Original Article

Effects of compressive stress combined with mechanical vibration on osteoclastogenesis in RAW 264.7 cells

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ABSTRACT

Objectives: To investigate the effects of compressive force and/or mechanical vibration on *NFATc1*, *DCSTAMP*, and *CTSK* (cathepsin K) gene expression and the number of tartrate-resistant acid phosphatase (TRAP)–positive multinucleated cells in RAW 264.7 cells, a murine osteoclastic-like cell line.

Materials and Methods: RAW 264.7 cells were subjected to mechanical vibration, compressive force, or compressive force combined with vibration. Cell viability and the numbers of TRAP-positive multinucleated cells were evaluated. *NFATc1*, *DCSTAMP*, and *CTSK* gene expressions were analyzed using real-time quantitative reverse transcription polymerase chain reaction.

Results: Compressive force combined with mechanical vibration significantly increased the numbers of TRAP-positive multinucleated cells but did not significantly affect cell viability. In addition, compressive force combined with mechanical vibration significantly increased *NFATc1*, *DCSTAMP*, and *CTSK* mRNA expression compared with compressive force or vibration alone.

Conclusions: Compressive force combined with mechanical vibration induces osteoclastogenesis and upregulates *NFATc1*, *DCSTAMP*, and *CTSK* gene expression in RAW 264.7 cells. These results provide more insight into the mechanisms by which vibratory force accelerates orthodontic tooth movement. (*Angle Orthod.* 2022;92:555–561.)

KEY WORDS: *Cathepsin K*; Compressive force; *DC-STAMP*; *NFATc1*; Osteoclastogenesis; Vibration

INTRODUCTION

Bone remodeling throughout orthodontic treatment is associated with various cellular activities involved in tooth movement. The study of these mechanisms may help to improve the quality of orthodontic treatment. Among the noninvasive methods tested, vibration combined with orthodontic force was demonstrated to accelerate tooth movement in both animal and human models.[1–3] Although some studies found no evidence that supplemental vibratory stimuli could significantly increase the rate of tooth movement,[4–6] many in vitro studies reported that mechanical stimuli in combination with low-magnitude, high-frequency vibration enhanced bone remodeling.[7–9] Application of compressive force combined with mechanical vibration to human periodontal ligament (PDL) cells upregulated prostaglandin E2 (PGE2), interleukin-6 (IL-6), IL-8, and receptor activator of nuclear factor-kappa B ligand (RANKL) and downregulated Runt-related transcription factor 2 (Runx2) and *OPG*.[7,9] In addition, compressive force combined with mechanical vibration upregulated *IL-1β* and *IL-6* and inhibited osteoprotegerin (*OPG*) expression in human alveolar bone osteoblasts.[8]

Osteoclasts are multinucleated cells derived from monocytes in the myeloid cell lineage. The formation of

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mature osteoclasts involves multiple processes, including differentiation of precursor cells into mononuclear cells and multinucleation by cell-to-cell fusion of mononuclear osteoclasts.[10] Osteoclast differentiation and function are regulated by a variety of mediators and cytokines secreted by many cells. Osteoblasts play a crucial role in osteoclastogenesis by expressing RANKL. RANKL induces osteoclastogenesis by binding to receptor activator of nuclear factor-kappa B (*RANK*), which consequently activates several molecules, including transcription factors.[11] *NFATc1*, a member of the nuclear factor activated T cells (*NFAT*) family of transcription factor genes, is regulated by RANKL via the *TRAF6* and *c-Fos* pathways.[12] *NFATc1* is essential for induction of several genes required for preosteoclast differentiation. Dendritic cell–specific transmembrane protein (*DC-STAMP*, encoded by *DCSTAMP*) is an osteoclastic gene upregulated by *NFATc1* and *c-Fos. DCSTAMP* is involved in preosteoclast differentiation but is also a key regulator of mononuclear osteoclast fusion. Osteoclasts isolated from *DCSTAMP* knockout mice were the only mononuclear tartrate-resistant acid phosphatase (TRAP)–positive cells in which no fusion occurred.[13] Even though *DCSTAMP* plays a role in the upregulation of osteoblastic activity, *DCSTAMP* expression cannot be detected in osteoblasts.[14]

