Inhibitory Effects of Water Extracts of Eucommiae Cortex and Psoraleae Semen Alone and in Combination on Osteoclast Differentiation and Bone

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[Abstract]

Objectives: The purpose of this study was to evaluate the effects of water extracts of Eucommiae cortex (EC), Psoraleae semen (PS), and their combination on receptor activator of nuclear factor-κappa-B ligand (RANKL)-induced osteoclast differentiation.

Methods: We assayed the protein expression levels of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), c-Fos, mitogen-activated protein kinases (MAPKs), and β-actin in cell lysates using western blotting. Similarly, mRNA expression levels of NFATc1, c-Fos, tartrate-resistant acid phosphate (TRAP), and glyceraldehyde-3-phosphate dehydrogenase, spermatogeni (GAPDH) from bone marrow macrophages (BMMs) were analyzed using reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, we determined the anti-osteoporotic effects of the water extracts of EC, PS, and their combination in a lipopolysaccharide (LPS)-induced bone-loss mouse model.

Results: The in vitro data revealed that the combination of EC and PS extract showed a more remarkable inhibition of osteoclast differentiation than each herb did alone. The combination downregulated the induction of c-Fos, NFATc1, and TRAP by suppressing the phosphorylation of p38 and c-Jun N-terminal kinases (JNKs) and inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Lastly, the in vivo data showed that PS reduced the LPS-induced bone erosion.

Conclusion: The result of this study suggests that EC and PS could be potential therapeutic agents for bone loss diseases such as osteoporosis.

※This study was supported by academic research grant of Wonkwang University in 2017
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Key words: Eucommiae cortex; Psoraleae semen; Osteoporosis; Osteoclast differentiation; Bone resorption

Received: 2016. 11. 28. Revised: 2017. 03. 16. Accepted: 2017. 05. 02. On-line: 2017. 05. 20.

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I. Introduction

Bone undergoes a continuous remodeling process of regeneration and degradation. Osteoblasts are involved in bone-forming while osteoclasts are associated with the bone resorption, and an imbalance between these two cell types can lead to the development of bone-related disorders such as osteoporosis. Medications available for osteoporosis in the modern field of medicine include bone resorption inhibitors and bone-forming agents. Most clinical treatments for osteoporosis involve the use of bone resorption inhibitors that have common side effects including gastrointestinal disturbance mediated by cells in the T cell lineage and monocytes, 3-day flu-like symptoms, increased bone mass during the early treatment period, jaw bone necrosis with long-term treatment, atypical subtrochanteric fracture, and delayed fracture recovery. These side effects have prompted research on alternative osteoporosis medications derived from natural substances, such as herbal medicines that have few side effects. Furthermore, a previous study has reported that various kinds of herbal medicines can increase the bone mass of the spinal column more significantly than existing medications for osteoporosis.

Although there has been no direct report on osteoporosis in the oriental medicine literature, osteoporosis appears to fall under the category of Golwi (骨萎), Golgeuk (骨極), Golbi (骨痹), and Golgo (骨枯) based on its clinical symptoms and patterns. In addition, EC and PS have function of Bosin (補腎) according to theories of physiology in oriental medicine, Sinjugol (腎主骨). Thus, in this study, we postulated that EC and PS would have therapeutic effects on osteoporosis.

Previous studies investigating the therapeutic effects of EC extracts on osteoporosis using scientific methods have only used ovariectomized white (albino) mice. Furthermore, a study reported that EC extracts inhibit the expression of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) by inhibiting the activation of p38, c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). However, the experiment conducted in that study used relatively high intracellular extract concentrations of 50–300 μg/mL.

A previous study, which reported the therapeutic effects of PS extracts in osteoporosis, only used a white mouse model of osteoporosis induced by estrogen deprivation via ovariectomy. Furthermore, the therapeutic effects of the PC extracts were assessed in vivo by assaying related biochemical markers including serum and urine components as well as and bone density.

Natural substances and their derivatives with inhibitory cellular effects on different stages of osteoclast differentiation have been investigated including deer antler and Taxilli ramulus extracts. There has not been any research on the cellular inhibitory effects of EC and PS extracts on osteoclast differentiation, inflammatory bone loss, or their mechanisms of action and combined effects. Therefore, the present study was aimed at investigating the inhibitory effects of single or combined administration of EC and PS extracts on osteoclast differentiation in a white mouse model of experimentally induced bone loss.

