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Herbal medicine Ninjinyoeito inhibits RANKL-induced osteoclast differentiation and bone resorption activity by regulating NF-κB and MAPK pathway

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ABSTRACT

Objectives: Osteoporosis is a systemic bone metabolism disorder characterized by decreased bone mass and strength. Osteoclasts (OCs) are giant multinucleated cells that regulate bone homeostasis by degrading bone matrix. Excessive OC differentiation and activity can lead to serious bone metabolic disorders including osteoporosis. Current treatments, including antiresorptive drugs, exert considerable adverse effects, including jaw osteonecrosis. Herbal medicines, such as Ninjinyoeito (NYT), may also offer efficacy, but with fewer adverse effects. In this study, we investigated NYT's effects on osteoclastogenesis.

Methods: Tartrate-resistant acid phosphatase (TRAP) staining and bone resorption assays were performed to examine NYT's effects on OC differentiation and function. OC-related gene expression at mRNA and protein levels was investigated to confirm NYT's inhibitory action against osteoclastogenesis. We also demonstrated involvement of signaling pathways mediated by IκBα and mitogen-activated protein kinases (MAPK) [extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38] and showed nuclear translocation of nuclear factor of activated T-cell cytoplasmic 1 (NFATc1) and nuclear factor kappa B (NF-κB) p65 during osteoclastogenesis.

Results: TRAP staining and bone resorption assays confirmed that NYT significantly inhibited OC differentiation and function. Western blot and RT-PCR results showed that NYT ameliorated osteoclastogenesis by suppressing mRNA and protein level expression of OC-related genes. Moreover, blots and immunocytochemistry (ICC) data clarified that NYT abrogates signaling pathways mediated by IκBα and MAPK (ERK, JNK, p38), and demonstrated nuclear translocation of NFATc1 and NF-κB p65 during OC differentiation.

Conclusions: These findings suggest NYT is an alternative therapeutic candidate for treating osteoporosis.

1. Introduction

Bone homeostasis is a physiological process that involves bone formation and resorption, regulated by osteoblasts and osteoclasts (OCs) [1]. Disruption in this balance can lead to various bone metabolic disorders. Osteoporosis is a systemic disorder of bone metabolism

Abbreviations: c-fms, colony-stimulating factor 1 receptor; c-fos, AP-1 Transcription Factor Subunit; CTSK, cathepsin K; DC-STAMP, dendritic cell-specific transmembrane protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MMP9, matrix metalloproteinase 9; NFATc1, nuclear factor of activated T-cell cytoplasmic 1; NF-κB, nuclear factor kappa B; OC-STAMP, osteoclast stimulatory transmembrane protein; p38, mitogen-activated protein kinase 14; RANK, receptor activator of nuclear factor kappa B; RANKL, receptor activator of nuclear factor kappa B ligand; TRAP, tartrate-resistant acid phosphatase; TRAF6, tumor necrosis factor receptor associated factor 6.

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characterized by decreased bone mass and strength, which increases the risk of bone fractures. Many therapeutic agents, such as anabolic and anti-resorptive drugs, have considerable adverse effects, including hypocalcemia, osteonecrosis of the jaw, and gastrointestinal disorders [2–4]. Recently, many herbal medicines have been considered alternative therapeutic candidates for treating osteoporosis and other bone metabolic disorders due to their lower cytotoxicity and broader pharmacological properties [5].

OCs are multinucleated giant cells derived from macrophage/monocyte linage cells and are responsible for bone remodeling by degrading the bone matrix [6]. Specifically, the binding of receptor activator of nuclear factor-κB ligand (RANKL) to its corresponding receptor (RANK) and macrophage colony-stimulating factor (M-CSF) to its receptor, colony-stimulating factor 1 receptor (c-fms), induce OC differentiation [7]. These bindings activate downstream signaling cascades [8], leading to the nuclear translocation of two core transcription factors: (1) nuclear factor of activated T-cell cytoplasmic 1 (NFATc1) and (2) c-fos [9]. Subsequently, OC-related genes, such as tartrate-resistant acid phosphatase (*TRAP*), Cathepsin K (*CTSK*), and matrix metalloproteinase 9 (*MMP9*) [10,11], are involved. For OC-induced bone resorption, OCs are required to adhere to the bone surface to create a sealing zone [12]. Following the adhesion of OCs to the bone surface, activated OCs form the ruffled border within the sealed zone, and secrete the acidic ions such as H^+ and Cl^- into the bone extracellular matrix (ECM) to establish the acidic milieu of the resorption lacuna and dissolve the mineralized ECM [13]. Destruction of the mineralized ECM exposes organic elements, which are subsequently degraded by several secreted lysosomal enzymes, such as TRAP, CTSK, and MMP9, thereby triggering bone resorption [14–16].

Ninjinyoeito (NYT), a traditional Japanese Kampo medicine known for its pharmaceutical effects, such as anti-inflammatory action, is useful for treating anemia and enhancing immunity [17,18]. NYT is composed of various herbal extracts such as *Ginseng*, *Glycyrrhiza*, *Japanese angelica root*, *Cinnamon bark*, *Polygala root*, *Citrus unshiu peel*, *Astragalus root*, *Schisandra fruit*, *Peony root* and *Rehmannia root*. NYT exerts anti-depressant action and reduces β-amyloid-induced axonal damage by enhancing the central noradrenergic system and neurotrophic factors [19,20]. Moreover, it suppressed the onset of arthritis, pain, and muscle atrophy in a rheumatoid arthritis mouse model [21]. Another study demonstrated that NYT inhibited fatty and fibrotic changes in the livers of mice with non-alcoholic steatohepatitis (NASH) [22]. A recent study reported that NYT suppresses OC differentiation in RAW264.7 cells, however, this study was emphasized on the effect of Unkeito in osteoclastogenesis [23], and the detailed mechanism of the NYT has not yet been elucidated. Therefore, we investigated the effect of NYT on osteoclastgenesis in this study.

