The Direct Effect of Low-Magnitude High-Frequency Mechanical Vibration on Osteoclast Formation from RAW267.4 Monocytes

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THE DIRECT EFFECT OF LOW-MAGNITUDE HIGH-FREQUENCY MECHANICAL VIBRATION ON OSTEOCLAST FORMATION FROM RAW264.7 MONOCYTES

By

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ABSTRACT

THE DIRECT EFFECT OF LOW-MAGNITUDE HIGH-FREQUENCY MECHANICAL VIBRATION ON OSTEOCLAST FORMATION FROM RAW264.7 MONOCYTES

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Low-magnitude high-frequency (LMHF) mechanical vibration has been demonstrated to enhance bone formation possibly through inhibition of osteoclastogenesis of bone. Earlier research has demonstrated that osteoclast formation from RAW264.7 monocytes was inhibited by a chewing cycle mimicking vibration through inhibition of dendritic cell-specific transmembrane protein (DC-STAMP). We hypothesize that application of LMHF mechanical vibration directly inhibits osteoclast formation from RAW264.7 monocytes possibly in a frequency specific manner.

RAW264.7 monocytes (ATCC) were cultured in alpha minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% Pen/Strep at 37°C and 5% CO_2. The cells were seeded at a density of 2000 cells/well in 96-well cell culture plates. After growth overnight, the cells were treated with 20 ng/ml recombinant receptor activator nuclear factor kappa-B ligand (RANKL) and refreshed every 2 days to induce osteoclast formation. In the meantime, the cells were subjected to a low-magnitude (0.3 g acceleration) mechanical vibration at various frequencies (0, 30, 60 and 90 Hz) respectively. For each frequency group, the vibration was applied for 1 hour per day for 5 consecutive days. By the end of the 5th day, the cells were rinsed with 1X PBS and fixed in 4% formaldehyde for 5 minutes. Tartrate-resistant Acidic Phosphatase (TRAP, a marker enzyme of osteoclast) staining was performed. The TRAP+ multi nuclei (> = 3) cells were counted and calculated. For statistical analysis, one-way ANOVA was used to test the differences among different frequency groups with Tukey *post hoc* comparison to compare between the groups, with p value being set at 0.05.

Three days after RANKL stimulation, osteoclasts started to form from RAW264.7 monocytes, with a peak observed on the 5th day. After 5 days, the cells underwent apoptosis and death. Compared to the control group (0 Hz), the 30 Hz but not 60 Hz and 90 Hz frequencies of vibration group showed statistically significant reduction of osteoclast formation by approximately 21% ($p < 0.05$, n = 6). No significant difference was found among the three frequency groups.

Low-magnitude high-frequency mechanical vibration directly inhibits osteoclast formation from RAW264.7 monocytes, which is frequency specific.

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CHAPTER I

INTRODUCTION

Throughout the human body, bone homeostasis is based on a balance of bone formation and bone resorption. Osteocytes, the main regulator of bone homeostasis, are responsible for maintaining bone mass through perception and response to mechanical cues including vibration. Several studies have demonstrated the beneficial effects of mechanical vibration in enhancing bone maintenance, formation, and healing in animals and humans. (Rubin *et al*., 2002; Judex *et al*., 2007; Garman *et al*., 2007; Oxlund *et al*., 2003; Xie *et al*., 2006; Rubin *et al.*, 2004; Verschueren *et al*., 2004)

Mechanical vibration is thought to have a positive effect on osteocytes, osteoblasts, and their bone marrow stromal precursor cells (BMSCs). The evidence includes: up-regulation of osteoblastic genes involved in bone formation and remodeling, direction of BMSCs lineage commitment to bias osteogenesis (You *et al*., 2008; Tan *et al*., 2007) and osteocytic signaling inhibition of osteoclastogenesis (Lau *et al*., 2010) all in the presence of mechanical vibration. It was recently demonstrated that mechanical vibration at a higher frequency of 60 Hz and 0.3 g acceleration loaded on the molars induced increased bone volumes, trabecular thickness, bone forming proteins, and a decrease in trabecular space in alveolar bone of rats (Alikhani *et al*., 2012), which is supported by a new study with a frequency at 30 Hz loaded on mouse molars that have been orthodontically moved. (Yadav *et al.* 2015).

There has been recent evidence supporting direct inhibition of osteoclasts by mechanical vibration. Osteoclasts, multinucleated hematopoietic cells of the monocyte/macrophage lineage (Lerner, 2004; de Vries *et al.*, 2009) are formed in several

steps, in which the receptor activator NF-κB ligand (RANKL)-mediated signaling pathway and downstream transcription factors play essential roles (Teitelbaum, 2007). Mechanical vibration (0.20 μm, 4 Hz for 1 h/day for 5 consecutive days) at a level that mimics mouse chewing cycles, directly inhibited osteoclastogenesis of RAW264.7 monocytes in the presence of RANKL (Kulkarni *et al.*, 2013). This is consistent with previous findings with low-magnitude high-frequency vibration (0.3 g, 45 Hz, 15 min/day) (Wu *et al.*, 2012).

The aim of our study is to explore the direct effect of low-magnitude high-frequency vibration, at various frequencies, on osteoclastogenesis in RANKL-induced RAW264.7 monocytes.

CHAPTER II

LITERATURE REVIEW

Osteoclasts and Osteoclastogenesis

Osteoclasts are multinucleated cells of monocyte lineage, arising from myeloid cells and well equipped to differentiate in a short period of time. The process of osteoclastogenesis is dependent on two cytokines. First, macrophage colony-stimulating factor (M-CSF) is critical for proliferating osteoclast progenitors. Second, NF-κB ligand (RANKL)-mediated signaling pathway and downstream transcription factors play essential roles in getting osteoclast formation from myeloid lineage and allows auto-regulation of osteoclastogenesis (Lerner, 2004; de Vries *et al.* 2009; Teitelbaum, 2007). As summarized in Figure 2-1 and Figure 2-2, RANKL originates mainly from osteoblastic cells and binds to RANK receptors on pre-osteoclasts, inducing the RANKL pathway and activates nuclear factor of activated T-cells-2 (NFAT2 or NFATc1) through a pathway involving a multitude of other factors (Figure 2-1). Another key factor in regulating osteoclastogenesis is osteoprotegerin (OPG), a decoy factor produced in osteoblasts and secreted to bind to RANKL, which determines the final effective amount of RANKL in action (Datta *et al.*, 2008) (Figure 2-2).

