Effects of Compressive Stress Combined with Mechanical Vibration on Osteoclastogenesis of RAW 264.7 Cells

Boontida Changkhaokham

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

Prince of Songkla University

2022

Effects of Compressive Stress Combined with Mechanical Vibration on Osteoclastogenesis of RAW 264.7 Cells

Boontida Changkhaokham

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

Prince of Songkla University

2022

Thesis Title Effects of Compressive Stress combined with Mechanical Vibration on Osteoclastogenesis of RAW 264.7 Cells

Author Miss Boontida Changkhaokham

Major Program Oral Health Sciences

Major Advisor:

.....
(Assoc. Prof. Dr. Chidchanok Leethanakul)

Co-advisor:

.....
(Assoc. Prof. Dr. Suwanna Jitpukdeebodintra)

Examining Committee:

.....Chairperson
(Prof. Dr. Prasit Pavasant)

.....Committee
(Assoc. Prof. Dr. Chidchanok Leethanakul)

.....Committee
(Assoc. Prof. Dr. Suwanna Jitpukdeebodintra)

.....Committee
(Assoc. Prof. Dr. Bancha Samruajbenjakun)

.....Committee
(Dr. Panomwat Amornphimoltham)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Oral Health Sciences

.....
(Prof. Dr. Damrongsak Faroongsarng)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

.....Signature

(Assoc. Prof. Dr. Chidchanok Leethanakul)

Major Advisor

.....Signature

(Assoc. Prof. Dr. Suwanna Jitpukdeebodintra)

Co-advisor

.....Signature

(Ms. Boontida Changkhaokham)

Candidate

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.

.....Signature

(Ms. Boontida Changkhaokham)

Candidate

ชื่อวิทยานิพนธ์ ผลของแรงกดร่วมกับแรงสั่น ต่อกระบวนการสร้างเซลล์สลายกระดูก ในเซลล์เพาะเลี้ยงแมคโครฟาจ (RAW 264.7)

ผู้เขียน นางสาวบุญธิดา ฉางข้าวคำ

สาขาวิชา วิทยาศาสตร์สุขภาพช่องปาก

ปีการศึกษา 2564

บทคัดย่อ

ผลของแรงต่อการกระตุ้นกระบวนการสร้างเซลล์สลายกระดูกมีความแตกต่างกันขึ้นอยู่กับ รูปแบบการให้แรง แต่อย่างไรก็ตามยังไม่มีการศึกษาใด ที่ศึกษาเกี่ยวกับการให้แรงสั่นร่วมกับแรงกด ในเซลล์ต้นกำเนิดเซลล์สลายกระดูก **<u>วัตถุประสงค์</u>** เพื่อศึกษาผลของการกระตุ้นด้วยแรงกด และ/หรือ แรงสั่น ต่อกระบวนการสร้างเซลล์สลายกระดูก ในเซลล์ RAW264.7 (เซลล์แมคโครฟาจจากหนู) **<u>วิธีการวิจัย</u>** เซลล์ RAW 264.7 ของหนู ถูกใช้เป็นเซลล์ต้นกำเนิดเซลล์สลายกระดูก ในการศึกษาเพื่อหาขนาดของแรงกด และแรงสั่นสะเทือน ที่เหมาะสม เพื่อนำมาใช้ในการให้แรงกดร่วมกับแรงสั่นสะเทือน เซลล์ RAW 264.7 ถูกกระตุ้นด้วยแรงกด ที่ขนาดต่างๆกัน (0.3 0.6 หรือ 0.9 กรัม ต่อตารางเซนติมตร) และถูกกระตุ้นด้วย แรงสั่นสะเทือนขนาดต่างๆกัน (30 เฮิรตซ์ หรือ 60 เฮิรตซ์) ที่ขนาด 0.49 กราวิตี้ ในการศึกษา เพื่อหาผลของการ กระตุ้นด้วยแรงสั่นสะเทือน ร่วมกับแรงกด เซลล์ RAW 264.7 ถูกกระตุ้นด้วยแรงกดและแรงสั่นสะเทือนที่เหมาะสม เป็นเวลา 20 นาทีต่อวันจำนวน 4 รอบ ต่อเนื่องเป็นเวลา 4 วัน หรือถูกกระตุ้นด้วยแรงสั่นสะเทือนร่วมกับแรงกด จากนั้นทำการวัดปริมาณการมีชีวิตของเซลล์ ด้วยวิธี เพรสโตบลู (Prestoblue) วัดปริมาณการแสดงออก ของยีนส์ เอ็นแฟทซีวัน ดีซีแสตมป์ และคาเทปซิน เค ด้วยวิธีควอนทิเททีฟ เรียลไทม์ พีซีอาร์ (quantitative real-time PCR) วัดจำนวนแทรป พอสิทีฟ มัลตินิวเคลียส เซลล์ (TRAP-positive multinucleated cells) ด้วยวิธีย้อมแทรป (TRAP staining) **<u>ผลการศึกษา</u>** เมื่อกระตุ้นด้วยแรงกดร่วมกับแรงสั่น มีผลส่งเสริมให้ จำนวน แทรป พอสิทีฟ

มัลตินิวเคลียส เซลล์ เพิ่มมากขึ้น เมื่อเปรียบเทียบกับกลุ่มที่ได้รับ แรงกด หรือแรงสั่น เพียงอย่างเดียว อย่างมีนัยสำคัญทางสถิติ ($P<0.05$) อีกทั้ง การให้แรงแต่ละชนิด ไม่มีผลต่อการมีชีวิต ของเซลล์ และเมื่อเปรียบเทียบกับการให้ แรงกด แรงสั่น หรือแรงกดร่วมกับแรงสั่น พบว่า เมื่อให้แรงกดและแรงสั่นร่วมกัน มีการส่งเสริมการแสดงออก ของยีนส์ เอ็นแฟทซีวัน ดีซีแสตมป์ และคาเทปซิน เค เพิ่มมากขึ้นอย่างมีนัยสำคัญทางสถิติ **สรุปผล** แรงกดร่วมกับแรงสั่นสะเทือน กระตุ้นกระบวนการ สร้างเซลล์สลายกระดูก อีกทั้ง เพิ่มยีนส์ เอ็นแฟทซีวัน ดีซีแสตมป์ และคาเทปซิน เค ในเซลล์ RAW 264.7 ผลการศึกษานี้ได้ให้ข้อมูลเชิงลึกมากขึ้นเกี่ยวกับกลไกการเร่งการเคลื่อนที่ของฟันด้วยแรงสั่นสะเทือน

Thesis Title Effects of Compressive Stress Combined with Mechanical Vibration on Osteoclastogenesis of RAW 264.7 Cells

Author Miss Boontida Changkhaokham

Major program Oral Health Sciences

Academic year 2021

ABSTRACT

The effects of mechanical stimulation on osteoclastic differentiation differ depending on the pattern of mechanical loading. However, there is still no knowledge about mechanical vibration combination with compressive force applied on osteoclast precursor cells. **Objectives** This study purposed to investigate the effects of compressive force combined with mechanical vibration or mechanical vibration alone on osteoclastogenesis in RAW 264.7 cells, a murine osteoclastic-like cell line. **Materials and Methods** Murine monocyte/macrophage RAW 264.7 cells were used as model osteoclast precursor cells. To determine the optimal compressive force and mechanical vibration. Various compressive force (0.3, 0.6 or 0.9 g/cm^2) was applied to RAW 264.7 cells and induced with either 30 Hz or 60 Hz at 0.49 g. To determine the effects of compressive force combination with mechanical vibration, RAW 264.7 cells were subjected to suitable compressive force or mechanical vibration for 20 min every 24 h for 4 days or combination of compressive force and vibration. Cell viability was assessed using Prestoblue assay. NFATc1, DC-STAMP and CTSK gene expression were measured by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) and the numbers of

TRAP-positive multinucleated cells (MNCs) were counted and analyzed **Results:** Compressive force combination with mechanical vibration significantly increased the numbers of TRAP-positive multinucleated cells when compared with compressed or vibrated group ($P<0.05$). Application of force on RAW 264.7 cells did not significantly affect cell viability. The combination of compressive force and vibration significantly increased NFATc1, DC-STAMP, and CTSK mRNA expression, compared to compressive force or vibration alone ($P<0.05$). **Conclusions:** Compressive force combined with mechanical vibration induces osteoclastogenesis and upregulates the expression of NFATc1, DC-STAMP and CTSK gene on RAW 264.7 cells. These results provide more insight into the mechanisms by which vibratory force accelerates orthodontic tooth movement.

ACKNOWLEDGEMENT

As the author, I am honored to special thank the Faculty of Dentistry, Prince of Songkla University for this wonderful opportunity.

My advisors, Assoc. Prof. Dr. Chidchanok Leethanakul and Assoc. Prof. Dr. Suwant to Jitpukdeebodintra, deserve my gratitude and appreciation for their direction, supervision, essential suggestions, and encouragement.

I appreciate the efforts of Ms. Suppaluk Wilairat, all the scientists and staff at the Research facilitation and development unit, Faculty of Dentistry, Prince of Songkla University, for their helpful suggestion and hospitality.

I also would like to thank my professors and all staffs at Orthodontic section, Faculty of Dentistry, Prince of Songkla University for being kind and helpful and my parents, sister and friends for all their love, patience and support.

Finally, I would like to acknowledge the Graduate School, Prince of Songkla University for providing me with all essentials for the achievement of my Ph.D.

Boontida Changkhaokham

CONTENTS

LIST OF TABLE

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

PDL = Periodontal ligament

LMHF = Low magnitude high frequency

RANK = Receptor activator of nuclear factor kappa B

RANKL = Receptor activator of nuclear factor kappa B ligand

OPG = Osteoprotegerin

mRNA = Messenger ribonucleic acid

PGE2 = Prostaglandin E2

Hz = Hertz

M-CSF = Macrophage-colony stimulating factor

TRAFs = Tumor necrosis factor receptor associated factors

IL-1 = Interleukin 1

IL-6 = Interleukin 6

IL-8 = Interleukin 8

PBS = Phosphate-buffered saline

°C = Celcius

h = Hour

CO_2 = Carbon dioxide

FBS = Fetal bovine serum

g = Gravity

min = Minute

qPCR = Quantitative real-time Polymerase chain reaction

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

C = Control

V = Vibration

CF = Compressive force

CFV = Compressive force combined with mechanical vibration

g/cm^2 = Gram per square centimeter

µl = Microliter

ng = Nanogram

U/µl = Unit per microliter

s = Second

TGF-ß = Transforming growth factor-ß

IGF-1 = Insulin-like growth factor-1

NFATc1 = Nuclear Factor of Activated T Cells 1

DC-STAMP = Dendritic cell-specific transmembrane protein

CTSK = Cathepsin K

TRAP = Tartrate-resistant acid phosphatase

BMU = Bone multi-cellular unit

CHAPTER 1

INTROD0UCTION

Background and rationale

Nowadays, demands of orthodontic treatment have increased dramatically. Patients who seek the treatment are not only teenagers but also adults. However, the treatment duration is very time-consuming approximately 2–3 years[1, 2] which lead to face with high risks of caries, external root resorption, diminished oral hygiene control and patient cooperation.[3-5]