2. Materials and methods

2.1. Antibodies and reagents

Isolation and purification of recombinant RANKL were prepared by the protocols described heretofore [24]. Rabbit polyclonal anti-CTSK antibody was purified as the protocol described heretofore [25]. The following antibodies were used in this study: mouse monoclonal anti-*c*-fms (sc-46662), mouse monoclonal anti-*c*-fos (sc-166940), mouse monoclonal anti-NFATc-1 (sc-7294), and mouse monoclonal anti-RANK (sc-374360)) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse monoclonal anti–NF–κB p65 (6956S), rabbit monoclonal anti-phospho-p38 (4511S)), rabbit polyclonal anti-p38 MAPK (9212S), rabbit monoclonal anti-phospho-IκBα (2859S), rabbit monoclonal anti-IκBα (4812S), rabbit polyclonal anti-phospho-JNK (9251S), rabbit polyclonal anti-JNK (9252S), rabbit polyclonal anti-phospho-Erk1/2 (9101S), rabbit polyclonal anti-Erk1/2 (9102S), andAlexa Fluor 488 goat anti-mouse IgG (4408S) from Cell Signaling Technology (Danvers, MA, USA); and HRP-conjugated GAPDH monoclonal antibody (HRP-60004) from Proteintech (Rosemont, IL, USA. The Osteo Assay Stripwell Plate was purchased from Corning, Inc. (Corning, NY, USA). The powder of NYT was purchased from Tsumura & CO, Japan. It was dissolved in minimum essential medium α (MEMα) (Wako Pure Chemicals, Osaka, Japan) and later centrifuged at 12000 rpm for 20 min and filtered by 0.45 μm Cellulose Acetate filter units (25CS045AS, Toyo Roshi Kaisha, Japan).

2.2. Cell culture

A murine monocytic cell line, RAW-D cells, gifted from Toshio Kukita (Kyushu University, Japan) [26,27] was cultured in MEM-αsupplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (10 mg/mL) in 5% CO_2 and 95% air humidified incubator at 37 °C. For OC differentiation from RAW-D cells, RAW-D cells were cultured in MEMα with RANKL (100 ng/mL) for 3 days. The timing of NYT addition to the medium was simultaneously with RANKL.

2.3. TRAP staining

Cells were fixed with 4% paraformaldehyde (PFA) at room temperature (RT) for 1 h, and subsequently treated with 0.2% Triton X-100 in PBS at RT for 5 min. Later, cells were stained for TRAP solution including 0.01% naphthol AS-MX phosphate (Sigma-Aldrich, Tokyo, Japan) and 0.06% fast red violet LB salt (Sigma-Aldrich, Tokyo, Japan) in the presence of 50 mM sodium tartrate and 50 mM sodium acetate (pH5.0). TRAP-positive multinucleated cells were counted under a light microscope, and the images were photographed by Olympus FSX100 microscope. The multinucleated cells containing three or more nuclei were considered as mature OCs.

2.4. Bone resorption assay

The bone resorption assay was performed as follows: RAW-D cells (1×10^3 cells/well) were seeded into Osteo Assay Stripwell plates with RANKL (500 ng/mL) stimulation for 10 days. Cells were washed with 5% NaClO and the images of bone resorption area were photographed by Keyence BZ-X800 fluorescence microscope and analyzed by Image J software.

2.5. Cell viability assay

Cells seeded in 96 well plate were incubated with Cell Counting Kit 8 (CCK-8, Dojindo, Kumamoto, Japan) for 1 h and then, the absorbance at 450 nm was measured by a microplate reader (HITACHI, Japan).

2.6. Western blot (WB) analysis

WB was performed as follows: whole cell lysates (WCL) were prepared using RIPA buffer (50 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) supplemented with proteinase inhibitor cocktail (Sigma-Aldrich Tokyo, Japan). WCL (15–50 μg) were run on 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The blots were blocked in Tris-buffered saline containing 0.05% Tween-20 and 5% skim milk for 1 h at RT, and subsequently probed with various antibodies at 4 °C overnight. After being washed, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. WB visualization was achieved using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA), according to the ECL substrate (Millipore, USA).

2.7. Immunocytochemistry (ICC)

The cells seeded and grown on glass coverslips were fixed with 4% PFA in PBS at RT for 1 h. After washing with PBS, the fixed cells were

