Effects of Low Magnitude High Frequency Mechanical Vibration Combined with Compressive Force on RANKL/OPG and Runx2 Expression in Human Periodontal Ligament Cells *in vitro*

Sutiwa Benjakul

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Oral Health Science

Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ ผลของการกระตุ้นด้วยแรงสั่นสะเทือนขนาดต่ำความถี่สูงร่วมกับแรงกดต่อการแสดงออกของจีนแรงค์แอลต่อจีนโอพีจี และ จีนรังซ์ทู ในเซลล์เนื้อเยื่อปริทันต์ของมนุษย์ในห้องปฏิบัติการ

ผู้เขียน นางสุทิวา เบญจกุล

สาขาวิชา วิทยาศาสตร์สุขภาพช่องปาก

ปีการศึกษา 2559

บทคัดย่อ

แม้ปัจจุบันมีการนำแรงสั่นสะเทือนมาใช้กระตุ้นการเคลื่อนที่ของฟัน ในขณะจัดฟัน แต่กลไกที่เนื้อเยื่อตอบสนองต่อแรงนี้ ยังไม่เป็นที่ทราบแน่ชัด **<u>วัตถุประสงค์</u>** เพื่อศึกษาผลของการกระตุ้นด้วยแรงสั่นสะเทือนขนาดต่ำความถี่สูงร่วมกับแรงกดในเซลล์เนื้อเยื่อปริทันต์ของมนุษย์ ในห้องปฏิบัติการ **<u>วิธีการวิจัย</u>** เซลล์เนื้อเยื่อปริทันต์ของมนุษย์ได้จากฟันกรามน้อยที่ถูกถอนจากกลุ่มตัวอย่าง 4 คน ในการศึกษาเพื่อหาความถี่ของแรงสั่นสะเทือนที่เหมาะสม เพื่อนำมาใช้ในการให้แรงสั่นสะเทือนร่วมกับแรงกด เซลล์เนื้อเยื่อปริทันต์ถูกกระตุ้นด้วยแรงสั่นสะเทือนขนาดต่ำ (0.3 กราวิตี้) ที่ความถี่ต่างๆ กัน (30, 60 หรือ 90 เฮิรตซ์) เป็นเวลา 20 นาทีต่อวัน จำนวน 3 รอบ ในการศึกษาเพื่อหาผลของการกระตุ้นด้วยแรงสั่นสะเทือนขนาดต่ำความถี่สูงร่วมกับแรงกด เซลล์เนื้อเยื่อปริทันต์ถูกกระตุ้นด้วยแรงสั่นสะเทือนที่ความถี่ที่เหมาะสม เป็นเวลา 20 นาทีต่อวัน จำนวน 3 รอบ หรือ ถูกกระตุ้นด้วยแรงกด 1.5 กรัมต่อตารางเซนติเมตร ต่อเนื่องเป็นเวลา 48 ชั่วโมง หรือถูกกระตุ้นด้วยแรงสั่นสะเทือนร่วมกับแรงกด จากนั้นทำการวัดปริมาณการมีชีวิตของเซลล์ด้วยวิธีเอ็มทีที (MTT assay) วัดปริมาณพรอสตาแกรนดินอีทู ด้วยวิธีอีไลซ่า (ELISA) และวัดปริมาณการแสดงออกของจีน แรงค์แอล, โอพีจี และ รังซ์ทู ด้วยวิธีควอนทิเททีฟเรียลไทม์พีซีอาร์ (quantitative real-time PCR) **<u>ผลการศึกษา</u>** แรงกด และแรงสั่นสะเทือนร่วมกับแรงกด มีผลลดการแบ่งตัวของเซลล์อย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับกลุ่มควบคุมที่ไม่ได้รับแรง แรงสั่นสะเทือน แรงกด และแรงสั่นสะเทือนร่วมกับแรงกด มีผลเพิ่มปริมาณพรอสตาแกรนดินอีทู และการแสดงออกของจีนแรงค์แอล แต่ไม่มีผลต่อการแสดงออกของจีนโอพีจี ส่งผลให้มีการเพิ่มขึ้นของสัดส่วนของจีนแรงค์แอลต่อจีนโอพีจี โดยพบการตอบสนองสูงสุดในกลุ่มที่ได้รับแรงสั่นสะเทือนร่วมกับแรงกด แรงกด และแรงสั่นสะเทือนร่วมกับแรงกด มีผลลดการแสดงออกของจีนรังซ์ทู **<u>สรุปผลการศึกษา</u>** แรงสั่นสะเทือนมีผลเพิ่มการแสดงออกของพรอสตาแกรนดินอีทู และจีนแรงค์แอล ทั้งทางตรง และมีผลเสริมผลที่เกิดจากการกระตุ้นด้วยแรงกดด้วย แต่ไม่มีผลเสริมผลหรือยับยั้งผลของแรงกด ต่อการแสดงออกของจีนโอพีจี และจีนรังซ์ทู เมื่อกระตุ้นด้วยแรงสั่นสะเทือนร่วมกับแรงกด

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ABSTRACT

Mechanical vibration can be used to accelerate tooth movement during orthodontic treatment, though the exact tissue responses remain unclear. **Objectives** This study aimed to investigate the effects of low magnitude high frequency (LMHF) mechanical vibration combined with compressive force on periodontal ligament (PDL) cells *in vitro*. **Materials and methods** Human PDL cells were isolated from extracted premolar teeth of four individuals. To determine the optimal frequency for later used in combination with compressive force, three cycles of low-magnitude (0.3 g) vibration at various frequencies (30, 60 or 90 Hz) were applied to PDL cells for 20 min every 24 h. To investigate the effects of LMHF vibration combined with compressive force, PDL cells were subjected to three cycles of optimal vibration frequency for 20 min every 24 h or 1.5 g/cm^2 compressive force for 48 h or vibration combined with compressive force. Cell viability was assessed using MTT assay, an ELISA was used to quantify PGE2 and quantitative real-time PCR was used to measure RANKL, OPG and Runx2 expression. **Results** Compressive force and vibration combined with compressive force significantly reduced cell viability. Vibration, compressive force and vibration combined with compressive force increased PGE2 production and RANKL expression but did not affect OPG expression, thus increasing the RANKL/OPG ratio. The highest level was observed in the cells exposed to vibration combined with compressive force. Compressive force and vibration combined with compressive force (but not vibration alone) reduced Runx2 expression. **Conclusions** Vibration directly increases and had the additive effects of compressive force on the PGE2 production and the expression of RANKL, but not OPG and Runx2, in PDL cells.

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Sutiwa Benjakul

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LIST OF ABBREVIATION AND SYMBOLS

LMHF	= Low magnitude high frequency
PDL	= Periodontal ligament
RANKL	= Receptor activator of nuclear factor kappa B ligand
OPG	= Osteoprotegerin
mRNA	= Messenger ribonucleic acid
PGE2	= Prostaglandin E2
Runx2	= Runt-related transcription factor 2
PDLSCs	= Periodontal ligament stem cells
PTH	= Parathyroid hormone
Hz	= Herz
%	= Percentage
IL-1	= Interleukin 1
IL-6	= Interleukin 6
cAMP	= Cyclic adenosine monophosphate
RANK	= Receptor activator of nuclear factor kappa B
TNF	= Tumor necrosis factor
M-CSF	= Macrophage-colony stimulating factor
TACE	= Metalloproteinase-disintegrin tumour necrosis factor-αconvertase
TRAFs	= Tumour necrosis factor receptor associated factors
h	= Hour
BMSCs	= Bone marrow-derived mesenchymal stromal cells
PBS	= Phosphate-buffered saline
NCM	= Normal culture medium
°C	= Celsius
CO_2	= Carbon dioxide
DMEM	= Dulbecco's modified essential medium
FBS	= Foetal bovine serum
U/ml	= Unit per milliliter

LIST OF ABBREVIATION AND SYMBOLS (continued)

