**Pectolinarigenin prevents bone loss in ovariectomized mice and inhibits RANKL-induced osteoclastogenesis via blocking activation of MAPK and NFATc1 signaling**

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**Running title: PEC attenuates RANKL-induced osteoclastogenesis**

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## Abstract

Osteoporosis (OP) is a metabolic disease caused by multiple factors, which is characterized by reduction of bone mass per unit volume and destruction of bone microstructure. Aberrant osteoclast function is the main cause of OP, therefore, regulating the differentiation and function of osteoclast is one of the treatment strategies for OP. Pectolinarigenin (PEC) is a medicinal implant isolated from *Fragrant Eupatorium*. Our experimental data showed that PEC was able to inhibit RANKL-induced osteoclastogenesis *in vitro*, by TRAcP staining, Fibrous actin (F-actin) ring formation and hydroxyapatite resorption assays. In terms of mechanism, PEC inhibited the osteoclastogenesis-related genes expression, including cathepsin K (*Ctsk*), matrix metalloproteinase 9 (*Mmp9)*, TRAcP (*Acp5*). Western blot demonstrated that PEC could significantly block the activation of RANKL-induced MAPK signaling cascades and was able to suppress the protein expression of NFATc1 and C-Fos. Meanwhile, the intracellular ROS levels were also reduced by PEC with a concentration-dependent manner. Further, PEC could prevent the ovariectomy-induced bone loss *in vivo*. Summarizing all, our data suggested that PEC inhibits osteoclast formation and function and RANKL signaling pathways, and thus could potentially be used in the treatment the osteoclast-related bone loss diseases.

Key words：Osteoporosis, osteoclast, Pectolinarigenin, MAPK

## Introduction

In mature bones, bone remodeling gradually replaces old and damaged bone tissue with new tissue, which provides source of calcium and phosphorus and maintains bone health. In order to adapt mechanical loading and growth, bone tissue is constantly self - reshaping to maintain mineral salt metabolism balance (Seeman, 2009). Remodeling of bone tissue involves two actions, including osteoblast mediated osteogenesis and osteoclast mediated bone distruction . Abnormal bone resorption or bone formation can lead to steady-state disorders of the skeletal system, such as Paget' s disease and loosening around the prosthesis resulting from joint replacement (AbuAmer et al., 2007; Lewis et al., 1993). Fracture is the most common

complication of osteoporosis. Once the fracture happens to the patients, life quality sharply declines and the risk of secondary fracture is significantly increased.

Osteoclasts are multinucleated macrophages originated from the hematopoietic stem cell (He et al., 2018). Macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κ-B ligand (RANKL) are required for the differentiation of osteoclasts (Arai et al., 1999; Novack, 2011). Binding of RANKL to the receptor activator of nuclear factor κ-B (RANK) recruits the tumor necrosis factor receptor associated factor 6 (TRAF6) which initiates downstream multiple signaling cascades, including NF - κB and MAPK pathways (Kikuta and Ishii, 2013; Takayanagi, 2007). Following activation, nuclear factor of activated T-cell (NFATc1) leads to the differentiation of the bone marrow-derived precursor cells towards osteoclasts (Takayanagi et al., 2002). In addition, reactive oxygen species (ROS) are commonly associated with cytotoxicity and cell survival (Carmody and Cotter, 2001). The destitute generation of ROS was also found to be consistent with poor RANKL-mediated osteoclast formation (Hyeon et al., 2013).

Pectolinarigenin (PEC) is a natural flavonoid compound, which was demonstrated to inhibit the growth of nasopharyngeal carcinoma cells (Wang et al., 2016). In addition, PEC can inhibit the growth and metastasis of osteosarcoma cells by interacting with the STAT3 pathway (Zhang et al., 2016). However, the role of PEC on the RANKL-induced osteoclastogenesis is not unknown.

