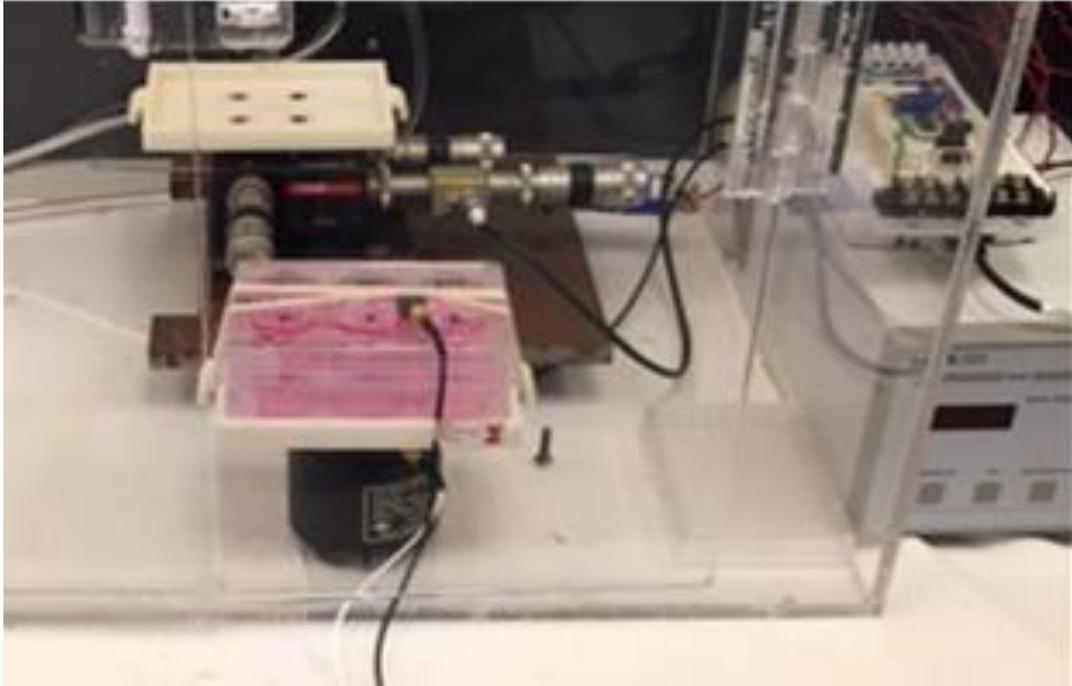


Figure 7: Enclosed thermal box at 37⁰C. Platform delivering vibration to plate of cells. Accelerometer attached on top of plate.

Experimental setup, groups and procedure

1) Differentiation Assay

Human PDL cells were cultured at 37 °C and 5% CO₂. A control group was cultured in DMEM supplemented with 10 % Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. The experimental group was cultured in an osteogenic medium containing β -glycerophosphate (10 mM), ascorbic acid (50 μ g/ml) and dexamethasone (0.1 μ M). After 2 days, the cells were seeded on 24-well culture plates at a density of 5×10^5 /well, and assigned within the plate to the study groups shown below (Table 1). IL-1 β working concentration was 1ng/ml.

Cell Differentiation Experimental Groups

Control medium	Control medium + IL-1 β
Differentiation medium	Differentiation medium + IL-1 β

Table 1: Experimental groups of PDL differentiation.

After confluence, the cells were either subjected to 0.3g/30Hz mechanical vibration or treated as sham control (0g/0Hz) for 1 hour per day over 21 consecutive days. Culture medium was refreshed every 3 days.

The level of differentiation of the PDL cells was examined using Alizarin Red staining. In details, the cells were washed with PBS and fixed with 10% formalin for 15 minutes at room temperature. Following, the cells were washed twice with distilled water and incubated at room temperature for 20 min with gentle shaking and addition of 10 % Alizarin (adjusted to 4.1-4.3pH with 0.5% NaOH). After incubation, the stained cells were washed four times with distilled water. The results of staining were observed under a reverse microscope and scanned into .tiff files by using Photoshop software (version CS6, 13.0.1). The stains were qualitatively analyzed.

2) Gene expression

Human PDL cells were maintained in 6-well plates with DMEM supplemented with 10 % Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin at 37 °C and 5% CO₂. The cells were used in gene expression experiments after confluence, approximately 1-2 days after seeding. The four experimental groups, all seeded in separate plates, are shown below (Table2).

Gene Expression Experimental Groups

(-) IL-1 β , (-)Vibration (<i>Control</i>)	(-) IL-1 β , (+)Vibration
(+) IL-1 β , (-)Vibration	(+) IL-1 β , (+)Vibration

Table 2: Experimental groups for PCR Analysis

The cells were placed into the vibration apparatus for 1 hour, subjected to 0.3g/30Hz vibration (+) or at 0g/0Hz vibration (-). Addition of IL-1 β at 10ng/ml occurred simultaneously with vibration. After 1 hour of either control or vibration conditions, Trypan blue exclusion assay was utilized to confirm cell viability. The cells were lysed and total RNA was extracted using Trizol (Bio-RAD) following the manufacture's protocol. The extracted total RNA was treated with DNAase (Life Technologies Corporation) to ensure sample integrity and remove any genomic DNA. The total RNA samples were stored at -20°C.