µg/ml	= Microgram per milliliter
mM	= Millimolar
µM	= Micromolar
g	= Gravity
min	= Minute
MTT	= The 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide
ELISA	= Enzyme-linked immunosorbent assay
qPCR	= Quantitative real-time Polymerase chain reaction
Con	= Control
V	= Vibration
C	= Compressive force
VC	= Vibration combined with compressive force
g/cm^2	= Gram per square centimetre
nm	= Nanometer
µl	= Microliter
ng	= Nanogram
DEPC-treated water	= Diethyl pyrocarbonate-treated water
U/µl	= Unit per microliter
s	= Second

CHAPTER 1

INTRODUCTION

Background and Rationale

Orthodontic treatment usually takes a long time to complete, which can lead to several complications.[1] Many attempts have been made to accelerate tooth movement, including physical,[2] pharmacological[3] and surgical approaches.[4-5] However, complications such as local pain, severe root resorption[6] and drug-induced side effects can occur, with varied results reported.[7]

Low magnitude high frequency (LMHF) mechanical vibration is a non-invasive method[8] that can be applied in conjunction with orthodontic treatment to increase the rate of tooth movement.[9] A number of vibratory devices are commercially available, such as AcceleDent (OrthoAccel Technologies, Inc., Houston, Tex, USA). Several *in vivo* studies have investigated the effects of vibration during the acceleration of tooth movement in animal models[10] and humans,[9, 11] but have reported conflicting results.[12] Moreover, the mechanisms of action of vibration on the surrounding tissues and cells, either periodontal ligament (PDL) cells or bone cells, have not been determined. Therefore, it would be interesting to investigate the mechanisms of action and cellular responses to vibration during application of orthodontic force *in vitro*.

Periodontal ligament (PDL) cells play a major role in initiation of the remodelling process during orthodontic tooth movement.[13] Compression of PDL is a prerequisite for tooth movement. The balance between Receptor activator of nuclear factor kappa B ligand (RANKL) and Osteoprotegerin (OPG) expression in PDL cells regulates bone remodelling during tooth movement. Compressive force upregulates RANKL messenger ribonucleic acid (mRNA) expression and protein via a prostaglandin E2 (PGE2)-dependent mechanism in PDL cells.[14] PGE2 is inflammatory mediator produced by PDL cells in response to mechanical stress that acts in autocrine and paracrine manners to stimulate RANKL expression and promote bone resorption.[14-15] The transcription factor Runt-related

transcription factor 2 (Runx2) also plays important roles in osteoblastic differentiation and bone deposition.[16] LMHF vibration has been reported to induce Runx2 mRNA expression and protein in periodontal ligament stem cells (PDLSCs).[17]

Although the effects of compressive force on the expression of PGE2, RANKL and OPG in PDL cells have been determined,[14, 18-19] the effects of vibration combined with compressive force on the expression of these osteogenic factors in PDL cells has not yet been assessed. This study aimed to investigate whether vibration enhances or inhibits the osteogenic factors effects of compressive force by regulating the secretion of PGE2 and expression of RANKL, OPG and Runx2 in PDL cells.

Review of Literature

Orthodontic tooth movement

Orthodontic tooth movement is initiated by the hard and soft tissues remodeling in dental and paradental areas. The orthodontic force applied on the teeth will alter the blood flow and the microenvironment around the PDL resulting in synthesis and release of many inflammatory mediators such as neurotransmitters, arachidonic acid metabolites, cytokines, growth factors and colony stimulating factors from the local cells. The increasing level of these mediators during orthodontic tooth movement results in the assumption that regulates the biological responses which occur after the orthodontic forces was applied. These substances initiate a cascade of signals that regulate many cellular responses by numerous cell types in and around teeth which provides a favorable microenvironment for bone deposition or resorption.[20]

Methods for accelerate orthodontic tooth movement

Since orthodontic treatment usually takes a long time to complete leading to several problems. It's important to accelerate the remodeling of alveolar bone during orthodontic treatment to shorten the time required, reduce the cost of treatment and

make more benefits to both patient and orthodontist. The rate of tooth movement depends on both of the physical characteristics of the applied force, and the biological responses from the PDL. Furthermore, this mechanism is modified by many other factors such as force magnitude, age, sexual hormone, bone density, genetic variability, and activation interval. To date, a number of attempts have been made to accelerate tooth movement.

The velocity of tooth movement mainly depends on the rate of bone resorption occurring at the compression side of the PDL, in the direction of orthodontic force applied to the tooth. Therefore, it is possible that administration of bone resorbing factors may increase bone-resorbing activity in the compressed PDL. Many studies have been done using these molecules exogenously such as PGE,[3, 21-22], 1,25-dihydroxyvitamin D 3,[23-24] parathyroid hormone (PTH),[25-26] and RANKL[27] to increase the rate of tooth movement in both animal experiments and humans. Although the effects of these methods have been investigated on animal experiments but clinical trials on humans are limited. Since avoiding systemic effects they must be local administered by injections that can be painful and cause discomfort to the patients. In addition, long term side effect was not tested.

Despite many biological approaches have been reported that they can increase the rate of tooth movement but complications, such as local pain, severe root resorption[6], and drug-induced side effects can occur. In addition, all of these methods are focus on only the activation of osteoclast on the resorption site of the alveolar bone. There is the question about the stability of these accelerations and no guarantee that when the resorption site is accelerated, the deposition site can catch up with it. This turned the trend to discover a physical approach that focuses on the activation of cellular activity of both osteoclast on the resorption site and osteoblast on the depositon site of the alveolar bone. These methods not only accelerate tooth movement but also increase the stability of post-orthodontic tooth movement. The idea of using physical approaches came from the concept of bone bending theory that applying orthodontic forces bend the bone and develop the bioelectrical potential. The negatively charged will take place at the concave site of the bone and favors osteoblastic activity, whereas the convex site

will be positively charged showed elevated osteoclastic activity.[20] This technique includes direct electric currents,[28-29] static magnetic field and pulsed electromagnetic field,[30-31] low-level laser radiation,[2, 32-39] and resonance vibration.[40-45]

The surgical technique has recently been studied in a number of publications. The increasing of bone turnover after bone grafting, fracture, and osteotomy has been known. Several surgical approaches such as interseptal alveolar surgery,[46] osteotomy and corticotomy,[47-50] and Piezocision technique[51] have been tried in order to increase the rate of tooth movement. This approach is the most clinically application and most tested with predictable and stable results. However, it is invasive, aggressive, and costly, and patients are not accepted to the surgery unless it is the only choice that is needed to have a good occlusion.

Vibratory stimulation and orthodontic tooth movement

Recently, externally applied resonance vibration with low magnitude (LM; <1g, where g=9.81 m/s^2), high frequency (HF; 20-90 Hz) and short duration have been investigated that can be enhancing bone remodeling and anabolic to bone tissue.[40-45, 52] In addition, from the bone bending theory, the bioelectrical potential is created when apply discontinuous forces, which leads to the idea of using cyclic forces and resonance vibrations combined with orthodontic force for accelerate orthodontic tooth movement. Furthermore, it is accepted that mechanical stimuli can activate the response of PDL cells and bone cells. There are many studies found that PDL cells and bone cells are able to sense mechanical vibratory stimulation directly and respond by alter gene expression and produce soluble factors that involve in bone remodeling process.[17, 53-55] It has been found that applying vibrations accelerated tooth movements in animal experiments[10, 56-58] and human.[9, 11, 59] Although vibration may accelerate orthodontic tooth movement with minimal side effects, knowledge of exactly how mechanical vibration accelerate orthodontic tooth movement is limited and their effects still have the controversial results. Some studies found that vibration has no effect on the acceleration of orthodontic tooth movement and there are studies found the opposite results.[60-62]

Moreover, the effects of vibration on tooth movement may depend on frequency, duration, amplitude, and site of application, but these previous studies used the different vibration protocols that lack of standardization to compare the results. Further studies needed to be examined to clearly identify the protocol that can be applied to get the maximum desired results. In addition, further studies in the field of molecular biology needed to explain the mechanisms at the cellular and genetic levels.

