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The effect of compressive force combined with mechanical vibration on human alveolar bone osteoblasts

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ABSTRACT

Objective: This study aimed to investigate the effects of compressive force combined with mechanical vibration on the expression of pro-inflammatory cytokines that promote osteoclastogenesis and related to orthodontic tooth movement acceleration in human alveolar bone osteoblasts *in vitro*.

Methods: Osteoblasts were subjected to compressive force (C), mechanical vibration (V), compressive force combined with mechanical vibration (CV), or no force as a control for 12, 24 and 48 h. Interleukin-1 beta (IL-1β), interleukin-6 (IL-6), receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) mRNA and protein expression were assessed using quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assays.

Results: In C and CV groups, IL-1β and IL-6 mRNA and protein expression were significantly higher and OPG mRNA and protein expression were significantly lower than control and V groups. However, the expressions were not different between C and CV groups. RANKL mRNA and protein expression were not different between any groups. While, OPG mRNA and protein expression in V group were significantly higher than control group.

Conclusions: Vibration neither enhanced nor inhibited the expression of IL-1β, IL-6, RANKL and OPG in compressed human alveolar bone osteoblasts.

1. Introduction

In dentistry, commercially available vibratory devices are employed as a non-invasive method to accelerate orthodontic tooth movement and shorten the orthodontic treatment time, in an attempt to reduce adverse effects such as dental caries, gingival recession and root resorption.[1,2] A short duration of low-magnitude, high-frequency mechanical vibration may increase alveolar bone formation and maintenance.[3] Some animal and human studies reported orthodontic force adjunctive with vibration accelerated tooth movement,[4,5] while other studies suggested vibration decreased or did not affect the rate of tooth movement.[6–8] Our recent *in vitro* study found periodontal ligament (PDL) cells received compressive force combined with vibration expressed higher level of Prostaglandin E2 (PGE2), IL-6, IL-8 and RANKL expressions and lower level of Runt-related transcription factor 2 (Runx2) and OPG expression than PDL cells received compressive force alone.[9,10] These may indicate that vibration stimulates osteoclastogenesis and possibly leads to acceleration of orthodontic tooth movement.

However, PDL cells are not the only cells presented in the periodontium and affected by orthodontic force. Osteoblasts also respond to the alteration of matrix strain which is caused by fluid flow change at bone lining cell and function as mechanosensors in the periodontium when force is applied to the teeth and bone bending process occurs.[11]

From previous *in vitro* studies, application of compressive force to osteoblasts upregulated the expression of interleukin-1 beta (IL-1β) and interleukin-6 (IL-6),[12,13] pro-inflammatory cytokines that exert both direct and indirect osteoclastogenesis effects on osteoclasts. IL-1β and IL-6 promote osteoclastogenesis by increasing receptor activator of nuclear factor kappa-B ligand (RANKL) and decreasing osteoprotegerin (OPG),[14,15] which was showed to promote orthodontic tooth movement in animal models.[16,17] On the contrary, application of vibrational force to osteoblasts was demonstrated to give opposite results by increasing OPG and decreasing RANKL expression which may inhibit osteoclastogenesis and diminish orthodontic tooth movement.[18] However, no study has yet investigated the effects of combined compressive force and mechanical vibrational force on human osteoblasts.

This study aimed to investigate the effects of compressive force

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combined with mechanical vibration on the expression of pro-inflammatory cytokines that promote osteoclastogenesis and related to orthodontic tooth movement acceleration; IL-1β, IL-6, RANKL and OPG, in human alveolar bone osteoblasts *in vitro*.

2. Materials and methods

2.1. Human osteoblast isolation and culture

The study protocols were approved by the Ethics Committee Board of the Prince of Songkla University, Songkhla, Thailand (EC5901-04-P-LR). Alveolar bone samples were obtained from four healthy adult donors (age, 17 to 25-years-old). Informed consent was provided for every donors prior to collection of discarded alveolar bone specimens from surgical removal procedure of lower impacted molars or orthognathic surgery of the mandible.

Mandibular alveolar bone was used for osteoblast isolation as described in previous studies.[19,20] Briefly, the periosteum and attached soft tissue were scraped out from the alveolar bone. The bone samples were vortexed three times in phosphate buffered saline (PBS) and maintained in α-minimum essential medium (αMEM; Gibco BRL, Grand Island, New York, United States of America) containing 10% fetal bovine serum (FBS; Gibco BRL), 1% penicillin-streptomycin (Gibco BRL) and 1% fungizone solution (Gibco BRL) at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide. Osteoblasts were obtained after 3–4 weeks and those in second to fourth passage were used in the experiment.