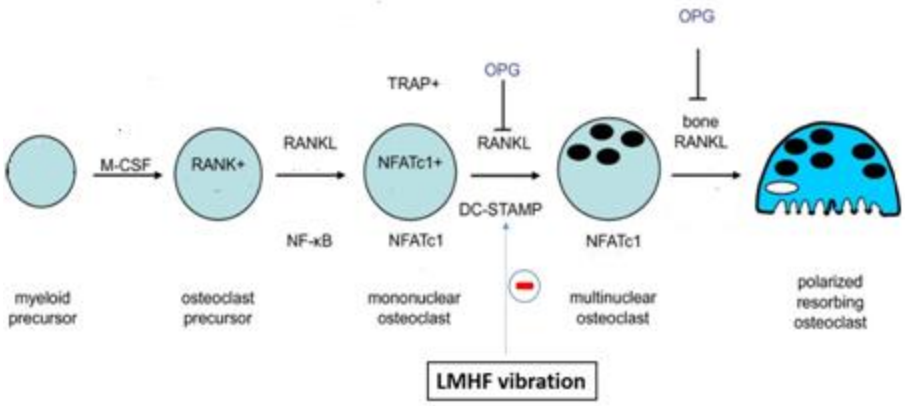

Figure 2-1: Osteoclastogenesis from myeloid cell lineage and the possible mechanism of directly regulating OC formation by mechanical vibration. (Kulkarni et al., 2013).

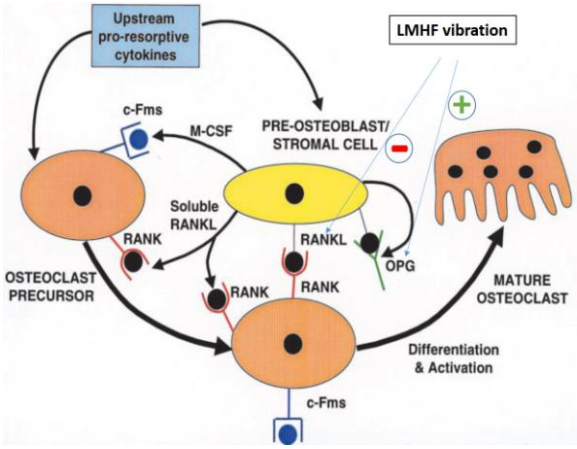

Figure 2-2: Current understanding of preosteoblastic/stromal cell regulation of osteoclastogenesis, and possible mechanism of indirectly regulating OC formation through osteocytes and osteoblasts. (Khosla S., 2001).

RAW264.7 cells are a murine osteoclastic-like cell line which can be induced to undergo transformation to macrophagic or osteoclastic cells. It is the RANKL-mediated pathway that irreversibly commits the cells to that pathway. These cells have been used as a model for osteoclastogenesis in most *in vitro* laboratory studies regarding LMHF vibration.

History of Mechanical Vibration on Bone Homeostasis

The basic multicellular unit of bone mainly consists of osteocytes, osteoblasts and multinucleated osteoclasts which are regulated by parathyroid hormone (PTH), growth hormone (GH), cytokines, biochemical stimuli and mechanical stressors.

Early work from Rubin's group provided promising results in improving the quality and maintenance of bone using low-magnitude high-frequency (LMHF) mechanical vibration. The LMHF vibration is defined as mechanical vibration at frequencies ranging from 10-100 Hz and at magnitudes typically of 0.3 g acceleration or less than 10 microstrain (με). Female sheep that underwent LMHF vibration for 20 minutes per day for 1 year showed significant increase in bone trabecular quantity and quality (Rubin *et al*., 2002). Site-specific LMHV of 45 Hz and 0.3 g in female mice demonstrated inhibition of trabecular bone resorption and maintenance of a high level of bone matrix quantity and quality (Xie *et al.,* 2006), increase in trabecular bone formation of the epiphysis (Garman *et al.*, 2007) and prevention of the ovariectomy-induced decrease in strength of the femur and tibia (Oxlund *et al.*, 2004). Similar results were demonstrated in high risk (under 65 kg) postmenopausal women who underwent whole body mechanical vibration of 20-90 Hz over a 1 year period and demonstrated a significant increase in bone mineral density, whereas controls showed a decrease in bone mineral density over the same time frame (Rubin *et al.* 2004). Increases in bone mineral density (BMD) of the hips and muscle strength were also demonstrated in postmenopausal women undergoing low-magnitude 35-40 Hz of vibration over a 6-month period (Verschueren *et al*., 2004).

Judex *et al.* (2007) provided further evidence of the LMHF vibration induced anabolic effects being frequency specific and independent of the magnitude of vibration. It was believed that the effects on bone cells was either dependent on increasing the number of loading cycles or an inherent preference of cells to specific frequencies.

These results have led to studies focusing on localized LMHF vibrations on the alveolar bone in dentistry. Alikhani *et al*. (2012) demonstrated that daily vibration of 60 Hz, 0.3 g for 5 minutes resulted in an increase in bone volume, density, trabecular thickness, collagen crosslinking, osteogenic proteins and gene expression (Figure 2-3).