PDL and PDL cells

Orthodontic tooth movement occurs by applying mechanical forces to the tooth, then the forces are transmitted to the alveolar bone through the PDL. PDL contains several cells populations comprising osteoblasts and osteoclasts on the bone side, whereas periodontal ligament fibroblasts, undifferentiated mesenchymal cells, macrophages, neural elements, endothelial cells, smooth muscle cells, and epithelial cell rests of Malassez in the body of the PDL, in addition cementoblasts on the root surface. All of these cells, the predominant cell type is the periodontal ligament fibroblasts. In this study, only periodontal ligament fibroblasts are defined as PDL cells.

PDL cells also play important role in alveolar bone remodeling during orthodontic tooth movement as well as osteoblasts and osteoclasts. PDL cells have the important functions to sense and respond to the forces generated by speech, mastication and orthodontic treatment. Commonly, ankylosed teeth, that lack of PDL, cannot be moved by mechanical force.[63] There are evidences that in the orthodontic tooth movement model, strains in the alveolar bone are generally lower than 0.02%[13, 64] which not sufficient to stimulate bone cells to trigger the bone remodeling process.[65] On the other hand, strains in the PDL have been reported to be in the range of 10–40%[13, 64] which above the threshold that required to stimulate fibroblasts to initiate the remodeling process (strain levels around 7–12%).[66-68] Assuming that strains in PDL and the response of PDL cells to mechanical stimulus play the important role in initiating modeling and remodeling process during orthodontic tooth movement.[13, 64, 69] PDL cells response to mechanical stimuli by form biologically active substances, such as cytokines

and enzymes for signaling the surrounding cells to modulate the resorption and formation of bone matrix.[14, 19, 69-78] External orthodontic forces are converted to intracellular signals that result in the resoption of bone by osteoclasts on compression site and the formation of the new bone by osteoblasts on tension site. The coordination of these two processes results in alveolar bone remodeling and tooth movement through the alveolar bone. PDL cells have been reported that it could recognize and respond to mechanical stimulation directly.[14, 19, 71-78] The in vitro studies using primary cultures from PDL tissue have indicated that both mechanical stress and strain induced intracellular mechanotransduction signals that alter gene expression in these cells. Some reports found that the PDL cells were able to induce osteoclastogenesis *in vitro*.[14, 76] In addition, the expression of RANKL and OPG in PDL cells has already been reported.[14, 19, 71, 74, 79] Furthermore, some PDL cells has the progenitor cells characteristics and are able to differentiate into osteoblast in response to the mechanical stress.[80]

Prostaglandin E2 (PGE2)

PGE2 is produced from arachidonic acid, the main component of phospholipids of the cell membrane, by the action of cyclo-oxygenase enzymes. PGE2 is one of key molecule that plays an important role in bone metabolism. Many studies have shown that bone resorption via osteoclasts is mediated by prostaglandins, especially PGE2[14, 81] which is produced by PDL cells in response to mechanical stress *in vivo*[82] and *in vitro*.[14, 83] In addition, during orthodontic tooth movement, PDL cells at the pressure side produce and secrete several inflammatory cytokines, such as interleukin 1 (IL-1) and interleukin 6 (IL-6) that can induce PGE2 production. PGE2 acts in an autocrine and paracrine manner to initiate the activity of adenylate cyclase and the accumulation of cellular cyclic adenosine monophosphate (cAMP) via the prostaglandin E4 receptor, inducing of the expression of RANKL, resulting in an increasing of bone resorption.[15, 84] The direct action of prostaglandins on an increasing of osteoclasts numbers and capacity to form a ruffled border and their effect on bone resorption has been reported in

clinical and animal studies.[85-87] Thus, a signaling cascade of these molecules in the PDL could regulate osteoclastogenesis through RANKL-RANK signaling.

On the other hand, like other bone resorbing agents, PGE2 also is a powerful skeletal anabolic factor that promotes osteoblastic cell differentiation and osteogenesis under numerous conditions, including mechanical stimulation[88-89] resulting in new bone formation, coupling bone resorption.

The RANK/RANKL/OPG system

The receptor activator of nuclear factor kappa B (RANK), RANKL and osteoprotegerin (OPG) are produced by numerous cell types and a variety of tissues, their expression effects involve in the three main biological systems: the osteoarticular, immune, and vascular systems.[90] The crucial role of the RANK/RANKL/OPG system in inducing bone remodeling process was recently elucidated.[90-91]

RANKL is a cytokine which in the group of the Tumor necrosis factor (TNF) super-family that plays the important role in the stimulation of differentiation and activation of osteoclast, and inhibition of osteoclast apoptosis.[91] It is expressed on osteoblasts and bone marrow stromal cells. Its expression in combination with macrophage-colony stimulating factor (M-CSF) is needed and sufficient for the complete differentiation of osteoclastic precursors into mature osteoclasts. Severe osteopetrosis and a total loss of osteoclasts have been reported in RANKL knock-out mice.[92] RANKL has both membrane-bound molecule and secreted from that is released from the cell surface by proteolytic cleavage with metalloproteinase-disintegrin tumour necrosis factor-α convertase (TACE).[93] Both forms of RANKL have the same function as ligands for RANK receptor. RANKL-RANK interactions lead to rapid differentiation of monocyte/macrophage lineage into osteoclasts. This interaction is also necessary activate the function and survival of mature osteoclasts.

RANK receptor is a transmembrane molecule that is expressed on the cell surface of osteoclast lineage cells. RANK has no soluble form.

OPG is also a cytokine which in the group of the TNF family and is produced by osteoblasts and bone marrow stromal cells. OPG is only secreted as a soluble protein because it lacks transmembrane and cytoplasmic domains. OPG acts as a decoy receptor that competes with RANK for RANKL binding, inhibits osteoclastogenesis. OPG has the biologic effects that inhibit the terminal stages of osteoclast differentiation, suppress the activation of matrix osteoclast, and induce osteoclast apoptosis.[94]

RANKL, RANK, and OPG expression are modulated by numerous osteotropic agents such as, hormones, growth factors and peptides, cytokines, and other factors.[90] In addition, there are many reports found that mechanical stimuli could modulate the expression of RANKL and OPG.[14, 19, 71, 73-74, 77, 95] Excess RANKL binds to the extracellular RANK domain on osteoclast precursors, leading to the recruitment of tumour necrosis factor receptor associated factors (TRAFs) to the intracellular domain of RANK. TRAFs 1–3, 5 and 6 are associate with the activation of several intracellular signaling pathways, that regulate osteoclast precursor fusion, differentiation into mature osteoclasts, and their subsequent activation for bone resorption and survival.[96] In contrast, excess OPG binds RANKL and prevents its interaction with RANK, decreasing numbers and function of osteoclasts. Thus, alveolar bone remodeling during orthodontic tooth movement is controlled by balance between RANKL and OPG that exerting a positive or negative control on the activation of RANK on osteoclasts.[90-91, 97]

In the field of orthodontics, Shiotani *et al.*[98] and Kim *et al.*[99] showed the presence of RANKL in periodontal tissues during experimental tooth movement of rat molars. Nishijima *et al.*[19] found a significant increasing of RANKL and decreasing of OPG in GCF of orthodontic patients during tooth movement compared to the control teeth at 24 h. There are many studies reported that compressive stress up-regulated RANKL expression and decreased OPG expression in human PDL cells *in vitro.*[14, 19, 71, 73-74, 77, 95] Furthermore, Kanzaki *et al.*[27] demonstrated that RANKL gene transfers to the periodontal tissue could activate osteoclastogenesis and increase the rate of experimental tooth movement in rats. In contrast, OPG gene transfer down-regulates

RANKL-mediated osteoclastogenesis and decreased the rate of experimental tooth movement.[100]

Runt-related transcription factor 2 (Runx2)

Runx2 is the transcription factor which in the group of the Runt family of transcription factors that is expressed at the onset of skeletal development by mesenchymal cells and is present in throughout of osteoblasts differentiation. Its expression is both necessary and sufficient for mesenchymal cell differentiation towards the osteoblast lineage.[16] To date, there are the reports found that LMHF mechanical vibration increases osteoblastic activity of preosteoblast,[53] osteocytes,[54] monocyte[55] and can promotes bone marrow-derived mesenchymal stromal cells (BMSCs)[101] and PDLSCs commitment to the osteoblast lineage by increasing of the expression of osteogenic gene such as Runx2.[17]

Objectives

To investigate whether vibration enhances or inhibits the osteogenic factor effects of compressive force by regulating the cell proliferation rate, the secretion of PGE2 and the expression of RANKL, OPG and Runx2 in PDL cells.

