Anti-osteoclastogenic effect of fermented mealworm extract by inhibiting RANKL-induced NFATc1 action

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Abstract. Augmented osteoclast activity and differentiation can lead to destructive bone diseases, such as arthritis and osteoporosis. Therefore, modulating osteoclastogenesis and differentiation may serve to be a possible strategy for treating such diseases. *Tenebrio molitor* larvae, also known as mealworms, are considered a good source of protein with nutritional value, digestibility, flavor and functional properties, such as antioxidant, anti-diabetic and anti-obesity effects. However, the role of mealworms in osteoclastogenesis remains poorly understood. The present study therefore investigated the effects of fermented mealworm extract (FME) on receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis in bone marrow-derived macrophages (BMMs) whilst also attempting to understand the underlying mechanism, if any. The cells treated with RANKL were used as the negative control. To prepare FME, defatted mealworm powder was fermented with a *Saccharomyces cerevisiae* strain, and then extracted with fermented alcohol. Cell viability of BMMs isolated from 5-week-old Institute of Cancer Research mice was measured using Cell Counting Kit-8 assay. Subsequently, the effects of FME on osteoclast differentiation were measured using tartrate-resistant acid phosphatase (TRAP) staining. In addition, expression of markers associated with osteoclast differentiation was assessed by reverse transcription-quantitative PCR. Expression of nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) was assessed by western blotting. TRAP staining revealed that FME inhibited osteoclast differentiation in a dose-dependent manner (10-100 μg/ml) without causing cytotoxicity. Specifically, the formation of osteoclasts appear to have been suppressed by FME as indicated by the reduction in the number of TRAP-positive multinucleated cells observed. Furthermore, FME treatment significantly decreased the mRNA expression of c-Fos, whilst also significantly decreasing the expression of NFATc1 on both protein and mRNA levels. c-Fos and NFATc1 are transcription factors that can regulate osteoclast differentiation. FME treatment also reduced the expression of genes associated with osteoclast differentiation and function, including *dendritic cell-specific transmembrane protein*, *osteoclast associated Ig-like receptor*, *Cathepsin K* and *TRAP*, compared with that in the control group. Subsequently, FME was found to effectively suppress RANKL-induced osteoclast differentiation compared with that by the non-fermented mealworm extract. These findings suggest that FME may confer anti-osteoclastogenic effects, providing insights into its potential application in treatment of osteoporosis.

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Introduction

Osteoporosis is a metabolic bone disorder that is characterized by a progressive decline in bone mass and reduction in bone strength, leading to fragile bones and an increased in the risk of fracture (1). Physiologically, it typically occurs due to an imbalance between osteoblast and osteoclast activity, with osteoclast activity becoming excessive and the resultant bone resorption outpacing bone formation during remodeling (1). According to results from a previous meta-analysis from 2000 to 2020, the worldwide prevalence of osteoporosis was 18.3% (95% CI, 16.2-20.7) (2). In particular, this disease is especially common among the elderly (aged >65 years), placing a problematic health burden on this population (3). Therefore, inhibition of osteoclastic activity has been proposed to be a potential strategy for the treatment or even prevention of osteoporosis (4). Hormone replacement therapy, bisphosphonates and inhibitor of the receptor activator of nuclear factor κB ligand (RANKL) are the most commonly applied methods for osteoporosis treatment (5). However, due to the side effects (such as muscle pain and dyspepsia) of these aforementioned drugs, efforts are being made to substitute them with naturally occurring compounds such as triphenyl hexene and methoxsalen (4,6,7).

Mealworms (*Tenebrio molitor* larvae) are important edible insects and serve as a potential protein source for animals and humans (8,9). Previous studies have shown that mealworms can exert anti-obesity (10), anti-diabetic (11) and antioxidant (12)

activities. In another previous study, Ham *et al* (13) found that the fermented extract of defatted mealworm (FME) had higher amounts of free amino acids and essential amino acids compared with those in their non-fermented (ME) counterparts. Mealworms are particularly rich in branched-chain amino acids (BCAAs), such as leucine, isoleucine and valine, which were previously found to be exceeded in quantity by FME (14). It has also been reported that FME treatment in *in vivo* models, including rats or mice, resulted in the attenuation of alcoholic and non-alcoholic hepatic steatosis and type 2 diabetes (13-15). In addition, Cui *et al* (16) reported that serum leucine and valine levels in patients were positively associated with total body bone mineral density (BMD) in Mendelian randomization analyses using a summary-level genome-wide association study. Therefore, the present study aimed to investigate the impact of FME on bone health, with specific focus on osteoclast differentiation. In addition, the present study aimed to elucidate its underlying mechanism of action.

Materials and methods

Preparation of ME and FME. ME and FME were provided from the Suncheon Research Center for Bio Health Care. Briefly, fat was first removed from freeze-dried mealworms for 5 days at -125˚C using edible hexane. Edible hexane (1 l) was added to freeze-dried mealworms (200 g) and stirred at 60 rpm for 24 h at 24˚C. This lipid extraction process was repeated three times. This extract was then filtered using polypropylene and the remaining solution was evaporated using a rotary evaporator (customized for a large capacity) at 24-25˚C and powdered. To prepare FME, defatted mealworm powder was fermented with a *Saccharomyces cerevisiae* strain (cat. no. KCTC 17299) provided by the Korean Collection for Type Cultures. The medium was prepared by mixing yeast, defatted mealworm powder and dextrose in a ratio of 1:2:2 (w/w). Specifically, yeast was inoculated ($1x10^6$ CFU/ml) into the medium and it was fermented for 72 h at 30±2˚C with 150 rpm shaking. The solvent used was distilled water, and the total volume per reaction was 1 l. Fermented alcohol (Ethanol Supplies World Co., Ltd.) was then added to the cultured medium at a ratio of 7:3 (fermented ethanol: cultured medium, v/v), and then the mixture was extracted under reflux cooling for 3 h at 85˚C and filtered under pressure (4.08 bar/60 PSI) at 24-25˚C using an ADVANTEC No. 2 filter paper (Toyo Roshi Kaisha, Ltd.). The resulting extract was evaporated with a rotary vacuum concentrator and freeze-dried for 72 h at -125˚C to obtain the extraction powder.