Cathepsin K, encoded by *CTSK,* is a potent lysosomal cysteine protease primarily secreted by mature osteoclasts that degrades collagen and matrix proteins during bone resorption. Several transcription factors stimulate *CTSK* gene expression; *NFATc1* strongly and independently stimulates *CTSK* activity. In addition, *CTSK* is the only protease secreted by osteoclasts that can degrade both the triple helix and telopeptides of type I collagen fibers. In addition to osteoclasts, *CTSK* is also expressed in various bone and nonbone cells. Mechanical stimulation can induce osteoblasts and osteocytes to express *CTSK*, which may contribute to bone homeostasis.[15]

The effects of mechanical stimulation on osteoclastic differentiation differ depending on the pattern of mechanical loading. In vitro studies of osteoclast precursor cells have shown that continuous compressive force positively affects osteclastogenesis.[16,17] In contrast, the application of tensile force to preosteoclasts stimulated with RANKL decreased the number of osteoclasts.[18] In terms of vibratory stimuli, treatment of preosteoclast cells with 0.3 g at 45 Hz for 15 min/d significantly decreased the number of RANKL-induced TRAP-positive multinucleated cells (MNCs) and inhibited osteoclast formation.[19] Another study showed that mechanical vibration (4 Hz, 1 hour) reduced *DCSTAMP* expression in osteoclast precursor cells and inhibited osteoclast formation.[20] However, relatively little is known about the effects of combined compressive and mechanical vibration on osteoclastogenesis. An animal study of orthodontic tooth movement reported that resonance vibratory stimulation enhanced RANKL expression and increased the number of osteoclasts in the PDL.[2] In addition, the use of a supplementary vibration device in an attempt to accelerate tooth movement in rats significantly increased *NF-κB* activation in osteoclasts, as well as the numbers of osteoclast precursors and osteoclasts on the bone surface.[3] Thus, this study aimed to investigate the underlying mechanisms of action of compression combined with low-magnitude, high-frequency vibration on osteoclastogenesis in vitro.

MATERIALS AND METHODS

Cell Culture Under Mechanical Stimuli

RAW 264.7 cells (TIB-71TM; American Type Culture Collection, Manassas, Va) were cultured in α-Minimal Essential Medium (Gibco BRL, Rockville, Md) containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% fungizone at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded overnight in 96-well plates at 2.0×10^3 cells/well. After obtaining a 70–80% confluent monolayer, cells were subjected to 0.6 g/cm^2 compressive force continuously (CF), mechanical vibration at 0.49 g at 60 Hz for 20 minutes per day (V), compressive force combined with mechanical vibration (CFV), or no force as a control (C). A plate was mounted onto the platform of a GJX-5 vibration calibrator and attached with modified acrylic cylinders for hydrostatic pressure–generated compressive force loading with no direct contact to the cells and allowing fluid leakage. After 4-day stimuli, a cell viability test was performed by using PrestoBlue Cell Viability Reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. PrestoBlue solution was mixed with fresh media at a ratio of 1:10, added to the cells, and incubated for 1 hour at 37°C. The absorbance was determined at 600 nm.

Mechanical-Induced Osteoclastogenesis and mRNA Expression

RAW 264.7 cells were treated with 50 ng/mL mouse recombinant RANKL and underwent stimuli with CF, V, CFV, or C for 4 days. Cells were fixed and stained using a TRAP staining kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The numbers of TRAP-positive MNCs (three or more nuclei per cell) were counted using a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 10× objective by two individuals who were blinded to the treatment of the cells. Total RNA

Table 1. Primers Used for Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Gene	Forward (5′-3′)	Reverse (5′-3′)	Accession No.
NFATc1	TTGGATTCTGACGAGCTGTG	GTGCAGCTGGATCAAGAACA	NM_001244933.1
CTSK	CAGCAGAACGGAGGCATTGA	CCTTTGCCGTGGCGTTATAC	NM_007802.4
DCSTAMP	CTAGCTGGCTGGACTTCATCC	TCATGCTGTCTAGGAGACCTC	NM_029422.4
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT	XM_003819132.3

was extracted from RANKL-induced RAW 264.7 cells in each group. Cells were lysed and RNA was isolated using innuPREP DNA/RNA Mini Kits (Analytic-Jena, Jena, Germany) according to the manufacturer's protocol. Then, cDNA was synthesized from 0.5 μg of total RNA by reverse transcription (Superscript III First Strand Synthesis System; Invitrogen) and amplified by real-time quantitative reverse transcription polymerase chain reaction using the primers shown in Table 1. The relative mRNA levels of *NFATc1*, *DCSTAMP*, and *CTSK* were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method.