Hypothesis

LMHF vibration does not enhance the effects of compressive force on the cell proliferation rate, PGE2 production, RANKL, OPG, the RANKL/OPG ratio and Runx2 expression.

Significance of the study

This study sheds light on the mechanisms by which PDL cells respond to vibration and vibration combined with compressive force and may support the ability of

vibration to accelerate tooth movement in clinical research. Moreover, it establishes a range of parameters for further *in vitro* and *in vivo* analyses.

CHAPTER 2

RESEARCH METHODOLOGY

Cell culture

This research was approved by the Institutional Ethics Committee Board of the Prince of Songkla University (EC5803-06-P-LR). Human PDL cells were isolated from the ligament tissues on the root of healthy premolar teeth, which extracted for orthodontic treatments from four individuals (2 males and 2 females; 17-20 years of age). The teeth were rinsed several times with phosphate-buffered saline (PBS). Remaining gingival tissue from the cervical portions was removed. Then tissues at the middle third of the tooth[102] were scraped with surgical blades and were cultured in normal culture medium (NCM) in humidified incubator with 5% CO_2 at 37°C. NCM consisted of Dulbecco's modified essential medium (DMEM; Gibco BRL, Grand Island, NY, USA), supplemented with 10% foetal bovine serum (FBS; Gibco BRL), 1% penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml; Gibco BRL) and 1% fungizone (250 µg/ml AmphotericinB; Gibco BRL). The PDL tissues at the apical and cervical parts of the root were discarded to avoid contamination with gingival fibroblasts and pulp cells in the culture. Cell preparations were established from each individual donor. All experiments were carried out using cell cultures at third to fifth passage and performed in triplicate using the four independently isolated cell preparations.

Morphological analysis and characterization of PDL cells

PDL cells were identified by spindle-shaped cell morphology, the expression of Scleraxis mRNA which is the ligament-specific marker,[103] the expression of Fibromodulin mRNA which specifically expressed in PDL cells but cannot detected in gingival fibroblasts and osteoblasts,[104] the expression of

Periostin mRNA which highly expressed in PDL cells but cannot be detected in gingival fibroblast,[105-106] and the ability to initiate an in vitro calcification after culture in osteogenic conditioned medium. To induce osteogenic differentiation, PDL cells were cultured in NCM supplemented with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 0.1 µM dexamethasone (Sigma-Aldrich, St Louis, MO, USA) for 21 days. Then stained the cells with 2% Alizarin Red stain solution (Sigma-Aldrich) and examined by phase contrast microscopy (Nikon Eclipse Ti-S; Nikon Instruments Inc., Melville, NY, USA) to observe calcium deposition.

Determination of optimal LMHF vibration

PDL cells were seeded in 35 mm culture dishes at 1 x 10^5 cells and cultured in NCM to 70-80% confluence, then changed the medium to DMEM with 2% FBS for 24 h to synchronize the cycle of the cell. Before the mechanical stimulus was applied, the culture medium was changed to NCM. Culture dishes were placed onto the platform of a GJX-5 vibration calibrator (Beijing Sending Technology, Beijing, China) that generates perpendicular mechanical vibration when the platform is parallel with the ground,[17] as illustrated in Figure 1A. Three cycles of low-magnitude (0.3 g) vibration at various frequencies (30, 60 or 90 Hz) were applied to PDL cells for 20 min every 24 h, the total experimental time was 48 h. Non-vibrated control cells were cultured in a same manner, but placed on a stationary platform for the same periods of time. The magnitude and frequencies of vibration were based on studies that reported positive bone remodelling.[40, 54]

Immediately after the end of mechanical vibration, cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay. PGE2 levels were quantified by an enzyme-linked immunosorbent assay (ELISA). The expression of RANKL and OPG was quantified using quantitative real-time Polymerase chain reaction (qPCR). The lowest frequency that led to a significant difference in the RANKL/OPG ratio compared to the control was selected as optimal frequency.

Application of LMHF vibration and compressive force

PDL cells were cultured and the cell cycle was synchronized as described above prior to the application of mechanical stimulus. Cells were divided into four groups by randomization: control without mechanical stimulation (Con), selected optimal vibration frequency (V), compressive force (C), and vibration combined with compressive force (VC). Vibration was generated with the same protocol as described above, at the selected optimal vibration frequency. Compressive force was generated at 1.5 g/cm^2 for 48 h using the method that modified from Kanzaki et al.[14] A glass cylinder containing acrylic mass was placed over the 70-80% confluent monolayer in each 35 mm culture dish, as illustrated in Figure 1B. Vibration combined with compressive force group was generated by mounted the cell culture of compressive force model onto the platform of a GJX-5 vibration calibrator, that generates the vibration with the same protocol as described above, at the selected optimal vibration frequency.

Immediately after the end of mechanical stimulation, cell viability was assessed using the MTT assay. PGE2 levels were quantified by ELISA. The expression of RANKL, OPG and Runx2 was quantified using qPCR.

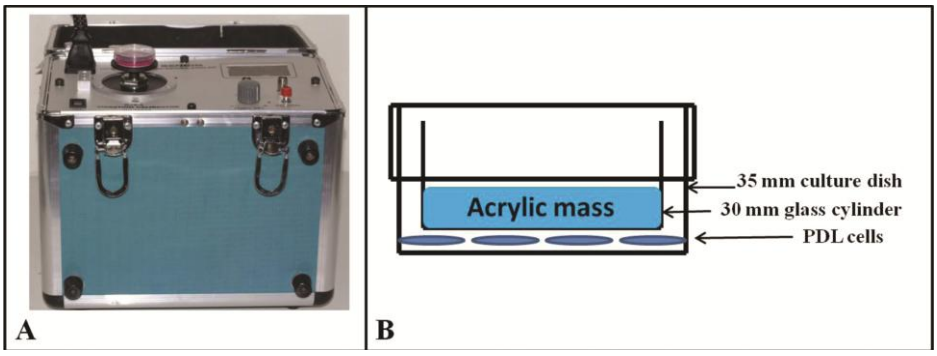

Fig.1: Model used to generate vibration and compressive force *in vitro*. (A) GJX-5 vibration calibrator generates mechanical vibration perpendicular to the bottom of the culture dish. (B) Illustration of how compressive force was applied. PDL cells were continuously compressed using a glass cylinder containing acrylic mass with a total force of 1.5 g/cm^2.

Cell viability assay

The cell viability was assessed using the MTT assay. Immediately after the completion of mechanical stimuli procedure, cell culture medium was aspirated and replaced with aliquots containing MTT solution (5 mg/ml; Sigma-Aldrich) of RPMI cell culture medium (Gibco BRL), incubated and protected from the light at 37°C, with 5% CO_2 for 3 h. Then, formazan crystals were solubilized in acid isopropanol. Quantification of the end product was performed at a wavelength of 570 nm using a Multiskan GO microplate spectrophotometer (Multiskan GO; Thermo Scientific, Waltham, MA, USA). Cell viability was calculated as percentage relative to the control.