II. Materials and Methods

1. Materials

The receptor activator of nuclear factor–kappa–B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) used in our research were purchased from Peprotech (London, UK). The 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay kit was purchased from Roche (Indianapolis, IN, USA). Phosphorylated–JNK (p–JNK), JNK, p–ERK, ERK,
p-p38, p38, and inhibitor of NF-κB (I-κB) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). c-Fos, NFATc1, and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Methods

1) Preparation of EC and PS extracts
The EC and PS material used in this study were from Yeongchun of Gyeongbuk Province and China, respectively, and were purchased from Omniherb Company. The EC and PS samples were evenly cut into pieces, macerated for 30 min, and boiled in a heating mantle for 2 h. Freeze-dried samples of the concentrates were used in our experiments, and their yields are shown in Table 1. Purified water (1 L) was added to 100 g of each herb and heated in a Glas-Col (Terre Haute, IN, USA) heating mantle for 2 h for the extraction. The obtained extracts were filtered, concentrated using a rotary vacuum evaporator, freeze-dried, and then powdered for use in the experiments. The extract combination was prepared by mixing the EC and PS extracts in a 1:1 ratio, followed by further extraction and processing using the procedure described above.

2) Osteoclast culture and differentiation inhibition
Bone marrow cells were obtained by euthanizing 5–week-old male ICR mice by dislocating the cervical vertebrae. The tibias and femurs were then separated, and a 1× antibiotic solution containing α-MEM medium containing 10% fetal bovine serum (FBS), antibiotics, and M-CSF (30 ng/mL) for 3 days. A cell suspension containing lymphocytes was removed, and the adherent cells were used as bone marrow macrophages (BMMs) of osteoclast progenitors. The BMMs were seeded into a 48-well plate at 3.5 × 10^4 cells/well and cultured with different concentrations of EC or PS extracts or their combination in a growth medium containing M-CSF (30 ng/mL) and RANKL (100 ng/mL). The growth medium was replaced 3 days later, and the cells were stained with tartrate-resistant acid phosphate (TRAP) solution 4 days later. Red violet-colored TRAP-positive cells were considered osteoclasts and those with three or more nuclei were recorded for statistical analysis.

3) Toxicity test
BMMs were seeded into a 96-well plate at 1 × 10^4 cells/well and cultured with M-CSF (30 ng/mL), followed by the extracts at different concentrations for 3 days. Then, 50 μL XTT was added to each well, and after a 4-h incubation, the optical density was measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, CA, USA).

4) Western blot analysis
The cultured cells were washed 2-3 times with 1× phosphate-buffered saline (PBS) to remove any residue and dissolved in a lysis buffer (50 mM Tris–chloride [Tris], 150 mM sodium chloride [NaCl], 5 mM ethylenediaminetetraacetic acid [EDTA], 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease

Table 1. Extraction Yield of Medicinal Herbs Used in the Study

<table>
<thead>
<tr>
<th>Korean name</th>
<th>Pharmaceutical name</th>
<th>Abbreviation</th>
<th>Yield (%)</th>
</tr>
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<tr>
<td>Duchung</td>
<td>Eucommiae Cortex</td>
<td>EC</td>
<td>11.1</td>
</tr>
<tr>
<td>Bogolji</td>
<td>Psoraleae Semen</td>
<td>PS</td>
<td>16.9</td>
</tr>
<tr>
<td>Duchung plus Bogolji</td>
<td>Eucommiae Cortex plus Psoraleae Semen</td>
<td>EC + PS</td>
<td>14.4</td>
</tr>
</tbody>
</table>
inhibitors). The solution was centrifuged (14,000 rpm, 20 min) to obtain the proteins, which were quantified using a DC protein assay kit (Bio-Rad, Hercules, CA, USA), and separated using 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, which was blocked with 5% skim milk in Tris–buffered saline plus Tween (TBS–T) to prevent nonspecific reactions, at 20°C for 1 h, followed by incubation with the primary antibodies at 4°C. Following the antibody reaction, the PVDF membrane was washed thrice with TBS–T every 10 min and then incubated with secondary antibodies (1:5000) at room temperature for 1 h. After washing the PVDF membrane thrice with TBS–T, an enhanced chemiluminescence (ECL) kit was used to develop and visualize the blots to assess the protein expression levels.