Statistical Analysis

Each experiment and data analysis was repeated independently at least three times. Values are presented as mean ± SD deviation. Differences between means were analyzed using one-way analysis of variance followed by Tukey multiple comparisons test and the Games-Howell test. Significance was defined as $P < .05$.

RESULTS

Combined Mechanical Stimuli Do Not Affect the 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 control cells ($P > .05$; Figure 1).

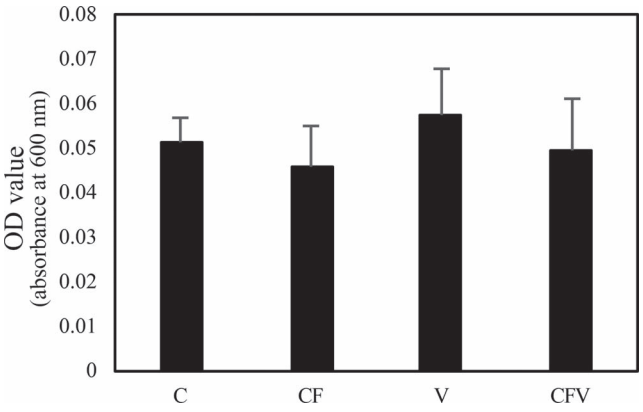

Figure 1. Viability of RAW 264.7 cells in the control group and cells exposed to various forces. No forces significantly affected the viability of RAW 264.7 cells. Values are mean ± SD, each assessed in triplicate. $P > .05$, n = 3.

Combined Mechanical Stimuli Increase the Numbers of TRAP-Positive Cells in RAW 264.7 Cells

TRAP-positive MNCs were observed in all groups. No significant difference was noted between groups C and V ($P > .05$). The number of TRAP-positive MNCs was highest in the CFV group and was significantly higher than the other groups ($P < .05$; Figure 2). The number of TRAP-positive MNCs was ranked and grouped from the highest to the lowest as follows: CFV > CF > V or C.

Combined Mechanical Stimuli Increase *NFATc1*, *DCSTAMP*, and *CTSK* mRNA Expression in Osteoclasts

NFATc1 expression was not significantly different between the CF and C groups. However, the CFV group highly upregulated *NFATc1* in RAW 264.7 cells, whereas group V resulted in only a slight upregulation of *NFATc1* mRNA ($P < .05$; Figure 3A).

The expression of *DCSTAMP* and *CTSK* mRNA was highest in the CFV group and was significantly higher than the other groups ($P < .05$; Figure 3B,C, respectively). No significant differences were observed between groups C and V ($P > .05$). The expression level was ranked and grouped from the highest to the lowest as follows: CFV > CF > V or C.

DISCUSSION

Matsuike et al.[21] observed TRAP-positive MNCs and an increase in the level of *DCSTAMP* mRNA expression of RAW 264.7 cells treated with 50 ng/mL of RANKL under 0.3, 0.6, and 1.1 g/cm^2 loading for 4 days. The same concentration of RANKL was used in this study. According to a pilot study, 0.6 g/cm^2 compressive force was selected. Vibratory stimulation with 0.49 g at 60 Hz was used as in previous studies on osteoblasts.[8,22] The vibration period of 20 min/d was used in clinical studies for tooth movement acceleration.[23,24] Cell viability assays in this study demonstrated that mechanical stimuli did not affect the viability of RAW 264.7 cells.

