Original Article

Int J Oral Biol 44:89-95, 2019
pISSN: 1226-7155 • eISSN: 2287-6618
https://doi.org/10.11620/IJOB.2019.44.3.89

International Journal of Oral Biology
IJOB
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Piperlongumine suppressed osteoclastogenesis in RAW264.7 macrophages

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Piperlongumine (PL) is a natural product found in long pepper (*Piper longum*). The pharmacological effects of PL are well known, and it has been used for pain, hepatoprotection, and asthma in Oriental medicine. No studies have examined the effects of PL on bone tissue or bone-related diseases, including osteoporosis. The current study investigated for the first time the inhibitory effects of PL on osteoclast differentiation, bone resorption, and osteoclastogenesis-related factors in RAW264.7 macrophages stimulated by the receptor activator for nuclear factor-κB ligand (RANKL). Cytotoxicity was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and osteoclast differentiation and bone resorption were confirmed by tartrate-resistant acid phosphatase (TRAP) staining and pit formation analysis. Osteoclast differentiation factors were confirmed by western blotting. PL exhibited toxicity in RAW264.7 macrophages, inhibiting osteoclast formation and bone resorption, in addition to inhibiting the expression of osteoclastogenesis-related factors, such as tumor necrosis factor receptor-associated factor 6 (TRAF6), c-Fos, and NFATc1, in RANKL-stimulated RAW264.7 macrophages. These findings suggest that PL is suitable for the treatment of osteoporosis, and it serves as a potential therapeutic agent for various bone diseases.

Keywords: *Piper longum*, Osteoclastogenesis, Osteoporosis, Macrophagy

Introduction

Bone is a dynamic organ, which support metabolism, structures, and minerals of the body [1,2]. Bones are formed by regenerated tissues that are modified through osteoclastic bone resorption and osteoblastic bone formation [1,2]. Bone resorption is involved in various physiological functions, such as removal of calcified cartilage during bone growth, modeling of bone during growth or adaptation, maintenance of mineral deterioration, removal of damaged skeletal bone, tooth eruption, and orthodontic tooth movement [3,4]. Osteoblasts rebuild bone by filling holes with collagen and create new bone by laying down calcium and phosphorus deposits (hydroxyapatite) [5]. Osteoclasts are multinucleated giant cells responsible for bone resorption, and mature osteoclasts are involved in the activation of bone resorption. Osteoclast differentiation requires cell–to–cell interactions of osteoclast precursor cells and osteoblasts and is controlled by the receptor activator for nuclear factor–κB ligand (RANKL) [6]. The osteoblast–osteoclast balance is regulated by a mixture of hormones and chemical elements [7].

Chinese medicine has been used for thousands of years in

Received August 8, 2019; **Revised** September 3, 2019; **Accepted** September 4, 2019
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Asia to treat fractures, joint diseases, and gonadal dysfunction. Although *in vitro* and *in vivo* experimental studies have been conducted to explore the potential underlying mechanisms of how herbal product act in target cells, tissues, or organs, more research is needed to shed light on these mechanisms [8]. Recent studies reported that bisphosphonate drugs for osteoporosis treatment were associated with various side effects. Therefore, there is growing interest in new natural substances that can replace these drugs [9–11].

Piperlongumine (PL) (5,6-dihydro-1-[(2E)-1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]-2(1H)-pyridinone) is a natural product found in long pepper (*Piper longum*) [12]. PL belongs to a family of alkaloids [12,13]. It has a variety of pharmacological effects, including antiplatelet aggregation, analgesia, and antifungal properties and hepatoprotective and antiasthmatic activity [14,15]. PL also has antitumor effects, with a marked cytotoxic effect against various types of tumor cells [12]. No studies have examined the effect of PL on bone tissue or bone-related diseases. In the present study, for the first time, we report that PL attenuated osteoclast differentiation and fusion.

Materials and Methods

1. Cell culture

A murine macrophage cell line, RAW264.7, was obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in HyClone™ Dulbecco Modified Eagle's Medium (DMEM; HyClone, Logan, UT, USA) with 10% heat-inactivated fetal bovine serum (FBS; HyClone) at 37℃ in a 5% CO_2 incubator. For osteoclast differentiation, the RAW264.7 cells were cultured in DMEM, supplemented with 10% fFBS and 10 ng/mL of recombinant RANKL (R&D Systems, Minneapolis, MN, USA) for 5 days.