For the non-fermented ME, yeast, defatted mealworm powder and dextrose were mixed in a ratio of 1:2:2 (w/w) without any incubation, and then fermented alcohol was immediately added to the mixture to proceed with the fermentation extraction process (Fig. 1).

The freeze-dried powder was dissolved in DMSO (300 μg/ml) and used for subsequent experiments.

Collection of bone marrow-derived macrophages (BMM) and osteoclast differentiation. BMMs were obtained from the femurs and tibiae of 5-week-old male ICR mice (n=2; weight, 25.85±0.35 g) purchased from Raon Bio (Yongin, Korea), as described in previous studies (17,18). The mice were housed in a controlled environment at 22±2˚C and 50±5% humidity and maintained in a 12-h light/dark cycle. The mice had free access to food and water. The mice were sacrificed by cervical dislocation, before their femurs and tibiae were carefully cleared of adherent soft tissues. The tip of each bone was then removed with forceps and the marrow was extracted by inserting a 1-ml syringe needle into the end of the bone and flushed with α-minimum essential medium (α-MEM) supplemented with 100 U/ml penicillin/streptomycin (all from Thermo Fisher Scientific, Inc.). After flushing, the cells were centrifuged at 400 x g for 5 min at 4˚C. Using Red Blood Cell Lysis Buffer (Merck KGaA), red blood cells were removed using centrifugation (400 x g for 5 min at 4˚C). After this step, the supernatant was removed, and α-MEM containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was added. After filtering through a 40-μm nylon mesh filter (BD Biosciences), the cells were immediately placed in the culture medium (α-MEM containing 10% FBS; Gibco; Thermo Fisher Scientific, Inc.) and cultured under 37˚C in a humidified atmosphere containing 5% CO_2. The cells from the two mice were pooled into the same culture. Previous studies have reported a typical yield of $7.5x10^7$-$1.5x10^8$ cells per mouse, although variations may occur depending on the specific conditions (17,19). The BMMs extracted from mouse bones were cultured for 3-7 days, during which the osteoclasts adhere whilst other substances and cells that do not attach fall off. During this period, cells were rinsed with fresh medium every 2 or 3 days and checked for cell status under a microscope. This process allows for isolation of pure osteoclasts (17,19). Animal experiments were conducted according to the ethical guidelines of the Sunchon National University Institutional Animal Care and Use Committee (approval no. SCNU_IACUC-2021-06).

For osteoclast differentiation, BMMs were seeded at a density of $1x10^4$ cells/well in a 96-well plate. The cells were incubated with 30 ng/ml recombinant murine macrophage colony-stimulating factor (M-CSF) provided by PeproTech, Inc., in α-MEM containing 10% FBS at 37˚C. The following day, the cells were treated with 10 ng/ml RANKL (R&D Systems, Inc.) along with either non-fermented ME or FME (1, 3, 10, 30 or 100 μg/ml) for 3 days at 37˚C. DMSO was used as a vehicle control in the RANKL-only treated cells. The concentrations used in the present study were based on a previous study (20).

Detection of osteoclast differentiation using tartrate-resistant acid phosphatase (TRAP) staining assay. After the differentiation process, cells in the 96-well plate were washed with PBS and subsequently fixed with formalin (3.7%) for 5 min at room temperature. Following fixation, the cells were treated with Triton X-100 (0.1%) for 10 min. Subsequently, the cells were treated with a TRAP solution (Sigma-Aldrich; Merck KGaA) for 10 min in a dark room at room temperature. TRAP-positive multinucleated cells with three or more nuclei were identified and counted as mature osteoclasts using light microscopy. The average of TRAP-positive multinucleated cells was determined from five locations per well and calculated.

Determining the cytotoxicity of FME on the BMMs. BMMs ($1x10^4$ cells/well) were cultured in 10% FBS-supplemented α-MEM containing M-CSF (30 ng/ml) for 24 h at 37˚C. The