Total RNA concentrations were measured by Spectrophotometer and 1 μ g of RNA was used for RT reactions. Reverse transcription (RT) reaction was accomplished using Oligo (dT) 20 primers and SuperScript II (Life Technologies Corporation) as instructed by the manufacturer. GAPDH, periostin and MMP-13 primers were obtained from Integrated DNA Technologies and verified for sequence quality using end-point PCR. Primer sequences are listed below in Table 3. cDNA obtained from reverse transcription reaction and primers were utilized to estimate gene expression using qPCR. qPCR was run using the StepOne real-time PCR system (Applied Biosystem). For qPCR analysis, reactions containing SYBR Green Mix with ROX (MIDSCI), 20 μ M of forward and reverse primers, and cDNA were loaded into the wells of a 48-well plate on ice. The

cycling parameters were 95°C for 10 minutes followed by 40 cycles of 95°C (15s), and 60°C (60s). A melting curve was observed to ensure amplification of a single product. All reactions were performed in triplicate and the mean threshold cycle (ct) for each gene product for each sample was used for analysis. The mRNA levels of target genes were normalized to that of the housekeeping gene, GAPDH. The entire experiment was repeated three times. Statistical analysis of ANOVA was performed.

Sequences of Primers utilized

MMP13 (F)	5'TTACCAGACTTCACGATGGCATT3'
MMP13 (R)	5'TCGCCATGCTCCTTAATTCC3'
Periostin (F)	5'GACTCAAGATGATTCCCTTT3'
Periostin (R)	5'GGTGCAAAGTAAGTGAAGGA3'
GAPDH (F)	5'GAAGGTGAAGGTCGGAGTC3'
GAPDH (R)	5'GAGATGGTGTGATGGGATTTC3'

Table 3: Primer sequences for MMP-13, periostin and GAPDH. Forward (F) and reverse (R) sequences listed.

CHAPTER IV

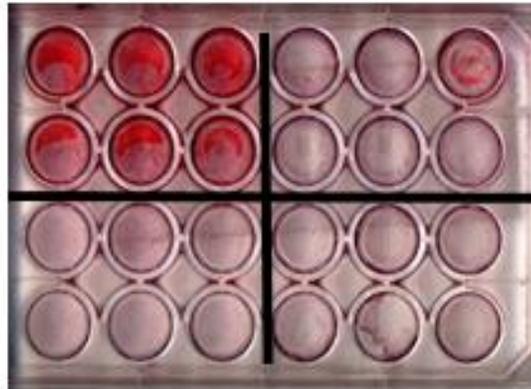
RESULTS

A) Differentiation Assay

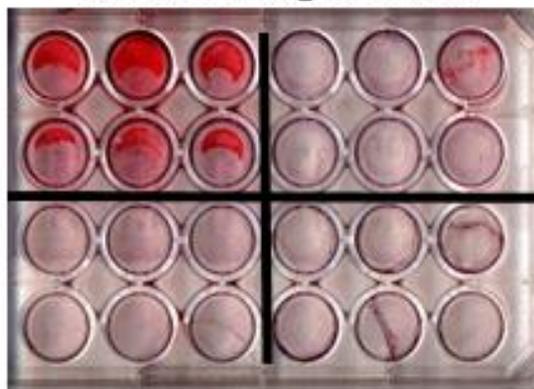
Results of Alizarin Red staining of the PDL cells subjected to IL-1 β and vibration are shown in Figure 8. PDL cells cultured in the control medium, DMEM with 10% FBS and 1% Penicillin/Streptomycin, produced no calcification in any treatment conditions. Whereas Alizarin Red staining shows high calcification in the cells cultured in osteogenic medium (differentiation medium). Addition of IL-1 β markedly decreased calcification seen in the cells cultured in osteogenic medium, however did not fully return to zero levels as seen in control medium cultured cells. Vibration at 0.3g/30Hz had no effect on the levels of calcification seen in all treatment groups, as seen in Figure 8.

Staining of PDL cells with Alizarin Red

PDL No Vibration



PDL 30Hz/0.3g Vibration



Experimental Conditions within Cell Culture Plates

Osteogenic medium	Osteogenic medium+1L-1 β
Control medium	Control medium +1L-1 β

Figure 8: Picture of plated cells stained with Alizarin Red. Stained cells show calcification.

Qualitative analysis was performed to compare samples, with a 4 degrees scale of (-) no calcification noted, (+) slight staining, (++) moderate staining, and (+++) complete staining of sample. The qualitative analysis is shown in Figure 9 and results summarized in a bar graph in Figure 10. The cells subjected to osteogenic medium had moderate calcification, reduced to slight levels with addition of IL-1 β . Vibration samples mirrored these levels. If 0.3g/30Hz vibration positively affects the cellular differentiation of PDL cells, an increase in calcification would be expected. However, no differences in calcification between PDL cells treated with 0.3g/30Hz vibration and without could be determined.