During application of controlled orthodontic force on teeth, there is a series of biological changes in micro-environment of paradental structure. The acute inflammatory responses initially occur through the migration of leukocytes during the vasodilatation. Osteoblasts and PDL cells under mechanical loading release signaling molecules stimulate osteoblasts to produce chemokines and RANKL. This signaling cascade induces chemotactic of osteoclast precursors recruitment. After osteoblast–osteoclast communications, these cells are differentiated into mature osteoclasts and eventually, initiate bone resorption. The vital biological factor for bone resorption results from osteoclast activation and the rate of tooth movement increases by any treatment that increases the rate of bone resorption.[6]

There are many methods to accelerate tooth movement, for example, techniques involved surgical, phramacological and physical approaches.[6, 7] However, while some methods such as corticotomy effectively increase of bone turnover, they

also are invasiveness, posing risks of surgical complications, and this approach influence only in the first few months.[8]

Lately, there are numerous reports depict methods to speed the rate of orthodontic tooth movement by mechanical vibration. However, there are some limitations and controversial. Previous studies showed that mechanical vibration combined with compressive force applied on periodontal ligament (PDL) cells might accelerate orthodontic tooth movement by increased of IL-6, IL-8 and PGE2.[9] On the other hand, vibration combined with compressive force did not significantly change the gene expression of RANKL, OPG, IL-1β and IL-6 on alveolar bone osteoblasts in human.[10]

This study is focused on mechanical vibration combined with compressive force applied in osteoclast precursor cells that mimic the environment in orthodontic treatment. Because these cells are importantly involved in the rate of tooth movement. In the previous study reported that compressive force promotes osteclastogenesis on osteoclasts by upregulation of NFATc1, DC-STAMP, TRAP, RANK and Cathepsin K in RAW 264.7 cells.[11-14] Additionally, the effect of vibration only inhibits osteoclast formation by decrease the expression of Cathepsin K, DC-STAMP receptor, MMP-9 and TRAP.[15, 16] However, there is still no knowledge about mechanical vibration combined with compressive force applied on osteoclast precursor cells.

The purpose of this study is to represent the molecular marker during the compression combined with mechanical vibration in osteoclast precursor cells (RAW 264.7 cells).

Review of literatures

Biology of tooth movement

Bone remodeling processes are induced when an orthodontic force that generates an aseptic inflammatory response is applied over the periodontium. The alterations to the homeostasis and circulation of the periodontal ligament (PDL) caused by inflammation produce zones of ischemia and vasodilatation, which various biological mediators such as chemokines, cytokines, neurotransmitters, growth factors, and hormones are released as a result. These chemicals stimulate cellular activity, allowing osteoclasts to resorb bone and osteoblasts to create bone.[17, 18]

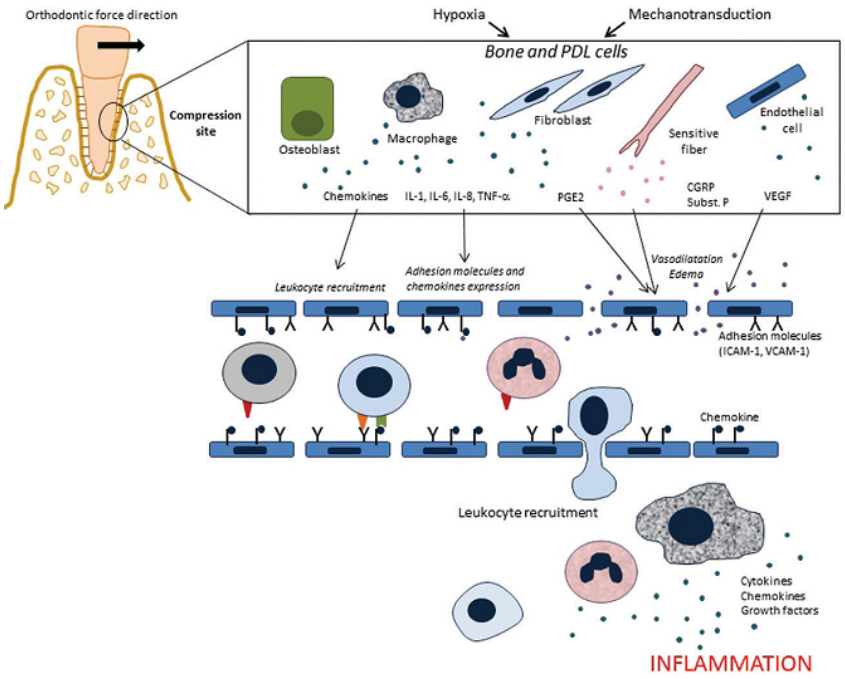

Figure 1: Orthodontic force triggers local hypoxia and inflammatory response in compression sites.[19]

On the compression side, orthodontic force triggers mechanotransduction and local hypoxia in fibroblast.[17] And also increases the expression of TNF-α, VEGF, IL-1β, IL-6 and IL-8. The physical strain also produce growth factors, PGE[19] and a variety of chemokines, such as CCL2, 3 and 5.[17] These chemokines are chemotactic factors is used for osteoclast precursors to migrate to osteolytic sites,[20] where the precursor cells differentiate into mature osteoclasts by osteoblast-osteoclast communication through membrane bound ligands and receptors, diffusion paracrine, and cell-bone matrix regulation.[21] Prostaglandin E2 (PGE2), IL-1β, IL-6, IL-8 and TNF-α are capable of stimulating osteoblasts to produce macrophage colony-stimulating factor (M-CSF) and RANKL[22, 23] which bind to their respective receptors, c-Fms and RANK, on osteoclast precursors to promote osteoclastogenesis.[21]

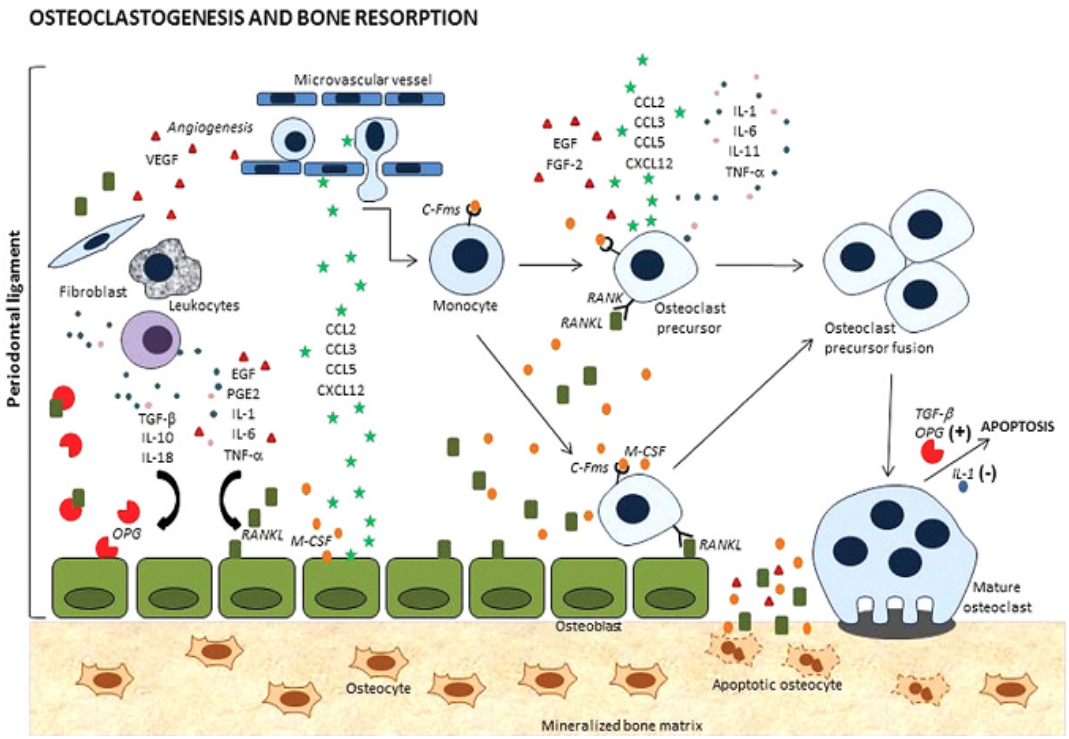

Figure 2: Osteoclastogenesis in the compression site during orthodontic tooth movement[19].

Bone formation and osteoblast differentiation occur at the tension site during orthodontic tooth movement. PDL cells are stretched and produce chemokines, cytokines, and growth factors, which encourage cell replication. The expression of transforming growth factor-β (TGF-β) and insulin-like growth factor-1 by local osteoblasts and osteocytes promotes the proliferation and differentiation of osteoblast precursors, as well as the production of new bone (IGF-1). During the period of maturation, alkaline phosphatase (ALP) and mRNA in osteoblasts are increased dramatically. In addition, anti-inflammatory cytokines, such as interleukin 10 (IL-10) and osteoprotegerin (OPG) are expressed by osteoblasts which are involved in bone formation and inhibit osteoclastogenesis. Bone resorption and formation occur in a dynamic equilibrium during orthodontic tooth movement. The contradictory effects of RANKL and OPG are crucial in bone turnover regulation.[17, 18]

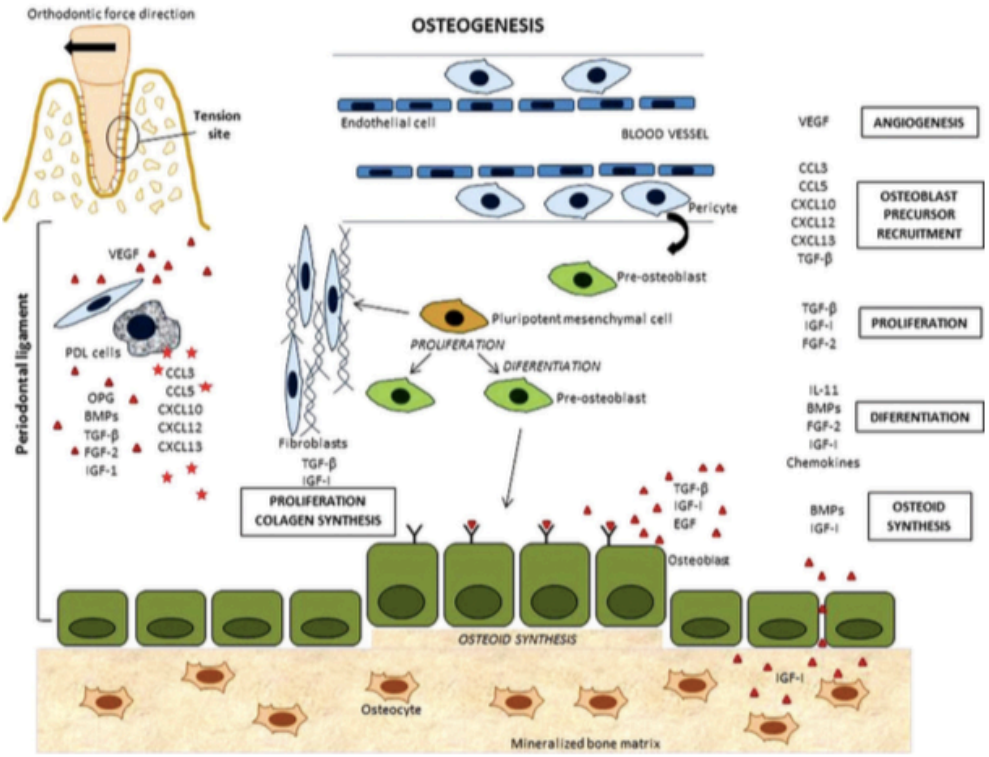

Figure 3: Biologic events in tension sites during orthodontic tooth movement.[19]

Osteoclast and bone remodeling cycle

The bone remodeling process involves both osteoclasts that resorb bone and osteoblasts that generate new bone. Osteoclasts derive from a hematopoietic precursor in the bone marrow.[24] M-CSF is required for mononuclear cell proliferation from the precursor population.[25] Pre-osteoclasts enter the bloodstream and migrate to the site of bone resorption. An immature osteoclast will fuse together in the presence of M-CSF and RANKL.[24, 25] Tartrate-resistant acid phosphatase (TRAP), beta-3 integrin and calcitonin receptor are all expressed by immature osteoclasts. To develop the cell into a mature osteoclast phenotype, RANKL and a host of transcription factors are necessary.