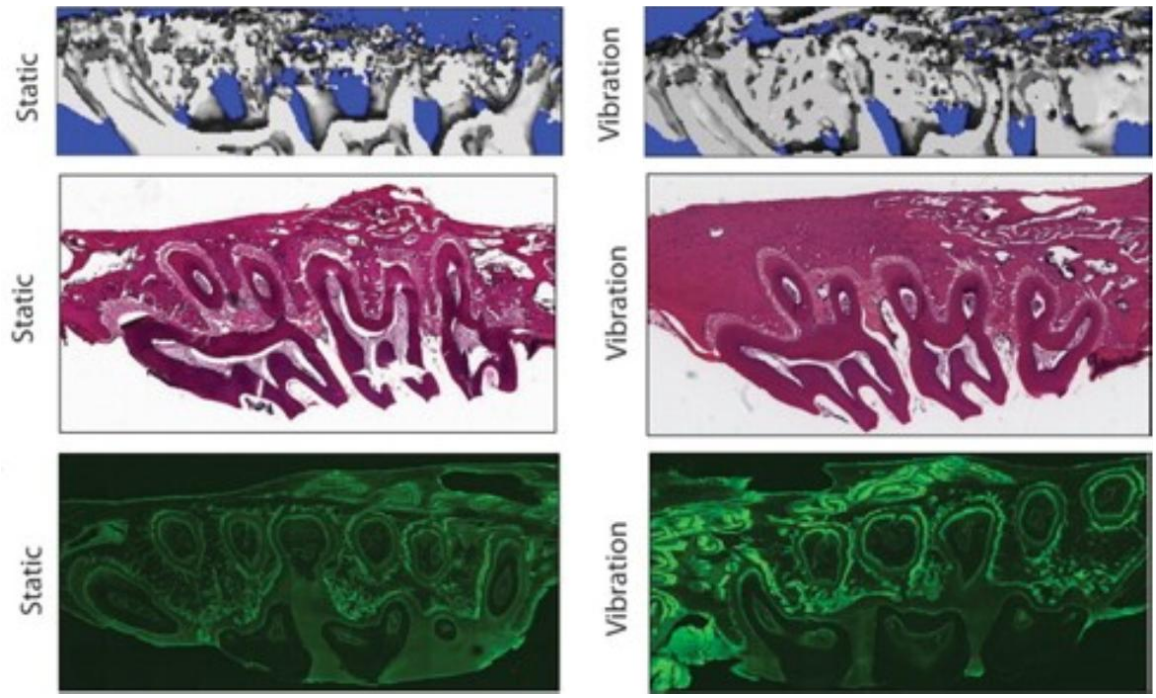

Figure 2-3: Localized LMHF on alveolar bone on the molars of mice (60 Hz, 0.3 g, 5 min). (From Alikhani *et al.* 2012)

Most recently, LMHF mechanical vibration of 30 Hz, 1 cN for 15 minutes for 7 days during relapse after orthodontically moved mice molars demonstrated similar results with increase in tissue volume, increase in PDL healing, decrease in osteoclast surface and numbers, and an overall anabolic effect on the bone (Yadav *et al.* 2015).

Mechanoreception of Osteocytes and Osteoblasts

The transformation of mechanical stress to biomechanical signals occurs mainly in osteocytes and osteoblasts and involves membrane proteins (including integrins, connexions and stretch-activated ion channels). Detection of stressors such as fluid shear stress leads to intracellular activation of signals, including integrin induced focal adhesion kinase (FAK) – mitogen-activated protein kinase (MAPK) signals (especially in osteocytes) in addition to upregulation of connexions that form channels allowing communication of cells with the extracellular matrix with other cells. (Datta *et al., 2008).*

Bone marrow precursors of osteocytes and osteoblasts have been demonstrated to transduce LMHF vibration signals to enhance bone formation. Pulsating fluid flow on osteocytes and osteoblasts resulted in a conditioned culture medium that prevented osteoclastogenesis (Tan *et al*., 2007) when co-cultured with pre-osteoclast cells. Lau *et al.* (2010) directly applied LMHF vibration on osteocytes (MLO-Y4, a mouse osteocyte cell line) and found a significant reduction in secretion of soluble factors, sRANKL and PGE_2 which produced an increase in number and size of osteoclasts formed. You *et al.* (2008) further showed both fluid flow and mechanical vibration led to less osteoclastogenisis from RAW264.7 monocytes regulated by osteocytes as there was a decrease in RANKL, increase in OPG, and possible other soluble factors that inhibited osteoclastic formation (Figure 2-2).

However, it has been demonstrated that unlike osteoclasts, the osteoblasts and osteocytes are not responding directly to the frequency, or the number of loading cycles but rather the resulting mechanical strain (Rubin *et al*., 2002, Simmons *et al*., 2003) and fluid shear stress (Kreke *et al.*, 2008; Sharp *et al.*, 2009; Kavlock and Goldstein, 2011) induced as a by-product of mechanical vibration. In summary, mechanoreception of osteoblasts and osteocytes to mechanical vibration alter osteoclastic activity.

Mechanoreception of Osteoclasts

It has long been hypothesized that transduction of mechanical signals by osteoblasts and osteoclasts is necessary to induce change in osteoclastic activity. Recently, the ability of osteoclasts to *directly* transduce mechanical signals without the presence of osteoblasts and osteocytes has been studied, specifically from LMHF mechanical vibration. Using similar LMHF vibration protocols (45 Hz, 0.3 g, 15 min/day) within the same range as previous studies, Wu *et al.* (2012) demonstrated a decrease in the number of RANKL-induced osteoclasts formed from RAW264.7 cells. This was measured using TRAP staining which stains the nuclei of osteoclasts with three or more nuclei indicative of successful osteoclastogenesis. They also noted a decrease in actin ring formation, mRNA expression on cathespin-K, MMP-9 as well as c-Fos protein, all of which are parts of the RANKL-induced cascade of osteoclastogenesis. Similar results were demonstrated by Kulkarni *et al.* (2013) with low magnitude vibration at a frequency that mimics mice chewing (4 Hz) for 1 h per day for 5 days after being treated with RANKL (20 ng/ml). In addition to the reduction in osteoclast formation, DC-STAMP gene and protein expression were reduced under mechanical vibration. This gene is necessary for fusion of the osteoclast cells (Figure 2-1). These two studies, together

with the *in vivo* studies previously referenced are able to provide some clues on the precise mechanism in which mechanical vibration has its effects within the RANKL cascade.

The way osteoclasts respond to mechanical vibration is different than that in osteoblasts and osteocytes. It is believed to be dependent on the frequency of oscillations, or the number of loading cycles and *in vivo* may be sensitive to specific frequencies that can be affected by the corresponding environment factors including hormones, age, and disease (Judex *et al.*, 2007). Again, in contrast to osteoblasts and osteocytes, mechanical strain (Sen *et al.*, 2011), fluid shear stress (Lau *et al*., 2010; Uzer *et al.*, 2012), and hydrostatic pressure, do not contribute to the mechanotransduction of vibration in osteoclasts. The precise mechanism is presently unknown and has been difficult to study because of the dynamic complexity of RANKL cascade and multiple factors involved in the process (Figure 2-1). Although various studies have found significant relationships between LMHF vibration and inhibition of osteoclastogenesis from RAW264.7 monocytes at various frequencies, none of them compared different frequencies to determine frequency specificity of the response. This in turn would lead to further investigation of the mechanisms involved that will help determine why this relationship exists whilst providing more insight into the general mechanisms involved in interference of the RANKL cascade.