2. MTT assay

The RAW264.7 cells (1×10^4 cells/well) were cultured in a 96-well plate. After 2 hours, the cells were treated with different concentrations (0.1 to 0.5 μg/mL) of PL for 24 hours. Subsequently, an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) solution (500 μg/mL) was added to the plate and incubated for 4 hours until the formation of formazan crystals. The formazan crystals were dissolved using dimethyl sulfoxide (DMSO; Sigma-Aldrich) for 10 minutes on a shaker. The absorbance of each sample was measured at 570 nm on an ELISA reader (Tecan, Männedorf, Switzerland).

3. Tartrate-resistant acid phosphatase staining

The RAW264.7 cells were treated with PL (0.1 to 0.5 μg/mL) in culture medium containing 10 ng/mL of RANKL and cultured for 5 days in a CO_2 incubator. The medium was replaced every day. After 5 days, the cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 10 minutes and then stained using a tartrate-resistant acid phosphatase (TRAP) activity staining kit (Sigma-Aldrich) according to the manufacturer's instructions. TRAP-positive cells were stained deep purple, and TRAP-positive multinucleated cells with three or more nuclei were counted under an Olympus CKX41 inverted light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (Nikon, Tokyo, Japan).

4. Pit formation

The RAW264.7 cells (5×10^4 cells/well) were cultured in an osteo assay plate (Corning, Corning, NY, USA) and treated with PL (0.1 to 0.5 μg/mL) in culture medium containing 10 ng/mL of RANKL. The medium was replaced every day. RAW264.7cells that differentiated into osteoclast-like cells in the osteo assay plate were removed with 5% sodium hypochlorite solution, and the plates were then washed twice with distilled water. The pit area resorbed by osteoclasts on the osteo assay plate was captured with a digital camera (Nikon) attached to an Olympus CKX41 inverted light microscope (Olympus).

5. Western blot analysis

The RAW264.7 cells (1×10^6 cells) were seeded in 100-mm culture dishes. After 2 hours, the medium was replaced with PL (0.1 to 0.5 μg/mL) in culture medium containing 10 ng/mL of RANKL for 5 days. The cells were then harvested, followed by lysis in 100 μL of RIPA buffer (Thermo Scientific, Rockford, IL, USA) and incubated at 4℃ for 1 hour. The cell lysates were collected at 13,200 RPM for 30 minutes at 4℃. Protein quantification, electrophoresis, and detection of protein expression were performed in the same manner as described in a previous study [16]. Primary antibodies against NFATc1, tumor necrosis factor receptor-associated factor 6 (TRAF6), c-Fos, and

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Beverly, MA, USA). Each blot was blotted with horseradish peroxidase-conjugated secondary antibody (1:5000). Immunoblotting with antibodies was performed using SuperSignal™ West Femto (Thermo Scientific, Rockford, IL, USA) enhanced chemiluminescence substrate and detected protein expressions with an Alpha Imager HP (Alpha Innotech, San Leandro, CA, USA). The total proteins of NFATc1, TRAF6, c-Fos, and GAPDH were then analyzed.

6. Statistical analysis

Statistical analyses were performed using Prism software version 5.0 (GraphPad, San Diego, CA, USA). A one-way ANOVA, followed by Dunnett's multiple comparison test was used to analyze cell viability, TRAP positivity, pit formation, and Western blot data. A p-value of < 0.05 was considered significant.

Results

1. Piperlongumine reduced the cell viability of RAW264.7 cells

To determine the appropriate concentration of PL in RAW264.7 macrophages, we used an MTT assay. The cells were treated with 0, 0.5, 1, 2.5, 5, and 10 μg/mL of PL for 12 and 24 hours. At concentrations greater than 1 μg/mL, PL was highly toxic in RAW264.7 cells, as follows: 0.5 μg/mL for 12 hours (97.8%) and 24 hours (60.8%), 1 μg/mL for 12 hours (86.3%) and 24 hours (34.4%), 2.5 μg/mL for 12 hours (82.7%) and 24 hours (21.2%), 5 μg/mL for 12 hours (54.9%) and 24 hours (17.5%), and 10 μg/mL for 12 hours (38.1%) and 24 hours (14.4%) (Fig. 1A). The survival rates of RAW264.7 cells treated with PL at a concentration of 0.5 μg/mL or less for 24 hours were 50% or more, as follows: 0.1 μg/mL (114.7%), 0.2 μg/mL (82.2%), 0.3 μg/mL (71.2%), 0.4 μg/mL (62.5%), and 0.5 μg/mL (57.2%). These results suggested that PL was highly toxic in RAW264.7 macrophages (Fig. 1B).