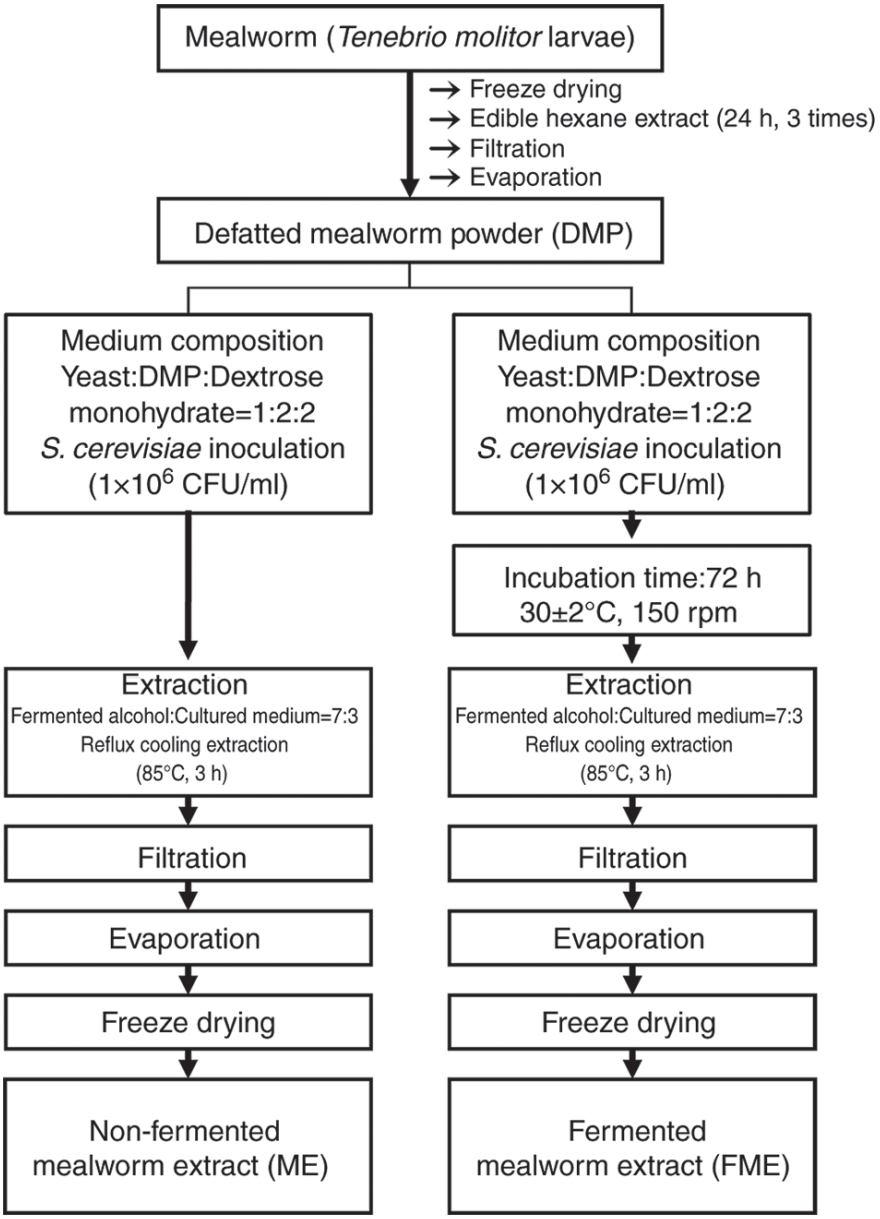

Figure 1. Preparation of non-fermented and fermented mealworm extracts. DMP, defatted mealworm powder.

next day, FME (10, 30 or 100 μg/ml) was added and incubated with the cells for 3 days at 37˚C, before cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Inc.). In each well, 100 μl of the culture supernatant was mixed with 10 μl of CCK-8 reagent and incubated for 4 h at 37˚C. Absorbance was measured at 450 nm at 1-h intervals. The sample treatment period was determined based on previous studies (21-23).

Reverse transcription-quantitative PCR (RT-qPCR). BMMs ($3x10^5$ cells/well) were incubated for 16-24 h at 37˚C with α-MEM containing 10% FBS. Following the initial incubation, the cells were treated with RANKL (10 ng/ml), M-CSF (30 ng/ml) and FME (30 or 100 μg/ml) at the same time, before being incubated for an additional 72 h at 37˚C. The total RNA was extracted from the BMM cells using the TRIzol reagent (Thermo Fisher Scientific Inc.) For the reverse transcription of the total RNA into cDNA, the ReverTra Ace™ qPCR RT master mix (Toyobo Life Science) was utilized. cDNA was synthesized by reacting 5X RT Master Mix, RNA template (1 μg/μl) and nuclease-free water following the manufacturer's protocol for 15 min at 37˚C, 5 min at 50˚C and 5 min at 98˚C. The mRNA expression levels were quantified using QuantiTect SYBR® Green RT-PCR kits (Qiagen GmbH) in a CFX96™ real-time system (Bio-Rad Laboratories, Inc.). Amplification was performed as follows: Initial denaturation at 95˚C for 15 min, followed by 40 cycles of 94˚C for 15 sec, 50˚C for 30 sec and 72˚C for 30 sec. The primers used for each gene are shown in Table I. An internal housekeeper gene *GAPDH* was used as described by Stephens *et al* (24). The mRNA expression was determined using the $2^{-\Delta\Delta Cq}$ method (25).

Western blot analysis. BMMs ($3x10^5$ cells/well) were seeded into a 6-well plate and incubated with α-MEM containing 10%

Table I. Primers used for reverse transcription-quantitative PCR.

Gene name	Forward/reverse (5'-3')	Species specificity	Amplicon size	Melting temperature, ˚C	RNA-seq identification No.
c-Fos	CCAGTCAAGAGCATCAGCAA/ AAGTAGTGCAGCCCGGAGTA	Mouse	247	60	NM_010234.3
Cathepsin K	ACTCCAGTCAAGAACCAGGG/ TCTTCTTGAGTTGGCCCTCC	Mouse	82	59	NM_007802.4
Dendritic cell-specific transmembrane protein	CCAAGGAGTCGTCCATGATT/ GGCTGCTTTGATCGTTTCTC	Mouse	255	59	NM_029422.4
Glyceraldehyde-3-phosphate dehydrogenase	AAGGTCATCCCAGAGCTGAA/ CTGCTTCACCACCTTCTTGA	Mouse	138	59	NM_001411843.1
Nuclear factor-activated T cells c1	AGGACCCGGAGTTCGACTT/ GTCGAGGTGACACTAGGGGA	Mouse	106	60	NM_001164112.1
Osteoclast-associated Ig-like receptor	GTCCTGTCGCTGATACTCCA/ CGCTGTTGGTGTGAAGTCAG	Mouse	87	59	NM_001290377.1
Tartrate-resistant acid phosphatase	AGGAAGAGCCTTCAAGTAAGTG/ CCACCCATGAATCCATCTTCT	Mouse	89	57	NM_001102405.1

