Abstract. Phlomis umbrosa Turcz (labiatae) has been suggested to promote bone growth. However, the anti-osteoporotic effects of P. umbrosa have not yet been elucidated. In the present study, the osteogenic effects of P. umbrosa were investigated in an osteoporosis model. ICR female mice were ovariectomized (OVX) to induce osteoporosis for 7 weeks. Treatment with 1, 10 and 100 mg/kg P. umbrosa was administrated orally to the OVX mice for 6 weeks. At the end of experiment, the microstructure of the capital femoral epiphysis was investigated. The levels of bone mineral density (BMD), bone mineral content (BMC) and serum osteocalcin concentration were evaluated. In addition, mineralized Saos-2 osteoblast cells were treated with 0.01, 0.1 and 1 µg/ml P. umbrosa to analyze the expression of osteoblast differentiation-associated factors. Hyperplasia of the growth plate in the femur was recovered by P. umbrosa treatment. BMD and BMC were significantly increased in P. umbrosa-treated femurs. Serum calcium concentration was increased following P. umbrosa treatment. In addition, the ratio of mineralization was markedly increased in P. umbrosa-treated differentiated osteoblasts along with increases in Runx2 levels. P. umbrosa conferred its osteogenic effects by upregulating Runx2 in osteoporosis. P. umbrosa may be a potential therapeutic material for the treatment of osteoporosis.

Introduction

Osteoporosis is a common bone disease characterized by a decrease in bone strength, an increase in fracture risk and microarchitectural deterioration of the bone tissue (1). A total of >9 million people worldwide suffer from osteoporosis, and its prevalence is increasing within aging societies (2). This skeletal disorder is directly associated with quality of life, as the first symptoms may be osteoporotic fractures in the vertebral column, rib, hip or wrist (3).

Several risk factors for osteoporosis, including estrogen loss, aging, vitamin D deficiency and low dietary calcium, have been widely observed in humans and other mammals (4). Pharmacological treatment for osteoporosis, including bisphosphonates, raloxifene and calcitonin has been suggested to cause side effects including fever, damage to the kidneys, joint pain and osteonecrosis (5). In addition, calcium supplements have occasionally demonstrated adverse effects including abdominal gas, bloating and constipation, although they are used for maintenance of bone remodeling and the prevention of osteoporosis and other bone diseases (6). Clinically, hormone replacement therapy (HRT) has been used to prevent bone loss in postmenopausal women (7). However, it has been revealed that HRT has side effects, including breast cancer, thromboembolic disease, musculoskeletal pain and gastrointestinal intolerance (8). Due to these limitations, the development of alternative anti-osteoporotic treatments is required.

Phlomis umbrosa Turcz (labiatae), a perennial herbaceous plant in Asia, has been traditionally used for treatment of bronchitis, colds, bleeding, arthralgia, rheumatic disease and bone fractures (9). Previous studies have suggested that P. umbrosa has anti-inflammatory, anti-nociceptive, anti-allergy and antioxidant activities (9,10). Notably, P. umbrosa exhibited beneficial effects on longitudinal bone growth rate in rats (11). However, the anti-osteoporotic effects of P. umbrosa have not been investigated yet.

The present study evaluated the therapeutic effects of P. umbrosa on osteoporosis in ovariectomized (OVX)-induced mice. In addition, the potential mechanisms of action of P. umbrosa extract were investigated in human osteoblasts-like Saos-2 cells.

Materials and methods

Preparation of P. umbrosa. P. umbrosa was purchased from Jungdo Herb, Inc. (Guri, Korea). A total of 100 g P. umbrosa was extracted with 1 liter distilled water for 24 h at room temperature (RT) with shaking. Following filtration,
extract was concentrated under decreased pressure with a rotary evaporator and lyophilized (yield=31.44%). The obtained powder was termed ‘PU’. A voucher specimen was deposited at the College of Korean Medicine of Kyung Hee University (Seoul, Korea).

PU was identified on the basis of its loganin and sweroside content by high-performance liquid chromatography (HPLC) with diode-array detection. The extract was dissolved in 70% methanol and sonicated for 30 min. Following filtration through a 0.2 µm filter membrane, 10 µl of aliquot was subjected to HPLC Agilent 1100 series (Agilent Technologies, Inc., Santa Clara, CA, USA). Chromatographic separation was achieved using a C18 column (250x4.6 mm, 5 µm; Shiseido, Osaka, Japan). Mobile phase A involved water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The separation temperature was set at 30°C and a flow rate of 0.45 ml/min. The peak on PU was synchronized with loganin and sweroside. The concentration of loganin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and sweroside (Sigma-Aldrich) in PU was 82.32 and 237.65 µg/mL, respectively.