Their functions are maintained stability and integrity of bone, consist of 3 steps, which are resorption, reversal and formation step. The remodeling process takes place in what are known as bone multicellular units (BMU). The remodeling cycle begins with the hormonal regulation of calcium homeostasis activating inactive osteoblasts (bone-lining cells). Proteolytic enzymes are produced and released by osteoblasts, which dissolve the osteoid that separates the osteoblastic cell layer from the mineralized bone. The osteoclasts which resorb bone then can adhere to the mineralized bone surface. The activation of receptors on osteoclasts, such as RANK and c-Fms, as well as the production of RANKL and M-CSF by osteoblasts, initiates the recruitment of osteoclasts into BMU. The osteoclasts leave the resorption site once the bone resorption lacunae have been molded, and mononucleated cells appear to clear up any remnants of the organic matrix after the osteoclasts have digested it. IGF-1 and TGF-β are growth factors which are secreted from the extracellular matrix of the bone and play an important role in the

recruitment and activation of osteoblasts to resorption lacunae. The remodeling process has been completed when the osteoblasts fill the resorption lacunae with new bone in an amount equal to that resorbed, and the mineralized bone matrix is covered by osteoid and a single layer of osteoblasts.[26]

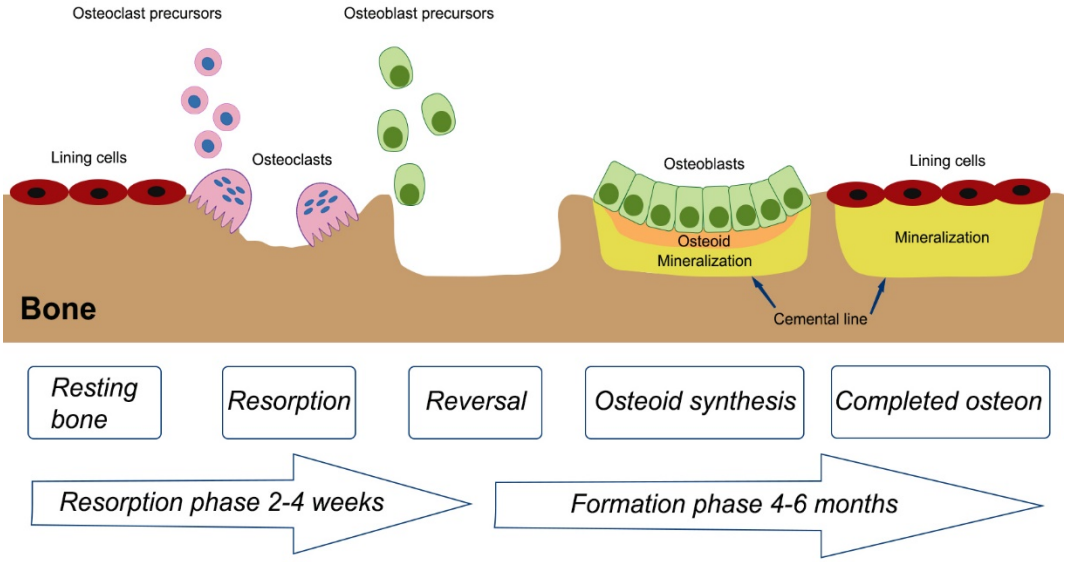

Figure 4: Step of bone remodeling [27]

Intercellular communication and regulation of osteoclastogenesis

The cell to cell communication in the bone is for the alteration of bone cell function. The osteoblastic lineage has a vital role in osteoclast formation and activities. The cytokine macrophage colony-stimulating factor (M-CSF) produced from stromal cells/osteoblasts and T lymphocytes, is a vital protein in regulate osteoclast differentiation and formation. M-CSF and its specific receptors, c-Fms, which are expressed on osteoclast precursors and activates the proliferation and prevents the apoptosis of early osteoclast precursors.[28]

RANKL and OPG have an important role in osteoclastogenesis. RANKL is expressed as membrane-bound, produced mainly by osteoblast/stromal cells and soluble RANKL, produced by activated T-cells and PDL cells. Osteoblasts are activated by PTH, PGE2, $1\alpha,25(OH)_2$ D_3 and IL-11. However osteoblasts also produce RANKL decoy receptor called OPG.[29] Moreover, IL-1 and IL-6, the products of stromal cells and monocytes, activate the osteoblasts to produce more RANKL but decrease OPG production. IL-1 and IL-6 also have direct effect to osteoclasts by work synergistically with RANKL.[30] So the RANKL/OPG ratio plays a vital role in orthodontic tooth movement

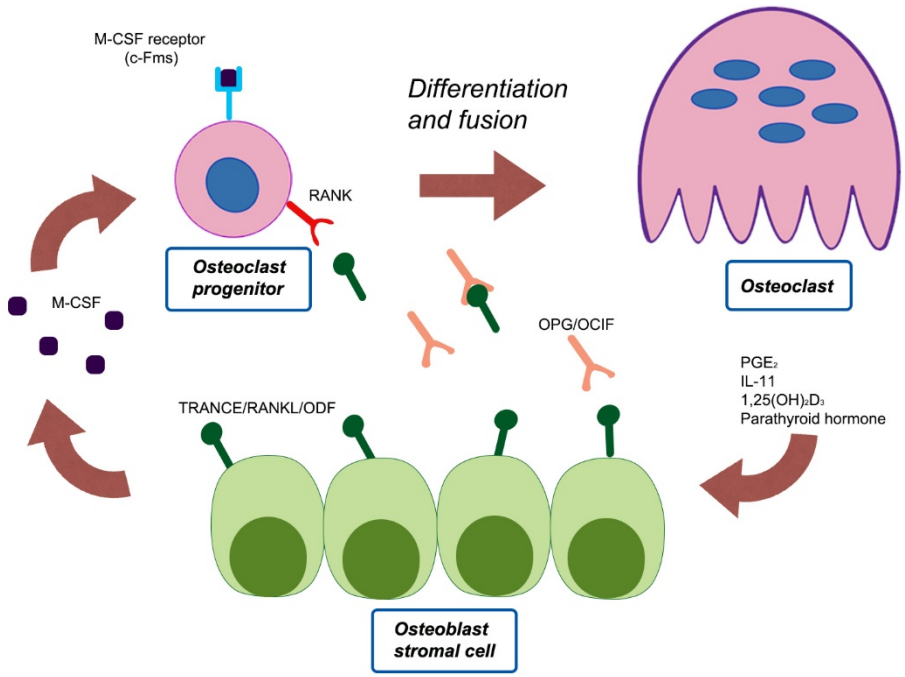

Figure 5: A mechanism of osteoblasts/stromal cells control osteoclastogenesis, modified from Yasuda et al.[31]

The expression markers of osteoclastogenesis

Numerous studies have shown that various molecule and transcription factors are essential in different stages of osteoclast survival differentiation and activation such as DC-STAMP, NFATc1 and ADAM8 are the important role in osteoclast fusion. Moreover, many various enzymes are the crucial for bone resorption such as carbonic anhydrase II, TRAP, Cathepsin K, and MMP-9 that consequently orthodontic tooth movement.

NFATc1

NFATc (Nuclear factor of activated T-cells cytoplasmic) is a transcription factor family which was originally recognized in T-cells. There are currently four members of the gene family identified (NFATc1 through NFATc4). In human OCs,

NFATc1 is the most commonly activated NFATc, with an expression exceeding that of NFATc2 through NFATc4. This transcription factor plays a major role in osteoclastogenesis. It is a master switch for regulating the downstream of RANKL and the differentiation of osteoclasts. Through calcium signaling and calcineurin, RANKL promotes and activates NFATc1. At the end stage of osteoclast differentiation, NFATc1 binds with Fos and Jun proteins to induces various target genes that responsibility for osteoclast differentiation and activation, including itself, such as TRAP, calcitonin receptor, Cathepsin K and ß3 integrin gene.[32]

DC-stamp

DC-STAMP (Dendritic cell–specific transmembrane protein), seven-transmembrane protein is released predominantly in osteoclasts and is expressed only after activated by RANKL in macrophage/monocyte lineage cells. DC-STAMP molecule has an impoetant role in the osteoclast precursors fusion and is important for the osteoclasts multinucleation in the presence of M-CSF and RANKL.[33] In the animal studies, mice with DC-STAMP knockout found few TRAP-positive multinucleated cells and increased bone density.[34]

Cathepsin K

Cathepsin K is a cysteine proteinase with type I and type II collagenase activity and is detected mostly in osteoclasts. Cathepsin K is a crucial matrix protein that is considered to play a mechanism in the breakdown of an organic elements during bone resorption. Cathepsin K is secreted beneath active osteoclasts into the resorption lacunae. Then type I collagen was breakdown.[35] The higher expression of Cathepsin K is increased by RANKL. Pycnodysostosis characterized by osteosclerosis

and short stature is caused by the congenital absence of Cathepsin K. Cathepsin K knockout mice develop osteopetrosis and exhibit pycnodysostosis-like symptoms.[36]

Methods to accelerate tooth movement

Long-term orthodontic treatment increased risk of gingivitis[37], root resorption[38] and dental caries[39]. Therefore, there is interested in accelerating the movement of teeth. In order to reduce the risks that may occur. Researchers have recently focused on surgical, biological and physical approaches to speed tooth movement and shorten orthodontic treatment times.[6]

Surgical approaches such as corticotomy[40] and piezocision[50] are based on the principle that an inflammatory cascade is initiated when bone is irritated surgically, which in turn promotes osteoclastogenesis and leads to faster tooth movement. Although the results are effective, surgical methods are invasive, and there is a possibility of injury to periodontal tissues as well as postoperative pain, therefore patient acceptability of these operations is low.[41]

Biological approaches such as injection of hormones (relaxin)[42], exogenous inflammatory mediators (prostaglandin E)[43] and vitamin D that induce bone resorption into periodontal tissue. Several studies have described pharmacological to accelerate tooth movement occurs by activation of osteoclasts. However, the daily administration or daily local injections are necessary because there are flushed out rapidly through the circulation. In addition to, these agents induce adverse effect such as pain and root resorption.[14]

Several physical approaches, including direct application of an electric current, electromagnetic field, low-level laser irradiation and LMHF vibration, have been proposed to speed tooth movement.[44]