In the study, we examined the invitro effect of PEC on osteoclast formation and activation, as well as signaling mechenisms, and we also determined the therapeutic potential of PEC on the the osteolytic condition in ovariectomized mice. We found that PEC inbited RANKL-induced osteoclast formation and function, ROS production, as well as MAPK and NFATc1 signaling pathways. Further, PEC prevented the ovariectomy-induced bone loss *in vivo*, suggesting a potential therapeutic effect for osteolysis.

## 2. Materials and methods

### 2.1. Materials and reagents

α-MEM and FBS were purchased from Gibco-BRL (Sydney, Australia). Pectolinarigenin (Fig.1A) was purchased from Chengdu Must Bio-Technology Co., Ltd (Chengdu, Sichuan Province, China). TRAcP staining kit, cell counting kit-8, cell dissociation solution and DAPI were procured from Sigma-Aldrich (St Louis, MO, USA). All primary antibodies were both purchased from Cell Signaling Technology (Danvers, MA). R&D Systems (Minneapolis, MN, USA) offered macrophage colony stimulating factor (M-CSF). Receptor activator of nuclear factor κ-B ligand (RANKL) was obtained as previously described (Xu et al., 2000). Beyotime (Jiangsu, China) offered ROS detection kit. Rhodamine-conjugated phalloidin was purchased from Molecular Probes, Inc. (Eugene, OR, USA).

### 2.2. Cell culture and *in vitro* osteoclastogenesis assay

Bone marrow macrophages (BMMs) used in this study was obtained by flushing the bone marrow from the femur and tibia of 6-week-old C57/BL6 mice. $6\times10^3$ BMMs seeded in each well of 96-well culture plates. After the cells were cultured overnight to adhere, BMMs were stimulated with complete medium supplemented with M - CSF (50 ng/ml) and RANKL (100 ng/ml), in the presence or absence of PEC (0, 1.25, 2.5, 5, 10 μM). After 5 days, mature osteoclasts were formed. After the cells were fixed and washed, the cells were stained using TRAcP staining kit, TRAcP positive multinucleated cells were counted as osteoclasts. Besides, we also explored the effects of PEC in osteoclast differentiation at different stage. Cells were plated into 96-well culture plates with M-CSF (50 ng/ml) and RANKL (100 ng/ml), and PEC was added on day 1, day 3 and day 5, respectively, or whole treatment period (day 1-5). Finally, the number of osteoclasts in every well was analyzed using Image J software.

### 2.3. CCK-8 cell proliferation and cytotoxicity assay

BMMs were plated in 96-well plates at a density of $6\times10^3$ cells per well and cultured with M-CSF (50 ng/mL) overnight. The following day, cells were treated with different concentrations of PEC (1.25, 2.5, 5, 10, 20 μM) for 48 h. And then CCK-8 solution (10 μl/well) was added into each well and incubated at 37 °C, 5%$CO_2$ for 2 h.

After incubation, the absorbance was measured at 450 nm using a microplate reader (Multiscan Spectrum; Thermo Labsystem, Chantilly, VA).

**2.4. Fibrous actin (F-actin) rings formation assay**

BMMs were seeded in 6-well culture plates with M-CSF (50 ng/ml) overnight to adhere. Mature osteoclasts were induced by M-CSF (50 ng/ml) and RANKL (100 ng/ml), with or without varying doses of PEC (5, 10 μM). After this, the plates were fixed in 4% paraformaldehyde, and 0.1% Triton X-100-PBS were used to permeabilizing osteoclast, followed by being blocked with 3% BSA in PBS for 2 h. The F-actin ~~ring~~belt was stained by rhodamine - conjugated phalloidin in dark for 1h, and the osteoclasts were subsequently washed with PBS for three times prior to the staining of DAPI for nuclei. Finally, images were captured under fluorescent microscope.

**2.5. Hydroxyapatite resorption pit formation assay**

Osteoclast activities were also examined via hydroxyapatite resorption assay. BMMs were plated into 6-well culture plates at a density of $1 \times 10^5$ and stimulated in the presence of 50 ng/ml M-CSF and 100 ng/ml RANKL. The osteoclasts were collected by cell dissociation solution at the early stage of osteoclast differentiation and translocated into hydroxyapatite-coated plates (Corning, Inc., NY, USA). The cells were incubated with M-CSF and RANKL in the presence or absence of PEC (5, 10 μM) until mature osteoclasts were formed, followed by being removed using sodium hypochlorite solution. The areas of Hydroxyapatite resorption pit were measured under the microscope (Nikon Corporation, Tokyo, Japan) and the areas of resorption were analyzed by Image J.