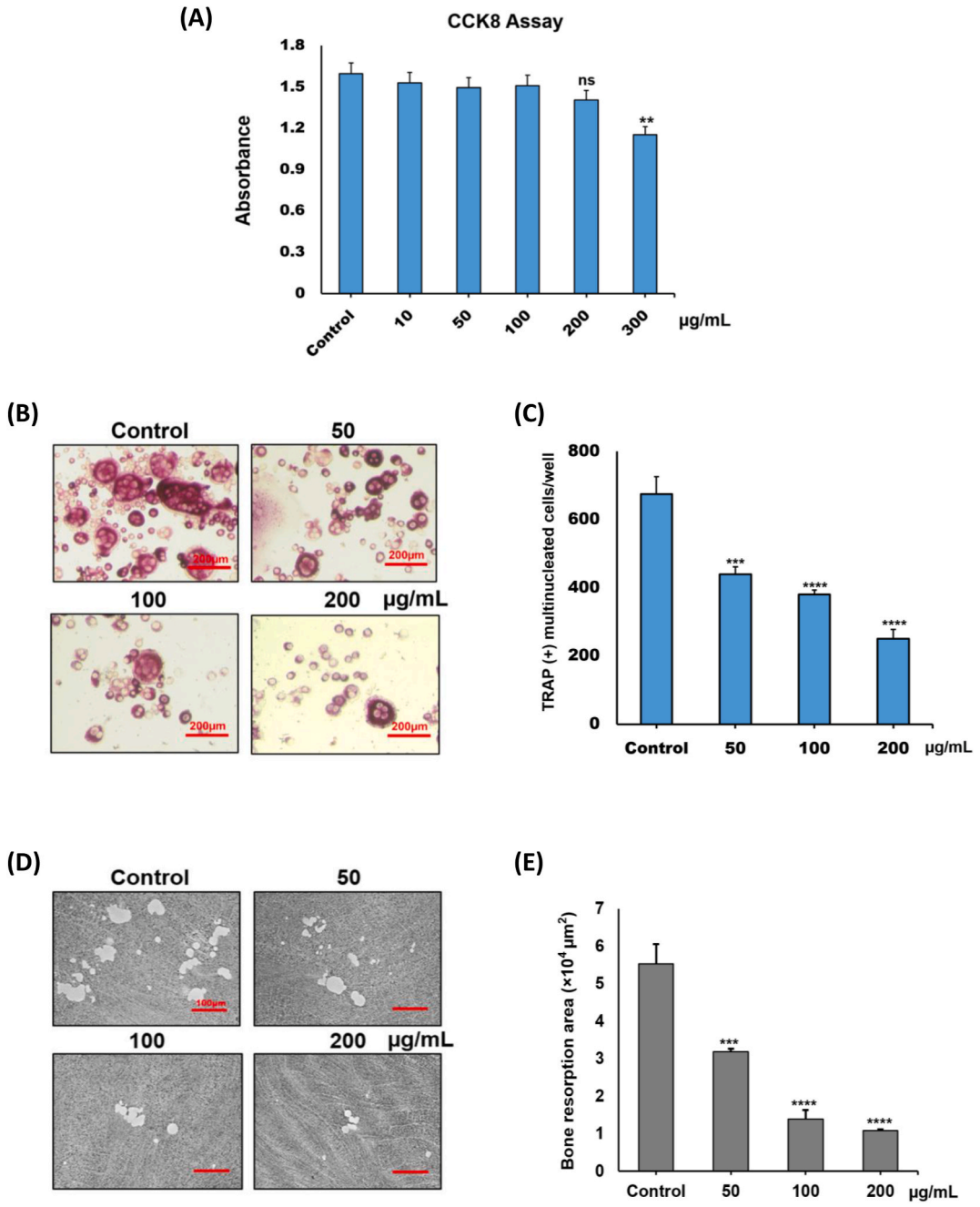

Fig. 1. NYT inhibits OC differentiation, and bone resorption activity without significant cytotoxic effect.
(A) Cell viability of RAW-D cells was tested by Cell Counting Kit (CCK-8) assay, ns, non-significant, $^{**}p < 0.01$.
(B) RAW-D cells were cultured with RANKL (100 ng/mL) and NYT for 3 days. TRAP staining was carried out to access OC formation derived from RAW-D cells.
(C) The number of TRAP-positive OCs having 3 nuclei or more than 3 and less than 10 nuclei per viewing field was counted, $^{***}p < 0.005$, $^{****}p < 0.001$, compared to the control.
(D) The images of bone resorption area induced by RAW-D cells derived OCs which were co-cultured with NYT and stimulated by RANKL (500 ng/mL).
(E) The bone resorption area was measured and analyzed using Image J software. Mean ± SD of triplicate samples. $^{***}p < 0.005$, $^{****}p < 0.001$.

permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were incubated sequentially with 0.1% bovine serum albumin (BSA) for 30 min and with primary antibodies at 4 °C overnight. The cells were washed, and stained with a secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA). Ultimately, nuclear staining with DAPI (Invitrogen Carlsbad, CA, USA) was performed. The samples were visualized using the Keyence BZ-X800 fluorescence microscope.

2.8. Quantitative real-time polymerase chain reaction (RT-PCR) analysis

For real-time PCR analysis, Trizol (Molecular Research Center, Cincinnati, OH, USA) was used for RNA extraction and purification from cultured cells. Following this, 0.1 μg of total RNA was reverse transcribed for cDNA synthesis using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR was done using MJ Mini (Bio-Rad), according to the manufacturer's instructions. The primer sets used are indicated in Table.

Primer (Name)	Forward sequence (5'→3')	Reverse sequence (5'→3')
RANK	CTTGGACACCTGGAATGAAGAAG	AGGGCCTTGCCTGCATC
NFATc1	TCATCCTGTCCAACACCAAA	TCACCCTGGTGTTCTTCCTC
c-fms	TTGGACTGGCTAGGGACATC	GGTTCAGACCAAGCGAGAAG
TRAP	TGGCTGAGGAAGTCATCTGAGTTG	GACCACCTTGGCAATGTCTCTG
CTSK	CAGCTTCCCCAAGATGTGAT	AGCACCAACGAGAGGAGAAA
OC-STAMP	TGGGCCTCCATATGACCTCGAGTAG	TCAAAGGCTTGTAAATTGGAGGAGT
DC-STAMP	CTAGCTGGCTGGACTTCATCC	TCATGCTGTCTAGGAGACCTC
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

2.9. Statistical analysis

Statistical significance was calculated using Microsoft Excel. The data were analyzed using a Kruskal-Wallis test; values of $p < 0.05$ were considered to indicate statistical significance. Data were expressed as means ± S.D. unless otherwise specified.