5) Reverse transcription–polymerase chain reaction (RT–PCR) analysis

Intracellular RNA was separated using 1 mL isolRNA lysis reagent (5PRIME) according to the manufacturer’s instructions. Briefly, 0.5 pg–1 μg of the separated RNA was used to synthesize cDNA using the ReverTra Ace® quantitative polymerase chain reaction (qPCR) reverse transcription (RT) kit (Toyobo, Japan). The synthesized cDNA was amplified using PCR with the primers listed in Table 2. Then, the amplified DNA was electrophoresed using a 1% agarose gel, stained with ethidium bromide (Et–Br), and subsequently visualized under ultraviolet (UV) light.

6) Animal experiment

To investigate the effects of the herbal extracts on bone loss in vivo, five 5–week–old male ICR mice were assigned to each of the following groups: EC, PS, combination, and PBS groups (0.4 or 0.2 mg per body weight in g). The mice were orally administered the preassigned treatment solutions for 10 days. To induce bone loss, lipopolysaccharide (LPS) was intraperitoneally injected into the mice in the experimental groups on day 4 and 7 after extract administration (day 1) while the control group mice were injected with PBS to reduce experimental errors. On day 10, the mice were euthanized, their femurs were harvested, fixed with 4% paraformaldehyde, and then three–dimensional (3D) images of the interior were captured using micro–CT (Bruker, Kartuizersweg 3B, 2550 Kontich, Belgium, Skyscan 1172).

7) Statistical analysis

The statistical analysis was performed thrice or more for each experimental group, and the results are presented as the means ± standard deviation.

| Table 2. Primers Used for Polymerase Chain Reaction (PCR) Amplification |
|-----------------------------|------------------|
| Primer | Sequence |
| c–Fos | Forward 5′- CTGGTGACGCCCACCTGTCGT-3′ |
| Reverse 5′- CTGGTGACGCCCACCTGTCGT-3′ |
| NFATc1* | Forward 5′- CAACGCCCTGACCACCGATAG-3′ |
| Reverse 5′- GGCTGCCCTCCGCTCTCATAGT-3′ |
| TRAP† | Forward 5′- ACTTCCCCAGCCCTTTACTAC-3′ |
| Reverse 5′- TCACGACATAGCCACACCG-3′ |
| GAPDH‡ | Forward 5′- ACCACAGTCGACCATCAG-3′ |
| Reverse 5′- TCCACACCCTTGTGCTGTA-3′ |

* NFATc1, nuclear factor of activated T-cells, cytoplasmic 1.
† TRAP, tartrate-resistant acid phosphate.
‡ GAPDH, glyceraldehyde-3-phosphate dehydrogenase, spermatogeni.
All experiments were repeated thrice or more, and only consistent results were used in the statistical analyses. For quantitative results, the Student’s t-test was used. The results of the animal experiments did not meet the assumption of normality since each group contained only five animals. Therefore, non-parametric Kruskal–Wallis, as well as Mann–Whitney tests, were performed to analyze results. The level of statistical significance was set at $p < 0.05$.

**III. Results**

1) Inhibitory effects of EC and PS water extracts in combination on RANKL-induced osteoclast differentiation

The differentiation of osteoclasts, which resorb bone, plays an important role in inducing osteoporosis. In this study, we investigated the effects of EC and PS extracts in combination on osteoclast differentiation. BMMs were treated with M-CSF and RANKL at concentrations of 5 and 10 μg/mL.
and cultured. The differentiation of BMMs into TRAP-positive multinucleated osteoclasts was observed in the control group, which was treated with M-CSF and RANKL only. However, osteoclast differentiation was inhibited in the EC and PS groups (Fig. 1A). The number of multinucleated osteoclasts after staining with TRAP was counted, and the results revealed that differentiation into multinucleated osteoclasts was significantly inhibited by the EC and PS extracts (Fig. 1B). In the subsequent experiments, the EC and PS extracts were used at a concentration of 10 μg/mL.

2) Effects of EC and PS extracts alone on RANKL-induced osteoclast differentiation

The combination of the EC and PS extracts inhibited osteoclast differentiation and, therefore, we investigated their individual effects on RANKL-induced osteoclast differentiation. BMMs were cultured with M-CSF and RANKL and treated with EC or PS extracts at concentrations of 5 or 10 μg/mL. In contrast to BMMs in the control group, which were treated with M-CSF and RANKL only, the EC group showed inhibition of the production of TRAP-positive multinucleated osteoclasts, Fur-
thermore, the differentiation of BMMs into mature, multinucleated osteoclasts was also inhibited. However, this inhibition was not significant compared with that observed in the combination group. The PS extract inhibited osteoclast differentiation; however, the cells treated at a concentration of 10 μg/mL exhibited cytotoxicity (Fig. 2).