2.2. Characterization of osteoblast cells

To confirm the osteoblastic phenotype of the isolated cells, osteoblasts were cultured in osteogenic stimulatory medium (α-MEM containing 10% FBS, 0.1% ascorbic acid, 0.2% dexamethasone and 0.1% β-glycerophosphate) for 28 days.[21] The Alkaline Phosphatase staining (Chemicon, Temecula, CA, United States of America) was done to assess osteoblast differentiation and Alizarin red staining (Sigma-Aldrich, St Louis, Missouri, United States of America) was applied to detect mineralized nodule formation following the manufacturers' instructions. Cell staining was examined by light microscopy.

2.3. Application of compressive force and mechanical vibration

Osteoblasts from each donor were individually transferred into six-well plates (3×10^5 cells per well). After reaching near-confluence cells were subjected to compressive force (C), mechanical vibration (V), compressive force combined with mechanical vibration (CV), or no force as a control as described below, and harvested to quantify mRNA or protein levels at 12, 24 and 48 h. The experiments were performed in triplicate for each donor cell line.

To stimulate with compressive force, osteoblasts in six-well plates were compressed continuously using the 32 mm-diameter plastic cylinder containing metal coins and an acrylic mass (2.0 g/cm^2). To stimulate osteoblasts with mechanical vibration, six-well plates were mounted onto a GJX-5 vibration sensor (Beijing Sending Technology, Beijing, China) parallel to the ground and exposed to vibration at an amplitude of 0.49 g and frequency of 60 Hz for 30 min (at 0, 24 and 48 h). This mechanical stimulus application protocol is a modification of the method described in previous studies.[20,22]

2.4. RNA extraction and real-time polymerase chain reaction (RT-PCR)

InnuPREP DNA/RNA mini kits (Analytic-Jena, Konrad-Zuse-Strasse 1, Jena, Germany) was used for the extraction of total RNA in accordance with the instructions of manufacturer. Total RNA was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, California, United States of America) then used in RT-PCR analysis.

Table 1
Real-time polymerase chain reaction forward and reverse primer sequences.

Gene	5'-forward primer-3'	5'-reverse primer-3'
IL-1β	CTGATGGCCCTAAACAGATGAAG	GTCGGAGATTCGTAGCTGGAT
IL-6	CGCCCCACACAGACAGCCAC	AGCTTCGTCAGCAGGCTGGC
RANKL	TCCCATCTGGTTCCCATAAA	GGTGCTTCCTCCTTTCATCA
OPG	GAAGGGCGCTACCTTGAGAT	GCAAACTGTATTTCGCTCTGG
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT

The primers sequences for *IL-1β, IL-6, RANKL, OPG* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) are presented in Table 1. RT-PCR analyses were performed on a Rotor-Gene Q (Qiagen, Qiagen Strasse 1, Hilden, Germany) using a SensiFAST SYBR No-ROX Kit (Bioline Inc, Taunton, Massachusetts, United States of America) in a single amplification program: activation at 95 °C for 2 min, denaturation at 95 °C for 5 s, followed by 40 cycles of annealing at 60 °C (*GAPDH, IL-1β, RANKL* and *OPG*) or 64 °C (*IL-6*) for 20 s and extension at 72 °C for 20 s. *GAPDH* was used as an internal control. The level of gene expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method.[23] The expression in experimental groups were presented as fold changes relative to control group.

2.5. Enzyme-linked immunosorbent assays

The culture media were assayed in duplicate using enzyme-linked immunosorbent assays (ELISA) to quantify IL-1β, IL-6, RANKL and OPG (DuoSet Human Immunoassay; R&D System, Minneapolis, Minnesota, United States of America). The total protein content in the culture media was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, Massachusetts, United States of America) according to the manuals provided. A microplate spectrophotometer (Multiskan GO; Thermo Scientific, Vantaa, Finland) was used to determine absorbance values. The protein expression levels were normalized to the total protein concentration and presented as pg/mg protein.

