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Effect of compressive force combined with vibration on CCL2 and CCL5 in human periodontal ligament cells

Supunsa Pongtiwattanakul [a], Chidchanok Leethanakul [a,b], Onnicha Rattanaporn [c], Peungchaleoy Thammanichanon [d], Sissada Tannukit [e,*]

[a] Orthodontic Section, Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand
[b] Center of Excellence for Oral Health, Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand
[c] Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand
[d] Institute of Dentistry, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand
[e] Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand

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ABSTRACT

Purpose: To investigate the effect of compressive force combined with vibration on expression of CC-chemokine ligand 2 (CCL2) and 5 (CCL5) in human periodontal ligament (hPDL) cells.
Methods: Human PDL cells were cultured and assigned into four groups: control (Con), compressive force 2.0 g/cm^2 for 24 h and 48 h (C), vibration 0.3 g 30 Hz for 20 min every 24 h (V), and compressive force combined with vibration (VC). At 24 h and 48 h, mRNA and protein levels of CCL2 and CCL5 were examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively.
Results: At 24 h and 48 h, CCL2 mRNA and protein levels in C and VC were significantly higher than Con. At 24 h, VC showed significantly higher CCL2 mRNA expression than C. However, there was no significant difference between CCL2 protein in C and VC at both time points. At 24 h and 48 h, CCL5 mRNA expression was significantly down-regulated in V and VC, whereas CCL5 protein was undetectable in all groups.
Conclusions: Application of compressive force combined with vibration resulted in the upregulation of CCL2 mRNA and protein levels, whereas CCL5 mRNA expression was down-regulated.

1. Introduction

Several cell types in the periodontal ligament tissue play important roles in response to various stimuli including mechanical loading and orthodontic forces.[1,2] Applied forces generate mechanical strain, which activates several cellular responses such as vascular changes and localized inflammation. Changes in oxygen and fluid flow in the periodontal ligament and alveolar bone induce localized cells to express various local mediators such as cytokines and chemokines. These in turn cause an acute inflammatory process during the early phase of tooth movement that occurs 1–2 days after the mechanical force is applied.[3]

The duration of conventional orthodontic treatment spans 2–2.5 years. Long-term treatment may pose potential harms such as root resorption, higher susceptibility to caries, and gingival recession.[4] Therefore, orthodontists use accelerated tooth movement to reduce treatment time and negative effects. Three commonly used methods of acceleration are biological, surgical, and physical approaches. Physical approaches use devices such as direct electric current, laser, and vibration.[4] The vibration is a painless method that has few side effects. The vibration device can be readily applied without harming the periodontal tissue, resulting in high patient satisfaction.[5] Previous studies reported an increase in various inflammatory mediators, including interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and prostaglandin E_2 (PGE_2) when compressive force combined with vibration was applied to human periodontal ligament (hPDL) cells.[2,6,7]

In the early phase of tooth movement, chemokines play important roles in the function of PDL cells in bone remodeling as well. The functions of chemokines include chemotaxis, activation of inflammatory and bone cells, induction of osteoclast recruitment, and participation in osteoclastogenesis.[1,8] Various chemokines play crucial roles in bone remodeling, such as CC-chemokine ligand 2 (CCL2) and CC-chemokine

* Corresponding author. Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University, Thailand.
E-mail address: sissada.t@psu.ac.th (S. Tannukit).

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ligand 5 (CCL5). CCL2 and CCL5 are key molecules frequently involved in the process of tooth movement. These chemokines play key roles in osteoclastogenesis, leading to bone resorption. Increased expression of CCL2 and CCL5 was shown to promote bone resorption in a particular area.[1,3] A significant increase in CCL2 expression was found in the periodontal tissue subject to orthodontic forces as well as other inflammatory tissues.[9,10] Previous studies have revealed that CCL2 can promote the chemotaxis of osteoclast precursors to the inflammatory site. It also stimulates cell fusion to form multinucleated osteoclasts.[11] CCL5 was shown to induce osteoclast and osteoblast migration to a localized area. The application of compressive force exhibited the most pronounced mRNA expression of CCL5 at 24 h post-treatment, whereas tensile force showed a notable up-regulation at 48 h.[3,12] There has not been any study on the effect of compressive force combined with vibration on these chemokines to date. The objective of this study was to examine the effect of compressive force combined with vibration on mRNA expression and protein levels of CCL2 and CCL5 in hPDL cells.