Qualitative Analysis of PDL Differentiation

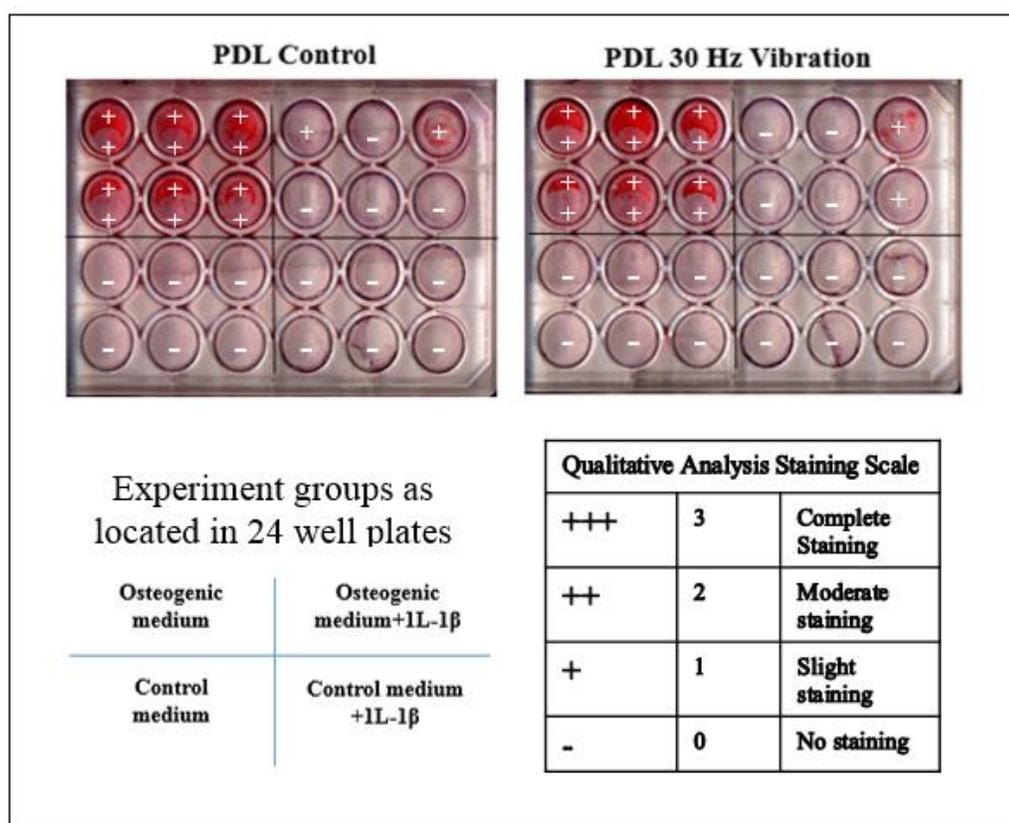


Figure 9: Qualitative Analysis of PDL differentiation, with listed scale

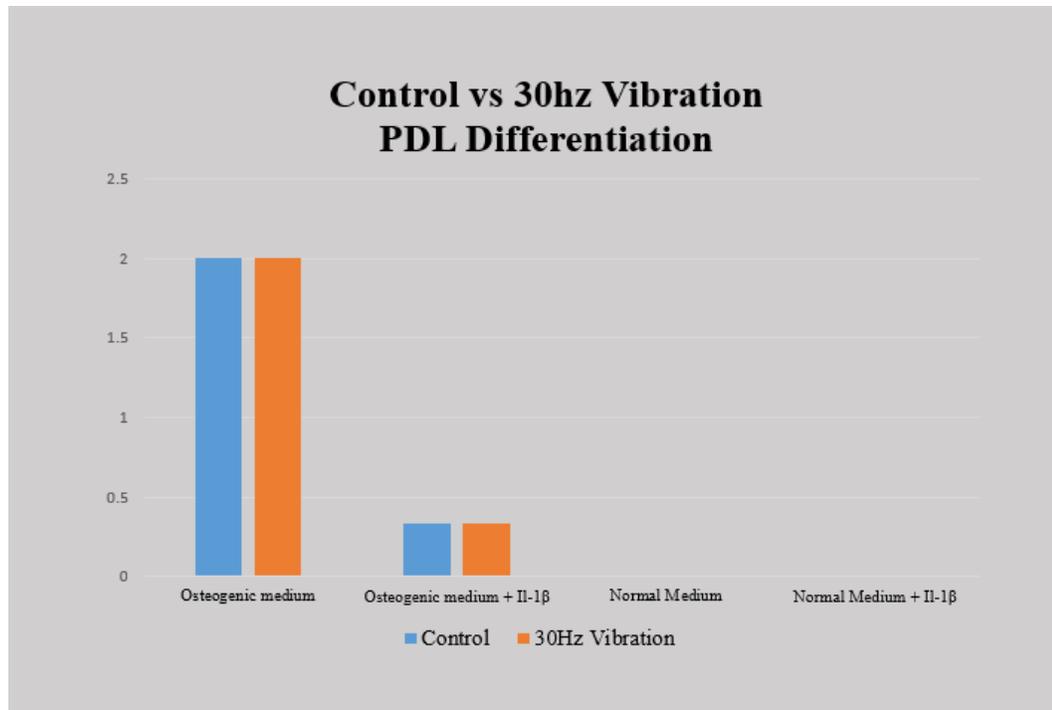


Figure 10: Qualitative analysis of PDL Differentiation

2) Gene Expression Results

In this experiment, we wanted to see the gene expressions of MMP-13 and periostin compared with the housekeeping gene GAPDH in PDL cells subjected to IL-1 β with or without vibration. Prior to utilization within the experiment, the quality of the primers, GAPDH, MMP-13 and periostin was verified. End-point PCR using TAQ polymerase enzyme showed distinct bands of product at expected size, as shown in Figure 11. End-point PCR also showed some contamination, so the samples were treated using DNase prior to qPCR.

Quality of primers; GAPDH, MMP-13, Periostin

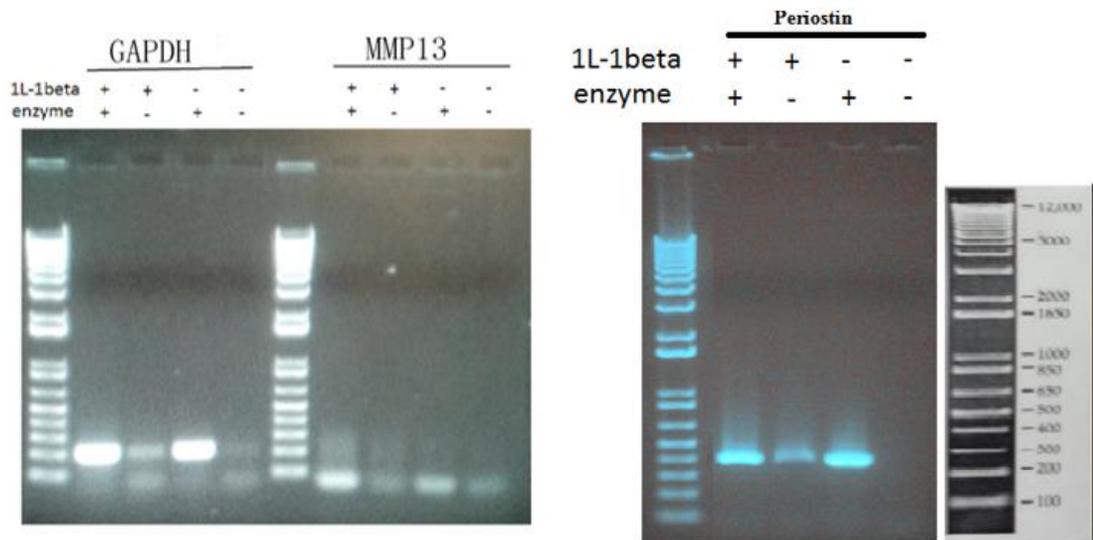


Figure 11: End-point PCR analysis of the PCR primers for GAPDH, MMP13, periostin. All three sets of intense bands at expected sizes confirmed the primers' reliability.

MMP-13 Expression

Gene expression of MMP-13 was calculated in relation to GAPDH. The expressions of MMP-13 and GAPDH data (ct values) from the three experimental trials are shown in Table 4. RQ levels of MMP-13 as related to GAPDH are shown in Table 5. Variability within trials produced big standard deviations.