**2.6. Intracellular reactive oxygen species generation assay**

BMMs were cultured with RANKL and M-CSF, with varying concentrations of PEC (5, 10 μM) for 48 h. And then the DCFH-DA diluted in pure α-MEM (without serum) (1:1000), was added into each well for 1 ml. The cells were incubated for 30 minutes at the conditions of 37℃, 5% $CO_2$ followed by ROS assay using ROS detection kit. Subsequently DCF fluorescence was detected by fluorescence microscope at an

excitation wavelength of 488 nm and at an emission wavelength of 525 nm. The average fluorescence intensity was analyzed using Image J software.

### 2.7. Western blotting

BMMs were plated into 6-well culture plates evenly. After adherence, the cells were incubated in serum-free medium for 3 h and then pre-treated with or without 10 μM PEC for 1 h. The cells were lysed using radioimmunoprecipitation assay buffer to collect the total protein after stimulated with RANKL (100 ng/ml) for 0, 5, 10, 20, 30, 60 minutes. To explore the PEC-treated long-term effects on osteoclastogenesis BMMs ($1 \times 10^5$ cells per well) were cultured in complete medium containing M-CSF (50 ng/ml) and RANKL (100 ng/ml), simultaneously stimulated in the presence or absence of PEC (10 μM) for stated time (0, 1, 3, 5 days). Protein was separated on sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose membranes. The NC membranes were blocked in 5% BSA for 1 h to interdict nonspecific bindings and then incubate with different primary antibodies (1:1000) at 4°C for 12 h. The next day, these NC membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibodies in dark for 1.5 h. After the membranes were washed with a mixture of tris-buffered saline (TBS) and Tween 20 for 3 times, the antibody reactivity was probed with Image-quant LAS 4000 (GE Healthcare, Silverwater, Australia) and the gray values were analyzed in image J.

### 2.8. Quantitative reverse transcription PCR assay

For real-time PCR, BMMs ($1 \times 10^5$ cells/well) were cultured in 6-well plates with RANKL and M-CSF for 5 days, with varying doses of PEC (5, 10 μM). Total RNA was obtained using Trizol reagent and we used RevertAid First Strand cDNA Synthesis Kit to synthesize the cDNA. The cDNA was then used to carry out the qPCR (RT-PCR), based on SYBR Green PCR MasterMix, and the expression levels of osteoclast-related messenger RNA were normalized to GAPDH expression and calculated by comparative Ct (ΔCt) method. The specific primers sequences were shown in Table 1.

### 2.9. Animal model construction and effect verification *in vivo*

All experimental procedures were performed in accordance with the guidelines of the Animal Care Committee of Guangxi Medical University. 10-week-old female C57/BL6 mice were divided into 4 groups (n=6) randomly, including ovariectomized (OVX) group (OVX mice injection with normal saline), sham group (sham operated control and injection with normal saline), low-dose group (OVX mice treatment with 5 mg/kg PEC), and high-dose group (OVX mice treatment with 10 mg/kg PEC). These mice were anesthetized with 10% chloral hydrate after fasting for 6 h and then the abdominal cavity was cut though the midline incision of the waist and back to remove bilateral ovaries and partial oviducts. After operation, the mice were put into cage to move freely and eat disinfectant pellets and purified water to recover for 7 days. Then, 5 mg/kg/day, 10 mg/kg/day PEC or saline was administered every two days over a 6-week-treatment period and the administration route was peritoneal injection. At the end of the experiment, 10% chloral hydrate were used to anesthetize the OVX mice before sacrificed, and the tibia was achieved.