2. Cytotoxicity of EC and PS extracts

To eliminate the involvement of cytotoxicity in the inhibitory effects of EC and PS extracts alone and in combination on osteoclast differentiation, an XTT assay was performed using BMMs treated with different concentrations. Based on the cell survival rate of the untreated control group that was considered to be 100%, the survival rates were 103.2%, 92.7%, 91.6%, and 81.0% in the EC group mice treated with extract concentrations of 5, 10, 20, and 40 μg/mL, respectively. Furthermore, survival rates of 80.6%, 73.5%, 69.9%, and 71.4% were found in the PS group mice treated with extract concentrations of 5, 10, 20, and 40 μg/mL. The combination treatment showed survival rates of 80.4%, 86.7%, 81.5%, and 73.5% at extract concentrations of 5, 10, 20, and 40 μg/mL. With a cell survival rate ≥ 70% as the minimum standard for no cytotoxicity, a slight cytotoxicity was observed following administration of the PS extract at 20 μg/mL while no cytotoxicity was observed in the EC and combination groups. Based on this result, we set the concentration of the EC and PS extracts at 5 μg/mL and that of the extract combination at 10 μg/mL. At this concentration, the extract combination exhibited synergistic effects over those of the EC and PS extracts while cytotoxicity was not observed (Fig. 3).

3. Inhibitory effects on RANKL-mediated expression of osteoclast differentiation-inducing factors

1) Effects of the combination of EC and PS extracts on RANKL-induced gene expression

RANK binds RANKL to induce the expression of transcription factors such as c-Fos and NFATc1 via various intracellular signaling pathways, thereby inducing the expression of TRAP, which is a marker of osteoclasts. To investigate the effects

![Fig. 3. Effect of Eucommiae cortex (EC), Psoraleae semen (PS), and EC + PS water extracts on cell viability](http://dx.doi.org/10.13045/acupunct.2017079)
of the EC and PS extracts alone and in combination on the gene expression of c-Fos, NFATc1, and TRAP, we performed RT–PCR. The results showed increased mRNA expression of c–Fos, NFATc1, and TRAP induced by RANKL on day 1–2. However, the mRNA expression of c–Fos, NFATc1, and TRAP was significantly inhibited in the combination group (Fig. 4).

2) Effects of EC and PS alone on RANKL–induced protein expression

The RT–PCR results showed increased mRNA expression of c–Fos, NFATc1, and TRAP induced by RANKL on Day 1–2. The mRNA expression of NFATc1 was significantly inhibited in the EC and PS groups. The mRNA expression of TRAP was significantly inhibited on Day 2 in the EC group, and on Day 1 in the PS group (Fig. 5).

4. Inhibitory effects on RANKL–induced expression of c–Fos and NFATc1 proteins

1) Effects of the combination of EC and PS extracts on RANKL–induced expression of c–Fos and NFATc1 proteins

RANKL induces C–Fos expression, thus inducing the expression of NFATc1, which plays an important role in osteoclast differentiation. Western blotting was performed to investigate the ef–
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Fig. 5. Eucommiae cortex (EC) and Psoraleae semen (PS) suppress receptor activator of nuclear factor–kappa–B ligand (RANKL)–induced c-Fos, nuclear factor of activated T–cells, cytoplasmic 1 (NFATc1), and tartrate–resistant acid phosphate (TRAP) expression

Bone marrow macrophages (BMMs) were pretreated with or without EC and PS (5 μg/mL) for 1 h and then stimulated with RANKL (100 ng/mL) for indicated times (1 day [24 h], 2 days [48 h]).

(A): mRNA expression was analyzed using reverse transcription–polymerase chain reaction (RT–PCR).

(B): Quantitative analysis using ImageJ program.

*Significant difference between control and extract treatment groups (* p < 0.05 and *** p < 0.001).

Error bars indicate standard deviation (SD); n = 3.

GAPDH, glyceraldehyde–3–phosphate dehydrogenase, spermatogeni.

Fig. 6. Eucommiae cortex (EC) plus Psoraleae semen (PS) water extracts suppress receptor activator of nuclear factor–kappa–B ligand (RANKL)–induced c–Fos and nuclear factor of activated T–cells, cytoplasmic 1 (NFATc1) expression

Bone marrow macrophages (BMMs) were pretreated with or without EC and PS water extracts (10 μg/mL) for 1 h and then stimulated with RANKL (100 ng/mL) for indicated times.