Many studies have focused at the effects of mechanical vibration; mechanical vibration has been found to improve bone density and promote bone growth.[45]In medicine, to prevent bone loss in high risk patient of osteoporosis, whole body vibration is used as a non-pharmacological intervention.[44, 46] However, a magnitude should be lesser than 1 g.[46]

Recently, the magnitude below 1 g (g = 9.8 m/s2) and frequency of mechanical vibration at 20-90 Hz has been used to accelerate tooth movement during orthodontic treatment.[47, 48]

Nevertheless, the mechanical vibration on tooth movement in both clinical studies and animal still controversial.[47-50]

Effects of mechanical vibratory on orthodontic tooth movement

There are many evidences showing that low-magnitude, high-frequency mechanical vibration enhances orthodontic tooth movement. From clinical trials, Kau et al. reported vibration (20 min/day, 30 Hz, 0.2N) can accelerate orthodontic tooth movement[47], Pavlin et al. reported 48.1% increase in rate of canine retraction by using vibration device (20 min/day, 30 Hz, 0.003 g)[45]. And later, Leethanakul et al. reported an increase of canine retraction rate by using vibratory stimuli (electric toothbrush, 15 min/day)[51]. Consistent with results from animal studies. Nishimura et al, found resonance vibration accelerate orthodontic tooth movement and do not increase the amount of root resorption in Wistar rats.[50]

On the other hand, some studies reported contrary results. From clinical trials, Woodhouse et al. found no evidence that supplemental vibrational force (20 min/day, 30 Hz, 0.25 N) can reduce the time required to achieve complete tooth alignment.[49] Miles et al found no clinical advantage in using the vibrational appliance (20 min/day, 111 Hz, 0.06 N) for the early resolution of crowding.[52] From animal models, Yadav et al. concluded that 20 Hz vibration did not increase the rate of orthodontic tooth movement in mice.[53] Kalajzic et al. reported that tooth movement in Sprague-Dawley rats was significantly inhibited by application of vibration (10 min/day, 30 Hz, 0.4 N).[48]

In summary, these studies showed that the effect of low-magnitude, high-frequency mechanical vibration on orthodontic tooth movement is still controversial in clinical trials and animal models. The conflicting results observed in previous studies may derive from the various vibration protocols, the mechanic of tooth movement and the measurement.

The varied results in these investigation could be attributable to the various tooth movement mechanics (anterior teeth retraction or canine distalization) or magnitude of forces. However, the study addressing the biological mechanism underlying these effects is still lacking.

Effects of mechanical vibratory in human cells

There are many in vitro studies that clarified the mechanisms of mechanical vibrations affect tooth movement. Several studies have demonstrated vibration promotes osteogenic behavior in osteoblasts, mesenchymal stem cells and periodontal ligament stem cells, such as increased expression of ALP, osteonectin and osteopontin mRNAs.[54, 55]

The application of vibration to accerelate tooth movement during orthodontic treatment has been mimicked in compressed cells in two in vitro studies. Benjakul et al. reported human periodontal ligament cells (hPDL) cells which vibrated with 30 Hz at 0.3g expressed higher levels of PGE2 and RANKL than control cells significantly.[9] The compressive force combination with mechanical vibration showed an increase in PGE2 and RANKL expression, but did not significantly increase the expression of OPG or Runx2 compared to control cells. Similarly, Phusuntornsakul., et al found that the mechanical vibration combination with compressive force synergistically upregulate IL-6, IL-8 and COX-2 gene and protein in hPDL cells and also increase the levels of PGE2, IL-6 and IL-8 in the condition medium via activation of the cyclooxygenase pathway.[56]

Additionally, the previous in vitro study about vibration of RAW 264.7 cells found that LMHFV (0.3g 45 Hz 15 min/day) inhibited osteoclasts formation by decreased the number of TRAP-positive MNCs.[11] Similarly, Kulkani et al, mechanical vibration (4 Hz 1 h) decreases the expression of DC-STAMP in osteoclast precursor cells following to the inhibition of osteoclast formation.[15]

Contrary, continuous compressive force (0.3, 0.6, 1.1 g/cm^2) increased DC-/OC-STAMP and NFATc1 gene expression and also enhanced the

number of TRAP-positive multinuclear cells.[13-16] It is interesting to study the underlying mechanism of compressive force and vibration on osteoclastogenesis.

Summary of the studies

All things considered, this in vitro study aim to investigate whether compressive stress combined with mechanical vibration induced RAW 264.7 cells could enhance the number of TRAP-positive multinucleated cells (3 or more 3 nuclei per cell) and the expression of osteoclastogenesis factor; NFATc1, DC-STAMP and Cathepsin K gene.

The 1st part of the study: To obtain the suitable magnitude of compressive stress and suitable frequency of mechanical vibration for investigate under compressive stress combined with mechanical vibration in RAW 264.7 cells.

Research question

1. Does the compressive stress express the TRAP-positive multinucleated in RAW 264.7 cells?

- Which gram of force is the suitable compressive stress for investigate under mechanical vibration combined with compressive stress in RAW 264.7 cells?

2. Does the mechanical vibration express the TRAP-positive multinucleated in RAW 264.7 cells?

- Which frequency of mechanical vibration is the suitable for investigate under mechanical vibration combined with compressive stress in RAW 264.7 cells?

Research objective

1. To count the number of TRAP-positive multinucleated cells (3 or more 3 nuclei per cell) in response to compressive stress.

2. To count the number of TRAP-positive multinucleated cells (3 or more 3 nuclei per cell) in response to mechanical vibration.

Hypothesis

1. The different compressive stress effects the expression of TRAP-positive multinucleated cells (3 or more 3 nuclei per cell).

2. The different frequency of the mechanical vibration effects the expression of TRAP-positive multinucleated cells (3 or more 3 nuclei per cell).

The 2nd part of the study:

1. To examine the compressive stress combination with the mechanical vibration on the number of TRAP-positive multinucleated cells (3 or more 3 nuclei per cell).

2. To examine the compressive stress combination with the mechanical vibration on the expression of NFATc1, DC-STAMP and Cathepsin K gene.

Research question

1. Does the compressive stress combined with mechanical vibration enhances or inhibits the number of TRAP-positive multinucleated cells (3 or more 3 nuclei per cell) in RAW 264.7 cells?

2. Does the compressive stress combined with mechanical vibration affect the expression of NFATc1, DC-STAMP and Cathepsin K gene in RAW 264.7 cells?

Research objective

1. To count the number of TRAP-positive multinucleated cells (3 or more 3 nuclei per cell) in response to compressive stress combined with mechanical vibration.

2. To measure gene expression of NFATc1, DC-STAMP and Cathepsin K gene in RAW 264.7 cells in response to compressive stress combined with mechanical vibration.

Hypothesis

1. The compressive stress combined with mechanical vibration affect RAW 264.7 cell in the expression of TRAP-positive multinucleated cells (3 or more 3 nuclei per cell).

2. The compressive stress combined with mechanical vibration affect RAW 264.7 cell in the expression of NFATc1, DC-STAMP and Cathepsin K gene.

Significant of the study

To provide the scientific knowledge of the effect of compressive stress combined with mechanical vibration-induced RAW 264.7 cells (macrophage cell line) in vitro on the expression of NFATc1, DC-STAMP and Cathepsin K gene and the number of TRAP-positive multinucleated cells.

CHAPTER 2

RESEARCH METHODOLOGY

Cell culture and osteoclast differentiation

The murine macrophage cell lines RAW 264.7 (TIB-71TM; American Type Culture Collection, Manassas, VA, USA) were used as osteoclast precursors in this study. Cells were cultured in α-minimal essential medium (α- MEM; Gibco BRL, Rockville, MD, USA) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% fungizone at 37 °C in humidified atmosphere containing 5% CO_2. To induce osteoclast formation, RAW 264.7 cells were seeded overnight in 96-well culture plate at 2.0×10^3 cells/well and were shifted to α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 1% fungizone and 50 ng/ml mouse recombinant RANKL. The medium was replaced each other day.

Application of compressive force

As a model of the pressure site of orthodontic tooth movement, RAW 264.7 cells were constantly compressed, using a modify method by Kanzaki et al.[22] (Figure 6) Briefly, modified acrylic cylinders were placed over the 70–80% the monolayers in the 96-well plates to generate a compressive force at 0.3, 0.6 or 0.9 g/cm^2 of compressive force for 4 days. Modified acrylic cylinders were washed with the detergent, 70% ethanol and then were sterilized by autoclave.

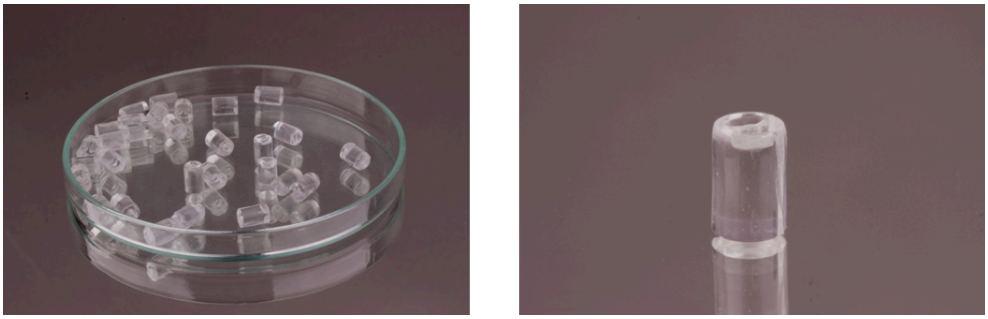

Figure 6: Modified acrylic cylinders

Application of mechanical vibration

Each 96-well plate cultured with RAW 264.7 cells were mounted on the platform of GJX- 5 vibration sensor (Beijing Sending Technology, Beijing, China). (Figure 7) The platform were on parallel with the ground. Those cells of the vibrate group were subjected to 30 or 60 Hz of vibratory stimulation at 0.49 g for 20 min every 24 h. The cells in control group were cultured in the same condition but were mounted on a stationary plate for the same time. The cells were returned to the incubator after the end of each cycle of mechanical vibration immediately.

Figure 7: GJX-5 vibration sensor

Determination of suitable magnitude of compressive stress

The murine monocyte/macrophage cell line RAW 264.7 were seeded at density of $2x10^3$ cells/well in 96-well plates with in α-MEM containing 10% FBS, 1%

penicillin-streptomycin and 1% fungizone, incubated at 37°C in a humidified atmosphere with 5% CO_2 until reach 70% confluence. Subsequently, the media are changed to α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 1% fungizone and 50 ng/ml mouse recombinant RANKL. Compressive force was applied by placing modified acrylic cylinder. Cells were randomly divided into four groups into

1. RAW 264.7 cells were not stimulated mechanically.
2. RAW 264.7 cells were stimulated by continuous compressive stress (0.3 g/cm^2, 4 days).
3. RAW 264.7 cells were stimulated by continuous compressive stress (0.6 g/cm^2, 4 days).
4. RAW 264.7 cells were stimulated by continuous compressive stress (0.9 g/cm^2, 4 days).

After the completion of each treatment, osteoclast formation assay and cell viability assay were performed in cells of all groups.

: To examine the effect of mechanical vibration on the expression of TRAP-positive multinucleated cells in RAW 264.7 cells and to obtain the suitable frequency.