3. Results

3.1. NYT inhibits OC differentiation & bone resorption activity without significant cytotoxicity

To evaluate the cytotoxic effect of NYT during RANKL-induced osteoclastogenesis, we performed a cell viability assay at different concentrations of NYT (50, 100, 200, and 300 μg/mL) using a CCK-8 assay. No significant cytotoxicity was observed up to a concentration of 200 μg/mL (Fig. 1A). Therefore, we selected concentrations of 50, 100, and 200 μg/mL for further studies.

To investigate the effect of NYT on RANKL-induced osteoclastogenesis, we examined the impact of different concentrations of NYT on OC differentiation in RAW-D cells treated with RANKL (100 ng/mL) using TRAP staining. Multinucleated cells with three or more nuclei were counted as TRAP-positive mature OCs. TRAP staining revealed that the number of TRAP-positive multinucleated OCs after NYT treatment was significantly decreased compared to that of the control group in a dose-dependent manner (Fig. 1B and C). Next, to confirm whether NYT inhibits bone-resorbing activity in mature OCs, we performed a pit formation assay with various concentrations of NYT (50, 100, and 200 μg/mL) using an Osteo Assay Stripwell plate. Similar to TRAP staining, the bone resorption area decreased in a dose-dependent manner compared to that of the control group (Fig. 1D and E). These results indicate that NYT significantly inhibits OC differentiation and physiological bone resorption activity in a dose-dependent manner.

3.2. NYT abrogates protein and mRNA expression of OC markers

To further examine the effects of NYT on OC differentiation, we analyzed the expression of OC marker proteins by WB and compared them to the control. Protein levels of several OC marker genes such as RANK, c-fms, CTSK, NFATc1, and c-fos were decreased following NYT treatment (100 and 200 μg/mL) (Fig. 2A and B). Among these proteins, NFATc1 is a master regulator of osteoclast differentiation and plays a critical role by translocating into the nucleus at the final stage of OC differentiation [28]. Our results indicate that NYT acts at the transcriptional level.

Moreover, we examined the mRNA expression levels of various OC markers, including RANK, NFATc1, TRAP, c-fms, CTSK, OC-STAMP, and DC-STAMP, following NYT treatment (100 and 200 μg/mL) using RT-PCR. Among these markers, OC-STAMP and DC-STAMP play major roles in osteoclast fusion and adhesion [29]. As expected, the mRNA levels of these markers decreased after NYT treatment (Fig. 2C). These findings indicate that NYT significantly abrogated the expression of OC markers at both the protein and mRNA levels.

3.3. NYT suppresses osteoclastogenesis via the NF-κB and MAPK pathways

According to the prior findings, we concluded that NYT suppresses osteoclastogenesis. We then examined the effects of NYT on RANKL-induced intracellular signaling pathways during OC differentiation. It has been discovered that OC differentiation is highly dependent on the nuclear factor kappa B (NF-κB) pathway [30]. Specifically, the binding of RANKL to its receptor RANK induces the recruitment of TRAF6, TGF-β-activated kinase (TAK1), leading to the subsequent phosphorylation of inhibitor of nuclear factor Kappa-B kinase (IKKβ). This process results in the phosphorylation of IκBα, which leads to the release of the p65 and p50 subunits, allowing them to translocate into the nucleus and stimulate target genes involved in OC differentiation [31]. We first analyzed the NF-κB pathway in a time dependent manner (0, 5, 10, 15, and 20 min). The blot results demonstrated that the phosphorylation level of IκBα (*p*-IκBα) decreased following NYT treatment (Fig. 3A and B).

The recruitment of TRAF6 to RANK forms a signaling complex containing TAK1 and TAK1-binding protein (TAB2), which activates all three mitogen-activated protein kinase (MAPK) pathways: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 [32]. RANKL activates ERK, JNK, and p38 through the activation of MEK1/2, MKK7, and MKK6, which then activate their downstream targets, including c-fos, AP-1 transcription factors, and MITF in OC precursors [33,34]. We investigated the phosphorylation of ERK, JNK, and p38 at the indicated time points (0, 5, 10, 15, and 20 min). WB results showed that the phosphorylation levels of ERK (p-ERK), JNK (p-JNK), and p38 (p-p38) were significantly decreased following NYT treatment (Fig. 3C and D). These findings indicate that NYT suppresses OC differentiation by inhibiting NF-κB and MAPK pathways.

3.4. Nuclear translocation of NFATc1 and NF-κB p65 can be ameliorated by NYT

Activation of most NFAT transcription factor family members (NFATc1, NFATc2, NFATc3, and NFATc4) is regulated by calcium/calmodulin signaling [28]. RANKL-mediated phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) results in the activation of spleen-associated tyrosine kinase (Syk) and Phospholipase C Gamma (PLCγ). Activated PLCγ mobilizes intracellular calcium, which activates the calmodulin-dependent phosphatase calcineurin. Calcineurin directly dephosphorylates serine residues in NFATc1, allowing its rapid translocation into the nucleus and subsequent activation [35,36]. Since NFATc1 translocation is crucial for OC differentiation, we analyzed the nuclear translocation of NFATc1 using ICC. As demonstrated in (Fig. 4A), the whitish area, indicating the nuclear translocation of NFATc1, was smaller in the NYT treatment group than in the control group. This suggests that NYT inhibits the nuclear translocation of NFATc1.