2. Piperlongumine suppressed receptor activator for nuclear factor-κB ligand-induced osteoclast differentiation and resorption in RAW264.7 cells

To study the inhibitory effect of PL on osteoclast differentiation, we used TRAP, an osteoclast marker. In differentiation medium containing RANKL (10 ng/mL), RAW264.7 cells were treated with PL (0.1 to 0.5 μg/mL) and cultured for 5 days. PL significantly inhibited the formation (TRAP-positive cells) of osteoclasts at concentrations from 0.1 to 0.5 μg/mL (0 μg/mL, 168.3 cells/well; 0.1 μg/mL, 105.2 cells/well; 0.2 μg/mL, 79.6 cells/well; 0.3 μg/mL, 31.3 cells/well; 0.4 μg/mL, 14.0 cells/well; 0.5 μg/mL, 5.8 cells/well) (Fig. 2).

To detect the bone resorption activity of the osteoclasts, we investigated resorption pit formation on a calcium-coated plate. The RAW264.7 cells were cultured for 5 days under the

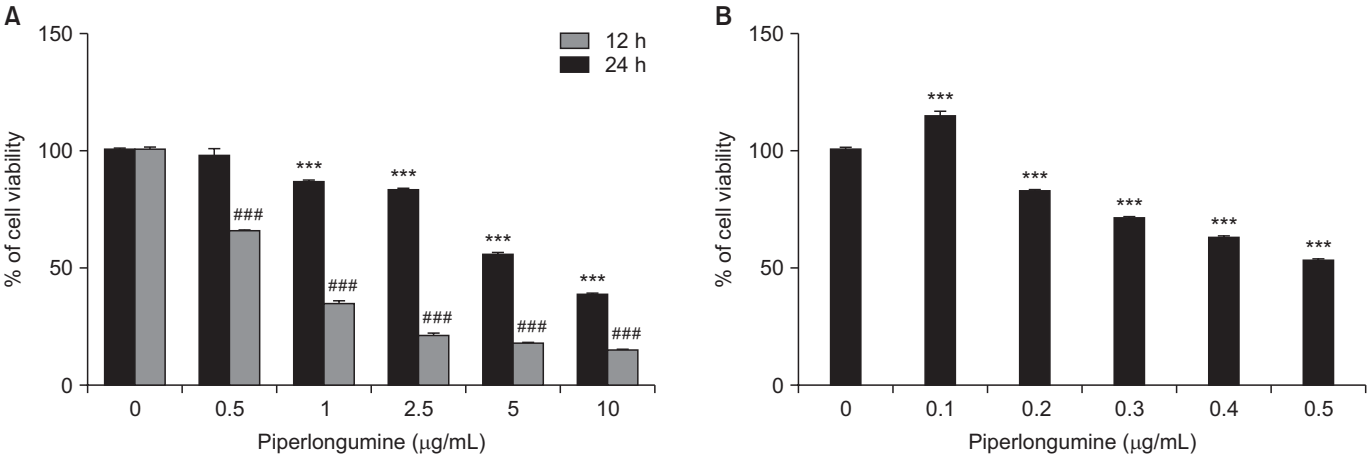

Fig. 1. Cytotoxic effect of piperlongumine in RAW264.7 cells. (A) Cell viability of RAW264.7 cell macrophages treated with high concentrations (0 to 10 μg/mL) of piperlongumine for 12 and 24 hours. The values represent the means ± standard deviations of three independent experiments. (B) Cell viability of RAW264.7 cell macrophages treated with low concentrations (0 to 0.5 μg/mL) of piperlongumine for 24 hours. The values represent the means ± standards of three independent experiments.
***$p < 0.001$ and $^{###}p < 0.001$ compared with the control.

same culture conditions as in TRAP staining. PL (0.1 to 0.5 μg/mL) clearly reduced the number and size of resorption pits (pit/well) in a dose-dependent manner (0 μg/mL, 86.3%; 0.1 μg/mL, 63.7%; 0.2 μg/mL, 42.7%; 0.3 μg/mL, 20.0%; 0.4 μg/mL, 10.2%; and 0.5 μg/mL, 8.7%) (Fig. 3). These results suggested that PL inhibited osteoclast formation and bone resorption via RANKL-induced osteoclastogenesis.