FBS at 37˚C for 16-24 h. Following the initial incubation, the cells were treated with M-CSF (30 ng/ml), RANKL (10 ng/ml) and FME (30 or 100 μg/ml) at the same time and incubated for an additional 72 h at 37˚C. At the end of the incubation period, the cells were washed with PBS and lysed in a lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA and 1% Triton) containing protease inhibitor (25 μg/ml leupeptin, 2 μg/ml aprotinin and 50 mM sodium fluoride) for 1 h at 4˚C. After lysis, the cells were centrifuged at 18,928 x g for 5 min at 4˚C and the supernatants were collected for western blotting. The protein concentrations in the supernatants were determined using the Bradford method (26). A total of 10 μg protein samples were separated using 10% SDS-PAGE and transferred onto nitrocellulose membranes. Subsequently, a blocking step was carried out at room temperature for 1 h using 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA). The membranes were then incubated overnight at 4˚C with antibodies against nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1; cat no. sc-7294; 1:200; Santa Cruz Biotechnology Inc.) and β-actin (cat no. A2066; 1:2,000; Sigma-Aldrich). Subsequently, the membranes were incubated with anti-mouse monoclonal (cat no. 7076) and anti-rabbit IgG (cat no. 7074) secondary antibodies (1:10,000; Cell Signaling Technology, Inc.) for 2 h at room temperature. The protein bands were visualized using a Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.), and the visualization of protein bands was assessed using a chemiluminescence image analyzer (Alliance Q9 advanced chemiluminescence imager). Densitometry analysis of the visualized protein bands was performed using the UVITEC Alliance Q9 advanced system (version 4.3.8; UVITEC) to quantify the protein expression.

Statistical analysis. All data are presented as the means ± SEM of three independent tests. Statistical analysis was performed using the SPSS version 26 software (IBM Corp.). The data were analyzed by two-way or one-way ANOVA followed by a Holm-Sidak post hoc test to examine the differences among the groups. The mRNA levels of *TRAP*, cathepsin K (*CTSK*), osteoclast-associated Ig-like receptor (*OSCAR*) and dendritic cell-specific transmembrane protein (*DC-STAMP*) were compared using the unpaired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

RANKL-induced osteoclast differentiation is inhibited more effectively by FME compared with non-fermented mealworm extract. FME was found to inhibit osteoclast differentiation more effectively compared with non-fermented ME at concentrations of 10 and 30 μg/ml. This difference was small at 1 and 3 μg/ml between FME- and ME-treated cells (Fig. 2A). However, the number of TRAP-positive multinucleated cells with three or more nuclei was reduced more effectively in FME-treated cells compared with that in the non-fermented ME-treated cells at concentrations of 1 to 30 μg/ml (Fig. 2B). To further evaluate the effects of FME on osteoclast differentiation, FME concentrations of 10, 30 and 100 μg/ml were next tested, to assess if these concentrations were capable of inducing cytotoxicity. These FME concentrations did not indicate cytotoxicity. Furthermore, the cell viability increased at 100 μg/ml FME compared with the control cells that were not treated with FME, although the difference was not statistically significant (Fig. 3B). FME exhibited a dose-dependent suppression of osteoclast differentiation (Fig. 3A). Notably, treatment with FME at concentrations >10 μg/ml significantly reduced the formation of osteoclasts, as evidenced by the significantly decreased number of TRAP-positive multinucleated cells with three or more nuclei (Fig. 3A).

RANKL-induced NFATc1 activation is suppressed by FME. To evaluate the mechanism underlying the anti-osteoclastogenic activity of the FME, the levels of the expression of key transcription factors involved in osteoclastogenesis, namely