One of the heterodimers of IκBα, p65, also known as RelA, has been investigated for its crucial role in OC differentiation. The two products of the canonical NF-κB pathway, p65 and p50, are released following IκBα phosphorylation and subsequently stimulate OC target genes within the

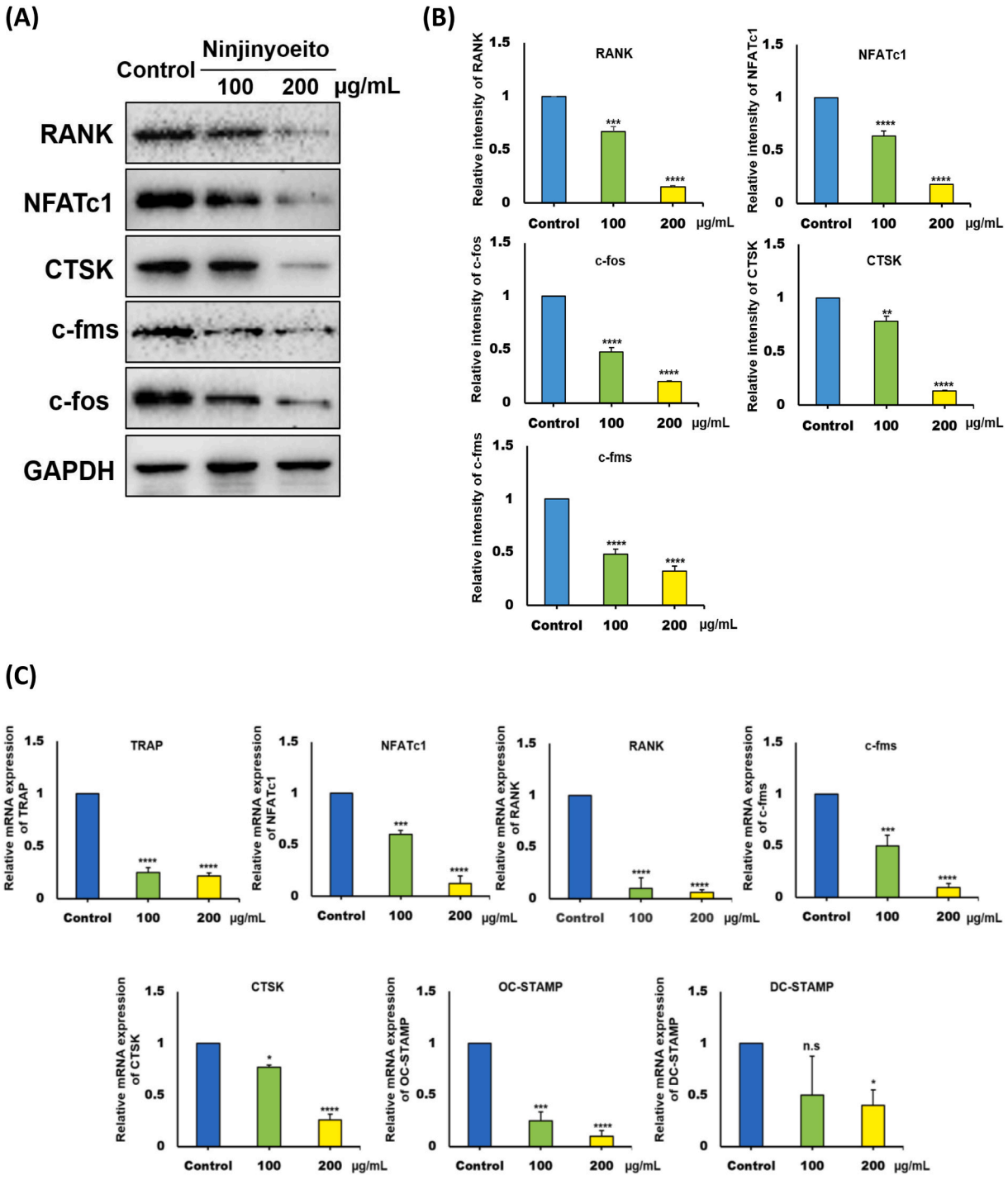

Fig. 2. NYT attenuates protein and mRNA expression of OC markers

(A) RAW-D cells were differentiated into OC by being cultured with RANKL (100 ng/mL) and NYT. The total expression levels of RANK, NFATc1, c-fms, c-fos, and CTSK were evaluated by immunoblotting. GAPDH was used as a loading control.

(B) Relative protein expression level of RANK, NFATc1, c-fms, c-fos, and CTSK, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$. The data were representative of three independent experiments.

(C) Relative mRNA expression level of *TRAP*, *NFATc1*, *RANK*, *c-fms*, *CTSK*, *OC-STAMP*, and *DC-STAMP*, ns, non-significant, $^{*}p < 0.05$, $^{***}p < 0.005$, $^{****}p < 0.001$. The data were representative of three independent experiments.

nucleus [37,38]. In this study, we investigated the nuclear translocation of NF-κB p65 using ICC. As in the previous analysis, we examined the whitish area (indicating the nuclear translocation of NF-κB p65) and the results demonstrated that NYT treatment decreased the nuclear translocation of NF-κB p65 (Fig. 4B). In addition, we investigated the protein expression level of NF-κB p65 during OC differentiation using WB. The protein level of NF-κB p65 was notably reduced in NYT treatment group (Fig. 4C and D). These findings indicate that NYT inhibits the nuclear translocation of both NFATc1 and NF-κB p65.