2. Methods

2.1. Cell culture

The experimental protocol was approved by the Human Research Ethics Committee (HREC), Faculty of Dentistry, Prince of Songkla University (EC6407-047). All subjects signed informed consent before the study participation. The hPDL tissue was collected from the middle 1/3 area of premolar roots from five participants aged 17–25 years old. The inclusion criteria were sound tooth and extraction with orthodontic reason. The exclusion criteria were participants with systemic disease, history of dental trauma or periodontal diseases, and previous orthodontic treatment. The extracted premolars were rinsed three times with phosphate-buffered saline solution (PBS). The hPDL cells were collected with surgical blade no.15, placed in 35-mm culture dishes with Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS), 1 % Penicillin-Streptomycin, and 1 % Fungizone and incubated in an incubator with 95 % air and 5 % CO_2 at 37 °C. Cells from passages number 3–5 were used in this study.

2.2. Alizarin Red staining

The osteogenic differentiation was assessed by culturing with osteogenic medium (DMEM supplemented with 10 nM dexamethasone, 200 μM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate (Sigma-Aldrich, St Louis, Missouri, USA) for 21 days. The medium was changed every three days. Cells cultured in DMEM were used as a control. Cells were stained with 2 % Alizarin Red (Sigma-Aldrich, St Louis, Missouri, USA) and visualized by phase contrast microscopy.

2.3. Experimental design

The protocol was modified from previous studies.[2,7] The hPDL cells were seeded into a 6-well plate at the density of 3×10^5 cells/well. The cells were then synchronized with DMEM containing 2 % FBS for 24 h before force application. Subsequently, the medium was replaced with DMEM supplemented with 10 % FBS and the cells were allocated into four groups: Control (Con), the cells received no treatment, Compressive force (C); the cells received only the compressive force using a plastic container (size 30 mm) with coins placed on its top to obtain a pressure of 2.0 g/cm^2 for 24 h and 48 h (Fig. 1A), Vibration (V); the cells were placed on a vibrator (GJX-5 vibration calibrator, Beijing, China) and received vibration at 0.3 g 30 Hz for 20 min every 24 h, for a total of 48-h period (Fig. 1B), and Compressive force combined with vibration (VC); the cells were subject to both compressive force and vibration at the same conditions with the C and V groups. Each group was performed in triplicate.

2.4. CCL2 and CCL5 mRNA expression

The total RNA was extracted using the PureLink™ RNA mini kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The RNA concentration was measured using Nano-300 Micro-spectrophotometer (Allsheng, Hangzhou, China). Total RNA of 500 ng was reverse-transcribed to cDNA using Superscript® VILO™ (Invitrogen, Carlsbad, USA) with a Rotor-Gene Q (Qiagen, Qiagen Strasse 1, Hilden, Germany). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on QuantStudio™ 5 (Thermo Fisher Scientific, Massachusetts, USA) using the SensiFAST™ SYBR® No-ROX Kit (Bioline Inc, Taunton, Massachusetts, USA) and the primers listed in Table 1. The PCR was carried out as follows: activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 52.9 °C (CCL2) or 56.9 °C (CCL5) for 1 min, and extension at 72 °C for 30s, and GAPDH as a control. The level of mRNA expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method and presented as fold changes relative to control.

2.5. Measurement of CCL2 and CCL5 proteins

Enzyme-linked immunosorbent assay (ELISA) was used to quantify CCL2 and CCL5 in the conditioned medium after interventions at 24 h and 48 h. Quantifications were performed using CCL2 and CCL5