The murine monocyte/macrophage cell line RAW 264.7 were seeded at density of 2x10^3 cells/well in 96-well plates with α-MEM containing 10% FBS, 1% penicillin-streptomycin and 1% fungizone, incubated at 37°C in a humidified atmosphere with 5% CO_2 until reach 70% confluence. Subsequently, the media were changed to α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 1% fungizone and 50 ng/ml mouse recombinant RANKL. Cells were randomly divided into three groups. Then, cells were randomly divided into

1. RAW 264.7 cells were not stimulated mechanically.

2. RAW 264.7 cells were stimulated by mechanical vibration (0.49 g, 30 Hz, 20 mins/day, 4 cycles of mechanical stimuli).

3. RAW 264.7 cells were stimulated by mechanical vibration (0.49 g, 60 Hz, 20 mins/day, 4 cycles of mechanical stimuli).

After the completion of each treatment, osteoclast formation assay was performed in cells of all groups.

<u>The 2nd part of the study</u>:

: To examine the effects of compressive stress combination with mechanical vibration on the number of TRAP-positive multinucleated (3 or more 3 nuclei per cell) cells.

: To examine the effects of compressive stress combination with mechanical vibration on NFATc1, CTSK and DC-STAMP gene expression

We used the suitable magnitude of compressive stress combined with frequency and magnitude of mechanical vibration from the 1st part of the study. Then, cells were randomly divided into

1. RAW 264.7 cells were not stimulated mechanically (C).

2. RAW 264.7 cells were stimulated by 0.6 g/cm^2 for 4 days (CF).

3. RAW 264.7 cells were stimulated by 0.49 g 60 Hz, 20 mins/day, 4 cycles (V).

4. RAW 264.7 cells were stimulated by 0.6 g/cm^2 combined with 0.49 g 60 Hz, 20 mins/day, 4 cycles (CFV).

After the completion each treatment, osteoclast formation assay, cell

viability, RNA extraction and quantitative real-time polymerase chain reaction for NFATc1, CTSK and DC-STAMP were performed in cells of all groups.

TRAP staining assay

After the 4 days of treatment, the cells were fixed and washed with 1 x PBS. TRAP staining kit was used (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The numbers of TRAP-positive MNCs (3 or more 3 nuclei per cell) were counted using a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 10X objective by two individuals who were blinded to the treatment of the cells.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

The total RNA of RANKL-treated RAW264.7 cells from each experimental group were isolated after the completion of mechanical stimulation procedure immediately. InnuPREP DNA/RNA mini kit (Analytic-jena, Germany) was used for cell lysis according to the manufacturer’s protocol. cDNA was synthesized from 10 μg of total RNA by reverse transcriptase (Superscript III First Strand Synthesis System; Invitrogen Life Technologies Co., Carlsbad, CA) and amplified using PCR technique. Primers for the NFATc1, DC-STAMP and CTSK were used in PCR. G lyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a housekeeping gene control in this study.

Table 1. Sequences of primers used for qPCR

Gene	Forward (5´-3´)	Reverse (5´-3´)	Accession number
NFATc1	TTGGATTCTGACGAGCTGTG	GTGCAGCTGGATCAAGAACA	NM_001244933.1
CTSK	CAGCAGAACGGAGGCATTGA	CCTTTGCCGTGGCGTTATAC	NM_007802.4
DC-STAMP	CTAGCTGGCTGGACTTCATCC	TCATGCTGTCTAGGAGACCTC	NM_029422.4
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT	XM_003819132.3

Cell viability assay

After mechanical stimulation for 4 days, the cell viability of each group of cells was evaluated using the PrestoBlue® assay (PrestoBlue® Cell Viability Reagent; Invitrogen) according to the manufacturer's instructions. The Prestoblue® solution was mixed with culture media at a ratio of 1:10, added to the cells, and incubated for 1 h at 37 °C. The cell viability was measured by absorbance at 600 nm.

Statistical analysis

All data are presented as the mean ± standard deviation of each group. To compare result between groups were carried out with SPSS software version 23.0 by one-way ANOVA using a multiple comparison TukeyHSD post hoc test; $P < 0.05$ was considered to indicate a significant difference.

CHAPTER 3

RESULTS

Part 1:

1.1 Effects of different magnitude of compressive stress and determination of optimal compressive force

The Cell viability in various compressive force

The cell viability in RAW 264.7 cells was examined by PrestoBlue assay between the control group and the cells after exposed force in various magnitude of compressive stress. Each magnitude of force did not affect the cell viability of RAW 264.7 cells ($P > 0.05$; Figure 8).

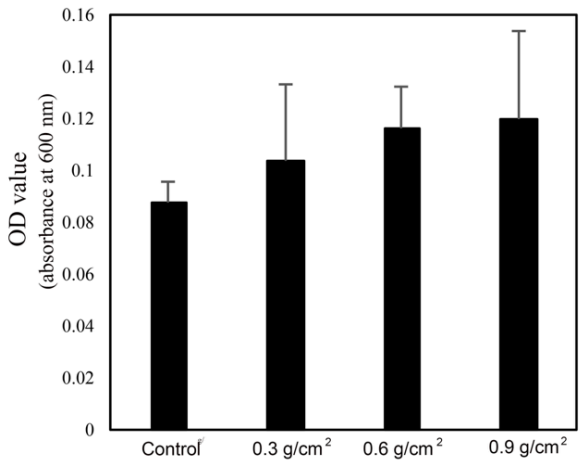

Figure 8: Cell viability of RAW 264.7 cells after subjected to various compressive force. Values are mean ± standard deviation, each assessed in triplicate ($P > 0.05$, n=3).

The number of TRAP-positive cells in various magnitude of compressive stress

RAW 264.7 cells were subjected by the different magnitudes of force for 4 days in the presence of 50 ng of RANKL. The 0.6 g/cm^2 and 0.9 g/cm^2 groups had no difference in the number of TRAP-positive cells ($P > 0.05$; Figure 9). On the other hand, 0.6 g/cm^2 group represented the most number of TRAP-positive cells and the second one is the 0.9 g/cm^2 group (Figure 9).

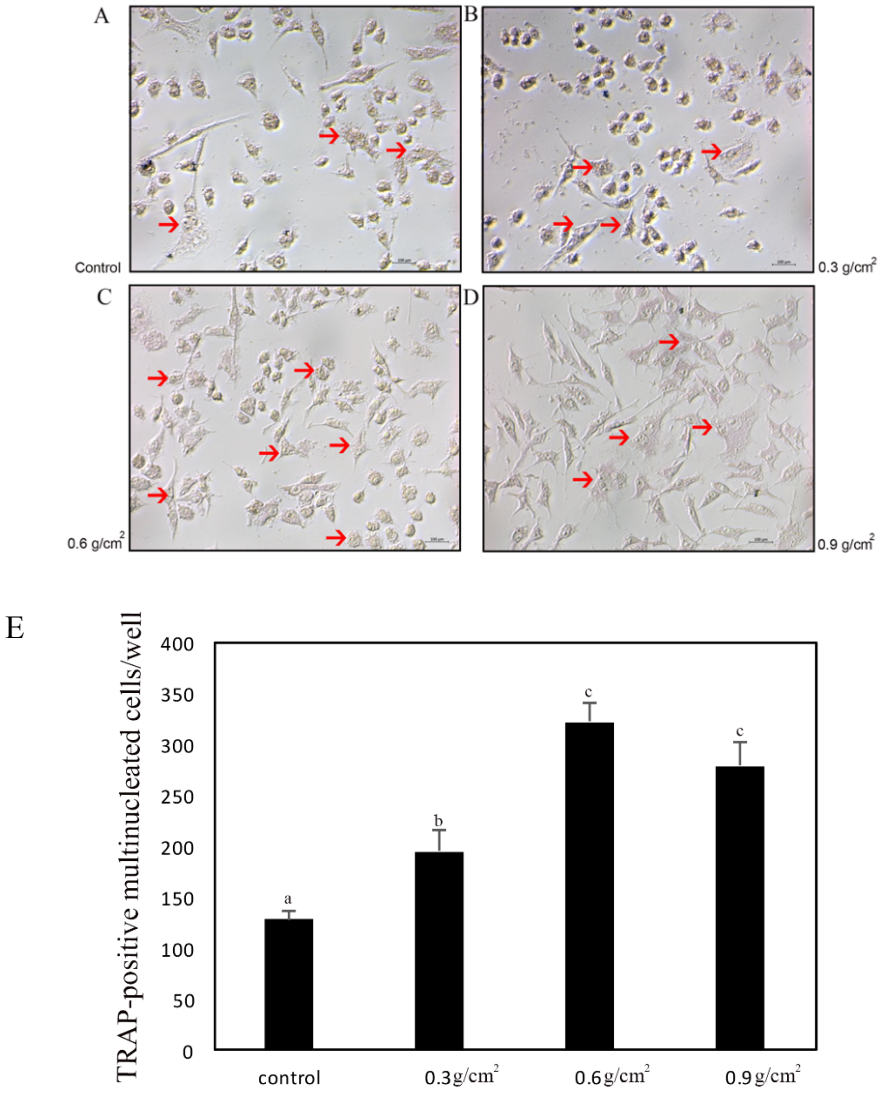

Figure 9: TRAP-positive cells of RAW 264.7 cells after subjected to various compressive force.

The red arrows indicate TRAP-positive MNCs (magnification = 40X, bar = 100 um). A: control group; B: 0.3 g/cm^2 group; C: 0.6 g/cm^{2}group; D: 0.9 g/cm^{2}group; E: A great number of TRAP-positive MNCs (≥ 3 nuclei) were observed in both 0.6 g/cm^2 and 0.9 g/cm^2 groups. Data are representative of three independent experiments. All values are shown as mean ± standard deviation. Significant differences between groups are indicated by different letters (a, b and c; $P < 0.05$, n = 3).

1.2 Effects of various vibration frequencies and determination of the optimal frequency

The number of TRAP-positive cells on the mechanical vibration

RAW 264.7 cells were subjected by 30 Hz and 60 Hz at 0.49 g. The highest number of TRAP-positive cells had shown in the 60 Hz group ($P < 0.05$; Figure 10C, arrows). Contrary, the 30 Hz and the control group had no difference in the number of TRAP-positive cells ($P > 0.05$; Figure 10A, 10B).