3. Piperlongumine inhibited osteoclastogenesis markers of receptor activator for nuclear factor-κB ligand-induced osteoclastogenesis

To determine the inhibitory effect of PL on osteoclastogenesis-related factors (i.e., TRAF6, NFATc1, and c-Fos), we used a Western blot assay. The RAW264.7 cells were treated with RANKL and PL (0.1 to 0.5 μg/mL) for 5 days. Compared with an untreated group (0 μg/mL of PL), PL reduced the protein expression of TRAF6, NFATc1, and c-Fos at a concentration of

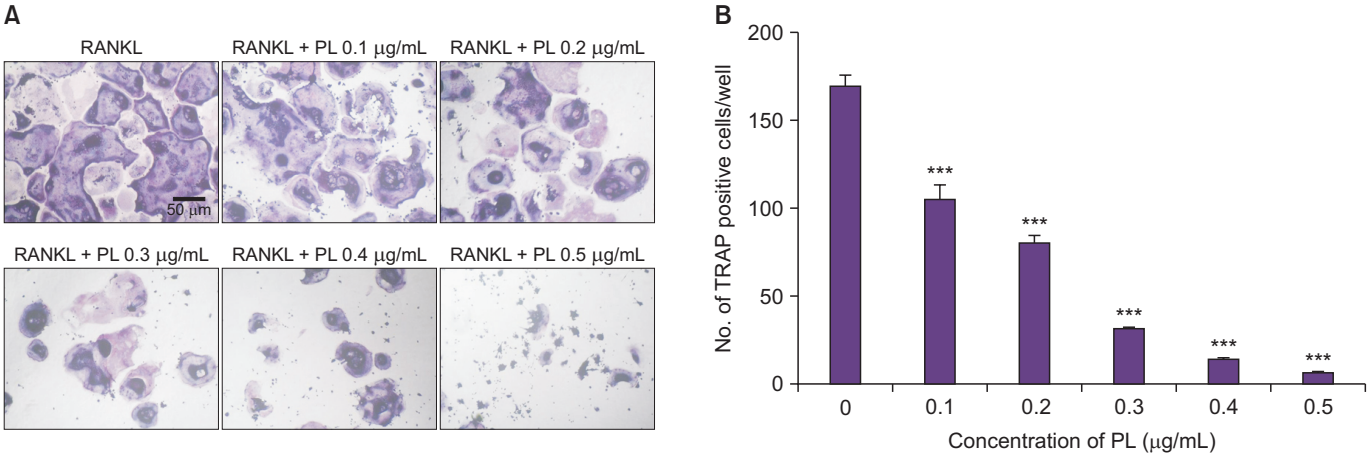

Fig. 2. Inhibitory effect of piperlongumine on osteoclast differentiation in RANKL-induced RAW264.7 cells. (A, B) Number of TRAP-positive multinucleated RAW264.7 cells containing three or more nuclei after treatment with 10 ng/mL of RANKL and 0.1 to 0.5 μg/mL of piperlongumine. The values represent the means ± standard deviations of three independent experiments.
RANKL, receptor activator for nuclear factor-κB ligand; PL, piperlongumine; TRAP, tartrate-resistant acid phosphatase.
***$p < 0.001$ compared with the control.