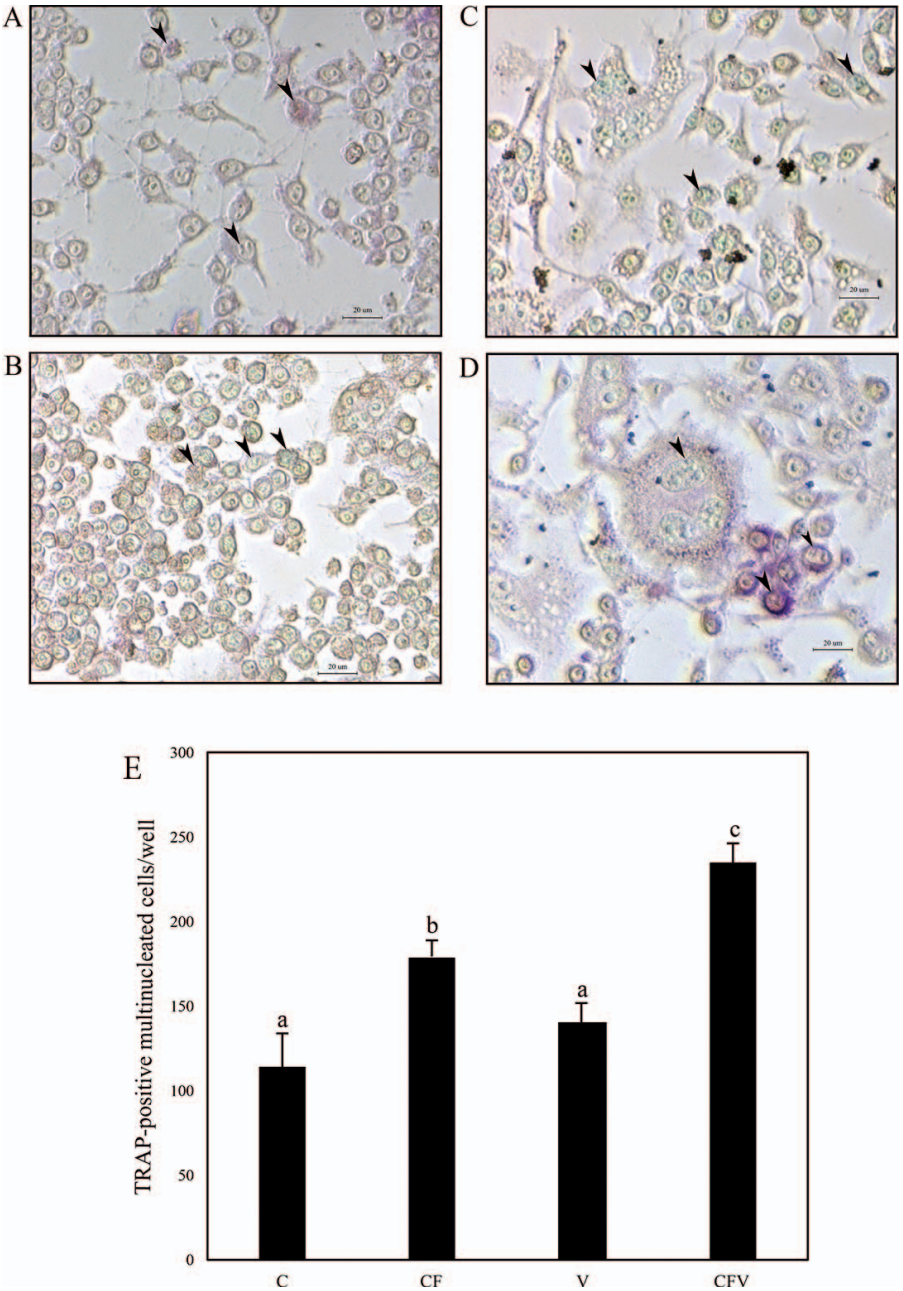

Figure 2. Combined compressive and vibratory force induces osteoclast differentiation in RAW 264.7 cells. The numbers of tartrate-resistant acid phosphatase (TRAP)–positive multinucleated cells (MNCs; ≥three nuclei) with purplish-red color staining were counted (magnification = 40×, bar = 20 um). The black arrows indicate TRAP-positive MNCs. (A) Control group (C); (B) compressed group (CF); (C) vibrated group (V); (D) combined compression and vibration group (CFV); (E) a greater number of TRAP-positive MNCs (≥three nuclei) were observed in the combined group. Data are representative of three independent experiments. All values are shown as mean ± standard deviation (SD). Significant differences between groups are indicated by different letters (a, b, and c; $P < .05$, n = 3).