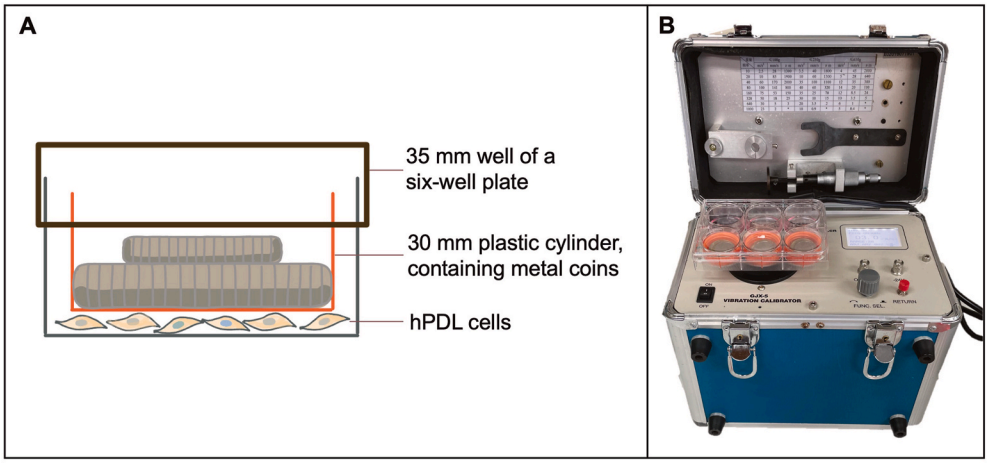

Fig. 1. Intervention: (A) Compressive force model using a plastic container with a coin placed on its top. (B) The GJX-5 vibration calibrator produces vibration that is perpendicular to the bottom of the culture plate.

Table 1
Primer sequences used for quantitative reverse transcription polymerase chain reaction.

mRNA	Forward (5′–3′)	Reverse (5′–3′)	T_M (°C)	Product size (bp)	Accession no.
CCL2	AGGAAGATCTCAGTGCAGAG	AGTCTTCGGAGTTTGCCTTTG	58.5	177	NM_002982.4
CCL5	CTCGCTGTCATCCTCATTGCTA	GCACTTGCCACTGGTGTAGAAA	60.7	150	NM_002985.3
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT	63.7	142	NM_002046.7

DuoSet® ELISA Kit (R&D system, Minneapolis, Minnesota, USA) following the manufacturer's instruction on Anthos Zenyth 200 Microplate Reader (Biochrom®, Massachusetts, USA) at 450 nm. The CCL2 and CCL5 concentrations were calculated from the standard curve.

2.6. Statistical analysis

The data was expressed as the mean ± SD and assessed normality using the Shapiro-Wilk test. The comparisons between groups were performed by Friedman's two-way ANOVA, and within group were performed by the Friedman's Rank test, followed by using the Bonferroni test for multiple comparisons. The significant difference was defined as $P < 0.05$.

3. Results

3.1. Cell morphology

The hPDL cells demonstrated spindle-shaped morphology (Fig. 2A) and grew at a higher rate compared to human gingival fibroblasts. After cultured with an osteogenic medium, the hPDL cells showed Alizarin red staining nodules (Fig. 2B).

3.2. Effect of mechanical forces on mRNA expression

CCL2 mRNA expression in C and VC was significantly higher than that in Con at both 24 h ($P = 0.041$, $P < 0.001$, respectively) and 48 h ($P = 0.019$, $P = 0.003$, respectively). At 24 h, VC showed significantly higher CCL2 mRNA expression than C ($P = 0.010$). However, there was no significant difference in CCL2 expression between 24 h and 48 h in all groups (Fig. 3A).

At 24 h, C showed the highest CCL5 mRNA expression, which was not statistically significantly different from Con (Fig. 3B). In addition, there was a significant difference between 24 h and 48 h in C ($P = 0.004$). When hPDL cells received only vibration (V) and combined forces (VC), there was a significant decrease in CCL5 mRNA expression compared to Con at both 24 h ($P < 0.001$ and $P = 0.006$, respectively) and 48 h ($P = 0.002$ and $P < 0.001$, respectively) (Fig. 3B).

3.3. Effect of mechanical forces on secreted protein

CCL2 protein levels in C and VC were significantly higher than those in Con at both 24 h ($P < 0.001$ and $P = 0.001$, respectively) and 48 h ($P < 0.001$ in both groups). When comparing between C and VC, there was no statistically significant difference at all time points. However, CCL2 protein in V significantly decreased compared to C and VC in both time points ($P \leq 0.001$) (Fig. 4). CCL5 protein was undetectable in all groups at both time points.