The tibia separated from mice was fixed with 4% paraformaldehyde for micro-CT scanning to obtain 2-dimensional and 3D bone microstructure images. Histomorphometric parameters of area-of-interest at the growth plate were determined by CTAn software (Bruker micro-CT, kontich, Belgium), including bone volume/total volume (BV/TV), bone surface area/total volume (BS/TV), and trabecular number (Tb. N). For histopathology, the fixed tibias were soaked in 12% EDTA (pH 7.4) for 2 weeks to decalcify and then embedded in paraffin to stain for the TRAcP activity. After staining, images enlarged at the magnifications of 40 and 100 times were taken by microscope. TRAcP positive cell number was analyzed using Image J software.

### 2.10. Statistical analysis

All results are representative of three independent experiments and are presented as mean ± SD. One - way ANOVA test and Student's t-test were used to examine the significance of differences among the results, with $p < 0.05$ being considered as significant (95% confidence intervals).

## 3. Results

### 3.1. PEC attenuated RANKL-induced osteoclast formation and function *in vitro*

The chemical structure of PEC is presented in Figure 1A. Firstly, using CCK-8 cell proliferation and cytotoxicity assay, we found that PEC did not induce cell death in osteoclatic precursor cells at concentration up to 20 μM (Figure 1, B). Next, to determine the effects of PEC on osteoclast differentiation, we performed two experiments including dose-dependent osteoclastogenesis assay and time-dependent osteoclastogenesis assay. As a result, PEC impaired RANKL induced osteoclastogenesis in a concentration-dependent manner, especially at the doses of 10 μM (Figure 2, A-C). The numbers and areas of TRAcP positive cells were decreased obviously by the treatment of PEC (10 μM) at the early stage of osteoclast differentiation (Day 1) (Figure 2, D-F). To further investigate whether the osteoclast functions were affected by PEC, we performed the F-actin formation assay and hydroxyapatite resorption assay. It was revealed that well-defined fibrous actin (F-actin) belt~~ring~~s were observed after RANKL stimulation while the average number of nuclei per cell and the areas of fibrous actin (F-actin) belts~~rings~~ each well were abolished substantially in the groups treated with PEC at the concentrations of 5 and 10 μM (Figure 3, A-C). The results of hydroxyapatite resorption assay were consistent with the degree of F-actin formation, and we used hydroxyapatite-coated plates to evaluate the ability of bone resorption after treated with PEC. We found that PEC could prevent the hydroxyapatite resorption pit formation (Figure 3, D-E). Hence, PEC attenuates RANKL-induced osteoclast formation and function *in vitro* without any overt cytotoxicity.

### 3.2. PEC depresses intracellular ROS level

As for detecting the intracellular ROS level, we used fluorescent probe DCFH-DA to examine whether PEC had any effect on RANKL-induced ROS generation. As demonstrated in Figure 3, the ROS production and the intensity of DCF fluorescence per positive cell in PEC-treated groups significantly decreased compared with the RANKL-stimulated group (Figure 3, F-G). Thus, the data indicated that PEC suppressed RANKL-induced reactive oxygen species production.

### 3.3. PEC suppressed osteoclast formation via MAPK signaling pathway

To expound the molecular mechanisms by which PEC inhibited RANKL-induced osteogenesis, we performed western blot analyses. The results of western blot showed that RANKL stimulation for 5 and 10 min markedly increased phosphorylation of p38 while this change was repressed in the PEC-treated group (Figure 4, A-B). In addition, phosphorylation of JNK was significantly inhibited at all time points by PEC (Figure 4, A-B). The effect of PEC on ERK phosphorylation was obscure (Figure 4, A-B) as well as NF-κB signaling, the inconspicuous upgradation of IκBα and the anti-phosphorylation of p65 were observed (Figure 4, C-D). These data indicated that PEC inhibited the activation of MAPK pathway especially by suppressing the activity of p38 and JNK but it has little effect on NF-κB signaling.