2.6. Statistical analysis

All values were expressed as mean ± standard deviation of four independent donors assessed in triplicate and analyzed by Kruskal-Wallis test and Mann-Whitney *U* test using SPSS software version 17.0 (SPSS, Chicago, Illinois, United States of America); statistical significance was determined as P values < 0.05.

3. Results

3.1. IL-1β mRNA and protein expression

IL-1β expression in C and CV groups were significantly higher than control and V groups at 12, 24 and 48 h for mRNA expression and at 24 and 48 h for protein expression ($p < 0.05$; Fig. 1A and B) and tended to increase over time. However, IL-1β mRNA and protein levels were not different between C and CV groups and between control and V groups at any time-points.

3.2. IL-6 mRNA and protein expression

IL-6 expression in C and CV groups were significantly higher than control and V groups at 12, 24 and 48 h for mRNA expression and at 48 h for protein expression ($p < 0.05$; Fig. 1C and D) and tended to increase over time. However, IL-6 mRNA and protein levels were not different between C and CV groups and between control and V groups at any time-points.

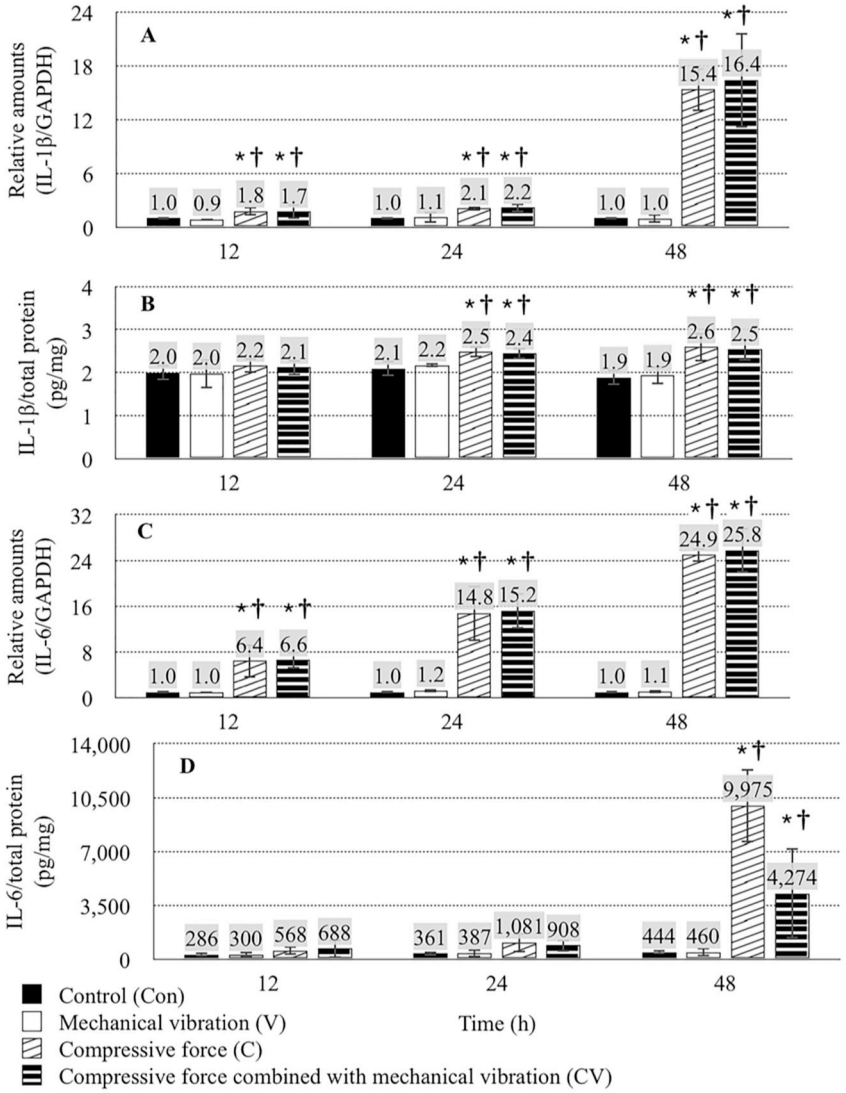

Fig. 1. Effect of exposure to compressive force, mechanical vibration, or compressive force combined with mechanical vibration for 12, 24 and 48 h on IL-1β (A: mRNA expression, B: protein expression) and IL-6 (C: mRNA expression, D: protein expression) in human osteoblasts. (* $P < 0.05$ compared with control group, † $P < 0.05$ compared with vibration group, Mann-Whitney U test).