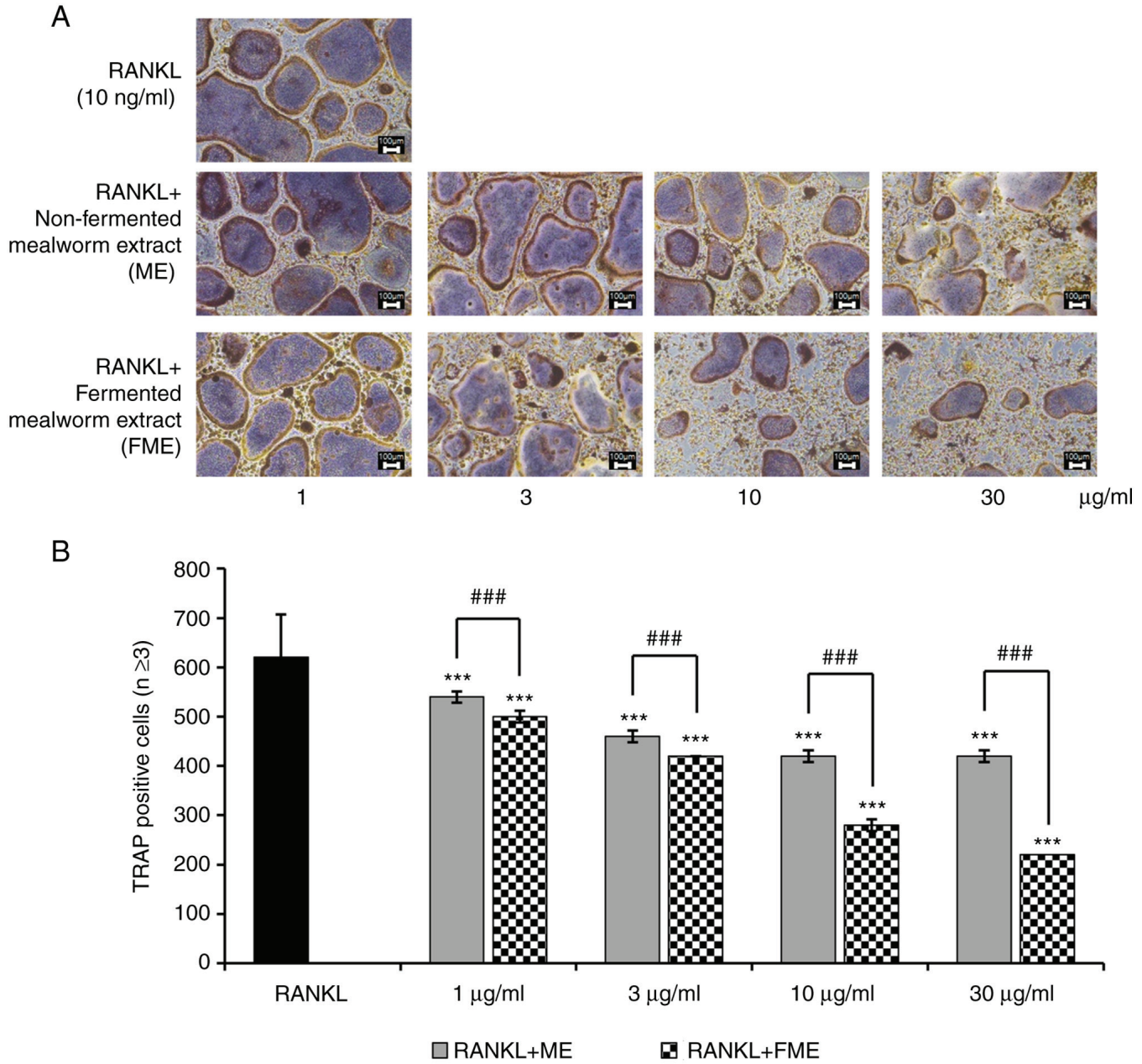

Figure 2. Comparison of the effects of ME and FME (1, 3, 10 and 30 μg/ml) on (A) RANKL-induced osteoclast differentiation and (B) TRAP-positive multinucleated cells with three or more nuclei. The results of three independent experiments are presented as the mean ± SEM values and analyzed by two-way ANOVA followed by Holm-Sidak post hoc analysis. Scale bars, 100 μm. ***P<0.001 vs. RANKL and $^{###}$P<0.001 vs. RANKL + ME. FME, fermented mealworm extract; ME, non-fermented mealworm extract; RANKL, receptor activator of nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphatase.

c-Fos and *NFATc1*, were examined. As shown in Fig. 4A, FME (100 μg/ml) significantly downregulated the expression of both transcription factors on day 1 compared with that in the RANKL-only group. Additionally, compared with that in the RANKL-only group, FME (30 μg/ml) significantly reduced *NFATc1* expression on day 0 (2-h reaction). Furthermore, compared with that in the RANKL-only group, *c-Fos* mRNA expression levels in FME 100 μg/ml showed a statistical difference on day 1 but none thereafter, whereas NFATc1 mRNA expression levels showed a statistical difference on days 0, 1 and 3 at 100 μg/ml of FME (Fig. 4A). Therefore, the present study next evaluated the impact of FME on the expression of the NFATc1 protein. The results revealed that FME exerted a dose-dependent downregulation of NFATc1 protein expression on each of the 3 days. In particular, there was a significant difference in concentration on day 2 (Fig. 4B).

Expression of osteoclast-specific marker genes is downregulated by FME treatment. NFATc1 has been previously reported to promote the expression of osteoclast-associated genes, including *TRAP*, *CTSK*, *OSCAR* and *DC-STAMP* (27,28). Therefore, the present study examined the effect of FME (100 μg/ml) on the mRNA expression of such genes. RANKL stimulation led to a time-dependent increase in the expression of the *TRAP*, *CTSK*, *OSCAR* and *DC-STAMP* (Fig. 5). However, treatment with FME significantly suppressed the mRNA expression of these genes compared with that in their RANKL-only counterparts on each of the 3 days (Fig. 5).