Cell lysates were analyzed using western blotting with antibodies for c–Fos, NFATc1, and β–actin.
pendent manner, and that of c-Fos was increased at 12 and 24 h. However, the expression of c-Fos and NFATc1 proteins was inhibited in the combination treatment group (Fig. 6).

2) Effects of EC and PS extracts alone on RANKL-induced expression of C-Fos and NFATc1 proteins

The expression of NFATc1 increased in a time-dependent manner 12, 24, and 48 h after RANKL treatment while that of c-Fos was increased 12 and 24 h after RANKL treatment. No change in the expression level of c-Fos and NFATc1 proteins was observed in the PS group, whereas the expression of NFATc1 protein was inhibited in the EC group (Fig. 7).

5. Effects on signaling pathways of osteoclast differentiation

1) Effects of the combination of EC and PS extracts on signaling pathways of RANKL-induced osteoclast differentiation

We investigated the effects of EC and PS extracts alone and in combination on major signaling pathways induced by RANKL to identify the mechanisms of osteoclast differentiation inhibition. BMMs were pretreated with the EC and PS extracts alone or in combination, followed by RANKL at 0, 5, 15, and 30 min, and MAPK phosphorylation was assessed. While phosphorylation of AKT, p38, and JNK was increased in the EC and PS groups, phosphorylation of p38 and JNK was simultaneously inhibited in the combination group. The proteolysis of IκB was also inhibited by RANKL (Fig. 8).

2) Effects of EC and PS extracts alone on signaling pathways of RANKL-induced osteoclast differentiation

BMMs were pretreated with EC and PS extracts, followed by RANKL at 0, 5, 15, and 30 min, and then MAPK phosphorylation was assessed. The phosphorylation of AKT and p38 was inhibited in the EC group, and that of p38 and JNK was inhibited in the PS group (Fig. 9).

6. Effects on LPS-induced inflammatory bone loss

A mouse model of inflammatory bone loss was used to investigate the in vivo effects of EC and PS.
Inhibitory Effects of Water Extracts of Eucommiae Cortex and Psoraleae Semen Alone and in Combination on Osteoclast Differentiation and Bone

Fig. 8. Eucommiae cortex (EC) plus Psoraleae semen (PS) water extracts inhibit receptor activator of nuclear factor–kappa-B ligand RANKL signaling pathways

Bone marrow macrophages (BMMs) were pretreated with or without EC and PS water extracts (10 μg/mL) and further stimulated with RANKL (100 ng/mL) for indicated times. Cells were lysed, proteins were resolved using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and subjected to western blot analysis.


Fig. 9. Eucommiae cortex (EC) and Psoraleae semen (PS) inhibit receptor activator of nuclear factor–kappa–B lig– and RANKL signaling pathways

Bone marrow macrophages (BMMs) were pretreated with or without EC and PS (5 μg/mL) and further stimulated with RANKL (100 ng/mL) for indicated times.

Cells were lysed, proteins were resolved using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and subjected to western blot analysis.

extracts alone and in combination, LPS (5 μg/g) was intraperitoneally injected into the mice once every 3 days, and then each extract was orally administered once daily at various concentrations. The extent of LPS–induced bone loss was assessed based on 3D images of the femur specimens obtained using the Skyscan BE/skyscan 1172 (Table 3).

The mean bone loss was 16.9% in the LPS–treated group compared to that in the control group. No bone loss was observed in groups treated with the single EC and PS extracts alone. The bone volume/tissue volume (BV/TV) was significantly increased in the group treated with the PS extract and LPS–stimulated, indicating a significant recovery of bone loss. No bone loss recovery was observed in the group treated with the extract combination (Fig. 10).

IV. Discussion

Osteoporosis is a systemic musculoskeletal disorder characterized by reduced bone mass and microstructure abnormalities and is a major geriatric disease that causes the bones to weaken and become brittle.26 The aging population has led to an increase in the number of patients with osteoporosis. Bone diseases such as osteoporosis are believed to be caused by excessive proliferation and activation of osteoclasts, which degenerate bone. Therefore, medications that can inhibit osteoclast activities have been widely used to treat osteoporosis. These medications include bisphosphonates, which mechanically inhibit bone resorption by osteoclasts and induce osteoclasts to undergo apoptosis.27 While bisphosphonates have been shown to be effective in the treatment of osteoporosis, they can result in serious side effects such as necrosis of the jaw bone in patients with periodontal diseases.28 For this reason, much attention has been focused on the development of osteoporosis medications from natural substances that tend to have few side effects. Natural substances derived from plants have been widely used to treat various diseases from ancient times. Identifying their mechanisms of action using modern research methods as a strategy to assess their values as osteoporosis medications would contribute to the development of osteoporosis medications from natural substances.