Clinical Implications

Implications of the potential inhibition of RANKL signaling cascade by LMHF vibration has led to questions about its applicability in models in which RANKL signal is pathologically amplified. All existing *in vitro* studies evaluating mechanisms of the

LMHF vibration induced responses in bone have been limited to models of healthy bone. *Porphyromonas gingivalis (Pg),* a gram-negative anaerobic bacterium, is one of the most responsible pathogens for chronic periodontitis. Mechanistically the lipopolysaccharide (LPS) from the cell wall of *Pg* bacteria is responsible for the inflammatory response via a multifaceted acceleration in RANKL signaling cascade. This has been demonstrated *ex vivo* in a model of rat mandibular slices in which LPS reduced bone sialoprotein and subsequently increased RANKL signaling and osteoclastogenisis (Sloan *et al.,* 2013). Kukita *et al.* (2013) studied sub-clones of RAW264.7 cells (RAW-D) and showed LPS did not act directly on RANKL, but increases its activity two ways. First, it activates toll-like receptors (TLR), especially TLR-2, which will then amplify RANKL expression from other cells including cementoblasts and other PDL cells, in addition to producing shingolipids that promote RANKL expression from osteoblasts. Secondly, within osteoclasts, the TLRs induce an increase in factors within the cascade, especially NFATc1 (responsible for the auto amplification loop and abolishment of the necessity of this initial RANKL signal) which increases the RANKL cascade activity (Figure 2-1). Ultimately, this overall amplification of RANKL activity not only increases osteoclastogenesis, but promotes the inflammatory response seen in periodontitis.

Complexities of this relationship are evident in the body of literature as it has recently been demonstrated that LPS can have opposing effects depending on its timing of stimulation on the osteoclast precursor monocyte. The mentioned effects only occur if the cell is firstly induced by RANKL alone and then monocyte is committed to the osteoclastic pathway. If not, this leads to multinucleated cells with phagocytic properties, but with no osteoclastic activity. (Zhang *et al*., 2011; Kajiya *et al*. 2010).

Orthodontic clinical implications have recently been studied in regards to relapse in orthodontics. Yadav *et al.*, (2015) applied mechanical vibration on mouse molars moved orthodontically to see whether mechanical vibration causes a difference in post-orthodontic movement. As a result, they found that mechanical vibration applied at 30 Hz and 1cN, 15 minutes per day for 7 days after removal of mesial force on mouse molars demonstrated a tendency to decrease relapse. There was a statistically significant increase in tissue density, sclerostin (which negatively regulates bone mass), decrease in osteoclast formation, with an overall anabolic effect on the bone. There was also maintenance in the thickness and integrity of the periodontal ligament compared to the control which showed sustained disruption of collagen fibers, post-orthodontic tooth movement. However, there was no difference in the movement of the molar compared to controls which may be influenced by other factors.

Hypothesis

Based on the findings of current studies, we hypothesize that LMHF directly inhibits osteoclastogenesis from RAW264.7 cells possibly in a frequency specific manner. To test our hypothesis, we examined osteoclastogenesis from RAW264.7 cells in response to different frequencies of LMHF mechanical vibration.

CHAPTER III

MATERIALS AND METHODS

Cell culture

RAW264.7 (ATCC, Manassas, VA) cells between 10 to 14 passages were used for the osteoclast formation assay. RAW264.7 cells were cultured up to near-confluence in 75 cm^2 culture flasks using α-MEM supplemented with 10% fetal bovine serum (FBS) (ATCC, Manassas, VA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Cellgro, Manassas, VA) at 37 °C and 5% CO_2 in cell culture incubator.

Mechanical vibration setup

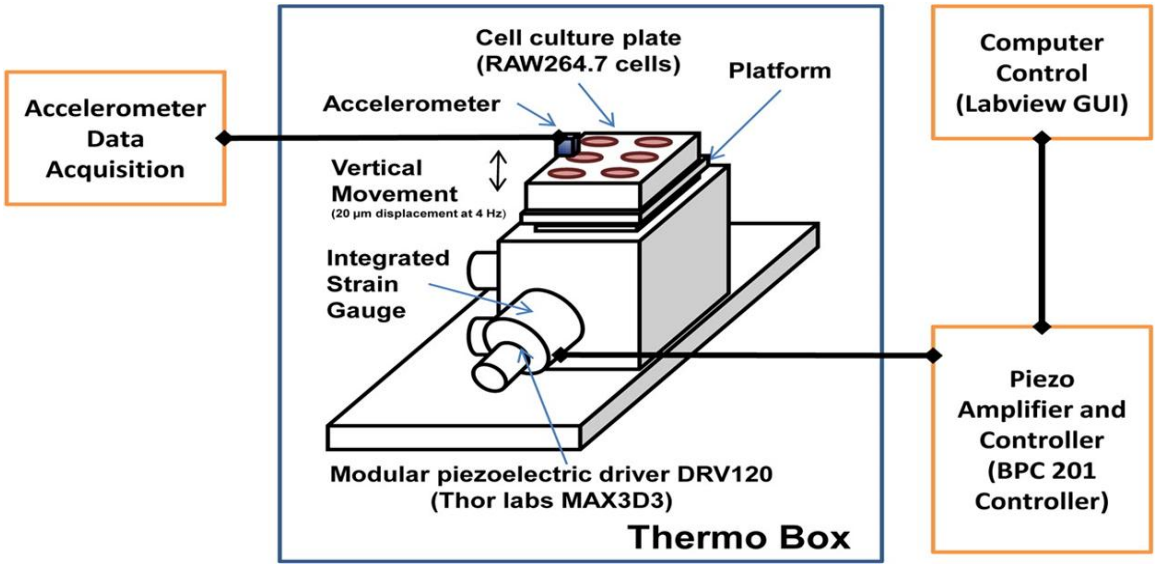

Figure 3-1: Mechanical vibration system composed of 1) vibration generator, 2) modulator, and 3) accelerometer.

A complete mechatronic test system in the experimental setup is outlined in Figure 3-1, containing mechanical and electrical components. A function generator

(Instek: Model FG 8015G) was used to generate a sinusoidal wave with a frequency of 30, 60, 90 Hz (Figure 3-2). This was connected to a current amplifier (Advanced Motion controls, Camarillo CA, Model Brush Type PWM Servo Amplifier) to deliver 0.3 g acceleration to the vibration plate (Figure 3-3). The signal generated was then measured by an accelerometer (Endevco) (Figure 3-3) on the z-axis on an oscilloscope (Hewlett Packard 150MHz Model 54602B) (Figure 3-2), to verify the frequency (in Hz) and the amplitude of 0.3 g. The whole system was powered by a 24 V, 4 Amp regulated power supply (CSi/Speco Model: PSR-4/24).