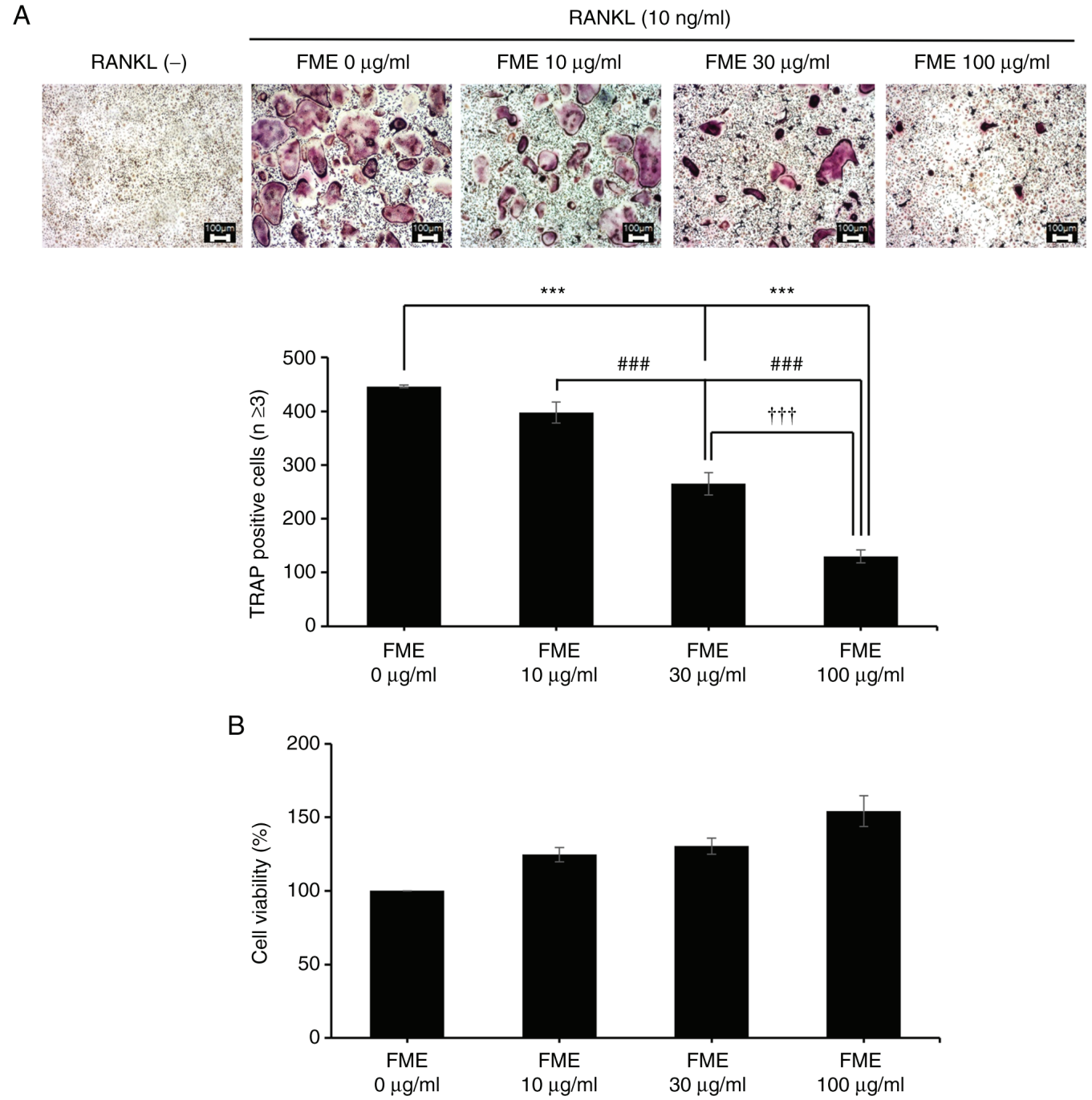

Figure 3. FME suppresses RANKL-induced osteoclast differentiation. (A) FME dose-dependently inhibited osteoclast differentiation and TRAP-positive multinucleated cells with three or more nuclei. Scale bars, 100 μm. BMMs were cultured with 30 ng/ml macrophage colony-stimulating factor for 24 h, before the indicated concentrations of FME were added and incubated for 3 days. (B) The effects of FME on the viability of BMMs were measured using a Cell Counting Kit-8 assay. The results of three independent experiments are presented as the mean ± SEM values and analyzed by one-way ANOVA followed by Holm-Sidak post hoc analysis. ***P<0.001 vs. 0 μg/ml FME, $^{###}$P<0.001 vs. 10 μg/ml FME and †††P<0.001 vs. 30 μg/ml FME. BMM, bone marrow-derived macrophages; FME, fermented mealworm extract; receptor activator of nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphatase.

These findings suggest that FME can suppressed the expression of osteoclast-associated genes, which are regulated by the NFATc1 signaling pathway.

Discussion

Osteoclasts are multinucleated cells of the macrophage lineage that can be derived from BMMs (29). They serve an important role in bone resorption (30). The process of osteoclast formation is regulated by genetic, humoral and mechanical signals (31). Among these, M-CSF and RANKL serve pivotal roles in stimulating osteoclast differentiation (30). The present study therefore induced osteoclast differentiation in BMMs using M-CSF (30 ng/ml) and RANKL (10 ng/ml). FME, compared with the non-fermented ME, was found to effectively suppressed the RANKL-stimulated osteoclast differentiation, as evidenced by TRAP staining. To assess whether the anti-osteoclastogenic effect of FME was associated with