3.3. RANKL mRNA and protein expression

RANKL mRNA and protein expression were not different among groups (Fig. 2A and B). However, RANKL mRNA and protein levels in C and CV groups tended to be higher than control and V groups at every time-point.

3.4. OPG mRNA and protein expression

OPG expression in C and CV groups were significantly lower than control and V groups at 12, 24 and 48 h for mRNA expression and at 24 and 48 h for protein expression ($p < 0.05$; Fig. 2C and D). However, OPG mRNA and protein levels were not different between C and CV groups at any time-points. Furthermore, OPG expression in V group were significantly higher than control group at 12, 24 and 48 h for mRNA expression and at 24 and 48 h for protein expression.

4. Discussion

Our recent studies[9,10] have found low-magnitude, high-frequency mechanical vibration was synergistic with compressive force in increasing the expression of pro-inflammatory cytokines and RANKL/OPG ratio in PDL cells and suggest its role in promoting orthodontic tooth movement. However, the mechanisms by which vibration accelerates tooth movement are not known in human osteoblasts. We examined IL-1β, IL-6, RANKL and OPG expression in alveolar bone osteoblasts exposed to compressive force combined with mechanical vibration in this study to explore the effects of vibration during orthodontic tooth movement on the pressure side. The results demonstrated that the application of compressive force combined with mechanical vibration to alveolar bone osteoblasts had the same effect on the measured parameters as the application of compressive force alone, while the application of mechanical vibration alone tended to enhance bone formation.

From previous studies,[3,18] vibration application to human osteoblasts had favorable effects on bone formation. Nevertheless, this study showed mechanical vibration alone had no effects on IL-1β, IL-6 or RANKL expression, but increased OPG mRNA and protein expression in human osteoblasts. Similarly, Hou et al. (2011)[18] reported mechanical vibration alone increased OPG mRNA and protein expression in a mouse osteoblastic cell line (MC3T3-E1 cells).

Several studies[12,13] reported application of compressive force alone to human osteoblasts promoted bone resorption. In this study model, compressive force of 2 g/cm^2 increased both IL-1β and IL-6 mRNA and