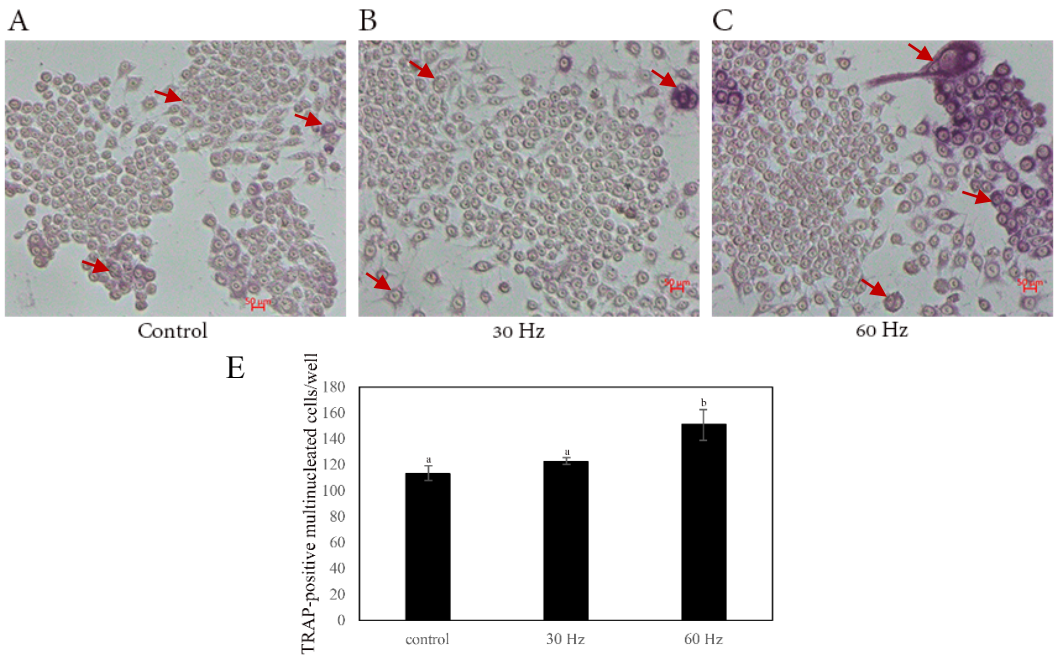

Figure 10: TRAP-positive cells of RAW 264.7 cells after being subjected to mechanical vibration.

The red arrows indicate TRAP-positive MNCs (magnification = 20X, bar = 50 um). A: control group; B: 30 Hz group; C: 60 Hz group; E: A great number of TRAP-positive MNCs (≥ 3 nuclei) were observed in the 60 Hz group. Data are representative of three independent experiments. All values are shown as mean ± standard deviation. Significant differences between groups are indicated by different letters (a and b; $P < 0.05$, n = 3).

Part 2:

Effects of mechanical vibration combined with compressive force on the cell viability of RAW 264.7 cells

Treatment with compressive force and/or mechanical vibration for 4 days did not significantly affect the viability of RAW 264.7 cells compared with the control group ($P > 0.05$; Figure 11).

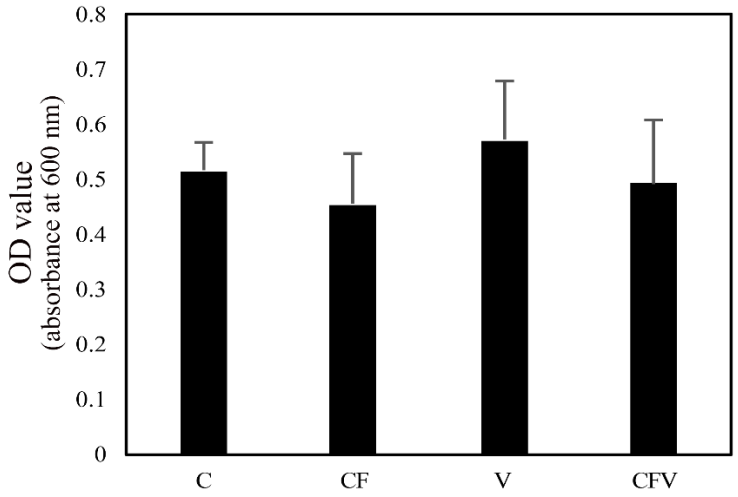

Figure 11: The viability of RAW 264.7 cells between the control group (C) and the cells after exposed to compressive force (CF), mechanical vibration (V) or compressive force combined with vibration (CFV). Values are mean ± standard deviation, each assessed in triplicate ($P > 0.05$, n=3).

Combined mechanical stimuli increase the numbers of TRAP-positive cells in RAW264.7 cells

TRAP-positive MNCs were observed in the control group, compressed group, vibrated group, and combined compressive force with vibrated group. The number of TRAP-positive MNCs was significantly higher in the compressed group than the vibrated and control groups, but was not significantly different between the vibrated and control groups. However, the combined compressive force and mechanical vibration group contained significantly high numbers of TRAP-positive MNCs compared with the other groups ($P < 0.05$; Figure 12E).

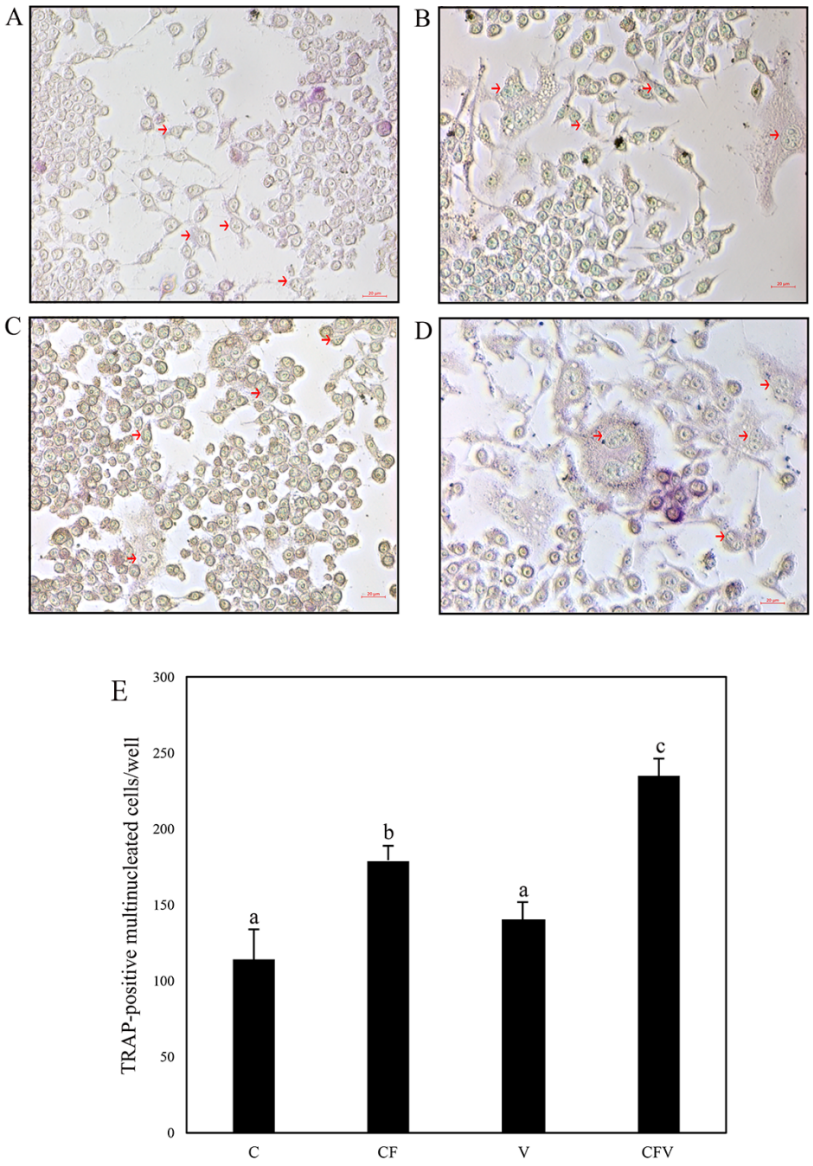

Figure 12: Combined compressive and vibratory force induces osteoclast differentiation in RAW 264.7 cells.

The red arrows indicate TRAP-positive MNCs. A: control group; B: compressed group; C: vibrated group; D: combined compression and vibration group; E: A greater number of TRAP-positive MNCs (≥ 3 nuclei) were observed in the combined group. Data are representative of three independent experiments. All values are shown as mean ± standard deviation. Significant differences between groups are indicated by different letters (a, b and c; $P < 0.05$, n = 3).

Combined mechanical stimuli increase NFATc1, DC-STAMP and CTSK mRNA expression in osteoclasts

NFATc1 mRNA expression in RANKL-treated RAW264.7 cells

NFATc1 expression was not significantly different between groups CF and C. However, the group CFV highly upregulated NFATc1 expression in osteoclasts, while group V only resulted in slight upregulation of NFATc1 mRNA ($P < 0.05$; Figure 13A). The expression level was ranked and grouped from the highest to the lowest as follows: CFV > V > CF, C.

DC-STAMP and CTSK mRNA expression in RANKL-treated RAW264.7 cells

DC-STAMP and CTSK mRNA were expressed highest in group CFV and was significantly higher than the other groups ($P < 0.05$; Figure 13B, 13C, respectively). No significant differences were observed between groups C and V ($P > 0.05$). The expression level was ranked and grouped from the highest to the lowest as follows: CFV > CF > V, C.

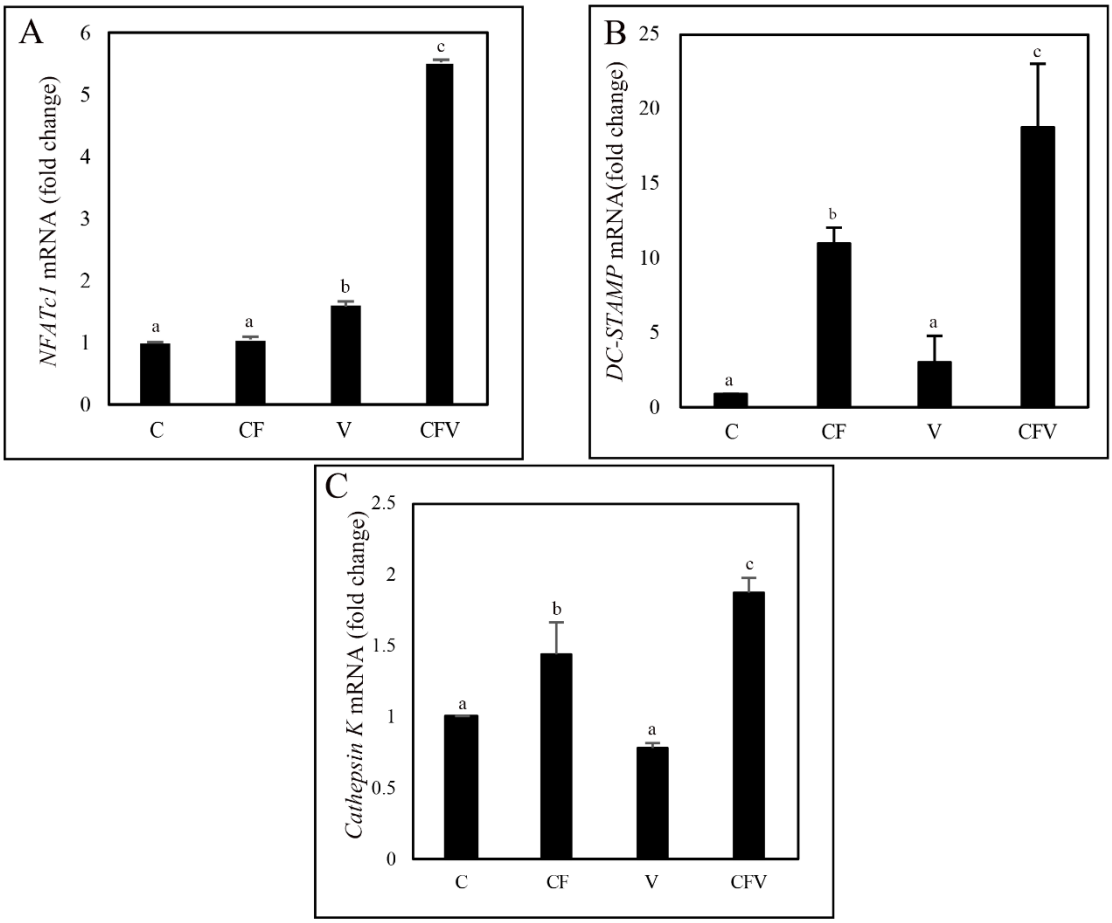

Figure 13: Effect of mechanical vibration combined with compressive force on mRNA expression in RANKL-treated RAW 264.7 cells. Real-time PCR analysis of A: NFATc1; B: DC-STAMP; C: CTSK. All data are mean ± standard deviation of triplicate experiments. Significant differences between groups are indicated by different letters (a, b and c; $P < 0.05$, n=3).