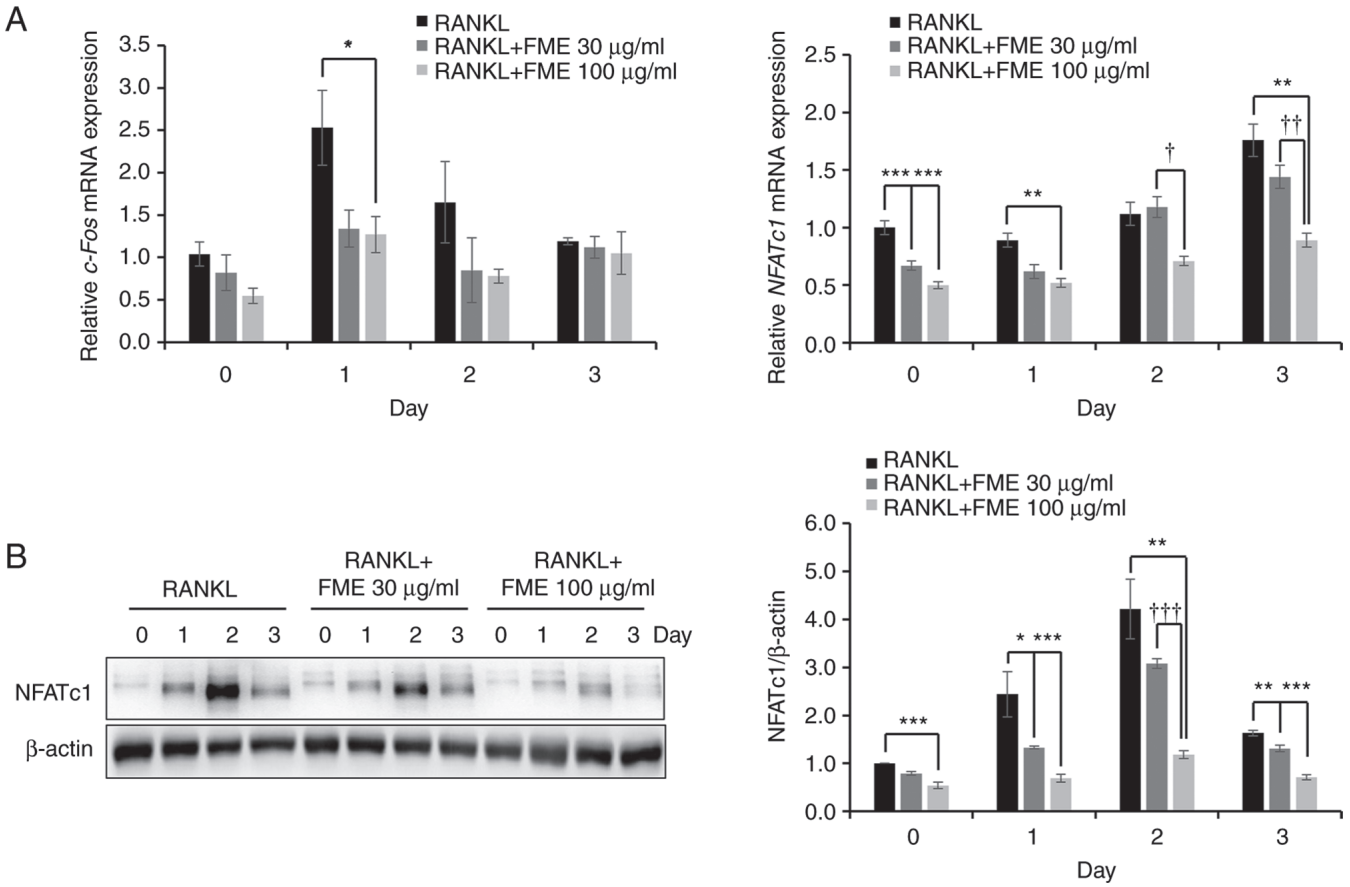

Figure 4. Effects of FME on the mRNA expression levels of *cFos* and *NFATc1* and NFATc1 protein expression in RANKL-induced cells. (A) *cFos* and *NFATc1* mRNA expression was measured by reverse transcription-quantitative PCR. *GAPDH* was used as an internal control. (B) Effects of FME on NFATc1 protein expression in RANKL-induced cells were measured by western blotting. Day 0 represents a 2-h reaction period. The results of three independent experiments are presented as the mean ± SEM values and analyzed by one-way ANOVA followed by Holm-Sidak post hoc analysis. *P<0.05, **P<0.01 and ***P<0.001 vs. RANKL. †P<0.05, ††P<0.01 and †††P<0.001 vs. 30 μg/ml FME. FME, fermented mealworm extract; RANKL, receptor activator of nuclear factor κB ligand, NFATc1, nuclear factor of activated T cells, cytoplasmic 1.

cytotoxicity, a cell viability assay was conducted for 3 days when stable cell growth was observed under the microscope. Given that the experiment period for differentiation inhibition spanned 3 days, the present study verified the absence of cytotoxicity throughout this duration. Although parameters in cell experiments can vary, such as the environment and cell condition, previous similar studies (21-23,32) also treated cells for 3 days using $1x10^4$ cells in 96-well plates under the same conditions and found no cytotoxicity at concentrations ≤100 μg/ml. FME exhibited a dose-dependent inhibition of osteoclast differentiation in the concentration range of 10-100 μg/ml, suggesting that it has an anti-osteoclastogenic effect without causing cytotoxicity.

The RANKL signaling pathway activates several transcription factors, such as the NF-κB, activator protein 1 (33) and NFATc1 (34). NFATc1 functions as a master transcription factor downstream of RANKL-RANK signaling and serves a critical role in osteoclast differentiation, formation and bone resorption (35,36). Therefore, the present study investigated whether FME can alter the activity of NFATc1 during RANKL-stimulated osteoclastogenesis. FME dose-dependently downregulated *NFATc1* gene and protein expression in response to RANKL stimulation. In addition, FME significantly downregulated *c-Fos* gene expression on day 1. c-Fos is an important transcription factor that is involved in the early stage of osteoclast differentiation whilst promoting NFATc1 action (37). These findings suggest that FME can inhibit the expression of key transcription factors involved in osteoclastogenesis, including *c-Fos* and *NFATc1*. In particular, the mRNA expression of NFATc1 continued to increase until day 3, whereas the corresponding protein expression levels peaked on day 2 and decreased on day 3. These observations are consistent with the results reported by Kim *et al* (38), which reported that the levels of NFATc1 protein expression decreased during late stage osteoclastogenesis due to its translocation from the cytosol into the nucleus in the presence of RANKL. They also suggested that NFATc1 continued to be transcribed until the end of osteoclastogenesis (38). However, a limitation of the present study is that it did not perform NFATc1 knockdown or overexpression.

Subsequent to the activation of NFATc1, a continuous upregulation in the expression of various factors has been previously reported, including CTSK, TRAP, DC-STAMP and OSCAR (39-42). CTSK, TRAP, DC-STAMP and OSCAR contribute to the differentiation of osteoclasts into mature osteoclasts and potentially worsen bone disorders (39-42). The present study revealed that RANKL stimulation markedly increased the mRNA expression levels of *CTSK*, *TRAP*,