Quantification of PGE2

The PGE2 levels in the culture media were determined by a commercially available kit (DuoSet® ELISA Development kit; R&D Systems, Minneapolis, MN, USA). In brief, 150 µl of supernatant from cultured cells and 50 µl of the Primary Antibody Solution were added to the plates that pre-coated with antibodies. Then, the plates were incubated on the horizontal orbital microplate shaker (KS-130-B KS 130 Basic Orbital Shaker; IKA Werke, GMBH & CO. KG, Germany) at room temperature for 1 h. After that, added 50 µl of PGE2 Conjugate and incubated at room temperature on the horizontal orbital microplate shaker (KS-130-B KS 130 Basic Orbital Shaker; IKA Werke) for 2 h. Then aspirated and washed the plates three times with Wash Buffer. After that, added 200 µl of Substrate Solution and incubated at room temperature for 30 min. Then, stopped color development by added 100 µl of Stop Solution and gently taped to ensure thorough mixing. The absorbance was measured immediately by using a Multiskan GO microplate spectrophotometer (Multiskan GO) at wavelength 450 nm with wavelength correction at 540 nm. The levels of PGE2 were calculated by comparison with the standard curve. Values were normalized to total protein content, assessed by Pierce™ BCA Protein Assay Kit (Thermo Scientific). Changes in experimental groups were expressed as fold changes relative to the control.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from cultured cells using innuPREP DNA/RNA mini kits (Analytic-Jena, Konrad-Zuse-Strasse 1, Jena, Germany) according to the manufacturer's protocol. The RNA purity and concentration were assessed using a spectrophotometer at 260 nm. Thereafter, the reverse transcription from total RNA to cDNA was performed using a SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Aliquots containing amounts 300 ng of total RNA were mixed with 1 µl of 50 µM Oligo(dT)20, 1 µl of 10 mM dNTP mix and added diethyl pyrocarbonate-treated water (DEPC-treated water) to total volume 10 µl. Pipetting the sample to ensure thorough mixing. Then, incubated the sample at 65^{o}C for 5 min and placed on ice for at least 1 min. After that, Added 10 µl cDNA Synthesis Mix (the mixing of 2 µl of 10xRT Buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUTtm (40 U/µl), 1 µl of SuperScript® III RT (200 U/µl)). Then incubated at 50^{o}C for 50 min, terminated reactions at 85^{o}C for 5 min and followed by chilled on ice. After that, added 1 µl of RNase H and incubated for 20 min at 37^{o}C. Then, the sample was used as template for qPCR.

Aliquots containing equal amounts of cDNA template were subjected to qPCR amplification on a Rotor-Gene® Q (Qiagen, Qiagen Str. 1, Hilden, Germany) using SensiFASTTM SYBR No-ROX Kit (Bioline Inc, Taunton, MA, USA) according to the manufacturer's protocol. The primers for Scleraxis,[103] Periostin,[107] RANKL,[108] OPG,[103] Runx2,[17] and GAPDH[109] are listed in Table 1. All primer sequences were described in previous reports. Appropriate intron spanning primers for PCR amplification of all genes were chosen in order to avoid co-amplification of genomic DNA. The polymerase activation started the PCR at 95^{o}C for 2 min, then denaturing at 95^{o}C for 5s, following by annealing at a temperature optimized for each primer pair (Table 1) for 10s and an extension at 72^{o}C for 20s for 35 cycles. The fluorescence data were analysed using Rotor-Gene Q software version 2.0.2 (Build 3) to determine *Ct* values. Ct values of interested gene were calculated in relation to GAPDH Ct values that served as an internal control. The internal control gene was validated that its expression was

unaffected by the experimental treatment. The levels of gene expression were calculated by the $2^{-\Delta\Delta_{Ct}}$ method. Changes in experimental groups were expressed as fold changes relative to the control. The efficiency for each specific primer was calculated base on the SYBR Green fluorescence curves and the standard dilution series by Rotor-Gene Q software. To ensure the presence of single amplification products, melting curves analysis was performed following PCR amplification to indicate the presence of a peak at the proper melting temperature for each gene sequence. In addition, 1.5% agarose gel electrophoresis of the PCR amplification products for each PCR reaction were performed.

Table 1: Primers used for real-time PCR

Gene		Product (BP)	Annealing temperature (^{o}c)
Scleraxis	F: 5'- ACACCCAGCCCAAACAGAT-3'	75	60
	R: 5'- TCTTTCTGTCGCGGTCCTT-3'		
Fibromodulin	F: 5'- GGGACGTGGTCACTCTCTG-3'	93	60
	R: 5'- CTGGGAGAGGGAGAAGAGC-3'		
Periostin	F: 5'-TGTTGCCCTGGTTATATGAG-3'	180	60
	R: 5'-ACTCGGTGCAAAGTAAGTGA-3'		
RANKL	F: 5'-TCCCATCTGGTTCCCATAAA-3'	260	60
	R: 5'-GGTGCTTCCTCCTTTCATCA-3'		
OPG	F: 5'-GAAGGGCGCTACCTTGAGAT-3'	102	62
	R: 5'-GCAAACTGTATTTCGCTCTGG-3'		
Runx2	F: 5'-CAGATGGGACTGTGGTTACTGT-3'	169	60
	R: 5'-GTGAAGACGGTTATGGTCAAGG-3'		
GAPDH	F: 5'-GCACCGTCAAGGCTGAGAAC-3'	142	62
	R: 5'-ATGGTGGTGAAGACGCCAGT-3'		

F, Forward primer; R, Reverse primer.

Statistical analysis

All data are presented as the mean ± standard deviation for the four independently isolated cell preparations assessed in triplicate. The Kruskal-Wallis test and Mann-Whitney *U*-test were performed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA); $P < 0.05$ was defined as statistically significant.

CHAPTER 3

RESULTS

Morphological analysis and characterization of PDL cells

The isolated cells exhibited a spindle-shaped morphology, expressed Scleraxis, Fibromodulin and Periostin mRNA and had the ability to undergo calcification *in vitro*, confirming they were PDL cells (Figure 2).

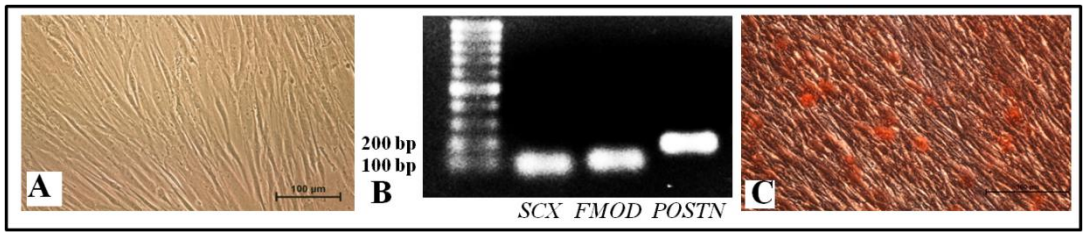

Fig.2: Characterization of the isolated PDL cells. (A) The cells exhibited a spindle-shaped cell morphology. (B) Expression of Scleraxis (SCX), Fibromodulin (FMOD) and Periostin (POSTN) mRNA on 1.5% agarose gel electrophoresis. (C) Alizarin Red staining after culture in osteogenic conditioned medium for 21 days.

Effects of different vibration frequency and determination of optimal vibration frequency

The vibration at all frequencies did not affect the viability of PDL cells (Figure 3A). PDL cells exposed to vibration at 30, 60 or 90 Hz had significantly higher levels of secreted PGE2 and RANKL mRNA expression than control cells ($P = 0.014$ and $P = 0.014$, respectively); however, cells exposed to all vibration frequencies expressed similar levels of PGE2 and RANKL (Figure 3B and 3C). Vibration did not significantly affect the expression of OPG mRNA (Figure 3D). Therefore, the RANKL/OPG ratio significantly increased at all vibration frequencies

(30 Hz; P = 0.013, 60 and 90 Hz; P = 0.014; Figure 3E). The frequency at 30 Hz was the lowest frequency that led to a significant difference in the RANKL/OPG ratio compared to the control, which was the optimal vibration frequency for combination with compressive force.