CHAPTER 4

DISCUSSION

This present study was the first, aimed to investigate pre-osteoclast cells by which mechanical vibration accelerates tooth movement. The RAW 264.7 cells, which are monocyte/macrophage-like cells obtained from BALB/c mice, have been reported as a great candidate for monocytes. They are highly effective in osteoclastogenesis.[57]

Matsuike et al.,[13] observed TRAP-positive multinucleated cells and an increase in the level of DC-STAMP mRNA expression of RAW 264.7 cells treated with 50 ng/mL of RANKL under 0.3, 0.6, 1.1 g/cm^2 loading for 4 days. The same concentration of RANKL was used in this study. According to the pilot study, 0.6 g/cm^2 compressive force and 60 Hz of vibratory stimulation were selected. Vibratory stimulation with 0.49 g at 60 Hz was used in our previous studies on osteoblasts.[10, 58] The vibration period of 20 min/day was used in clinical studies for tooth movement acceleration.[45, 47] Cell viability assays demonstrated mechanical stimuli in this study did not affect the viability of RAW 264.7 cells.

Sakamoto et al.,[59] previously reported that application of 0.5 g, 48.3 Hz vibration for 1 minute enhanced pre-osteoclast proliferation at 48 hours, but did not affect differentiation into osteoclasts. The vibration did not significantly induce differentiation of TRAP-positive cells, as there was no significant difference between the number of TRAP-positive cells in the control and vibrated group. Vibration has been shown to prevent the loss of long bone in many clinical studies.[60] Wu et al.,[16]

suggested low magnitude, high-frequency vibration inhibited RANKL-induced osteoclast differentiation.

Immunohistochemical analysis of a rat model showed whole-body vibration decreased RANKL expression, which implies that vibratory stimulation inhibits RANKL activity.[61] In this study, RAW 264.7 cells were treated with RANKL throughout the experiments. Although vibration slightly increased NFATc1 expression, it did not significantly alter DC-STAMP or CTSK mRNA expression. Kulkarni et al.,[15] reported vibrations downregulated DC-STAMP gene and protein expression in osteoclast precursor cells. Wu et al.,[16] also showed low magnitude, high-frequency vibration attenuated *RANKL*-induced upregulation of c-Fos in RAW 264.7 cells. The c-Fos pathway plays an important role in the regulation of DC-STAMP expression, which may explain the decrease in DC-STAMP mRNA expression observed in the vibration group in this study. We found vibration reduced the expression of the osteoclast-specific gene CTSK, which is characteristically associated with the function of mature osteoclasts. An *in vitro* study of bone marrow-derived osteoclasts treated with supernatant from cultivated osteoblasts showed micro-pulse vibration inhibited osteoclastic activity, including CTSK expression.[62] These findings may help to elucidate the role of vibration in the regulation of various stages of osteoclastic function.

The present study shows that DC-STAMP and CTSK were expressed at similar levels in all treatments. High levels of both DC-STAMP and CTSK were observed in response to a compressive force, with or without vibration. Numerous *in vitro* studies clearly indicate compressive force stimulates the expression of many osteoclast-specific genes involved in osteoclast differentiation and function in RAW 264.7 cells.[11-14] Additionally, many studies have demonstrated high expression of NFATc1 in

response to compressive force in RAW 264.7 cells.[12, 13, 33] Takayanagi et al.,[33] observed continuous expression of NFATc1 mRNA and protein in bone marrow-derived monocyte/macrophage precursor cells until TRAP-positive and multinucleated phenotypes were detected. Induction of NFATc1 peaked at more than 20-fold higher than baseline levels at 48 hours after RANKL stimulation and was sustained thereafter. However, we suggest the lack of a relationship between NFATc1 expression and compressive force level in this study may be due to the downregulation of NFATc1 expression before day 4.

Our recent study found that the application of compressive stress combination with mechanical vibration to human osteoblasts had no additional effect on the pro-inflammatory cytokines expression or the RANKL/OPG ratio compared to compressive force alone.[10] However, other studies showed compressive force and mechanical vibration synergistically upregulated the expression of RANKL and inflammatory mediators in PDL cells.[56, 63] In the present study, compressive force and vibration had an obvious synergistic effect on NATc1 mRNA expression. Moreover, the combined stimuli tended to increase DC-STAMP and CTSK expression compared with compression alone. These results suggest that compressive force combined with mechanical vibration may stimulate both PDL fibroblasts and pre-osteoclasts to participate in osteoclastogenesis.

The present study demonstrates the effect of compressive force and/or vibration on the number of TRAP-positive cells and NFATc1, DC-STAMP, and CTSK mRNA expression in RANKL-induced RAW 264.7 cells. We found that mechanical vibration synergistically promotes the expression of genes involved in osteoclastogenesis in the presence of compressive force stimulation. However, we

suggest additional studies with extended time points in order to explore the chronological sequence and peak levels of each mRNA and the number of TRAP-positive cells. Moreover, the role of PDL cells in osteoclastogenesis under compressive force combined with mechanical vibration should be considered in further studies.

CHAPTER 5

CONCLUSIONS

Mechanical vibration (0.49 g, 60 Hz) and combined mechanical vibration and compressive force (0.6 g/cm^2) had no effect on the viability of RAW 264.7 cells. Mechanical vibration combination with compressive force significantly increased NFATc1, DC-STAMP, and Cathepsin K gene expression in osteoclasts. Also, compressive force combination with mechanical vibration had additive effects on TRAP-positive MNCs (≥ three nuclei).

REFERENCES

1. Fink DF, Smith RJ. The duration of orthodontic treatment. Am J Orthod Dentofacial Orthop. 1992;102(1):45-51.

2. Fisher MA, Wenger RM, Hans MG. Pretreatment characteristics associated with orthodontic treatment duration. Am J Orthod Dentofacial Orthop. 2010;137(2):178-86.

3. Geiger AM, Gorelick L, Gwinnett AJ, Benson BJ. Reducing white spot lesions in orthodontic populations with fluoride rinsing. Am J Orthod Dentofacial Orthop. 1992;101(5):403-7.

4. Bishara SE, Ostby AW, editors. White spot lesions: formation, prevention, and treatment. Seminars in Orthodontics; 2008;14(3):174-82.

5. Julien KC, Buschang PH, Campbell PM. Prevalence of white spot lesion formation during orthodontic treatment. Angle Orthod. 2013;83(4):641-7.

6. Nimeri G, Kau CH, Abou-Kheir NS, Corona R. Acceleration of tooth movement during orthodontic treatment-a frontier in orthodontics. Prog Orthod. 2013;14:42.

7. Long H, Pyakurel U, Wang Y, Liao L, Zhou Y, Lai W. Interventions for accelerating orthodontic tooth movement: a systematic review. Angle Orthod. 2012;83(1):164-71.

8. Sanjideh PA, Rossouw PE, Campbell PM, Opperman LA, Buschang PH. Tooth movements in foxhounds after one or two alveolar corticotomies. Eur J Orthod. 2009;32(1):106-13.

9. Benjakul S, Jitpukdeebodintra S, Leethanakul C. Effects of low magnitude high frequency mechanical vibration combined with compressive force on human periodontal ligament cells in vitro. Eur J Orthod. 2018;40(4):356-63.

10. Chatmahamongkol C, Pravitharangul A, Suttapreyasri S, Leethanakul C. The effect of compressive force combined with mechanical vibration on human alveolar bone osteoblasts. J Oral Biol Craniofac Res. 2019;9(1):81-5.

11. Hayakawa T, Yoshimura Y, Kikuiri T, Matsuno M, Hasegawa T, Fukushima K, et al. Optimal compressive force accelerates osteoclastogenesis in RAW264.7 cells. Mol Med Rep. 2015;12(4):5879-85.

12. Ikeda M, Yoshimura Y, Kikuiri T, Matsuno M, Hasegawa T, Fukushima K, et al. Release from optimal compressive force suppresses osteoclast differentiation. Mol Med Rep. 2016;14(5):4699-705.

13. Matsuike R, Tanaka H, Nakai K, Kanda M, Nagasaki M, Murakami F, et al. Continuous application of compressive force induces fusion of osteoclast-like RAW264.7 cells via upregulation of RANK and downregulation of LGR4. Life Sci. 2018;201:30-6.

14. Matsuike R, Nakai K, Tanaka H, Ozaki M, Kanda M, Nagasaki M, et al. Continuous Compressive Force Induces Differentiation of Osteoclasts with High Levels of Inorganic Dissolution. Med Sci Monit. 2019;25:3902-9.

15. Kulkarni RN, Voglewede PA, Liu D. Mechanical vibration inhibits osteoclast formation by reducing DC-STAMP receptor expression in osteoclast precursor cells. Bone. 2013;57(2):493-8.

16. Wu SH, Zhong ZM, Chen JT. Low-magnitude high-frequency vibration inhibits RANKL-induced osteoclast differentiation of RAW264.7 cells. Int J Med Sci. 2012;9(9):801-7.

17. Krishnan V, Davidovitch Z. Cellular, molecular, and tissue-level reactions to orthodontic force. Am J Orthod Dentofacial Orthop. 2006;129(4):469.e1-32.

18. Wise GE, King GJ. Mechanisms of tooth eruption and orthodontic tooth movement. J Dent Res. 2008;87(5):414-34.

19. Andrade I, Taddei SRdA, Souza PEA. Inflammation and Tooth Movement: The Role of Cytokines, Chemokines, and Growth Factors. Semin Orthod. 2012;18(4):257-69.

20. Li X, Qin L, Bergenstock M, Bevelock LM, Novack DV, Partridge NC. Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts. J Biol Chem. 2007;282(45):33098-106.

21. Matsuo K, Irie N. Osteoclast-osteoblast communication. Arch Biochem Biophys. 2008;473(2):201-9.

22. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res. 2002;17(2):210-20.

23. Meikle MC. The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. Eur J Orthod. 2006;28(3):221-40.

24. Bar-Shavit Z. The osteoclast: a multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell. J Cell Biochem. 2007;102(5):1130-9.

25. Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, et al. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med. 1999;190(12):1741-54.

26. Lerner UH. Bone remodeling in post-menopausal osteoporosis. J Dent Res. 2006;85(7):584-95.

27. Kearns AE, Kallmes DF. Osteoporosis primer for the vertebroplasty practitioner: expanding the focus beyond needles and cement. AJNR Am J Neuroradiol. 2008;29(10):1816-22.