MMP-13 and GAPDH CT values in Periodontal Ligament cells

Gene	MMP13 expression (CT value)				GAPDH (CT value)			
	control	1L-1beta	30HZ	1L-1beta+30HZ	control	1L-1beta	30HZ	1L-1beta+30HZ
Experiment 1	29.7148952	28.93675613	30.48834038	29.69169235	12.5826149	12.5210848	12.79488659	12.88311386
	29.6096573	28.2482872	30.1764946	29.77604675	12.5137587	12.5115824	12.55266285	12.91366482
	29.5644512	28.30071831	30.22827148	29.80355072	12.3266697	12.4743214	12.78187275	12.87083817
Experiment 2	30.1281872	30.09852982	30.4459343	30.82317352	13.3841295	13.2928915	13.77114391	12.94978046
	30.3315239	30.27981949	30.24276161	29.97358322	13.647212	13.2179289	13.4272728	13.58940697
	30.1771507	30.07518196	31.02029037	31.66429901	13.43262	13.1940222	13.54966927	14.10658836
Experiment 3	34.1930504	31.58631325	32.79443741	34.03709412	19.7268829	13.8476286	15.93914318	16.04094505
	34.3978195	30.95522499	32.85752487	33.47303391	19.9504318	13.6206617	15.56635571	15.82611847
	34.3025894	31.17357635	32.78982925	33.34088898	19.1603184	13.986618	15.73820972	16.2337513

Table 4: CT values of MMP-13 and GAPDH expressions. Four groups were tested: no vibration, no 1L-1 β (control), 1L-1 β , 30Hz vibration, 1L-1 β + 30Hz vibration. Three experiments, with three qPCR data points per experiment are shown above.