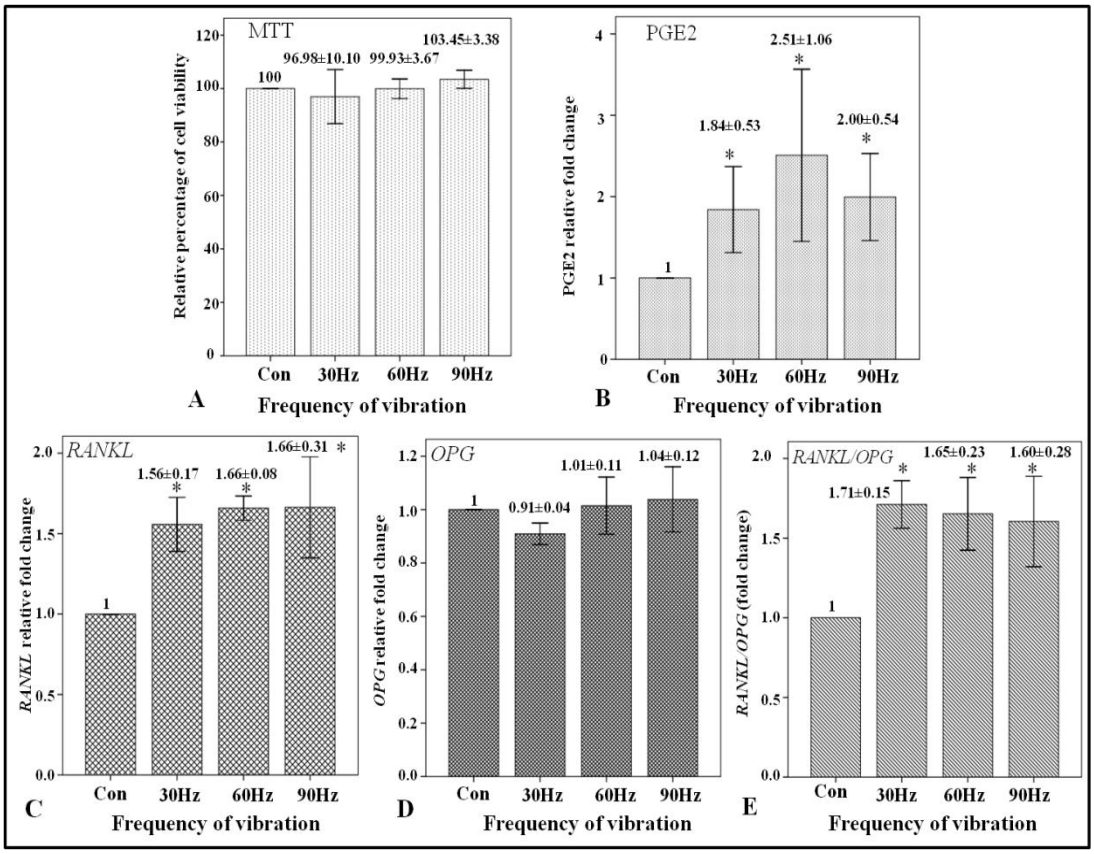

Fig.3: The cell viability, PGE2 secretion and gene expression of RANKL, OPG and the RANKL/OPG ratio in human PDL cells after exposed to three cycles of vibration at 30, 60 or 90 Hz at 0.3 g for 20 min every 24 h *in vitro*. The vibration at all frequencies did not affect the viability of PDL cells (A), PGE2 levels (B) and RANKL mRNA expression (C) significantly increased in the cells exposed to any vibration frequencies compared to the control, OPG mRNA expression was not changed at all vibration frequencies (D), the RANKL/OPG ratio significantly increased at all vibration frequencies (E). Values are mean ± SD of the four cell lines isolated from different individuals, each assessed in triplicate (*P < 0.05, Mann-Whitney U-test).

Effects of LMHF vibration and compressive force

Compressive force and vibration combined with compressive force significantly reduced cell viability compared to the control (P = 0.014 and P = 0.014, respectively; Figure 4A). However, mechanical stimuli did not result in obvious morphologic changes in any treatment group (Figure 4B).

PGE2 was increased in PDL cells exposed to mechanical stimuli - either 30 Hz vibration or compressive force; the highest level was observed in the cells exposed to vibration combined with compressive force (Figure 4C). Compared to the control, cells exposed to compressive force and vibration combined with compressive force expressed significantly higher levels of RANKL (P = 0.014 and P = 0.014, respectively; Figure 4D), while OPG expression was not affected (Figure 4E). Therefore, cells exposed to compressive force and vibration combined with compressive force had significantly higher RANKL/OPG ratio than the control (P = 0.014 and P = 0.014, respectively; Figure 4F). In addition, the RANKL/OPG ratio in the vibration combined with compressive force group was significantly higher than the RANKL/OPG ratio in the group subjected to compressive force alone (P = 0.021; Figure 4F).

Vibration alone (30 Hz) did not affect the expression of Runx2 compared to the control. In contrast, compressive force and vibration combined with compressive force significantly downregulated Runx2 compared to control cells (P = 0.014 and P = 0.014, respectively; Figure 4G).