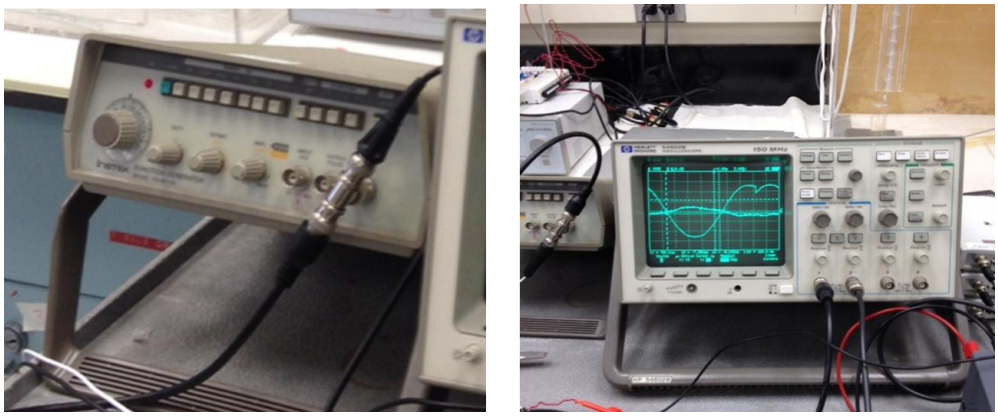

Figure 3-2: Left: Function generator controlling magnitude and frequency of mechanical vibration. Right: Oscilloscope to verify the output of the LMHF vibration.

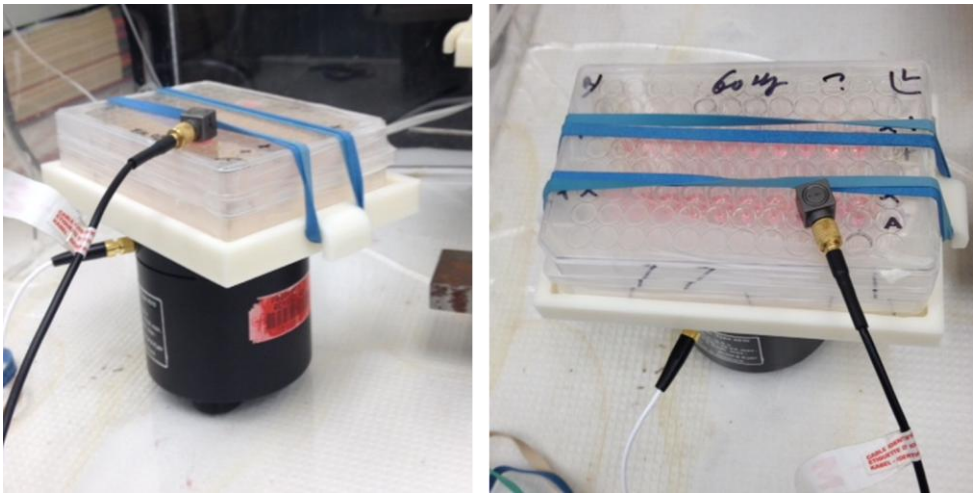

Figure 3-3: Left: Vibration delivery plate. Right: Accelerometer measuring the delivery of LMHF mechanical vibration on the 96-well plate in the 60 Hz group.

Mechanical vibration application

RAW264.7 monocytes were harvested using a cell scraper and seeded at 2.0×10^3 cells/well in 96-well tissue culture plates in α-MEM with 10% FBS and antibiotics. To induce osteoclast formation, RAW264.7 cells were incubated with 20 ng/ml mouse recombinant RANKL (R&D systems, Minneapolis, MN) overnight to prime the cells to commit to osteoclast formation, and refreshed every 2 days for 5 days. Concurrently, each group was subjected to a frequency of 30, 60, 90Hz, with 0.3 g acceleration of vibration for 1 h per day for 5 consecutive days. The cells in the control group were treated under the same condition as the cells in the vibration group but without turning on the vibration. The plates were sealed with parafilm "M" (American Can Company, Greenwich, CT) immediately prior to vibration to stabilize the pH value of the medium during vibration and removed subsequently prior to return back to the incubator.

Osteoclast formation assay

After the 5 days of culture and treatment, the cells were fixed in 4% formaldehyde in 1 x PBS for 5 minutes. Fixed cells were washed with 1 x PBS, and stained for tartrate-resistant acid phosphatase (TRAP) according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). The number of TRAP-positive multinucleated (3 or more nuclei per cell) cells was counted using a Leica DM IL microscope (Leica, Wetzlar, Germany) equipped with a 10× objective.

Statistical analysis

Each single experiment was repeated for at least 6 times. Data were presented as mean ± SD in graphs. Differences between the means were statistically analyzed using one-way ANOVA with Tukey's *post hoc* comparison, and the significance was considered when p value was less than 0.05.