MMP-13 expression in PDL cells

Gene	MMP13 expression (RQ value)			
	control	1L-1beta	30HZ	1L-1beta+30HZ
Experiment 1	1	2.23	0.74	1.22
Experiment 2	1	0.875434101	0.833545744	0.684223771
Experiment 3	1	0.15	0.192	0.134
mean	1	1.0851447	0.588515248	0.679407924
SD	0	1.055738557	0.346563065	0.543016017

Table 5: MMP-13 expression in PDL cells, normalized to GAPDH levels.

Looking at data trends, IL-1 β increased MMP-13 expression but was similar to the control group, only 0.1 fold higher. With addition of vibration, MMP-13 expression decreased in both conditions. Without IL-1 β , a decrease of 0.4 fold and with IL-1 β , 0.3 fold. This is summarized in graphical format in Figure 12. Due to variability within the trials, ANOVA analysis led to no statistical significance and a p value of 0.72, Table 6.

MMP-13 expression in PDL cells subjected to IL-1 β and 30Hz Vibration

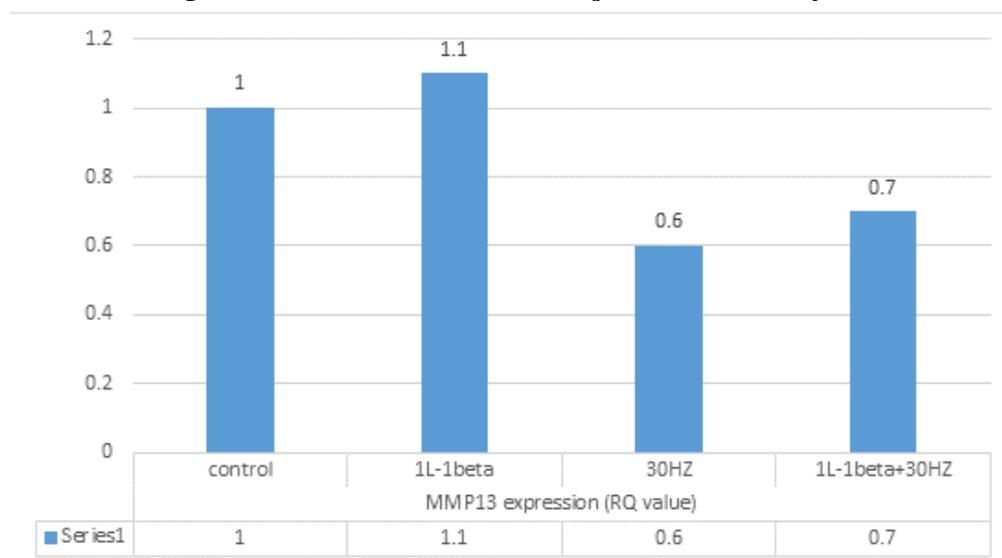


Figure 12: Data Trends, MMP-13 expression in PDL cells, normalized to GAPDH.

MMP-13 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.524	3	.175	.457	.720
Within Groups	3.059	8	.382		
Total	3.583	11			

Table 6: MMP-13 ANOVA analysis. No statistically significant findings, $p = .720 (\leq 0.5)$

Periostin Expression

Gene expression of periostin was also compared in relation to GAPDH.

Expressions of periostin and GAPDH data from the three experimental trials are shown in

Table 7. RQ levels of periostin as related to GAPDH are shown in Table 8.

Periostin and GAPDH CT values in Periodontal Ligament Cells

Gene	Periostin expression (CT value)				GAPDH (CT value)			
	control	1L-1beta	30HZ	1L-1beta+30HZ	control	1L-1beta	30HZ	1L-1beta+30HZ
Experiment 1	17.567028	16.7887516	17.46600914	17.81301689	12.5826149	12.5210848	12.79488659	12.88311386
	17.6833286	16.50580597	17.49196053	17.92863274	12.5137587	12.5115824	12.55266285	12.91366482
	17.4103794	16.71880531	17.62155151	17.91352272	12.3266697	12.4743214	12.78187275	12.87083817
Experiment 2	18.5072842	18.92373085	18.44428444	18.06038094	13.3841295	13.2928915	13.77114391	12.94978046
	18.9631214	18.40853691	18.51723289	18.53885078	13.647212	13.2179289	13.4272728	13.58940697
	18.5418396	18.30044174	18.27308464	18.05931664	13.43262	13.1940222	13.54966927	14.10658836
Experiment 3	21.5647411	16.93522072	18.46227646	18.69525337	19.7268829	13.8476286	15.93914318	16.04094505
	21.6881485	16.27373886	18.409935	18.69450951	19.9504318	13.6206617	15.56635571	15.82611847
	21.8255539	16.22326851	18.35246277	18.75188065	19.1603184	13.986618	15.73820972	16.2337513

Table 7: CT values of Periostin and GAPDH expressions. Four groups were tested: no vibration, no IL-1 β (control), 1L-1 β , 30Hz vibration, IL-1 β + 30Hz vibration. Three experiments, with three qPCR data points per experiment are shown above.