### 3.4. PEC abrogated RANKL-induced activation of NFATc1, C-Fos and expression of NFATc1-targeted gene

We next investigated the effect of PEC on NFATc1 and C-Fos activation, which were downstream of MAPK signaling pathway. Under the interference by PEC, the NFATc1 and C-Fos proteins dramatically declined after stimulating with RANKL for three days (Figure 4, E-F). Following these results, we conjectured that PEC could reduce the expression of various osteoclast marker genes. As demonstrated in RT-PCR, the expression level of NFATc1-targed genes such as *Ctsk*, *Atp6v0d2*, *Acp5* and *Mmp9* were down-regulated by PEC in a dose-dependent manner (Figure 4G-J). Thus, these results revealed that PEC was able to inhibit NFATc1, C-Fos protein expression and relevant osteoclast-specific gene expression regulated by NFATc1.

### 3.5. Anti-osteoclast activity of PEC in ovariectomized mice

Finally, we employed an animal model to further investigate the anti-osteoclast activity of PEC *in vivo*. The ovariectomized mice were generated and treated with PEC for 6 weeks, and the 3D reconstruction images of the proximal tibia in PEC-treated group revealed that PEC markedly prevent OVX-induced osteolysis. In contrast, the samples isolated from OVX group and sham-operation group exhibited loss of bone trabecular (Figure 5, A). The change of bone histomorphometric parameters also demonstrated that PEC inhibited OVX-induced bone loss. Notably,

the bone surface area/total volume (BS/TV), trabecular bone volume percentage (BV/TV), as well as the trabecular number (Tb. N) were increased in PEC-treated group sample (Figure 5, B-D). And the conclusion that PEC could prevent OVX-induced osteoclastogenesis *in vivo* was also confirmed in histopathology assay. Consistent with the above results, our data showed that TRAcP positive cells were obviously reduced in PEC-treated group (Figure 5, E-G) compared with the OVX group and sham-operation group. In brief, our findings indicated that PEC has inhibitory effect against the over-activation of osteoclasts in ovariectomized mice.

**Discussion**

Bone metabolism is a dynamic process, and with the changes of mechanical stress, hormones and cytokines, dynamic reconstruction is maintained by bone formation and bone distruction. By this way, the bone tissue has normal shape and functions (RV et al., 2018). Osteoporosis is a metabolic disorder associated with systemic bone degeneration (Bar-Shavit, 2010). The exorbitant bone resorption was caused by over-activation of osteoclasts, and increased osteoclastogenesis and function is one of the key causes of osteoporosis (Zhou et al., 2016). In the treatment for osteoporosis, inhibition of osteoclast formation is still remaining as the potential target. Current treatments are clinically challenged with side-effects, including osteonecrosis with bisphosphonate and increased risk of breast cancer (Perazella and Markowitz, 2008; Rachner et al., 2011). Of note, A large number of studies have shown that isoflavones have significant anti-osteoporosis effects (Wang et al., 2017; Vitale et al., 2013), such as flavonoids isolated from soybeans are phytoestrogen-like compounds that were previously used as estrogen receptor modulators (Juma et al., 2012). Pectolinarigenin, a flavonoid, has inhibitory activities on melanogenesis (Lee et al., 2017). In this study, we demonstrated the anti-osteoclast activity of PEC *in vitro* and prevented bone loss in ovariectomized mice. Based on the structure of PEC (Figure 1A), it is possible to further develop different ramifications to treat osteoporosis.

As for the molecular mechanisms of RANKL-induced osteoclastogenesis, multiple pathways, such as NF-κB, AKT and MAPK, were all significant (Dejardin, 2006). TRAF-6 is a necessary upstream effector in RANKL signaling pathway and is recruited by trimers formed by the binding of RANK and RANKL, leading to subsequent phosphorylation of IKK. Following activation of IKK, IκB-α was degraded and NF-κB dimers, such as NF-κB p65, were released and then translocated to the nucleus, modulating osteoclastogenesis gene transcription (Soysa and Alles, 2009). In our study, PEC had little effect on RANKL –induced NF-κB signaling pathway, since it failed to inhibit the degradation of IκB-α and the phosphorylation of p65.