Sakamoto et al.[25] previously reported that the application of 0.5-g, 48.3-Hz vibration for 1 minute enhanced preosteoclast proliferation at 48 hours but did not affect differentiation into osteoclasts. 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.[26] Wu et al.[19] suggested that low-magnitude, high-frequency vibration inhibited RANKL-induced osteoclast differentiation.

Immunohistochemical analysis of a rat model showed that whole-body vibration decreased RANKL expression, which implies that vibratory stimulation inhibits RANKL activity.[27] In this study, RAW 264.7

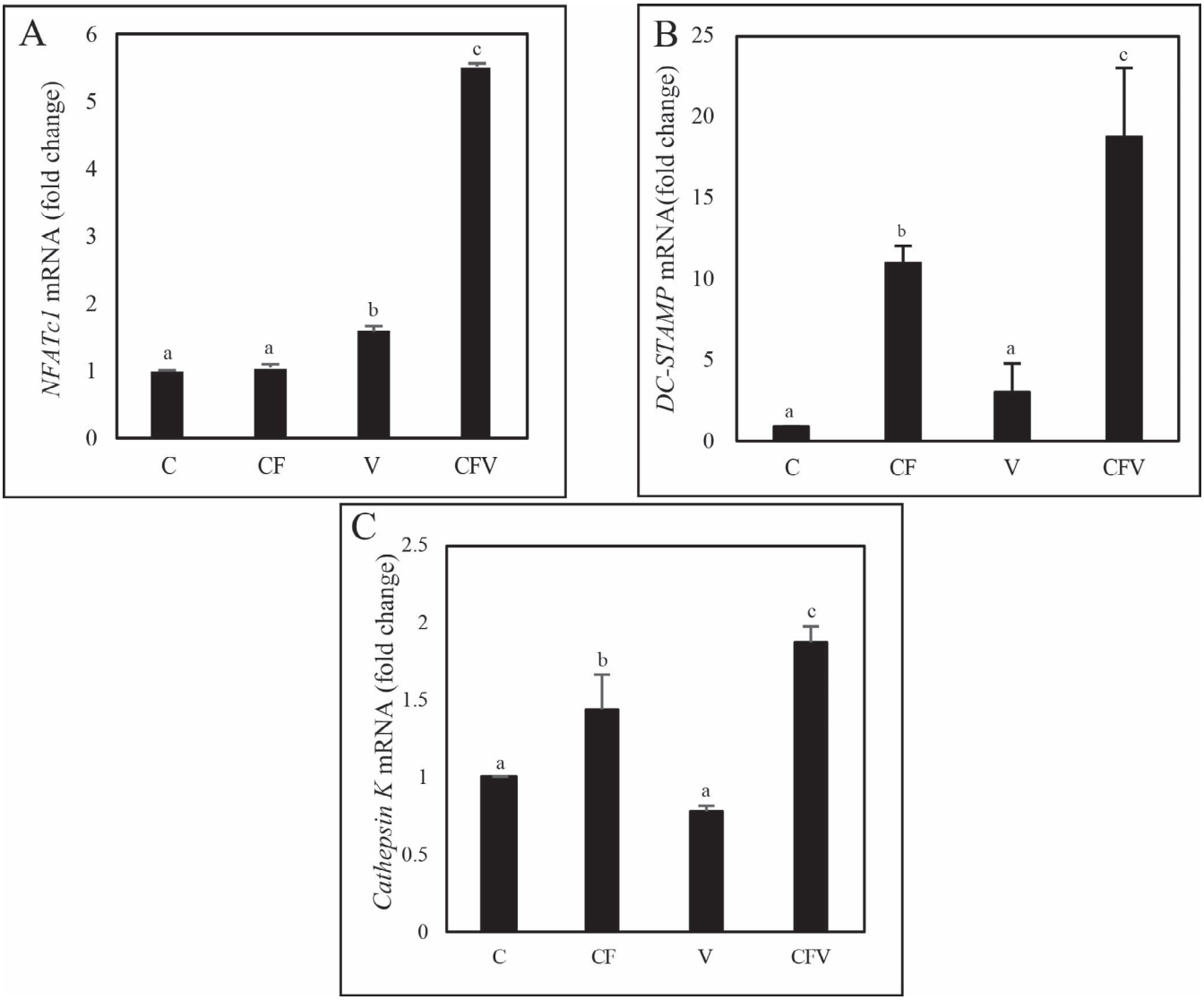

Figure 3. Effect of mechanical vibration combined with compressive force on *NFATC1*, *DCSTAMP*, and *CTSK* mRNA expression in RAW 264.7 cells. All data are mean ± SD of triplicate experiments. Significant differences between groups are indicated by different letters (a, b, and c; $P < .05$, $n = 3$).

cells were treated with RANKL throughout the experiments. Although vibration slightly increased *NFATc1* expression, it did not significantly alter *DCSTAMP* or *CTSK* mRNA expression. Kulkarni et al.[20] reported that vibration downregulated *DCSTAMP* gene and protein expression in osteoclast precursor cells. Wu et al.[19] also showed that 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 regulation of *DCSTAMP* expression, which may explain the decrease in *DCSTAMP* mRNA expression observed in the vibration group. In this study, 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 that micro-pulse vibration inhibited osteoclastic activity, including *CTSK* expression.[28] These findings may help to elucidate the role of vibration in the regulation of various stages of osteoclastic function.

The present study showed that *DCSTAMP* and *CTSK* were expressed at similar levels in all treatments. High levels of both *DCSTAMP* and *CTSK* were observed in response to compressive force, with or without vibration. Numerous in vitro studies clearly indicated that compressive force stimulates the expression of many osteoclast-specific genes involved in osteoclast differentiation and function in RAW 264.7 cells.[1617,21,29] In addition, many studies demonstrated high expression of *NFATc1* in response to compressive