EC is the dried bark of Eucommia ulmoides Oliv., which belongs to the Eucommiaceae family. The bark is peeled off the trees in April and May, cut, stacked, and dried until the inner side turns purplish brown, EC is mild, non–toxic (溫無毒), and

<table>
<thead>
<tr>
<th>CTR</th>
<th>EC 200</th>
<th>PS 200</th>
<th>LPS+</th>
</tr>
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<tbody>
<tr>
<td>BV/TV (%)</td>
<td>30.9±0.0</td>
<td>28.6±0.4</td>
<td>31.5±0.9</td>
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<td>Trabecular thickness(㎛)</td>
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<td>16.3±0.5</td>
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<td>Trabecular separation(㎛)</td>
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<tr>
<td>Trabecular number(1/㎛)</td>
<td>0.020±0.0</td>
<td>0.019±0.0</td>
<td>0.019±0.0</td>
</tr>
</tbody>
</table>

* CTR, control group
† EC, Eucommiae Cortex
‡ PS, Pseudaloe Semen acidaed
§ LPS, lipopolysaccharide

BV/TV, bone volume/trabecular volume

http://dx.doi.org/10.13045/acupunct.20170707
has a sweet and slightly spicy taste (甘微辛). It effects on (歸經) the kidney and liver meridian (肝腎經) and strengthens muscle and bone (强筋骨) and, therefore is used to treat numbness and weakness of feet and knees (足膝痿弱) and other diseases. It is combined with Psoraleae semen to treat chills and pain in the waist and knees (腰膝酸痛) and musculoskeletal weakness (筋骨無力) caused by liver and kidney yang deficiencies (肝腎不足).

Psoraleae fructus is the dried ripe fruit of *Psoralea corylifolia* L., which is an annual herb that belongs to the Leguminosae family. It is harvested in September and then dried. Psoraleae fructus is mild and non-toxic (溫無毒) with a bitter and spicy taste (辛苦). It effects on (歸經) the kidney and spleen meridian (腎脾經), and adding Yang energy

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Fig. 10. Skyscan analysis of lipopolysaccharide (LPS)-induced bone loss in mice

(A) Mice were orally treated with Eucommiae cortex (EC), Psoraleae semen (PS), and EC + PS extracts (200 or 400 mg·kg⁻¹·day⁻¹) or 1× phosphate-buffered saline (PBS) daily for 10 days. LPS (5 μg/g body weight) or 1× PBS was administered intraperitoneally on day 4 and 7, and the femurs were harvested on day 10 after EC, PS, and EC + PS extract treatments. Radiographs were captured using Sky-scan.

(B) Bone volume/tissue volume (BV/TV), trabecular thickness, separation, and number were analyzed using histomorphometric results.

*Statistically different from LPS group. (*p < 0.05).
to kidney--Gi with warm medicine (溫腎助陽). Therefore, it is used to treat cold feeling and pain in the waist and knees (腰膝冷痛). It is combined with EC to treat kidney yang deficiency and back pain.  

Considering the description of osteoporosis as "腎主骨髓" in 《素問·陰陽應象大論》, as "腎主骨" in 《素問·宣明五氣》, and as "腎之合骨也" in 《素問·五藏生成論》, Sinheo (腎虛) can be recognized as the main cause of osteoporosis, and medications that Bosin (補腎) may be effective in the treatment of osteoporosis, a geriatric disease. We hypothesized that EC and PS, both of which enhance kidney yang, can effectively treat osteoporosis. Therefore, we investigated the inhibitory effects of the EC and PS extracts alone and in combination on osteoclast differentiation and bone resorption.

M–CSF and RANKL are cytokines that are essential for the differentiation of stem cells into multinucleated osteoclasts. RANK binds with RANKL to promote the expression of c–Fos and NFATc1 via intracellular signaling pathways, thereby inducing the expression of TRAP, which is a marker of osteoclasts. TRAP is the only bone resorption factor expressed by mature osteoclasts and can be used to determine their differentiation stage. TRAP–positive cells were stained to investigate the inhibitory effects of EC and PS extracts and their combination on osteoclast differentiation and bone resorption.