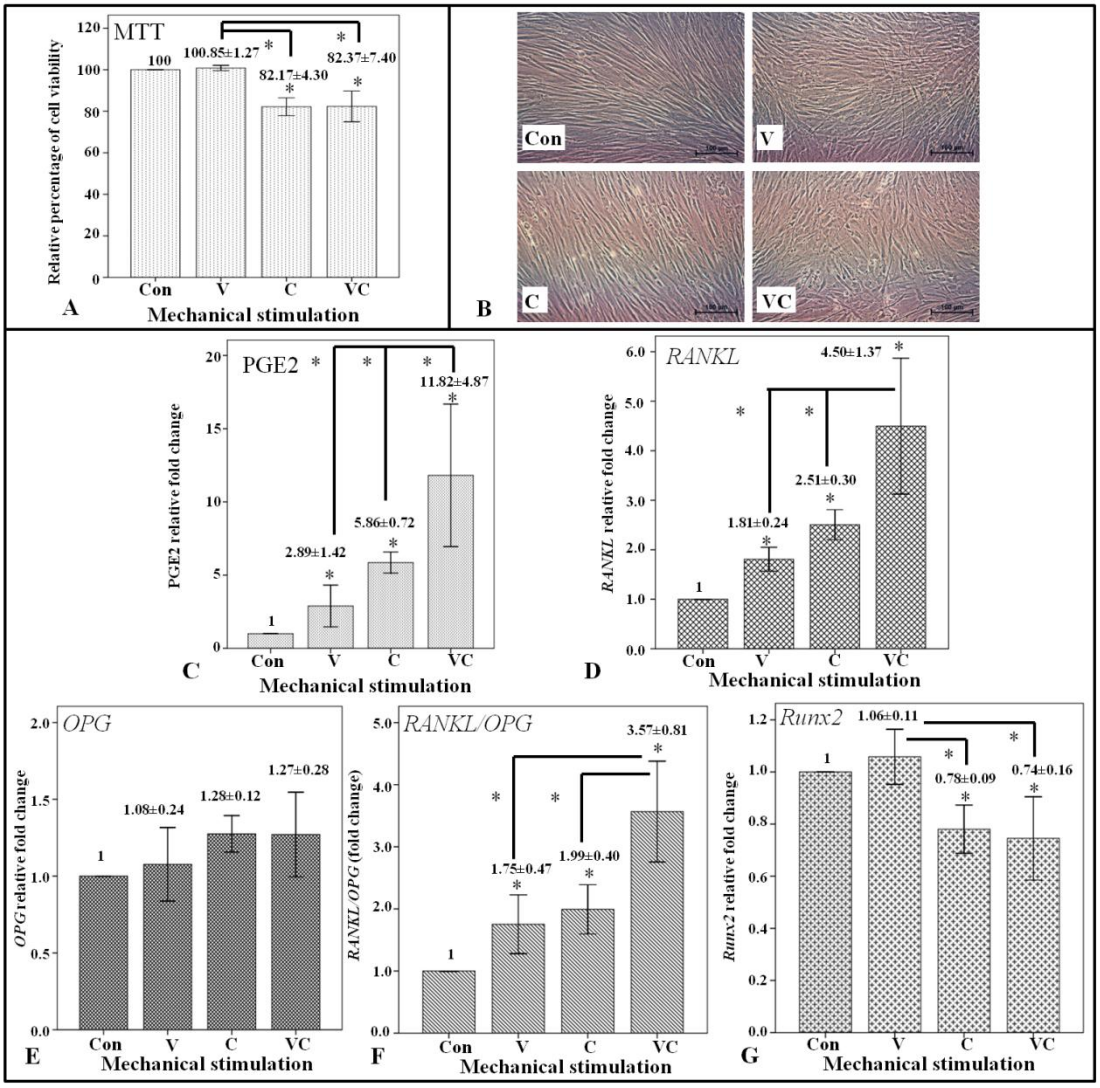

Fig.4: The cell viability, cell morphology, PGE2 secretion and gene expression of RANKL, OPG, the RANKL/OPG ratio and Runx2 in PDL cells after exposed to three cycles of vibration at 30 Hz at 0.3 g for 20 min every 24 h (V), 1.5 g/cm^2 compressive force for 48 h (C), or vibration combined with compressive force (VC) *in vitro*. Compressive force and vibration combined with compressive force significantly reduced cell viability (A), cell morphology observed with phase contrast microscopy found no obvious morphologic changes of the cells in all groups (B), PGE2 levels (C) and RANKL mRNA expression (D) increased in the cells exposed to any mechanical stimuli, OPG mRNA expression was not changed in all groups (E), the RANKL/OPG ratio significantly increased in the cells exposed to any

mechanical stimuli; the highest level was observed in the cells exposed to vibration combined with compressive force (F), Runx2 mRNA expression was not changed in the cells exposed to vibration, while significantly decreased in the cells exposed to compressive force and vibration combined with compressive force (G). Values are mean ± SD of four cell lines isolated from different individuals, each assessed in triplicate (*$P < 0.05$, Mann-Whitney U-test).

CHAPTER 4

DISCUSSION

To examine the mechanism by which vibration accelerates tooth movement, we applied vibration in combination with compressive force to human PDL cells, mimicking the application of vibration on the compression side of the tooth during orthodontic treatment. To the best of our knowledge, there are no reports on the effects of vibration in combination with compressive force on the production of PGE2, the RANKL/OPG ratio and Runx2 expression in human PDL cells. Based on the study of Kanzaki *et al.*,[14] RANKL expression increased in a force-dependent manner, with the peak response observed at 2 g/cm^2 compressive force. We used a lower force (1.5 g/cm^2) to investigate whether vibration enhanced or inhibited the effects of compressive force on the RANKL/OPG ratio. In addition, the effects of vibration depend on the frequency.[45] We selected the frequency at 30 Hz as the optimal vibration frequency for combination with compressive force, which was the lowest frequency that led to a significant difference in the RANKL/OPG ratio compared to control cells. This study showed that vibration enhanced PGE2 secretion, RANKL and the RANKL/OPG ratio expression in compressed PDL cells, but had no effect on Runx2 expression.

PDL cells may respond directly to vibration by increasing RANKL expression or indirectly upregulate RANKL in response to increased release of PGE2. A previous study reported that vibration increased RANKL and the rate of tooth movement in a rat model,[10] implying vibration promoted the osteoclastogenesis and bone resorption required to initiate tooth movement. Therefore, the vibration-induced upregulation of PGE2 and the RANKL/OPG ratio observed in this study may explain the ability of vibration to accelerate tooth movement in clinical research.[9, 11, 59] However, the vibration-induced upregulation of RANKL mRNA expression levels in this study does not have to indicate the increasing of osteoclastic resorption or orthodontic tooth movement. Further studies

to investigate the effects of vibration on the protein levels, the osteoclastic activities, and the acceleration of tooth movement *in vivo* are needed to confirm our findings.

In this study, compressive force and vibration combined with compressive force significantly reduced cell viability but had no effect on cell morphology; which was similar to previous studies.[110-111] Indeed, compressive force can be increased up to 2 g/cm^2 with no any damage to the cells.[112-113] Our study and previous reports indicated that mechanical stimuli affected cell proliferation but did not damage PDL cells. However, we found slightly reduced in viable cell number than the previous reports,[110-111] which may be resulted from the loss of cell during removal of a glass cylinder used to generate compressive force. Moreover, the application of vibration in combination with compressive force did not increase the reduction in cell proliferation observed under compressive force alone.

Compressive force increased PGE2 secretion and RANKL expression in PDL cells; which was similar to previous reports.[14, 19, 103, 110] The effects of compressive force on the OPG expression were still controversial. In this study, compressive force had no significant effect on OPG expression, in agreement with previous studies.[14, 112] However, one study reported compressive force (0.5 to 4.0 g/cm^2) upregulated OPG in human PDL cells,[73] while another reported exposure to compressive force downregulated OPG in PDL cells.[19] Overall, it appears that compressive force increases PGE2 secretion and upregulates RANKL in PDL cells. However, it is possible that the expression of OPG to compressive force depends on several factors, including force magnitude, duration and inter-individual variations. Further investigation using a larger number of samples and/or different compressive force protocols is needed to establish the mode of OPG production in PDL cells exposed to compressive force.

Runx2 was downregulated after exposure to compressive force and vibration combined with compressive force. Currently, there are no reports on these effects in human PDL cells. A previous study reported static compressive force significantly downregulated Runx2 mRNA expression in osteoblast-like cells.[114] Moreover, Diercke *et al.*[115] reported static compressive force significantly induced ephrin-A2 expresssion in PDL cells which can suppress the osteoblastogenesis gene

expression such as Runx2 and decrease sign of osteoblastic differentiation in osteoblasts. Collectively, it is possible that the application of compressive force can downregulates Runx2 expression and inhibited the differentiation of PDL cells into osteoblasts in the similar manner as in the osteoblasts.

Vibration with various frequencies had no significant effects on the viability of PDL cells, in agreement with previous report in mouse osteoblast-like cells.[116] In contrast, Zhang *et al*.[17] reported that exposure of PDLSCs to vibration periodically over 3 days reduced the cell proliferation as it increased cell differentiation. This discrepancy may be due to differences in the cell types, culture conditions and vibration protocols used.

All vibration frequencies tested significantly increased PGE2 production and RANKL expression, but had no significant effect on OPG expression. Lau *et al*.[54] showed that application of vibration with the same magnitude and frequency to osteocytes for 1 h significantly decreased PGE2 and RANKL mRNA and protein expression, with had no effect on OPG. These inconsistent results may be due to cell types and/or different durations of vibration. The vibration-induced increases in PGE2 expression and the RANKL/OPG ratio were similar for all frequencies tested. Further studies at a wider range of frequencies are necessary to evaluate if the response of PDL cells to vibration is frequency-dependent. PGE2 and RANKL are known to stimulate osteoclast formation and bone resorption;[15, 84] therefore, this study indicates application of vibration may promote bone resorption during orthodontic treatment. In contrast, previous studies reported LMHF vibration enhanced bone formation in human PDLSCs,[17] mouse osteoblast-like cells[116] and rat bone marrow-derived mesenchymal stromal cells;[101] these differences may reflect the use of different research models. The PDL cells used in this study may respond to mechanical stimuli in a different manner to bone cells[18, 110] or different vibration protocols may induce varied responses. Indeed, the response of cells to vibration may be also dependent on several other factors, such as the magnitude,[53, 116] frequency,[17, 116-117] duration[101, 118] and schedule of mechanical stimuli.[119] In agreement with a previous study,[17] Runx2 expression slightly increased, though not significantly, in PDL cells exposed to vibration at 30 Hz. It is possible that the

vibration protocol used in this study was not sufficient to upregulate Runx2 expression and could not induce PDL cell differentiation.