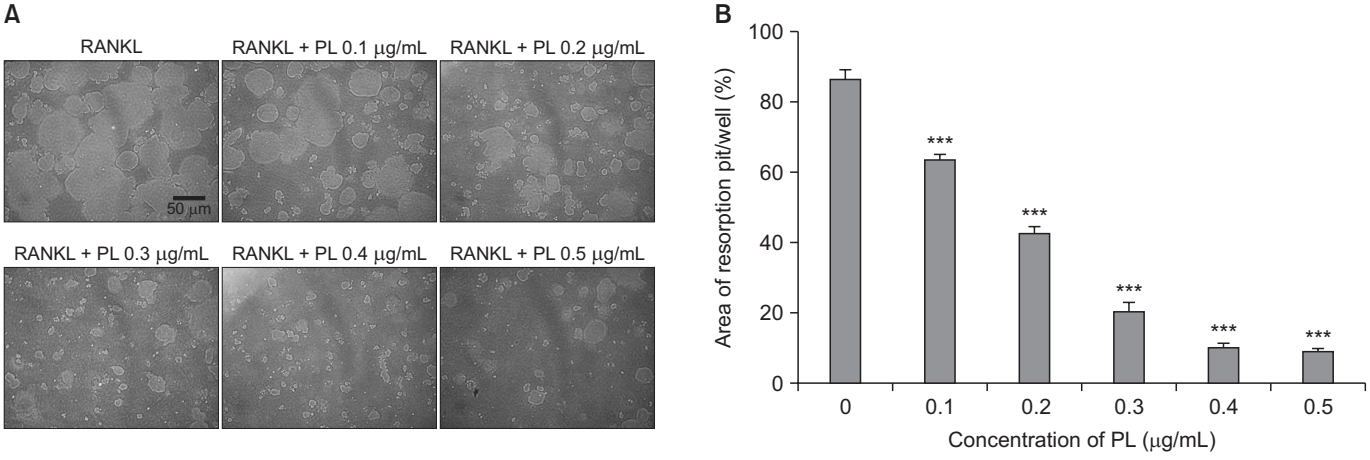

Fig. 3. Inhibitory effect of piperlongumine on bone resorption in RANKL-induced RAW264.7 cells. Resorption pit formation and resorption areas (A) are expressed as the area of pit formation with respect to the total area (B). The values represent the means ± standard deviations of three independent experiments.
RANKL, receptor activator for nuclear factor-κB ligand; PL, piperlongumine.
***$p < 0.001$ compared with the control.