While osteoclast differentiation was also inhibited in cells treated with each extract of EC or PS alone, this inhibition was not significant compared to that observed in the combination group. Although EC and PS extracts inhibited osteoclast differentiation, their combination had stronger effects than each extract did alone. Furthermore, while the PS extract showed cytotoxicity, the EC extract and combination treatment showed no cytotoxicity, suggesting that EC may mitigate the cytotoxicity of PS. The combination of the EC and PS extracts may provide important insights that could contribute to the development of osteoporosis medications.

The c–Fos protein, whose expression is induced by RANKL, induces the expression of NFATc1 and plays an important role in osteoclast differentiation. Western blotting was performed to investigate the effects of EC and PS extracts and their combination on the expression of c–Fos and NFATc1 proteins. The expression of NFATc1 was increased in a time–dependent manner 12, 24, and 48 h after RANKL treatment. The expression of c–Fos was increased at 12 and 24 h after RANKL treatment: however, the expression of c–Fos and NFATc1 was inhibited in the combination group (Fig. 4). On the other hand, no change in the expression level of c–Fos and NFATc1 was observed in the PS group while the expression of NFATc1 was inhibited in this group. The extract combination inhibited osteoclast differentiation more effectively than the single ES or PS extracts did alone, possibly because the EC extract likely enhanced the inhibitory effects of the PS extract on the expression of NFATc1 proteins.

C–Fos is a transcription factor that is expressed within 30 min after RANKL stimulation. C–Fos increases the expression of NFATc1, which subsequently induces the expression of the osteoclast–specific marker genes such as TRAP and cathepsin K to induce osteoclast differentiation. Inhibiting the expression of c–Fos reduces that of NFATc1, which leads to reduced expression of TRAP, an osteoclast–specific marker. We performed RT–PCR to assess the expression level of the c–Fos, NFATc1, and TRAP genes and observed increased mRNA expression of c–Fos, NFATc1, and TRAP induced by RANKL on day 1–2. However, the mRNA expression of c–Fos, NFATc1, and TRAP was significantly inhibited in the combination group. Although the EC and PS extracts significantly inhibited the expression of c–Fos, NFATc1, and TRAP, which are the key genes involved in the process of osteoclast differentiation induced by RANKL, the inhibition of NFATc1 expression by EC and PS extracts was not significant compared to that by the extract combination. The expression of c–Fos was
not inhibited by EC and PS extracts. This explains why the inhibition of osteoclast differentiation was more evident in the combination group than it was in the EC and PS groups, and indicates that the combination of EC and PS extracts inhibits the key mechanism of osteoclast differentiation.

In osteoclast precursor cells, the cytoplasmic domain of RANK binds TRAFs following exposure to an RANKL–induced stimulus to activate proteins in various signaling pathways. P38, JNK, and ERK, which are MAPKs, are known to be involved in important signaling pathways of osteoclast differentiation. MAPKs play an important role in the expression of c-Fos, SB203580 and SP600125, which inhibit P38 and JNK, respectively, also inhibit RANKL–induced expression of cFos and, thus, the activation of p38 and JNK could be considered an important intermediate process in osteoclast differentiation. The inhibition of different stages of the RANKL signaling pathway to inhibit osteoclast production could facilitate the development of a therapeutic approach for osteoporosis. We assessed the effects of EC and PS extracts and their combination on the activation of signal transducers to investigate their mechanism of inhibition of NFATc1. The combination of EC and PS extracts inhibited the activation of p38 and JNK, suggesting that the inhibition of NFATc1 expression by the extract combination may be associated with the activation of p38 and JNK. Phosphorylation of AKT and p38 was inhibited in the EC group while that of p38 and JNK was inhibited in the PS group. This suggests that the effects of the PS extract on the relevant signaling pathways were greater than those of EC in the extract combination.

NF–κ B is a transcription factor involved in differentiation, survival, and activation of various cells and is known to be involved in the expression of NFATc1 in osteoclasts. I–κ B binds NF–κ B in the cytoplasm to prevent it from traveling to the nucleus while RANKL destroys I–κ B to enable NF–κ B to translocate the nucleus. Continuous expression of I–κ B inhibits NF–κ B activity and ultimately inhibits differentiation into osteoclasts. We investigated the effects of EC and PS extracts and their combination on the activation of NF–κ B. The level of I–κ B was reduced at 5 and 10 min as I–κ B was degraded by RANKL. However, in the combination group, the level of I–κ B was unchanged over time. Therefore, the inhibition of NFATc1 expression by the combination of the EC and PS extracts may be associated with inhibition of NF–κ B activation.