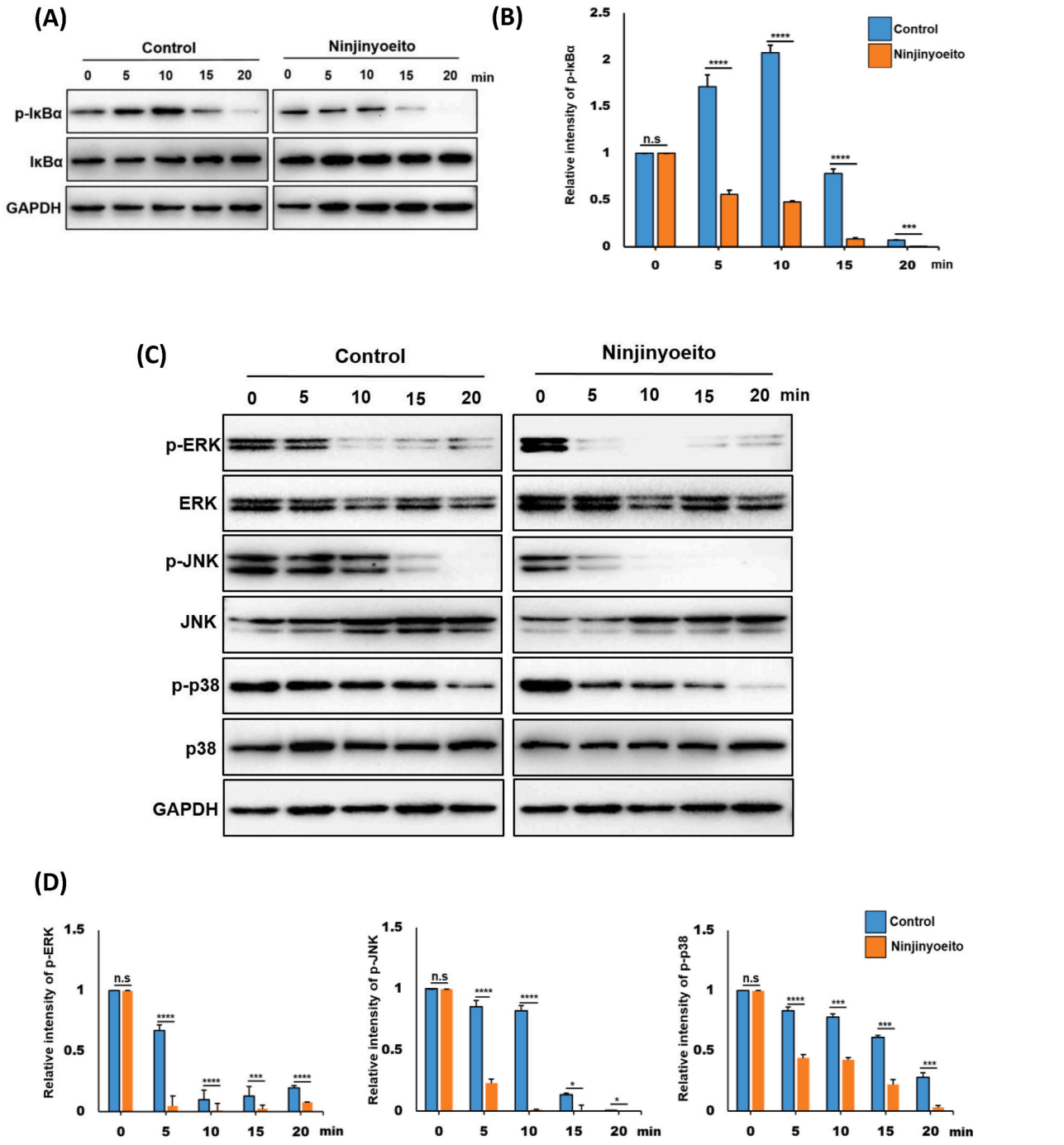

Fig. 3. NYT inhibits OC differentiation via abrogating NF-κB and MAPK pathway

(A) RAW-D cells were incubated with serum (−/−) culture media and with or without NYT in the absence of RANKL for 2 h. After RANKL (200 ng/mL) supplementation, the cells were incubated for the indicated times, and subsequently collected. The cell lysates were subjected to SDS-PAGE followed by immunoblotting for the detection of total expression levels of IκBα, phosphorylated-IκBα (p-IκBα), and GAPDH was used as a loading control.

(B) Relative expression level of p-IκBα corresponding to the indicated time (0, 5, 10, 15, 20 min), ns, non-significant, ***$p < 0.005$, ****$p < 0.001$. The data were representative of three independent experiments.

(C) RAW-D cells were incubated with serum (−/−) culture media and with or without NYT in the absence of RANKL for 2 h. Cells were incubated for the indicated times after RANKL supplementation, and then collected. The cell lysates were subjected to SDS-PAGE followed by immunoblotting for the detection of total expression levels of ERK, p-ERK, JNK, p-JNK, p38, p-p38, and GAPDH was used as a loading control.

(D) Relative expression level of p-ERK, p-JNK, p-p38 in corresponding to the indicated time (0, 5, 10, 15, 20 min), n.s, non-significant, *$p < 0.05$, ***$p < 0.005$, ****$p < 0.001$. The data were representative of three independent experiments.