CHAPTER IV

RESULTS

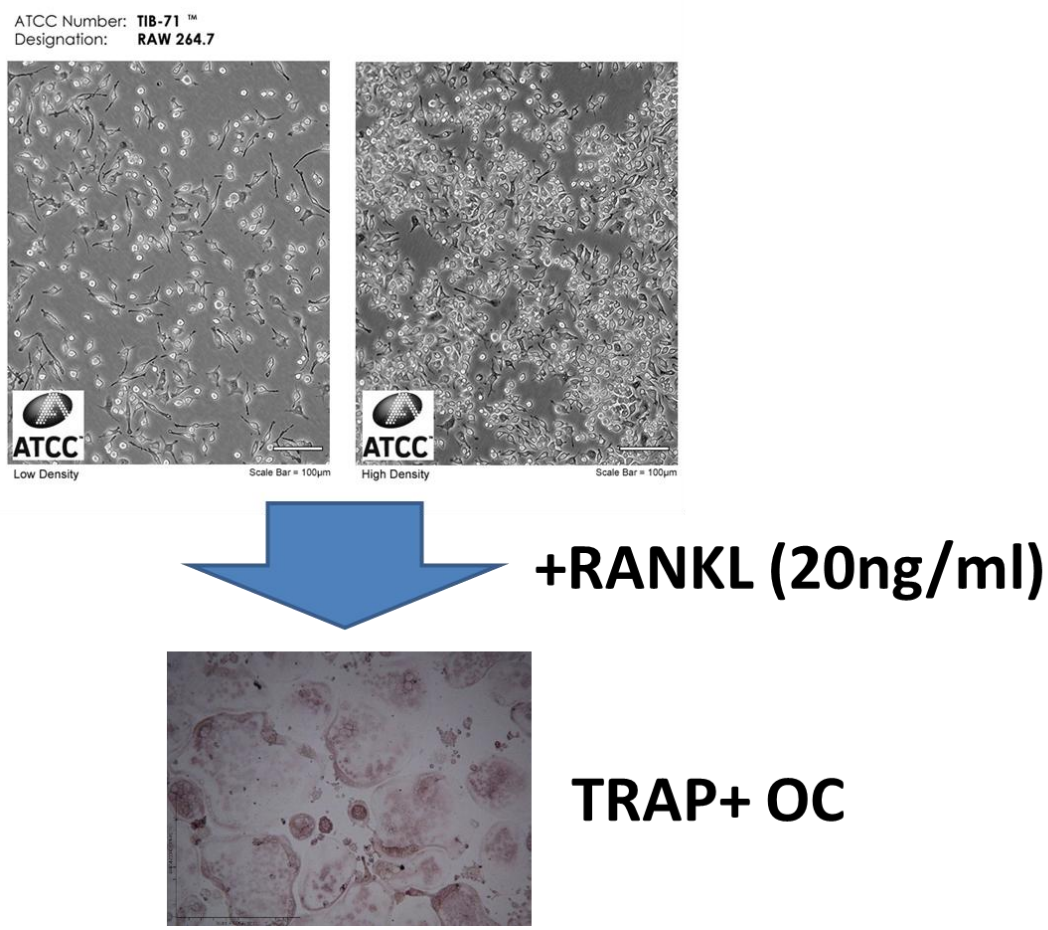

Figure 4-1: Under stimulation of RANKL, RAW264.7 monocytes merge to form multinucleated (>=3 nuclei) TRAP positive osteoclasts.

Table 4-1: Number of osteoclast formed with 0 ng/ml of RANKL in control group.

RANKL	Trial	Total
0 ng/ml	**1**	0
	2	0
	3	0
	4	0
	5	0
	6	0

Table 4-2: Number of osteoclasts formed under various frequencies of LMHF mechanical vibration.

RANKL	TRIAL	0 Hz	30 Hz	60 Hz	90 Hz
20 ng/ml	**1**	171	135	204	181
	2	180	132	189	155
	3	199	157	175	169
	4	219	205	155	204
	5	201	166	169	221
	6	213	159	186	193
	Ave	197	159	180	187
	SD	19	26	17	24

Table 4-3: Descriptive statistics for the number of osteoclasts formed under 0.3 g magnitude and frequencies of 0 Hz (Group 1), 30 Hz (Group 2), 60 Hz (Group 3), 90 Hz (Group 4).

Descriptives

OC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
1	6	197.1667	18.57328	7.58251	177.6752	216.6581	171.00	219.00
2	6	159.0000	26.35906	10.76104	131.3379	186.6621	132.00	205.00
3	6	179.6667	17.10750	6.98411	161.7134	197.6199	155.00	204.00
4	6	187.1667	23.93672	9.77213	162.0466	212.2867	155.00	221.00
Total	24	180.7500	24.87927	5.07846	170.2444	191.2556	132.00	221.00

Table 4-4: ANOVA analysis for osteoclast formation under LMHF vibration

ANOVA

OC

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	4709.500	3	1569.833	3.296	.042
Within Groups	9527.000	20	476.350		
Total	14236.500	23			

Table 4-5: Tukey comparison for osteoclast formation under LMHF vibration. Statistically significant reduction of osteoclast formation occurs only at 30Hz compared to controls (0 Hz)

Multiple Comparisons

Tukey HSD

					95% Confidence Interval	
VAR00001	VAR00001	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
1	2	38.16667*	12.60093	.031	2.8975	73.4359
	3	17.50000	12.60093	.520	-17.7692	52.7692
	4	10.00000	12.60093	.856	-25.2692	45.2692
2	1	-38.16667*	12.60093	.031	-73.4359	-2.8975
	3	-20.66667	12.60093	.380	-55.9359	14.6025
	4	-28.16667	12.60093	.148	-63.4359	7.1025
3	1	-17.50000	12.60093	.520	-52.7692	17.7692
	2	20.66667	12.60093	.380	-14.6025	55.9359
	4	-7.50000	12.60093	.932	-42.7692	27.7692
4	1	-10.00000	12.60093	.856	-45.2692	25.2692
	2	28.16667	12.60093	.148	-7.1025	63.4359
	3	7.50000	12.60093	.932	-27.7692	42.7692

*. The mean difference is significant at the 0.05 level.