Periostin expression in PDL cells

Gene	Periostin expression (RQ value)			
	control	1L-1beta	30HZ	1L-1beta+30HZ
Experiment 1	1	1.87	1.19	1.05
Experiment 2	1	0.916033447	1.278030753	1.425864577
Experiment 3	1	0.669	0.668	0.659
mean	1	1.151677816	1.045343584	1.044954859
SD	0	0.634229021	0.329740032	0.383457182

Table 8: Periostin expression in PDL cells, normalized to GAPDH levels.

Periostin expression in all groups was very similar. Compared with control, periostin expression in the presence of IL-1 β slightly increased by 0.15 fold. With addition of vibration a 0.04 fold increase occurred with and without IL-1 β . This is summarized in graphical format in Figure 13. As stated only slight difference in periostin expression was found across all groups and ANOVA analysis confirmed no statistical significance and a p value of 0.971, Table 9.

Periostin expression in PDL cells subjected to IL-1 β and 30Hz Vibration

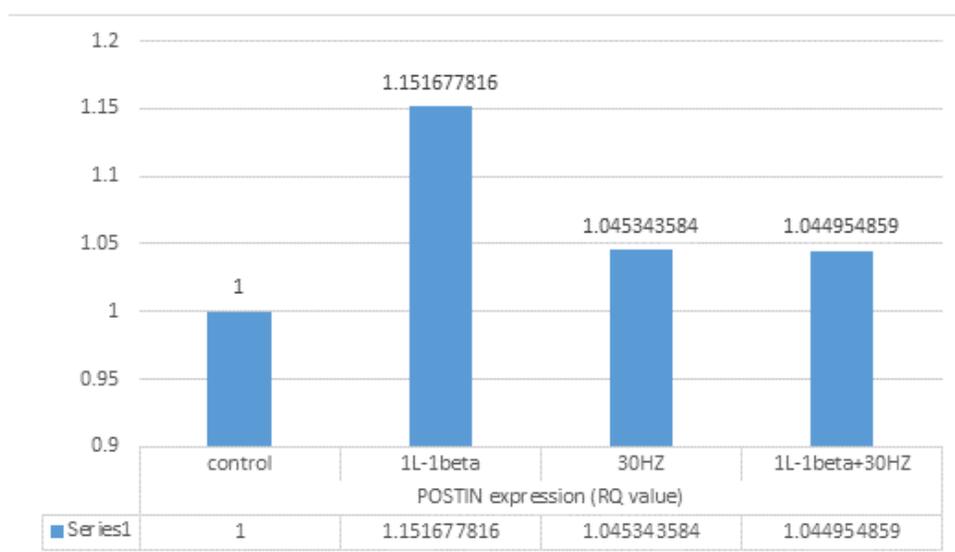


Figure 13: Periostin expression in PDL cells, normalized to GAPDH levels.

Periostin ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.037	3	.012	.076	.971
Within Groups	1.316	8	.165		
Total	1.353	11			

Table 9: Periostin ANOVA analysis. No statistically significant findings, p=.971 (≤ 0.5)

CHAPTER V

DISCUSSION

Experimental Setup

The study goal was to see the effects of vibration on cellular differentiation and inflammation under orthodontic tooth movement. Periodontal ligament cells (PDL) were chosen due to their essential role in response to mechanical force and capacity for renewal and repair during orthodontic tooth movement (Lekic, 1996). As an important player in the events of alveolar bone remodeling, PDL cells are an ideal population of cells to study. Periodontal ligament cells are primarily fibroblasts but also can include osteoblasts, osteoclasts, epithelial cell rests of Malassez, macrophages, undifferentiated mesenchymal cells, neural elements, cementoblasts and endothelial cells (Lekic, 1996). Previous studies of differentiation, however, utilized PDL cells from animals, or PDL stem cell populations only, which can be limitations. Hence our study utilized cells obtained from ScienCell Laboratories which were human PDL in origin with a heterogeneous mix of cells as seen within an in vivo periodontal ligament.

Other aspects of our study also sought to provide the most pertinent view of orthodontics. IL-1 β was utilized as our model of orthodontic stress, as IL-1 β is a key cytokine that is released early to response to mechanical stress (Krishnan, 2006). Vibration frequency of 30Hz and magnitude of 0.3g paralleled the output of AcceleDent, the FDA approved vibration treatment adjunct currently on the orthodontic market. To study the mRNA changes of cytokines i.e. MMP-13 and periostin, a short treatment time of 1 hour was chosen to see immediate and pronounced molecular effects of vibration. Whereas, a longer treatment time for the differentiation experiment of 21 days was

utilized to gain insight on how cells react to extended treatment, as occlusal vibration in orthodontics is also extended, usually 20 min a day over the course of an entire treatment, usually 12-24 months.