4. Discussion

This study aimed to investigate the expression of CCL2 and CCL5 in hPDL cells subject to compressive force combined with vibration. Previous study demonstrated that hPDL cells were able to withstand a pressure of 3.0 g/cm^2 for 48 h without causing cellular damage.[13] Therefore, we opted for a compressive force of 2.0 g/cm^2 to avoid detrimental effects to cells.[2] The hPDL cells play pivotal roles as the primary responders to mechanical stimulation and hPDL tissues contain various cell types including fibroblasts, osteoblasts, and stem cells, that have different roles and capabilities.[14] Therefore, hPDL cells are essential for tissue homeostasis and cellular response. In this study, the PDL cells exhibited a spindle-shaped morphology, resembling gingival fibroblasts. Nonetheless, PDL cells differ from gingival fibroblasts in several aspects, including differentiation potential. Unlike gingival fibroblasts, PDL cells were able to form mineral nodules when cultured with an osteogenic medium. This feature has promising application for regenerative medicine.[7,15]

The hPDL cells in C, which mimicked the conditions of orthodontic force on the compression side, showed markedly increased expression of CCL2 mRNA compared to Con. This is consistent with previous studies that reported upregulation of CCL2 mRNA expression during the early phase of mechanical loading.[10] CCL2 was up-regulated in the early phase of tooth movement and gradually decreased over one week. In addition to response to mechanical forces, substances-induced inflammation and pathogens can modulate CCL2 expression as well.[9,16,17] This study showed the highest level of CCL2 mRNA expression in VC that is in line with previous studies that reported the combined effect of vibration and compressive forces on up-regulation and secretion of many inflammatory cytokines.[5–7] However, the CCL5 mRNA expression was down-regulated in VC and appeared unchanged in C. This finding differs from the study by Lee et al., where compressed hPDL cells showed significantly increased CCL5 mRNA expression at 24 h.[12] The discrepancy was probably due to a higher degree of compressive force and different culture medium used in the previous study. Another possible

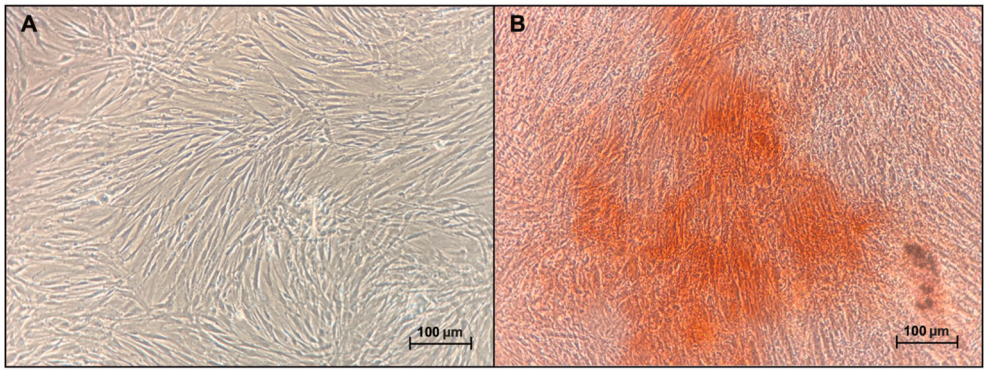

Fig. 2. Human periodontal ligament (hPDL) cell morphology: (A) hPDL cells in control and (B) Alizarin Red staining.

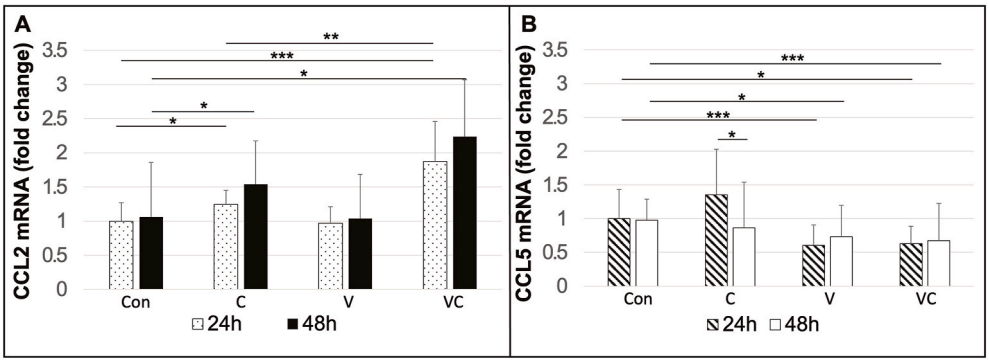

Fig. 3. mRNA expression in experimental and control groups at 24 h and 48 h from 5 participants. The data was presented as fold changes relative to a control (Con): (A) CCL2 mRNA expression, (B) CCL5 mRNA expression. The Friedman two-way ANOVA was performed with Bonferroni correction. (*$P < 0.05$, **$P = 0.01$, ***$P < 0.001$).