However, as the cellular response is dependent on several factors, additional studies with larger sample sizes and using different vibration protocols are necessary to confirm our findings and to define the ideal intensity and frequency of vibration. Indeed, other studies have reported conflicting results,[12, 60] which may reflect variations between cell types/species and different vibration protocols. In addition, the in vivo responses to mechanical stimulation are likely to be more complex than the in vitro responses of single cell types. Further molecular studies are required to investigate the mechanisms underlying the cellular responses to mechanical stimulation.

In conclusion, this research sheds light on the mechanisms by which PDL cells respond to vibration and vibration combined with compressive force. This study establishes a range of parameters for further in vitro and in vivo analyses. However, the vibration-induced upregulation of RANKL mRNA expression levels in this study does not have to indicate the increasing of biological effects. We will investigate these effects on the protein levels in the future work to confirm our findings. Moreover, LMHF vibration may indirectly induce RANKL expression via a signalling pathway related to PGE2 in PDL cells. We aim to investigate the effects of PGE2 on the expression of RANKL and characterise this transduction pathway in future work.

CHAPTER 5

CONCLUSION

LMHF vibration had no effect on the viability of PDL cells, *in vitro*. PDL cells respond directly to 30, 60 and 90 Hz vibration by increasing PGE2 production, and upregulating RANKL leading to a higher RANKL/OPG ratio. LMHF vibration had the additive effects of compressive force on PGE2 production, RANKL and the RANKL/OPG ratio upregulation, but had no effect on OPG and Runx2 expression.

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APPENDICES

ที่ ศธ 0521.1.03/1003

คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์
15 ถนนกาญจนวณิชย์
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หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า

โครงการวิจัยเรื่อง ผลของการกระตุ้นด้วยการสั่นสะเทือนจากแรงขนาดต่ำความถี่สูงร่วมกับแรงกดต่อการแสดงออกของจีนแรงค์แอลต่อจีนโอพีจี และ จีนรังซ์ทู ในเซลล์เนื้อเยื่อปริทันต์ของมนุษย์ในห้องปฏิบัติการ

รหัสโครงการ EC5803-06-P-LR

หัวหน้าโครงการ รองศาสตราจารย์ ดร.ทพญ.ชิดชนก ลีธนะกุล

ผู้ร่วมโครงการวิจัย ทันตแพทย์หญิงสุทิวา เบญจกุล

สังกัดหน่วยงาน ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมในการวิจัย (Research Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาศึกษาการวิจัยในคนของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นสากล ได้แก่ Declaration of Helsinki, the Belmont Report, CIOMS Guidelines และ the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

ในคราวประชุมครั้งที่ 7/2559 **เมื่อวันที่** 21 กรกฎาคม 2559

ให้ไว้ ณ วันที่ **16 กันยายน 2559**

(รองศาสตราจารย์ ดร.ทพญ.ศรีสุรางค์ สุทธปรียาศรี)
ประธานคณะกรรมการจริยธรรมในการวิจัย

หมายเหตุ :- ออกแทนหนังสือรับรองฉบับที่ ศธ 0521.03/485 ลงวันที่ 24 เมษายน 2558 เนื่องจากคณะกรรมการสอบโครงร่างวิทยานิพนธ์มีมติให้เปลี่ยนแปลงชื่อโครงการวิจัย และระเบียบวิธีวิจัย

Cell viability

Subject No.	Relative percentage of cell viability		
	30 Hz	60 Hz	90 Hz
1	100.66 ± 9.76	100 ± 7.85	106.08 ± 17.47
2	100.67 ± 5.42	100.20 ± 6.89	105.99 ± 19.30
3	82.07 ± 12.49	94.95 ± 1.78	98.93 ± 2.19
4	104.51 ± 12.30	103.78 ± 8.19	102.81 ± 12.06
Mean	96.98	99.93	103.45
SD	10.10	3.67	3.38

PGE2

Subject No.	PGE2 level per total protein (pg/mg)			
	Control	30 Hz	60 Hz	90 Hz
1	151.68 ± 1.16	196.00 ± 1.84	238.58 ± 10.78	211.31 ± 7.37
2	16.55 ± 2.76	42.39 ± 3.42	39.70 ± 4.82	36.96 ± 0.41
3	24.48 ± 4.33	44.15 ± 4.31	50.31 ± 1.41	42.94 ± 4.86
4	81.30 ± 5.06	139.41 ± 9.70	325.80 ± 25.82	211.80 ± 35.85

RANKL

Subject No.	Gene expression level (fold change relative to control sample)		
	30 Hz	60 Hz	90 Hz
1	1.50	1.55	1.76
2	1.78	1.66	1.20
3	1.57	1.72	1.91
4	1.38	1.70	1.78
Mean	1.56	1.66	1.66
SD	0.17	0.08	0.31

OPG

Subject No.	Gene expression level (fold change relative to control sample)		
	30 Hz	60 Hz	90 Hz
1	0.94	1.16	1.16
2	0.94	0.95	0.91
3	0.88	0.92	0.96
4	0.87	1.02	1.12
Mean	0.91	1.01	1.04
SD	0.04	0.11	0.12

RANKL/OPG ratio

Subject No.	Gene expression level (fold change relative to control sample)		
	30 Hz	60 Hz	90 Hz
1	1.59	1.34	1.52
2	1.89	1.74	1.32
3	1.78	1.87	2.00
4	1.59	1.66	1.59
Mean	1.71	1.65	1.60
SD	0.15	0.23	0.28

Cell viability

Subject No.	Relative percentage of cell viability		
	V	C	VC
1	101.77 ± 7.99	85.58 ± 2.43	89.66 ± 13.17
2	99.64 ± 0.99	83.76 ± 6.16	78.34 ± 9.13
3	99.88 ± 4.88	83.48 ± 2.34	87.43 ± 5.40
4	102.11 ± 3.77	75.88 ± 2.16	74.04 ± 4.71
Mean	100.85	82.17	82.37
SD	1.27	4.30	7.40

PGE2

Subject No.	PGE2 level per total protein (pg/mg)			
	Control	V	C	VC
1	81.55 ± 2.59	105.19 ± 2.00	548.56 ± 37.68	661.25 ± 39.10
2	56.05 ± 1.54	263.47 ± 48.73	341.71 ± 41.24	584.33 ± 92.22
3	77.84 ± 17.51	243.98 ± 24.03	429.31 ± 2.38	759.00 ± 85.60
4	42.23 ± 9.29	104.42 ± 58.50	214.53 ± 21.61	800.95 ± 74.20

RANKL

Subject No.	Gene expression level (fold change relative to control sample)		
	V	C	VC
1	2.04	2.79	4.69
2	1.79	2.68	3.41
3	1.92	2.10	6.36
4	1.48	2.46	3.52
Mean	1.81	2.51	4.50
SD	0.24	0.30	1.37

OPG

Subject No.	Gene expression level (fold change relative to control sample)		
	V	C	VC
1	1.29	1.23	1.48
2	0.83	1.15	0.87
3	0.91	1.43	1.41
4	1.28	1.29	1.32
Mean	1.08	1.28	1.27
SD	0.24	0.12	0.28

RANKL/OPG

Subject No.	Gene expression level (fold change relative to control sample)		
	V	C	VC
1	1.58	2.27	3.17
2	2.16	2.33	3.92
3	2.11	1.47	4.51
4	1.16	1.90	2.67
Mean	1.75	1.99	3.57
SD	0.47	0.40	0.81

Runx2

Subject No.	Gene expression level (fold change relative to control sample)		
	V	C	VC
1	0.98	0.71	0.62
2	1.15	0.87	0.85
3	1.15	0.69	0.60
4	0.95	0.85	0.91
Mean	1.06	0.78	0.74
SD	0.11	0.09	0.16

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List of Publication and Proceeding

Benjakul S, Leethanakul C, Jitpukdeebodintra S. Effects of low magnitude high frequency mechanical vibration on the expression of RANKL & OPG in human periodontal ligament cells. Multidisciplinary Approach in Dentistry 2016, The Royal College of Dental Surgeons of Thailand, December 15-17, 2015.