Cellular Staining Results

The differentiation aspect of our study confirmed that the human PDL cells can be differentiated, capable of calcification and osteogenesis, as seen when PDL cells were cultured in osteogenic medium. This is consistent with the findings from Wolf et al who showed transplanted human PDL cells into mice can show osteogenic and cementogenic differentiation. The transplanted cells in their study showed osteogenic markers for alkaline phosphatase, osteopontin, PTH-receptor 1 and osteoclastin as well as early mineralization by calcium and phosphate staining (Wolf, 2013). Lin et al, went further into detail about how this process occurs. After tooth extraction in rats, Lin et al monitored PDL fibroblasts and their role in osteogenesis. After extraction PDL cells began to proliferate highly, peaking at 24 hours. Between 1-2 days, cells began to migrate into the coagulum and by 3 days were producing collagen fibers and dense connective tissue. By day 4-5 some PDL fibroblasts had differentiated into osteoblasts and began to lay down new bone (Lin 1994).

With the addition of IL-1 β as our model of orthodontic tooth movement, we see a marked decrease of calcification. A significant decrease of calcification was seen in the sham control plate when IL-1 β was added and similar decrease was seen in the 30Hz vibration group. This shows that IL-1 β inhibits differentiation. This is to be expected as inflammation factors, such as IL-1 β , have global catabolic effects. While IL-1 β is

important for bone resorption, it inhibits bone formation (Iwasaki, 2001). By lowering the differentiation potential of PDL cells, IL-1 β concentrations could decrease the rate of bone formation. Any intervention to offset this potential could lead to an avenue to increase the rate of orthodontic tooth movement.

Interestingly, however, the addition of vibration at 30Hz produced no significant change in the amount of differentiation compared to control, with or without IL-1 β addition. This leads to a conclusion that 0.3g/30Hz vibration does not have the ability to affect differentiation either in control cells or mechanically stressed cells. This is contrary to a previous study from Zhang et al, who showed increasing osteogenic differentiation with application of vibration. However in the study, Zhang et al utilized a more general cell, human PDL stem cells and different vibration amounts. While our study focused on 30Hz, and 0.3g acceleration, which is the amount of AcceleDent, their study had a frequency of 50Hz and tested magnitudes of 0.1g to 0.9g. 0.3g and 50Hz was found to have the highest differentiation of the PDL stem cells (Zhang, 2014). It is possible that by increasing our vibration frequency level a change in differentiation could be seen. In fact the same researchers, lend evidence to this theory as they also tested human PDL stem cell differentiation to 0.3g acceleration and various frequencies from 10-180Hz. There, 30Hz and 0.3g acceleration which is the same utilized in our experiment was found to have no significant difference to the control in both alkaline phosphatase activity, osteogenic gene expression and osteogenic protein expression. Only higher frequencies, with a peak of 50-60Hz/0.3g produced positive results for osteogenesis (Zhang, 2012).

Although Zhang et al has studied PDL stem cell's differentiation responses to vibration, it is worth note that they did not investigate whether a change would be seen in an inflammatory environment, as seen with orthodontic tooth movement. With addition of IL-1 β , an orthodontic model of stress, our intent was to see if a change in differentiation could be seen in an inflammatory environment. However our results indicate no change of PDL differentiation with or without IL-1 β . This could indicate that vibration of 0.3g/30Hz has no measurable impact on healthy or inflammatory-stressed PDL cells.

Gene Expression Results

Orthodontic tooth movement relies on recruitment of cells within the PDL to respond to mechanical stress and differentiate into cells capable of tissue remodeling. Cytokine mediators within orthodontic tooth movement help orchestrate these cellular responses to tooth movement. These cytokines have many regulatory and signaling functions. We were interested in two specific cytokines, periostin and MMP-13.

Periostin has roles in the bone remodeling and differentiation of cells during tooth movement. Periostin preserves bone mass and promotes bone remodeling (Conway, 2013). Higher levels of periostin increase osteoblast proliferation, and differentiation (Zhu, 2009). Since one of our aims is to determine if vibration has an effect on differentiation of PDL cells, periostin was chosen.

MMP-13 is a collagenase and an inflammatory cytokine. By measuring MMP-13 mRNA expression within the cell, we hoped to see if vibration had an effect on overall cellular inflammation. While inflammation cytokines are needed to facilitate orthodontic

tooth movement, higher than physiologic levels of inflammation can bring up side effects including pain, root resorption and exasperate periodontal disease. Within orthodontics, it would be positive if vibration therapy could increase the rate of orthodontic tooth movement while simultaneously reducing the overall level of inflammation.

As can be seen, the qPCR analyses of MMP-13 and periostin produced large variations within the three experiments which limited our results and consequently produced no statistically significant conclusions. However, general trends can be seen to which future study can be oriented towards. Analysis of MMP-13 using PDL cells showed similar expression in both control and IL-1 β treated cells. These similar levels could be due to a lack of quality, concentration or treatment time of IL-1 β . With addition of vibration, a parallel decrease is seen with and without addition of IL-1 β . This may show that vibration can lower cellular inflammation. These findings parallel other studies. Xu et al looked at cyclic tensile strain on chondrocytes and found that low vibration of 0.5 Hz acted as an antagonist of IL-1 β , decreasing inflammation (Xu, 2000). Similarly, Lavagnino et al showed decrease of MMP-1, interstitial collagenases expression in tendon cells when subjected to 0.17 Hz to 1.0 Hz (Lavagnino 2003). Our preliminary findings that vibration may decrease MMP-13 production in PDL cells are interesting and further studies are warranted.