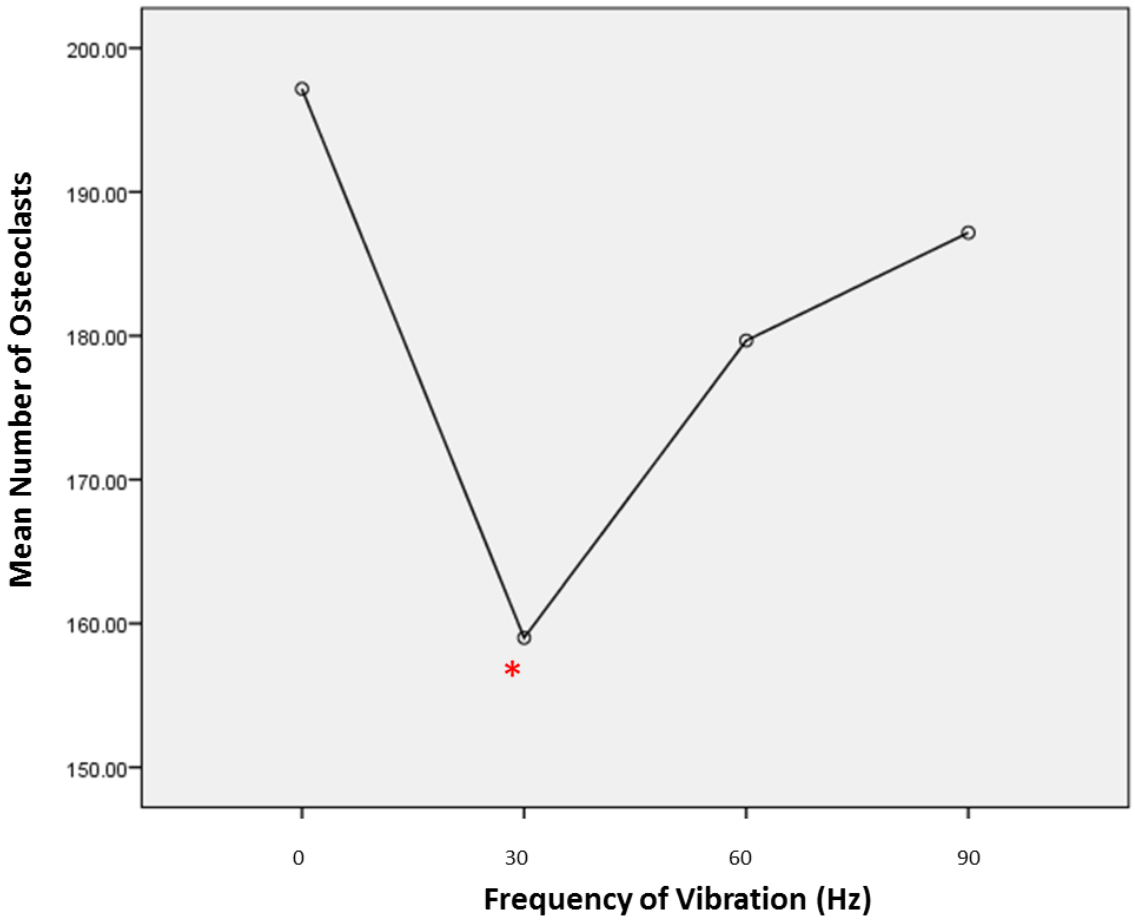

Figure 4-2: LMHF mechanical vibration significantly inhibits osteoclast formation from RAW264.7 monocytes by 21% at 30Hz (n = 6, * ***p*** = 0.031).

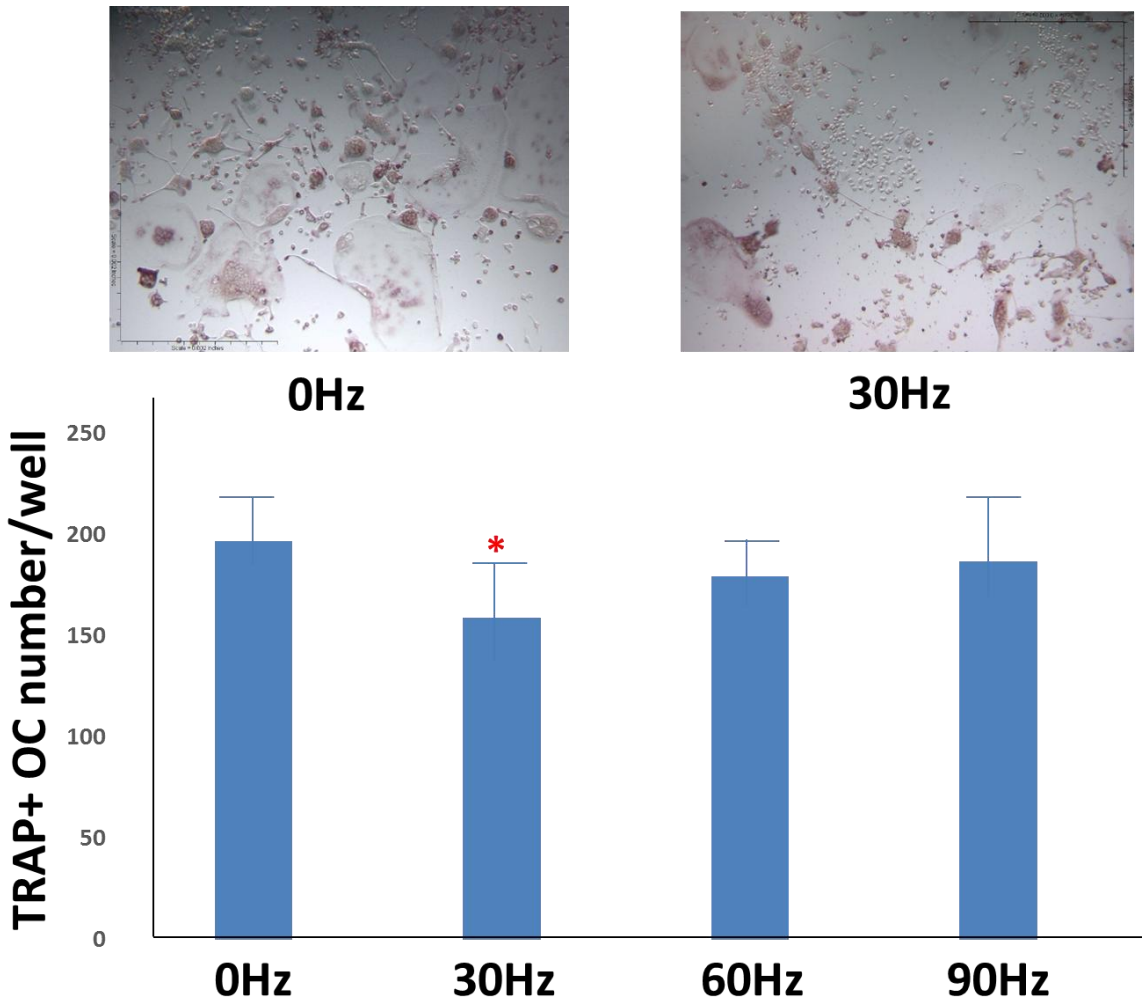

Figure 4-3: Reduction in osteoclast formation as demonstrated in cellular cultures stained with TRAP. LMHF mechanical vibration inhibits osteoclast formation from RAW264.7 monocytes by 21% at 30Hz, which is statistically significant. (n = 6, * ***p*** = 0.031)

CHAPTER V

DISCUSSION

In this study it was demonstrated that mechanical vibration directly inhibited osteoclast formation from RAW264.7 monocytes in a frequency specific manner. Overall, mechanical vibration reduced osteoclast formation ($p<0.05$, $n=6$). The 21% reduction in osteoclastogenesis was only significant in the 30 Hz group ($p<0.05$, $n=6$) when compared to the control (Table 4-4 and Table 4-5). Although decreases were shown at 60 and 90 Hz, these were not significant when compared to the control, as seen in Figure 4-2 and Figure 4-3. There was also no difference amongst the three frequency groups (Table 4-5). Our results support those of Wu *et al*. (2012), Kulkarni *et al.* (2013) and most recently Yadav *et al*. (2015) that show osteoclast precursor cells are directly responsive to mechanical vibration. Unique to our study, these results indicate that the relationship with LMHF vibration and its effects on osteoclastogenesis may be frequency specific as overall there was a decrease at all levels of frequency, but only 30 Hz was statistically significant, however this precise value may vary as there was no statistical significance amongst the different frequency groups.