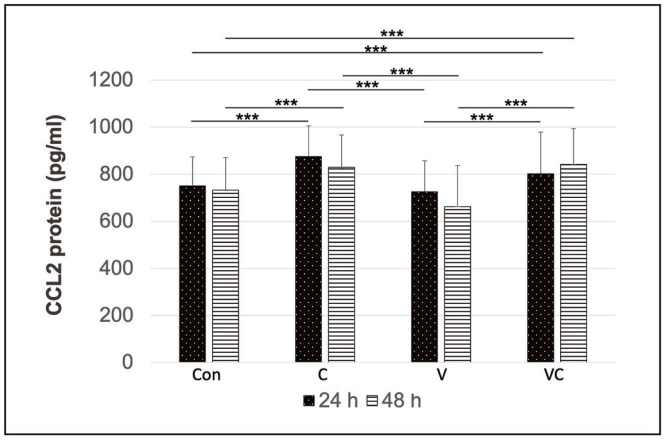

Fig. 4. CCL2 protein in experimental and control groups at 24 h and 48 h from 5 participants. The data was presented as mean ± SD. The Friedman two-way ANOVA was performed with Bonferroni correction. (*$P < 0.05$, **$P < 0.01$, ***$P \leq 0.001$).

explanation is the inter-subject variability that causes different cellular response.[18] In the present study, the expression of CCL5 mRNA in all groups was similar to the basal level. This finding suggested that CCL5 may not be the primary chemokine that responds to mechanical forces in orthodontic tooth movement. This was similar to the previous study in which hPDL cells were subject to tensile force for 24 h and 48 h. The CCL5 mRNA expression and secreted protein were not significantly different from Con, and the amount of CCL5 protein was less than CCL2 protein as well.[17]

Application of vibration to hPDL cells did not modulate the expression of CCL2 mRNA and down-regulated expression of CCL5 mRNA. This finding may arise from a potential impact of vibration on cell proliferation.[19] In addition, the study that used different vibration frequencies to 3T3-L1 preadipocytes found that vibrations at 20 Hz and 30 Hz resulted in significant decrease of total DNA content. This decreased DNA content may affect the level of mRNA expression.[20] Previous studies reported that vibration at 30 Hz had no effect on the expression of IL-1β and TNF-α, suggesting that using vibration alone may not be sufficient to induce or directly affect the expression of these cytokines. A synergistic up-regulation of mRNA expression of IL-1β and TNF-α was evident in application of compression and vibration at 30 Hz.[2] It was postulated that vibration combined with compressive force may induce distortion in hPDL cells, thereby enhancing the effect of the membrane-cytoskeleton phenomenon that modulates cytokine expression.[21] In addition, it is probable that vibration could amplify the effect by reactivation of compressive force. This reactivation resembles to an intermittent compressive force, which contributes to stabilization of cytokine expression.[2,22] Furthermore, it was reported that low-frequency vibrations increased the displacement of forces and caused cellular distortion, thereby stimulating the intracellular mechanotransduction pathway that results in upregulation of inflammatory cytokines/chemokines.[23]

In this study, CCL2 mRNA expression was significantly up-regulated in the C and VC groups at 24 h and 48 h. Conversely, the secreted protein in both groups remained unaltered. One of the possible explanations is the exclusive detection of protein with extracellular secretion. The process of secreted protein is a highly regulated and complex events that involve multiple intracellular mechanisms.[24] After mRNA is synthesized, it undergoes post-transcriptional modifications and is transported to the cytoplasm. The cellular protein level depends on multiple parameters including mRNA stability, translational efficiency, and protein degradation. Furthermore, secretory protein is governed by a multitude of mechanisms such as protein modifications, vesicular transport, and exocytosis. Some proteins are exclusively secreted in response to signals or stimuli.[24] These reasons may be attributed to the disparity between the CCL2 mRNA and secreted protein levels.

Application of compressive force of 2.0 g/cm^2 and mechanical vibration at 30 Hz on hPDL cells exhibited increased expression of CCL2 mRNA and protein levels at 24 h and 48 h post-treatment. In contrast, CCL5 mRNA expression was down-regulated at both time points. To better understand the molecular mechanism of alveolar bone remodeling driven by orthodontic force, further studies on various chemokines involved in this event and their communication network are necessary.

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Consent

The authors have stated that “All subjects signed informed consent before the study participation” in the methods section of the manuscript.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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