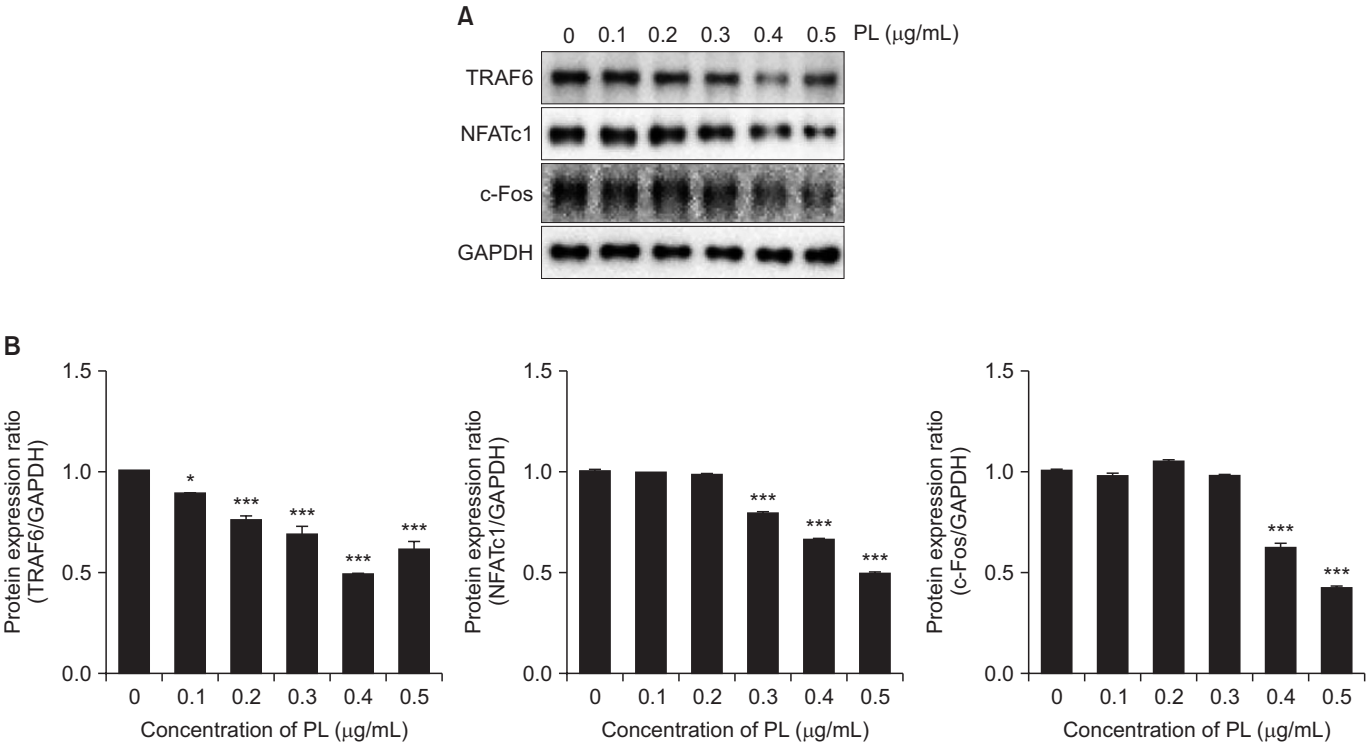

Fig. 4. Suppression of osteoclastogenesis transcription factors in receptor activator for nuclear factor-κB ligand-induced RAW264.7 cells. (A) Expression of osteoclastogenesis-related proteins according to Western blotting. (B) A graph of quantified protein expression is shown below the Western blotting image. The expression data were normalized using GAPDH. The values represent the means ± standard deviations of three independent experiments.
PL, piperlongumine; TRAF6, tumor necrosis factor receptor-associated factor 6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
*p < 0.05 and ***p < 0.001 compared with the control.

0.3 μg/mL or higher (Fig. 4). Therefore, PL suppressed osteoclastogenesis markers.

Discussion

New therapies and supplements are urgently needed to manage and promote bone health due to the rapid growth of the aging population, increased life expectancy, and associated financial burden on the health care system and individuals [17]. If bone resorption by osteoclasts becomes more active than bone formation by osteoblasts, bone density decreases due to an imbalance in the bone remodeling process, leading to bone diseases, such as osteoporosis, and deterioration of bone mass and microstructure [18]. Various treatments, including hormone replacement therapy (e.g., parathyroid hormone), bisphosphonates, and denosumab, have been proposed for osteoporosis, but all are associated with side effects when used for long term treatment [19]. Increasingly, natural products and herbal medicines are attracting attention because they have therapeutic potential in reducing bone loss and maintaining bone health [20]. For example, bioactive compounds found in the plants *Herba epimedii*, *Rhizoma drynariae*, *Fructus psoraleae*, and *Cortex eucommiae* include icariin and naringin, both of which are effective in preventing and treating osteoporosis [21]. The ideal osteoporosis treatment should promote osteoblast differentiation and inhibit the formation and destruction of osteoclasts [22]. In this respect, an agent is needed that improves bone quality while reducing the side effects of existing osteoporosis treatments. In the present study, we demonstrated for the first time that PL, a natural product found in long pepper (*P. longum*), inhibited RANKL-induced osteoclastogenesis in RAW264.7 macrophages.

Osteoblast cells express RANKL and macrophage colony-stimulating factor (M-CSF), which interact with receptors in monocytes and macrophages to upregulate osteoclastogenic molecules, resulting in the formation of multinuclear osteoclasts due mononuclear/macrophage fusion [23]. Osteoclasts are produced in an acidic microenvironment degrading the substrate and characterized by the expression of TRAP [24]. TRAP staining is used as a histochemical marker for the detection of osteoclasts, macrophages, dendritic cells, and immune cells, and it can be easily detected using light microscopy [24-

26]. The proton pump is activated in the ruffled membrane of mature osteoclasts in contact with the substrate to create an acidic microenvironment to melt the bone matrix and form a "pit-like" structure [23]. In this study, treatment with RANKL alone induced macrophage fusion/differentiation into multinucleated osteoclasts in RAW264.7 macrophages, and combination treatment with RANKL and PL inhibited TRAP-positive osteoclasts and bone resorption pits in a dose-dependent manner.

RANK, RANKL, and M-CSF induced osteoclast formation in the presence of co-stimulatory signals. RANKL induced the activation of TRAF6 and the c-Fos pathway, which were amplificate the NFATc1. Once this process accumulated, it produced osteoclast differentiation-associated transcription factors [27].

In the present study, PL significantly inhibited the expression of osteoclastogenesis-related factors, such as TRAF6, c-Fos, and NFATc1. Therefore PL may actively inhibit osteoclast differentiation-related factors, which lead to osteoclast differentiation and maturation in RANKL-stimulated RAW264.7 macrophages. These findings suggest that PL may be a suitable treatment for osteoporosis and that it may serve as a potential therapeutic agent for various bone diseases.

Acknowledgements

This work was supported by a 2-Year Research Grant of Pusan National University.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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