A study using an LPS–induced rat model of inflammation, which is commonly used in research on bone loss and destruction in chronic inflammatory environments, reported that osteoclast differentiation is promoted by LPS. Furthermore, the authors reported that their observations were attributable to the LPS–induced expression of tumor necrosis factor (TNF)–α, which subsequently increased the expression of the proto–oncogene tyrosine–protein kinase Src (c–src). Bone loss results from the effects of inflammatory cytokine expression, which is increased by LPS as well as inflammatory mediators such as prostaglandin E2 (PGE2). Results in this study have suggested that single extracts of EC and PS and their combination inhibit osteoclast differentiation to promote a recovery from bone loss. Therefore, we investigated the effects of single EC and PS extracts and their combination in an animal model of LPS–induced inflammation.

EC has been reported to increase bone density and bone strength in white rat models of induced osteoporosis when coadministered with PS and treadmill exercise and, therefore, could be effective in the treatment of osteoporosis. EC administered to ovariectomized white rats has been observed to increase the trabecular bone volume. PS has been observed to reduce the serum level of osteocalcin and increase the level of phosphorus and calcium in bones in ovariectomized white rats and, thus, has been reported to be effective in the prevention and treatment of osteoporosis. Based on these observations, we investigated the effects of EC and PS extracts and their combination on LPS–induced bone loss. The results showed that
single extracts of EC and PS and their combination did not promote recovery of LPS-induced bone loss. The BV/TV was increased in the PS group, indicating a significant recovery of bone loss compared to the group treated with LPS alone. No significant changes in the trabecular thickness, separation, number were observed although slight improvements were evident. This finding may be associated with the previous observation where NFATc1 expression and phosphorylation of p38 and JNK were inhibited in the PS group. Since EC has previously been reported to increase bone density in an ovariectomized model, it appears that EC affects bone metabolism via pathways that are not associated with inflammation.

In summary, EC and PS inhibited osteoclast differentiation, and their inhibitory effects were significantly stronger when the extracts were combined. The mechanisms of action of the EC and PS extracts were associated with the inhibition of NFATc1 expression by the inhibition of p38, JNK, and NF-κB activation. However, in the in vivo LPS-induced bone loss model, a more significant recovery of bone loss was observed in animals treated with PS, which inhibited p38 and JNK phosphorylation and the expression of NFATc1 proteins, than in those treated with the extract combination. This study is meaningful because to the best of our knowledge, it is the first study to report the inhibitory effects of the combination of EC and PS extracts on osteoclast differentiation and bone resorption. Further investigation on the synergistic effects of ES and PC extracts, their combined ratio, and whether the signaling pathways they affect are independent or not is necessary.

V. Conclusion

We investigated whether single extracts of EC and PS and their combination had inhibitory effects on RANKL-induced osteoclast differentiation as well as bone resorption.

1. Based on the TRAP assay results, RANKL-induced osteoclast differentiation was more significantly inhibited by the combination of EC and PS extracts than by each of the extracts alone.

2. The EC and PS extracts at a concentration of 5 µg/mL showed no cytotoxicity and, therefore, were used in the experiment. The extract combination was prepared by mixing the EC and PS extracts in a 1:1 ratio and was used at a concentration of 10 µg/mL since no cytotoxicity was observed at this concentration.

3. The EC extract inhibited the phosphorylation of AKT and p38 in RANKL-induced pathways to inhibit the expression of the NFATc1 gene, thereby inhibiting osteoclast differentiation.

4. The PS extract inhibited the phosphorylation of AKT and p38 in RANKL-induced pathways to inhibit the gene expression of NFATc1, thereby inhibiting osteoclast differentiation.

5. The EC and PS extract combination inhibited the phosphorylation of p38 and JNK in RANKL-induced pathways and the activation of NFATc1, thereby inhibiting osteoclast differentiation.

In the in vivo experiment, the EC and PS extract combination had no effects on recovery from LPS-induced bone loss and the BV/TV was increased in the PS- and LPS-treated group, indicating a significant recovery of bone mass compared to the recovery in the group treated with LPS only.

In conclusion, the EC and PS extracts inhibited osteoclast differentiation through different routes. The inhibition of osteoclast differentiation was enhanced when the extracts were coadministered. Only the single extract of PS was effective against bone loss caused by inflammation.
VI. References

22. Heo JK, Hwang DS, Lee JM, Lee CH, Jang JB,