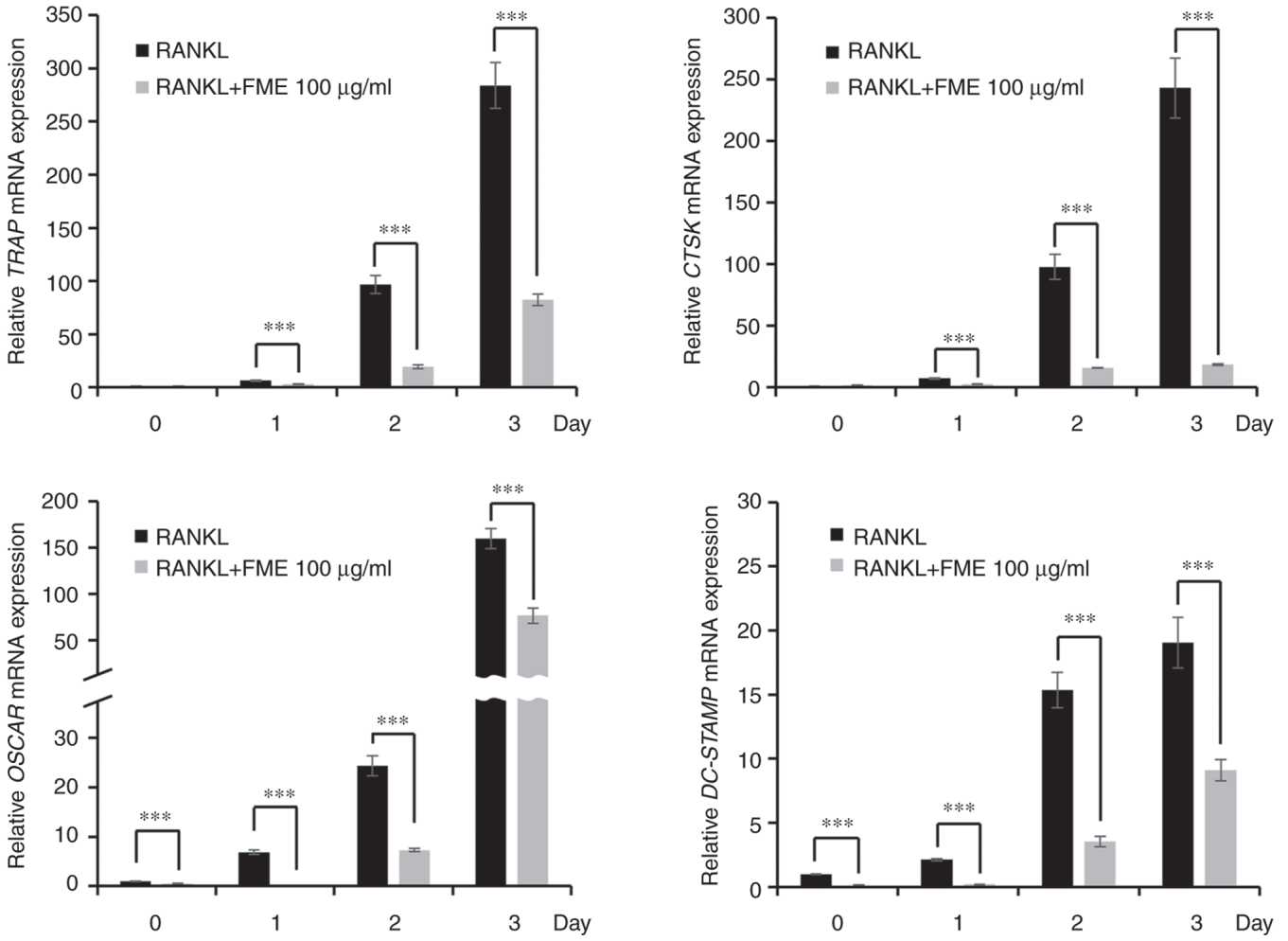

Figure 5. Effects of FME on the expression of osteoclastogenic-associated marker genes *TRAP*, *CTSK*, *OSCAR* and *DC-STAMP*. Reverse transcription-quantitative PCR was performed. Day 0 represents a 2-h reaction period. The results of three independent experiments are presented as the mean ± SEM values and analyzed using an unpaired Student's t-test. ***P<0.001 vs. RANKL. FME, fermented mealworm extract; RANKL, receptor activator of nuclear factor κB ligand; *TRAP*, tartrate-resistant acid phosphatase; *CTSK*, cathepsin K; *OSCAR*, osteoclast associated Ig-like receptor; *DC-STAMP*, dendritic cell-specific transmembrane protein.

DC-STAMP and *OSCAR* in a time-dependent manner. However, FME treatment significantly reduced the expression of these genes from day 0 to 3. This suggest that FME has the ability to suppress the expression of osteoclast-associated genes involved in bone resorption and fusion of mononuclear pre-osteoclasts. Notably, FME treatment at concentrations of 30 and 100 μg/ml resulted in a significant reduction in the number of TRAP-positive multinucleated cells. The fusion of mononuclear pre-osteoclasts is a critical step in the formation of mature multinucleated osteoclasts (27,43), which can lead to increased bone resorption activity and decrease in bone mass (27,44). Specifically, DC-STAMP serve an essential role in the fusion of mononuclear osteoclasts (27,45). The reduction observed in the number of TRAP-positive multinucleated cells suggests that FME may interfere with the fusion process and consequently inhibit osteoclast maturation and bone resorption.

FME was previously found to have higher amino acid content compared with non-fermented ME, with leucine and alanine being the most abundant amino acids present (14). A recent study reported a negative correlation between plasma valine, leucine, isoleucine and alanine levels with BMD in elderly women (46). By contrast, in men, plasma tryptophan levels exhibited an inverse correlation with BMD (46). Another study previously demonstrated that although BCAAs can activate osteoclast differentiation, at higher concentrations BCAAs were actually found to inhibit this process (47). This suggests the presence of a negative feedback mechanism for BCAAs (47). However, the effects of amino acids on osteoclast differentiation remain poorly understood and further investigation is required to determine the specific mechanism and impact of different amino acids on osteoclast differentiation. Another limitation of the present study is that it did not identify specific compounds and their potential effects on osteoporosis. Additionally, to further substantiate the anti-osteoclastogenic effects of FME and their effects on NFATc1 activity, experiments using NFATc1 inhibitors as opposed to expression manipulation should be conducted.

Taken together, results from the present study suggest that FME has a potential inhibitory effect on osteoclast differentiation and formation. This effect may be associated with the downregulation of NFATc1 activity and the suppression of osteoclastogenic marker expression, such as CTSK, TRAP, DC-STAMP and OSCAR. These results suggest that FME is a

promising intervention method for the prevention or treatment of osteoporosis. However, since the present study was only conducted *in vitro*, it is necessary to evaluate its efficacy and safety through *in vivo* studies and clinical trials in animal and human subjects.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MKL conceived the study and edited the manuscript. JRH carried out all of the assays in the study and drafted the manuscript. All authors have read and approved the final manuscript. JRH and MKL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The experimental protocols were approved by the Sunchon National University Institutional Animal Care and Use Committee (approval no. SCNU_IACUC-2021-06; Suncheon, Korea) and were carried out on the basis of relevant national and international guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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