MAPK signaling cascade was also important for the regulation of osteoclast formation, including ERK, JNK and p38 pathways (Hagemann and Blank, 2001). In

this pathway, phosphorylation of JNK induces the activation of AP-1 to targeting the AP-1 regions of DNA, and initiates the transcription of osteoclast marker genes such as MMPs and TRAcP, thus stimulating the differentiation of osteoclast (Vaira et al., 2008). Significantly, p38 pathway is also a vital pathway in osteoclast differentiation (Matsumoto et al., 2000). Inhibition of p38 signaling pathway attenuates osteoclast differentiation and local bone resorption (Choi et al., 2012). We found that PEC markedly depressed the MAPK signaling cascade, especifically on the phosphorylation of JNK and p38, while had no significant effect of ERK signaling pathway.

NFATc1 and c-Fos activated by MAPK are important downstream regulators of osteoclast formation (Nishikawa et al., 2010). As described above, AP-1 is activated by phosphorylation of JNK, while c-Fos is a crucial component of AP-1. Mutagenesis of c-Fos prevented osteoclast formation and leads to osteopetrosis (Grigoriadis et al., 1994). In addition, NFATc1 serves as a primary regulator of gene expression of terminal RANKL-induced osteoclastogenesis. Previous studies found that NFATc1-deficient embryonic stem cells (ESCs) failed to form mature osteoclast (Takayanagi, 2007), and mice lacing NFATc1 developed osteopetrosis (Takayanagi et al., 2002). In the current study, we demonstrated that PEC inhibited the protein levels of NFATc1 and c-Fos. Notably, NFATc1 plays a key role in the regulation of the osteoclast markers gene expression such as *Ctsk*, *Atp6v0d2, MMP9 and Acp5* (Feng et al., 2009; Kim et al., 2008; Matsuo et al., 2004). Consistent with the RANKL-induced activation of NFATc1 was depressed, the expression of these NFATc1-targeted genes was also inhibited by treatment of PEC, resulting in obstruction of osteoclast formation. Furthermore, the F-actin also plays an essential role on osteoclast function (Garbe et al., 2012; Teitelbaum, 2000). We found that PEC was effective on suppressing F-actin ring formation and osteoclast bone resorption in a concentration-dependent manner.

Currently, growing evidence has suggested that RANKL-induced intracellular ROS levels are associated with osteoclast formation (Kim et al., 2017; Lee et al., 2005; Yip et al., 2005). Osteoclast differentiation is promoted in the Nrf2-deficient mice, because RANKL-induced ROS was increased (Hyeon et al., 2013). After stimulation of hydrogen molecules, RANKL-induced osteoclastogenesis was attenuated via

down-regulation of intracellular ROS generation (Li et al., 2014). Besides, ormeloxifene inhibited osteoclast formation by repressing production of RANKL-induced ROS (Kharkwal et al., 2012). Thus, we investigated the effect of PEC on intracellular ROS and found that the accumulations of ROS were dramatically decreased by PEC treatment in a dose-dependent manner.

PEC had anti-osteoclast activity *in vitro*, following which, we examined the effects of PEC *in vivo*. Our data suggested that PEC protected against bone loss in OVX mice. As consequences of animal experiment, the reduction of TRAcP positive osteoclasts cell number and the improved quantity of bone trabecula, suggesting that PEC protected against bone loss in OVX mice. Collectively, although the PEC interrupted the osteoclastogenesis by inhibiting the activation of MAPK signaling cascade and NFATc1 *in vitro*, the effects of PEC on osteoblast formation and function is yet to be determiened, which is necessary in future studies. Our study indicated that PEC could act as a potent restrainer of RANKL-induced osteoclastogenesis against diseases caused by over-activation of osteoclast such as osteoporosis.

**Acknowledgements**

This work was supported in part by National Natural Science Foundation of China (81501910), the Natural Science Foundation of Guangxi Province (2015GXNSFCA414001 , 2015GXNSFDA139019), the Guangxi Collaborative Innovation Center for Biomedicine talent cultivation (GCICB-TC-2017001). This study was also supported in part by grants from the Australian Health and Medical Research Council (NHMRC, APP1107828, APP1027932, APP1163933).

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