4. Discussion

RANKL, a critical stimulator of osteoclastogenesis, is expressed in various tissues, including skeletal muscle, skin, bone, brain, and lymphoid organs. The RANKL-RANK signaling pathway plays a crucial role in bone remodeling by regulating OC differentiation and influencing OC-independent mechanisms, such as T cell–dendritic cell interactions in the immune system. This pathway activates the canonical NF-κB pathway and induces key transcription factors, including c-fos [39]. In addition, the RANK and ITAM costimulatory signaling pathways

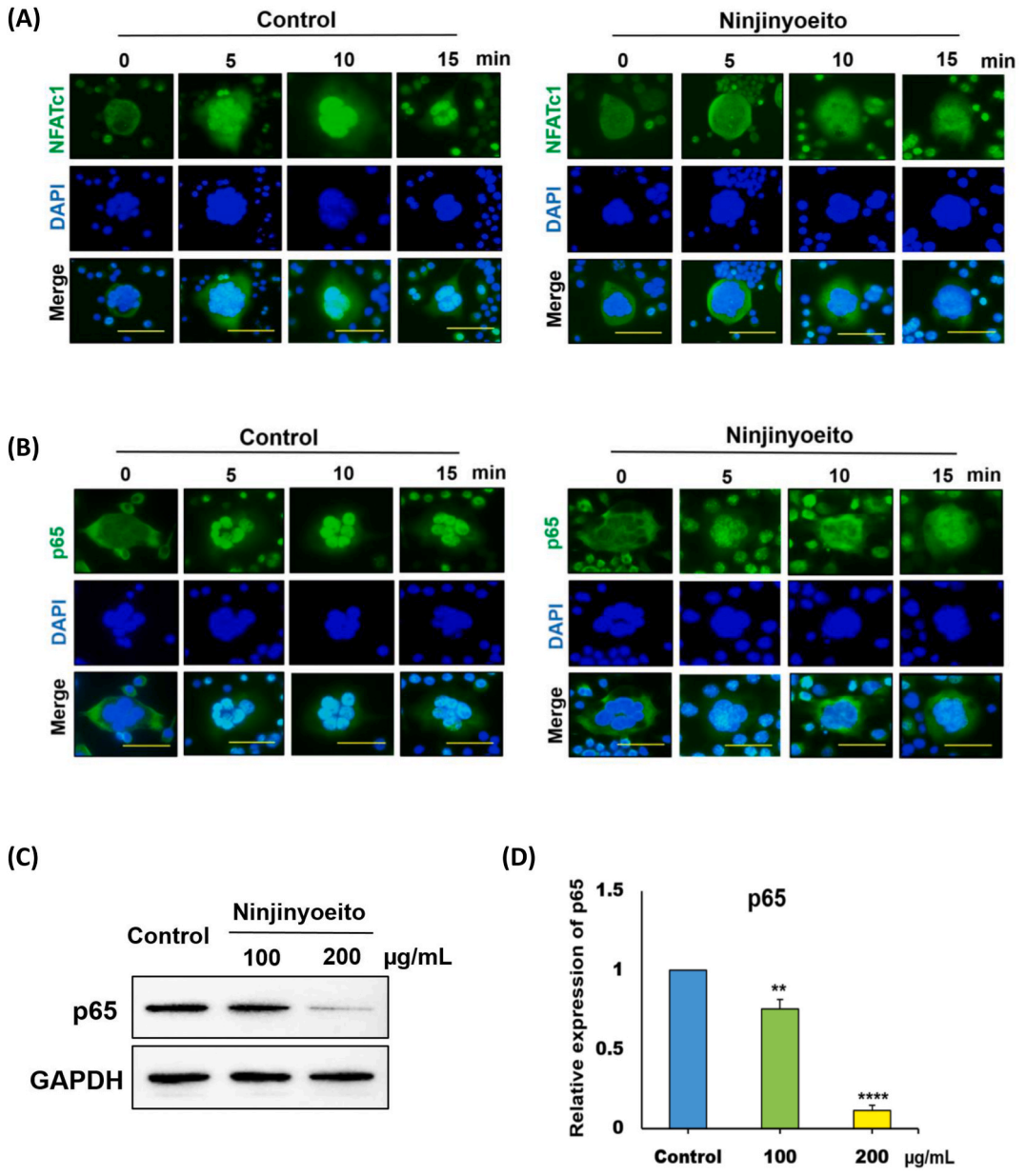

Fig. 4. NYT suppresses nuclear translocation of NFATc1, NF-κB p65 and protein expression of NF-κB p65

(A) Nuclear translocation of NFATc1 was detected by ICC. Green color represents subcellular NFATc1 expression and whitish area represents nuclear localization of NFATc1, (Scale bar=50 μm). The data were confirmed with three independent experiments.

(B) ICC results showing nuclear translocation of p65. The green color represents subcellular NF-κB p65 expression and the whitish area represents nuclear localization of NF-κB p65, (Scale bar=50 μm). The data were confirmed with three independent experiments.

(C) RAW-D cells were co-cultured with NYT and differentiated into OCs with RANKL (100 ng/mL). The protein level of NF-κB p65 was determined by immunoblotting. GAPDH was used as a loading control.

(D) Relative protein expression of NF-κB p65 in control and NYT treated samples, *$p < 0.05$, ****$p < 0.001$. The data were representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

synergistically induce the expression of NFATc1, a master regulator of osteoclastogenesis. NFATc1 is primarily regulated by calcium and calcineurin. Calcineurin inhibitors such as FK506 and cyclosporine A, strongly inhibit RANKL-induced OC differentiation by inhibiting NFATc1 translocation into the nucleus both *in vitro* and *in vivo* [28]. Similar to RANKL-RANK signaling, the binding of M-CSF to its receptor c-fms can induce OC differentiation [8]. These signaling pathways subsequently co-stimulate the expression of OC-related genes, such as *TRAP* and *CTSK*. In this study, we found that NYT inhibited OC differentiation (Fig. 1B and C) and bone-resorbing activity (Fig. 1D and E) by suppressing the protein and mRNA expression of key OC markers (Fig. 2).