force in RAW 264.7 cells.[12,21,29] Takayanagi et al.[12] observed continuous expression of *NFATc1* mRNA and protein in bone marrow–derived monocyte/macrophage precursor cells until TRAP-positive and multinucleated phenotypes were detected. The induction of *NFATc1* peaked at more than 20-fold higher than baseline levels at 48 hours after RANKL stimulation and was sustained thereafter. However, 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.

A recent study found that the application of compressive force combined with mechanical vibration to human osteoblasts had no additional effect on the expression of proinflammatory cytokines or the RANKL/*OPG* ratio compared with compressive force alone.[8] However, other studies showed that compressive force and mechanical vibration synergistically upregulated the expression of RANKL and inflammatory mediators in PDL cells.[9,30] In the present study, compressive force and vibration had an obvious synergistic effect on *NATc1* mRNA expression. In addition, the combined stimuli tended to increase *DCSTAMP* and *CTSK* expression compared with compression alone. These results suggested that compressive force combined with mechanical vibration may stimulate both PDL fibroblasts and preosteoclasts to participate in osteoclastogenesis.

The present study demonstrated the effect of compressive force and/or vibration on the number of TRAP-positive cells and *NFATc1*, *DCSTAMP*, and *CTSK* mRNA expression in RANKL-induced RAW 264.7 cells. It was found that mechanical vibration synergistically promoted the expression of genes involved in osteoclastogenesis in the presence of compressive force stimulation. However, additional studies with extended time points are recommended to explore the chronological sequence and peak levels of each mRNA and the number of TRAP-positive cells. In addition, the role of PDL cells in osteoclastogenesis under compressive force combined with mechanical vibration should be considered in further studies.

CONCLUSIONS

- Mechanical vibration (0.49 g, 60 Hz) and combined mechanical vibration and compressive force had no effect on the viability of RAW 264.7 cells.
- Mechanical vibration combined with compressive force significantly upregulated *NFATc1*, *DCSTAMP*, and *CTSK* gene expression in osteoclasts.
- Compressive force and mechanical vibration synergistically increased the numbers of TRAP-positive MNCs ($\geq$three nuclei).

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