This dose specificity could help potentially explain significant reduction in RANKL-induced osteoclastogenesis observed across various different LMHF levels in previous studies compared to the range (0-90Hz) in our study. Wu *et al*. (2012) showed vibration at 45 Hz and 0.3 g for 15 minutes reduced osteoclastogenesis and down-regulated c-Fos, whereas Kulkarni *et al.* (2013) recently demonstrated this with 4 Hz and 20 μm displacement for 1 hour, through down-regulation of DC-STAMP protein

production. LMHF vibration of 60 Hz and 0.3 g vibration at the molars in healthy rats had a gradient effect and increased alveolar bone volumes, osteogenic genes, collagen crosslinking, bone formation particles, and mineral density (Alikhani *et al.*, 2012). A very recent study with 30 Hz for 15 min and 1 cN of force on orthodontically moved mouse molars in relapse demonstrated a decrease in osteoclast number and surface in addition to an increase in tissue volume (Yadav *et al*, 2015).

The response at the various frequency levels may be explained by Judex *et al.* (2007) who showed that the effects of mechanical vibration were mainly dependent on increases in the frequency of oscillations or the number of loading cycles on bone cells, not the magnitude of the vibration. Furthermore, *in vivo* these cells may have an inherent preference to specific frequencies that can be altered by the environment, including hormones, disease and age. The variations in the vibration protocol, culture medium and environment set up across the different studies may explain the different levels of significance amongst the studies. In our study, significance only at 30 Hz compared to the control indicates frequency specificity of RAW264.7 monocytes in this particular protocol. However, the lack of statistical significance between the three treatment groups warrants further study to determine the exact range of specificity for these cells as factors such as limited sample size, in addition to the increments in frequency being too large to accurately decipher precise changes at various levels of LMHF mechanical vibration. Internal studies of frequencies above this range in frequency do not indicate significant levels of change compared to 90 Hz and this is supported by previous studies which defined LMHF as ranging between 10 and 100 Hz (Judex *et. al* 2007). Frequencies below this range however, i.e. 4 Hz (Kulkarni *et al*., 2013), warrant inclusion in future

comparative studies. Larger sample groups, consistent design and frequency levels of treatment groups that are closer in range (i.e. 10 Hz difference between each group) can aid in investigating ideal range of frequency specificity for LMHF vibration on RAW264.7 monocytes, and eventually determine if this would be clinically significant. To the best of our knowledge, this is the first study to compare the frequency dependency of the direct effect of LMHF mechanical vibration on osteoclastogenesis, thus further study is warranted.

The precise biological mechanism is presently unknown and has been difficult to study given the dynamic complexity of the RANKL cascade and multitude of factors that play a role. It is currently unknown how and why certain levels of frequency alter the RANKL cascade, but our study implicates dose specificity. Identifying the precise ideal range of frequency would aid in investigating the underlying mechanisms by measuring changes in various factors in the RANKL cascade at this determined frequency compared to controls and other frequencies. Previous study of osteoclast precursor cells *in vitro* show that mechanical strain (Sen *et al.*, 2011), fluid shear stress (Lau *et al*., 2010; Uzer *et al.*, 2012), in addition to the negligible hydrostatic pressure, do not contribute to the mechanotransduction of vibration on these cells. Furthermore, comparisons with vastly studied mesenchymal cells, including osteoblast and osteocytes, may yield limited results. Although mesenchymal cells and hematopoietic cells both reside in bone marrow *in vivo*, they possibly respond to different types of mechanical load. Mesenchymal cells were shown not to respond to 60 Hz and 0.3 g (similar to our study), but do respond to mechanical strain (Rubin *et al*., 2002, Simmons *et al*., 2003) and fluid shear stress (Kreke *et al.*, 2008; Sharp *et al.*, 2009; Kavlock and Goldstein, 2011). Thus, vibration may then

only directly affect hematopoietic cells, inhibiting osteoclast formation and ultimately resulting in anabolic bone activity.

The Kulkarni study (2013) with similar protocol as ours but at 4 Hz provided promising evidence of a significant reduction in DC-STAMP (responsible for fusion of the pre-osteoclastic cells) post-mechanical vibration of RAW264.7 monocytes and subsequent inhibition of osteoclastogenesis. Extending our current study to measure levels of DC-STAMP can allow us to hypothesize the mechanisms of mechanoreception and transduction of the precursor cells and would allow comparisons to determine potential relationships with this (or other RANKL cascade factors) and frequency specificity.

There may be potential clinical implications of our findings. Due to its anabolic effects, hypothetically the LMHF vibration could result in reduced time for orthodontic retention when studied in mice. Yadav *et al.,* (2015) recently showed that 30 Hz at 1 cN applied for 15 minutes for 7 days after removal of mesial force on mouse molars - demonstrating a biological tendency to decrease relapse. Although the difference in molar movement was not significant, compared to the control, there was a statistically significant increase in tissue density, sclerostin (which negatively regulates bone mass), decrease in osteoclast formation, with overall anabolic effect on the bone. There was also maintenance in the thickness and integrity of the periodontal ligament compared to the control, which showed sustained disruption of collagen fibers post-orthodontic tooth movement. Further study would be required to determine a potential frequency specificity of these effects as well as determination if these results are clinically significant.

Other implications include managing periodontal pathology specifically that of lipopolysaccharide (LPS) from the cell wall of one of the primary pathogens, *P. gingivalis*. The interaction with RANKL and LPS *ex vivo* in mandibular slices of rats (which showed increased in osteoclastogenesis in the PDL) (Sloan *et al.*, 2011) and precursor RAW264.7 cells (increase in the RANKL cascade, especially NFATc1) (Kukita *et al.,* 2013) has been investigated and can lead to more information on the mechanism of vibration inhibiting osteoclastogenesis. Specifically, with this pathological amplification of RANKL activity, a study model similar to ours could investigate if LMHF vibration can reduce or prevent the increase in RANKL activity and osteoclastogenesis from RAW264.7 monocytes. Although difficult to study because of the complexity in the cascade, establishing this relationship can warrant further investigation on the effects of LMHF vibration on RANKL *in vivo* and potential clinical applications in adjunct therapeutics.

Conclusion

1. Low-magnitude (0.3 g acceleration) high-frequency (10-100 Hz) mechanical vibration directly inhibits osteoclast formation *in vitro*, which is statistically significant at 30Hz, but not 60 and 90 Hz. The unknown mechanism of this phenomenon needs to be further investigated.

2. Potential clinical implications include biological enhancement of orthodontic retention and adjunct therapeutics in bone maintenance in periodontitis.

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