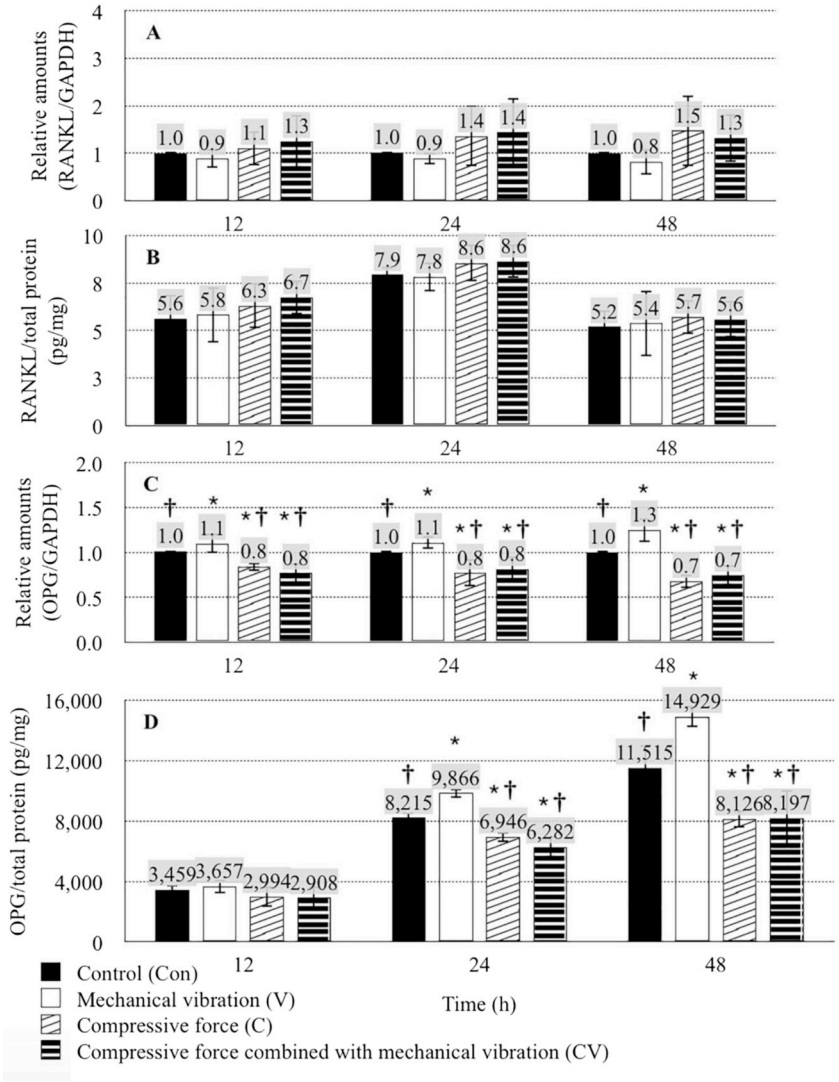

Fig. 2. Effect of exposure to compressive force, mechanical vibration, or compressive force combined with mechanical vibration for 12, 24 and 48 h on RANKL (A: mRNA expression, B: protein expression) and OPG (C: mRNA expression, D: protein expression) in human osteoblasts. (* $P < 0.05$ compared with control group, † $P < 0.05$ compared with vibration group, Mann-Whitney *U* test).

protein expression and downregulated OPG mRNA and protein in human osteoblasts. Upregulation of IL-1β and IL-6 protein expression were delayed compared to induction of the mRNAs, reflecting the lag between transcription and translation. Similarly, Koyama et al. (2008)[12] reported 2 g/cm^2 compressive force increased IL-1β and IL-6 mRNA and protein expression at 24 h in a human osteosarcoma cell line (Saos-2 cells). Also, Tripuwabhrut et al. (2012)[13] reported compressive force increased *IL*-6 mRNA in human osteoblasts at 24 h. The varied in early induction of *IL*-6 mRNA and delayed induction of IL-6 protein expression by compressive force observed between studies[12,13] could be related to the use of different cell types,[24] the methods of applying compressive force and the experimental time-points.

We observed compressive force increased IL-1β and IL-6 mRNA and protein expression, whereas it did not significantly alter RANKL mRNA and protein expression and downregulated OPG mRNA and protein expression in human osteoblasts. The delayed induction of IL-1β and IL-6 protein expression in osteoblasts exposed to compressive force may be not sufficient to increase RANKL expression at the same time-point. Our results are consistent with a previous study in which compressive force of 2 g/cm^2 did not affect RANKL mRNA expression in osteoblasts, while higher compressive force of 4 g/cm^2 was able to increase RANKL mRNA expression.[25] However, a previous clinical study demonstrated that high levels of IL-1β in gingival crevicular fluid were detected during human orthodontic tooth movement.[5] These might indicate that PDL cells may respond to compressive force and upregulate IL-1β protein, and in turn, activate osteoblasts.

In this study, the application of compressive force combined with mechanical vibration to human osteoblasts had no effect on IL-1β, IL-6, RANKL or OPG mRNA and protein expression further than the application of compressive force alone. However, Nishimura et al. (2008)[5] found vibration combined with orthodontic force increased RANKL immunostaining in periodontal ligament compared with application of orthodontic force alone in a rodent model. These might suggest that compressive force combined with mechanical vibration may not affect osteoblasts but affects other cells in the periodontium such as periodontal ligament fibroblasts.[18]

This is the first study to quantify mRNA and protein expression levels in human osteoblasts after application of three types of force with data collections at 12, 24 and 48 h. Our findings expand the current knowledge of the effects of combined mechanical forces on the osteoblasts, also the effects were compared between combined and single individual forces. However, we suggest a study with extended time-points to confirm the effects of force on osteoblasts. Moreover, the expression of the membrane-bound form of RANKL by immunofluorescent

staining should be considered in further studies.

In conclusion, the application of mechanical vibration to human alveolar bone osteoblasts enhanced OPG expression but had no effects on IL-1β, IL-6 or RANKL expression. While the application of compressive force combined with mechanical vibration enhanced IL-1β and IL-6 expression, inhibited OPG expression but had no effect on RANKL expression in human alveolar bone osteoblasts, which were not different from the application of compressive force alone.

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Conflicts of interest

The authors report no conflicts of interest.

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