The qPCR results of periostin expression in this study are more complicated to explain. In our experiments, a slight increase in periostin is seen with addition of IL-1 β , however similar to control values are seen with addition of vibration with and without IL-1 β treatment. No values produced statistically significant results and no trend could be determined. This could be related to the following factors. First, periostin is related to

the differentiation of cells during tooth movement. As seen previously, 0.3g/30Hz vibration did not produce any change in PDL osteogenic differentiation when stained with Alizarin Red. Since there were no significant results nor any general trends seen within periostin expression, it could once again be evidence that vibration at this level is not capable of producing differentiation of PDL cells. Secondly, periostin is a complex molecule, with many different roles of which many are not fully understood. Cobo states that periostin seems to have different expression based on cell type and degree of mechanical stress (Cobo, 2016) and as stated previously even within tooth movement differing amounts of periostin occur within different areas of the PDL (Wilde, 2003). Thus perhaps our experimental model is not adequate to study periostin expression in orthodontic tooth movement. Continued studies may benefit from utilizing a different cytokine to focus on.

Limitations of the Study

During this study there are limitations that could have affected our results. First, orthodontic tooth movement was simulated in vitro by utilizing IL-1 β . While this model is warranted, as IL-1 β is produced during initial orthodontic tooth movement and has been shown to regulate bone resorption and bone formation by mechanical stress (Davidovitch, 1988 and Preiss, 1994), orthodontic tooth movement is complex and many factors, cytokines and cells are actively involved. In such, IL-1 β is a simplistic model, although mimicking all conditions that occur in vivo is not possible. Secondly, IL-1 β was utilized in concentrations of 1 ng/ml for differentiation and 10 ng/ml for qPCR analysis. There is limited research available to determine in vivo concentrations of IL-1 β

during orthodontic tooth movement and studies that exist attempt to measure gingival crevicular fluid (GCF), a process that is inexact. Preiss reported that the concentration of IL-1 β in healthy patients GCF to be within the range of 22.8 ng/ml – 150 ng/ml and 85.8 ng/ml - 882.2 ng/ml in periodontitis patients (Preiss 1994). This would signal to us that our concentration of IL-1 β was too low. However a study of orthodontic tooth movement on IL-1 β in canines showed a lower concentration. In this study teeth showed a concentration of 0.10 ng/ml of IL-1 β in the GCF of orthodontically moved teeth (Leethanakul, 2016). Different concentrations of IL-1 β in vivo reported in literature, leads to confusion on how much to utilize as a study model. Future studies may utilize higher concentrations or longer treatment time of IL-1 β on the PDL cells.

The nature of our qPCR results was also a limiting factor of this study. Variations within our qPCR results was seen. This could have been from many factors and due to the numerous steps from cell culture to qPCR analysis, any variability or inconsistency is of great detriment. This variations led to big standard deviations and no statistically significant conclusions. Such large end stage variability may point to errors in the beginning of cell culture. Future experiments under close laboratory control are needed to obtain a larger sample size with more consistency.

Clinical Implications and Conclusions

Low magnitude, high frequency vibration is an interesting therapeutic adjunct or treatment that many medical fields are studying. There are studies that show the positive results of vibration in treating osteoporosis, maintaining strength when a patient is disabled, during fracture healing, and for tendon and muscle healing (Edwards 2015).

Low magnitude, high frequency vibration in the field of orthodontics may have benefits such as increasing osteogenesis and decreasing inflammation. The implications of these could help many people who seek orthodontic care. The negative aspects of orthodontics such as increased caries and gingival hypertrophy due to poor oral hygiene combined with extended treatment times, and inflammation modulated side effects such as pain, root resorption and periodontal breakdown could be reduced if the rate of orthodontic tooth movement could be increased.

Our study focused specifically on the magnitude and frequency of the most common vibration treatment adjunct available in orthodontics, AcceleDent at 30Hz, 0.3g. AcceleDent claims to increase the rate orthodontic tooth movement and decrease pain. While other studies focus on different frequencies and magnitudes, this study sought to limit focus to this commercially available treatment adjunct to make findings clinically relevant. In our study, the addition of vibration at 0.3g/30Hz did not change the differentiation of PDL cells, both with and without an inflammatory environment. Thus if AcceleDent does indeed produce faster orthodontic tooth movement, this study implies that PDL differentiation is not the mechanism. Also our gene expression results indicate that inflammation in PDL cells may be reduced with vibration, however our data trends were not statistically significant. Hence, these data alone do not support reduction in inflammation as a mechanism that pain could be decreased. However further experiments focusing on reducing experimental variability are warranted at investigating vibration's effect on PDL inflammation.

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