MAPKs are proline-directed serine/threonine kinases that play a crucial role in cell growth, differentiation, and apoptosis. Three major MAP kinase subfamilies have been identified in mammalian cells: (1) ERKs, (2) JNKs, and (3) p38 MAP kinases [40]. One study found that mice with a genetic deletion of *erk1* exhibited reduced OC formation *in vivo*, suggesting that ERK1 plays a crucial role in OC differentiation [41]. Additionally, a specific inhibitor of p38α and β (SB203580) suppressed RANKL-mediated OC differentiation in RAW264.7 cells, while OC precursor cells derived from *jnk1*-deficient mice–but not from *jnk2*-deficient mice–exhibited reduced differentiation ability [42,43]. ERKs, JNKs, and

p38 MAPKs play various roles in the formation, maturation, activation, and resorption of OCs. In our study, we found that NYT also suppressed these signaling pathways (Fig. 3), suggesting that NYT may have effects beyond its originally intended use.

Ginsenosides, active components of NYT, have been utilized for anti-aging, anti-inflammatory, and anticancer treatments. Ginsenoside Rg2, in particular, has been shown to inhibit osteoclastogenesis by down-regulating the NFATc1, c-fos, and MAPK pathways [44]. Additionally, another study demonstrated that ginsenoside compound K attenuates ovariectomy (OVX)-induced osteoporosis by suppressing RANKL-induced osteoclastogenesis and oxidative stress [45]. Paeoniflorin, another major constituent of NYT and a natural product derived from *Paeonia lactiflora*, has anti-inflammatory, analgesic, and diuretic properties. It has been reported to regulate osteoclastogenesis and osteoblastogenesis by modulating NF-κB signaling pathway [46]. Another study revealed that paeoniflorin ameliorates collagen-induced arthritis by suppressing the NF-κB signaling pathway during OC differentiation [47]. In our study, we observed an inhibitory action of Paeoniflorin against osteoclastogenesis in a dose dependent manner and this was also confirmed to be due to the abrogation of IκB signaling (supplemantary data). Canonical and non-canonical NF-κB signaling is induced by RANKL and tumor necrosis factor (TNF). RANKL and TNF induce canonical signaling by recruiting TNF receptor (TNFR)-associated factor 6 (TRAF6) and TRAF2/5, respectively, to their receptors. This recruits a complex consisting of IκB kinase, including IKKα, IKKβ, and IKKγ (NF-κB essential modulator, NEMO), which induces the phosphorylation and degradation of IκB-α and the release of p65/p50 heterodimers, which then translocate to the nucleus [48,49]. This induces the expression of c-fos and NFATc1. Non-canonical signaling is activated by IKKα following its phosphorylation by NF-κB-inducing kinase (NIK). NIK then phosphorylates IKKα leading to the processing of p100 in the proteasome to form p52 and the subsequent formation of RelB heterodimers [50]. Ginsenosides and Paeoniflorin are the major components of NYT, and each play a role in regulating osteoclastogenesis. However, the molecular mechanisms underlying these effects remain unclear. In this study, we found that NYT regulates osteoclastogenesis by inhibiting the nuclear translocation of NFATc1 and NF-κB p65, which are the key factors in osteoclastogenesis (Fig. 4).

In clinical trials for osteoporosis, therapeutic agents such as alendronic acid and risedronic acid, which inhibit the mevalonate pathway, are commonly used to suppress OCs. The mevalonate pathway provides prenyl pyrophosphates, which are essential for the activity of GTPases that promote OC differentiation. However, these drugs target only a single stage of OC differentiation and are associated with serious adverse effects, including osteonecrosis of the jaw. Hence, there is a need to explore alternative treatments for osteoporosis. Herbal medicines, such as NYT, have been as potential alternatives. Interestingly, NYT has demonstrated an inhibitory effect on osteoclastogenesis without significant cytotoxicity. Another advantage of NYT is its therapeutic action against physical weaknesses. Physical weakness is often observed in older people and postmenopausal women with metabolic disorders, such as osteoporosis. In this regard, NYT possesses double therapeutic efficacy by simultaneously improving physical strength and treating osteoporosis. These results suggest that NYT could be considered as an alternative candidate for osteoporosis treatment.

In Kampo medicine, NYT serves as a complementary agent. Other complementary agents include Hochuikitou and Juzentaihoto. Juzentaihoto contains components similar to those of NYT; thus, it is possible that it may possess the same inhibitory action against OC differentiation. This Kampo medicine shows potential for further investigation as a therapeutic candidate.

In this study, we evaluated the inhibitory effects of NYT on OC differentiation and elucidated the mechanisms and pathways involved through various experiments. Further *in vivo* studies are required.

5. Conclusion

In the present study, NYT significantly inhibited OC differentiation and bone resorption by downregulating the protein and mRNA expression of various OC markers. Furthermore, we elucidated that NYT ameliorates osteoclastogenesis by inhibiting the phosphorylation of IκBα and MAPKs (ERK, JNK, p38). In addition, NYT abrogated the nuclear translocation of NFATc1, a master regulator of osteoclastogenesis, and NF-κB p65, an important subunit of IκBα. Furthermore, NYT demonstrated minimal cytotoxic effects on OC precursor cells. Thus, NYT has potential as a therapeutic drug with fewer adverse effects for treating osteoclast-related bone metabolic disorders, such as osteoporosis.

Ethical approval

No approval from the ethics committee was required for this study.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Kaung Htike: data collection, Formal analysis, data interpretation, figures, Writing – review & editing. **Kunihiro Yoshida:** Formal analysis, figures, technical assistance. **Takanori Eguchi:** Funding acquisition. **Katsuki Takebe:** Writing – review & editing. **Xueming Li:** technical assistance, literature search. **Yaxin Qu:** technical assistance, literature search. **Eiko Sakai:** Writing – review & editing. **Takayuki Tsukuba:** Writing – review & editing. **Kuniaki Okamoto:** designed the experiments, data interpretation, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.job.2024.09.007.

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