28. Ng KW, Romas E, Donnan L, Findlay DM. Bone biology. Baillieres Clin Endocrinol Metab. 1997;11(1):1-22.

29. Khosla S. Minireview: the OPG/RANKL/RANK system. Endocrinology. 2001;142(12):5050-5.

30. Kwan Tat S, Padrines M, Theoleyre S, Heymann D, Fortun Y. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev. 2004;15(1):49-60.

31. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A. 1998;95(7):3597-602.

32. Troen BR. The role of cathepsin K in normal bone resorption. Drug News Perspect. 2004;17(1):19-28.

33. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell. 2002;3(6):889-901.

34. Ikeda F, Nishimura R, Matsubara T, Tanaka S, Inoue J, Reddy SV, et al. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. J Clin Invest. 2004;114(4):475-84.

35. Matsumoto M, Kogawa M, Wada S, Takayanagi H, Tsujimoto M, Katayama S, et al. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression

during osteoclastogenesis through association of NFATc1 and PU.1. J Biol Chem. 2004;279(44):45969-79.

36. Brömme D, Lecaille F. Cathepsin K inhibitors for osteoporosis and potential off-target effects. Expert Opin Investig Drugs. 2009;18(5):585-600.

37. Ristic M, Vlahovic Svabic M, Sasic M, Zelic O. Clinical and microbiological effects of fixed orthodontic appliances on periodontal tissues in adolescents. Orthod Craniofac Res. 2007;10(4):187-95.

38. Khoroushi M, Kachuie M. Prevention and Treatment of White Spot Lesions in Orthodontic Patients. Contemp Clin Dent. 2017;8(1):11-9.

39. Andrade I, Jr., Sousa AB, da Silva GG. New therapeutic modalities to modulate orthodontic tooth movement. Dental Press J Orthod. 2014;19(6):123-33.

40. Wilcko MT, Wilcko WM, Pulver JJ, Bissada NF, Bouquot JE. Accelerated osteogenic orthodontics technique: a 1-stage surgically facilitated rapid orthodontic technique with alveolar augmentation. J Oral Maxillofac Surg. 2009;67(10):2149-59.

41. Aksakalli S, Calik B, Kara B, Ezirganli S. Accelerated tooth movement with piezocision and its periodontal-transversal effects in patients with Class II malocclusion. Angle Orthod. 2016;86(1):59-65.

42. McGorray SP, Dolce C, Kramer S, Stewart D, Wheeler TT. A randomized, placebo-controlled clinical trial on the effects of recombinant human relaxin on tooth movement and short-term stability. Am J Orthod Dentofacial Orthop. 2012;141(2):196-203.

43. Yamasaki K, Shibata Y, Imai S, Tani Y, Shibasaki Y, Fukuhara T. Clinical application of prostaglandin E1 (PGE1) upon orthodontic tooth movement. Am J Orthod. 1984;85(6):508-18.

44. Rubin C, Recker R, Cullen D, Ryaby J, McCabe J, McLeod K. Prevention of postmenopausal bone loss by a low-magnitude, high-frequency mechanical stimuli: a clinical trial assessing compliance, efficacy, and safety. J Bone Miner Res. 2004;19(3):343-51.

45. Pavlin D, Anthony R, Raj V, Gakunga P. Cyclic Loading (Vibration) Accelerates Tooth Movement in Orthodontic Patients: A Double-Blind, Randomized Controlled Trial. Semin Orthod. 2015;21(3):187-94.

46. Verschueren SM, Roelants M, Delecluse C, Swinnen S, Vanderschueren D, Boonen S. Effect of 6-month whole body vibration training on hip density, muscle strength, and postural control in postmenopausal women: a randomized controlled pilot study. J Bone Miner Res. 2004;19(3):352-9.

47. Kau C, Nguyen JT, English J. The clinical evaluation of a novel cyclical force generating device in orthodontics. Orthod Pract. 2010;1(1):1-4.

48. Kalajzic Z, Peluso EB, Utreja A, Dyment N, Nihara J, Xu M, et al. Effect of cyclical forces on the periodontal ligament and alveolar bone remodeling during orthodontic tooth movement. Angle Orthod. 2014;84(2):297-303.

49. Woodhouse NR, DiBiase AT, Johnson N, Slipper C, Grant J, Alsaleh M, et al. Supplemental vibrational force during orthodontic alignment: a randomized trial. J Dent Res. 2015;94(5):682-9.

50. Nishimura M, Chiba M, Ohashi T, Sato M, Shimizu Y, Igarashi K, et al. Periodontal tissue activation by vibration: intermittent stimulation by resonance vibration accelerates experimental tooth movement in rats. Am J Orthod Dentofacial Orthop. 2008;133(4):572-83.

51. Leethanakul C, Suamphan S, Jitpukdeebodintra S, Thongudomporn U, Charoemratrote C. Vibratory stimulation increases interleukin-1 beta secretion during orthodontic tooth movement. Angle Orthod. 2016;86(1):74-80.

52. Miles P, Smith H, Weyant R, Rinchuse D. The effects of a vibrational appliance on tooth movement and patient discomfort: a prospective randomised clinical trial. Aust Orthod J. 2012;28:213-8.

53. Yadav S, Dobie T, Assefnia A, Gupta H, Kalajzic Z, Nanda R. Effect of low-frequency mechanical vibration on orthodontic tooth movement. Am J Orthod Dentofacial Orthop. 2015;148(3):440-9.

54. Kim IS, Song YM, Lee B, Hwang SJ. Human mesenchymal stromal cells are mechanosensitive to vibration stimuli. J Dent Res. 2012;91(12):1135-40.

55. Chellaiah M, Kizer N, Silva M, Alvarez U, Kwiatkowski D, Hruska KA. Gelsolin deficiency blocks podosome assembly and produces increased bone mass and strength. J Cell Biol. 2000;148(4):665-78.

56. Phusuntornsakul P, Jitpukdeebodintra S, Pavasant P, Leethanakul C. Vibration enhances PGE(2) , IL-6, and IL-8 expression in compressed hPDL cells via cyclooxygenase pathway. J Periodontol. 2018;89(9):1131-41.

57. Hartley JW, Evans LH, Green KY, Naghashfar Z, Macias AR, Zerfas PM, et al. Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. Retrovirology. 2008;5:1.

58. Pravitharangul A, Suttapreyasri S, Leethanakul C. Iliac and mandible osteoblasts exhibit varied responses to LMHF vibration. Cell Biol Int. 2018;42(10):1349-57.

59. Sakamoto M, Fukunaga T, Sasaki K, Seiryu M, Yoshizawa M, Takeshita N, et al. Vibration enhances osteoclastogenesis by inducing RANKL expression via NF-**K**B signaling in osteocytes. Bone. 2019;123:56-66.

60. Sen EI, Esmaeilzadeh S, Eskiyurt N. Effects of whole-body vibration and high impact exercises on the bone metabolism and functional mobility in postmenopausal women. J Bone Miner Metab. 2020;38(3):392-404.

61. Pichler K, Loreto C, Leonardi R, Reuber T, Weinberg AM, Musumeci G. RANKL is downregulated in bone cells by physical activity (treadmill and vibration stimulation training) in rat with glucocorticoid-induced osteoporosis. Histol Histopathol. 2013;28(9):1185-96.

62. García-López S, Villanueva RE, Massó-Rojas F, Páez-Arenas A, Meikle MC. Micro-vibrations at 30 Hz on bone cells cultivated in vitro produce soluble factors for osteoclast inhibition and osteoblast activity. Arch Oral Biol. 2020;110:104594.

63. Benjakul S, Leethanakul C, Jitpukdeebodintra S. Low magnitude high frequency vibration induces RANKL via cyclooxygenase pathway in human periodontal ligament cells in vitro. J Oral Biol Craniofac Res. 2019;9(3):251-5.

APPENDICES

ที่ ม.อ. 651.6/081

คณะทันตแพทยศาสตร์
มหาวิทยาลัยสงขลานครินทร์
15 ถนนกาญจนวณิชย์
อ.หาดใหญ่ จ.สงขลา 90112

หนังสือแจ้งผล
การพิจารณาโครงการวิจัย

วันที่ 2 กรกฎาคม 2563

เรื่อง แจ้งผลการพิจารณาโครงร่างการวิจัย

เรียน รองศาสตราจารย์ ดร.ทพญ.ชิดชนก ลีธนะกุล

ตามที่ท่านเสนอโครงการวิจัยเพื่อขอรับพิจารณาจริยธรรมการวิจัยในมนุษย์

รหัสโครงการ EC6307-026

เรื่อง (ภาษาไทย) ผลของแรงกดร่วมกับแรงสั่นต่อกระบวนการสร้างเซลล์สลายกระดูกในเซลล์เม็ดเลือดขาวแมคโครฟาจ (RAW 264.7)

(ภาษาอังกฤษ) Effects of compressive stress combined with mechanical vibration on osteoclastogenesis of RAW 264.7 cells.

หัวหน้าโครงการ รองศาสตราจารย์ ดร.ทพญ.ชิดชนก ลีธนะกุล

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ ได้ทบทวนโครงร่างการวิจัย ผลการพิจารณา คือ
โครงการวิจัยเข้าข่ายยกเว้นการพิจารณาจริยธรรมการวิจัยในมนุษย์ (Exempt Determination)
จึงออกใบรับทราบ เมื่อวันที่ 2 กรกฎาคม 2563

ทั้งนี้ ได้บรรจุในวาระการประชุมคณะกรรมการพิจารณาจริยธรรมการวิจัยในมนุษย์ ครั้งที่ 7/2563 วาระที่ 3.5.1 เพื่อให้รับทราบแล้ว

จึงเรียนมาเพื่อโปรดทราบ

(รองศาสตราจารย์ ดร.ทพ.ไชยรัตน์ เฉลิมรัตนโรจน์)
ประธานกรรมการจริยธรรมการวิจัยในมนุษย์
คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

หมายเหตุ

- ผู้วิจัยไม่ต้องรายงานความก้าวหน้าต่อคณะกรรมการจริยธรรมการวิจัยในมนุษย์ และไม่ต้องต่ออายุโครงการ แต่ยังคงต้องรายงานความก้าวหน้าต่อแหล่งทุนวิจัย
- ท่านยังคงต้องส่งรายงานสรุปผลการวิจัยแก่คณะกรรมการจริยธรรมการวิจัยในมนุษย์ เมื่อดำเนินการวิจัยเสร็จสิ้น

VITAE

Name Miss Boontida Changkhaokham

Student ID 6210830005

Education Attainment

Degree	Name of Institution	Year of Graduation
Doctor of Dental Surgery	Western University	2015

Scholarships and Awards during Enrolment

Graduate School Research Scholarship, Prince of Songkla University 2017-2021

Faculty of Dentistry Scholarship, Prince of Songkla University 2017-2021

Work Position and Address

Mae Fah Luang University, Chiang Rai, Thailand

E-mail: boontida.tra@mfu.ac.th

List of Publication and Proceeding

Boontida Changkhaokham. Continuous versus released compressive force on osteoclastogenesis of RAW 264.7 cells. International Symposium for Interface Oral Health Science 2022; 15-16 Jan 2022 Tohoku University Graduate School of Dentistry