Animals and treatments. ICR mice were purchased from Raonbio, Inc. (Yongin, Korea). Female 6-week-old ICR mice were housed at 22±1°C in an atmosphere with 55±10% humidity in a 12 h light: dark cycle with ad libitum access to a standard chow diet (Orient Bio., Inc., Seongnam, Korea) and water. The animal experiments were approved by the Institutional Animal Care and Use Committee of Kyung Hee University Laboratory Animal Center [approval no. KHUASP (SE)-15-079].

The mice were randomized into 7 groups (n=7; total=49 mice): Sham-operated mice (Sham group); OVX mice treated orally with vehicle (OVX group); OVX mice injected intraperitoneally with 10 µg/kg 17β-estradiol (E2 group); OVX mice treated orally with 150 µg/kg calcium chloride (Ca group); OVX mice treated orally with 1 mg/kg PU (PU1 group); OVX mice treated orally with 10 mg/kg PU (PU10 group); and OVX mice treated orally with 100 mg/kg PU (PU100 group). E2 and Ca were used as positive controls. All treatments started at 7 weeks following OVX surgery, and lasted for 6 weeks. At 13 weeks after the experiment began, the animals were sacrificed, and blood was collected by cardiac puncture. The right and left femurs were obtained.

Histological analysis. The right femur was fixed in 10% neutralized formalin for 18 h at RT and demineralized with 0.1 M ethylenediaminetetraacetic acid aqueous solution for 1 month. Following demineralization, femur samples were dehydrated by using xylene and consecutive ethanol concentrations (70, 80, 90, 95 and 100%) at 10 min each. Sagittal sections of the paraffin-embedded tissues were sliced at a 7 µm thickness. The slides were stained with hematoxylin for 5 min and eosin solution for 5 sec at RT according to kit instructions (Sigma-Aldrich). Histological changes were monitored using the Leica Microscope DML B2/11888111 equipped with a Leica camera DFC450 (Leica Microsystems, Buffalo Grove, IL, USA) at x100 magnification.

Measurement of bone mineral density (BMD) and bone mineral content (BMC). Following sacrifice, the left femur was collected and cleaned by removing the attached muscles and connective tissues. The sample was stored in 10% neutralized formalin until use. The levels of BMD and BMC in the left femur were determined by dual-energy X-ray absorptiometry with an InAlzyer instrument (Medikors, Seongnam, Korea).

Serum analysis. Samples were prepared from blood collected by cardiac puncture in heparinized tubes. The collected blood was centrifuged at 27,000 x g for 30 min, and then the supernatant was stored at -80°C until use. The concentration of serum calcium was measured using the Calcium Colorimetric Assay kit (AdipoGen Life Sciences, Shizukuo, Japan) according to the manufacturer's protocol. The concentration of calcium in the serum was measured at 570 nm absorbance using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Cell culture. The human osteoblastic Saos-2 cell line (Korean Cell Line Bank, Seoul, Korea) was routinely grown in Dulbecco's modified minimal essential medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1% penicillin and 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere containing 5% CO2 and 95% humidity. The cell culture medium was changed every 3-4 days. To confirm the cytotoxicity of PU, Saos-2 cells were incubated with culture medium containing different concentrations of PU extract (0.01, 0.1 and 1 µg/mL) for 10 days. Subsequently, 2 mg/mL MTT solution was added for 4 h. Dimethyl sulfoxide was then added, and cell viability was measured at an absorbance of 570 nm.

Mineralized matrix formation assay. The cells were seeded in 6-well plates at density of 0.8x104 cells/well and stabilized for 24 h. To induce osteoblast differentiation, 50 µg/mL L-ascorbic acid (AA; Thermo Fisher Scientific, Inc.) and 10 mM β-glycerophosphate (β-GP; Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) were added into osteogenic culture medium for 10 days. The culture medium was changed every 3-4 days. Then, the cells were fixed in 10% formalin for 10 min and stained with the 40 mM Alizarin Red-S (pH 4.2; Sigma-Aldrich; Merck KGaA) for 15 min, all at RT. The plates were observed under the Leica Microscope DML B2/11888111 equipped with a Leica camera DFC450 at x100 magnification. For quantification of Alizarin red S, 500 µl citrate solution containing 20% methanol and 10% acetic acid was added for 20 min at RT, and the absorbance of supernatants was measured at 570 nm using an ELISA reader (Molecular Devices, LLC., Downingtown, PA, USA).

Western blot analysis. The Saos-2 cells were lysed with radio-immunoprecipitation assay lysis buffer (BioPrince, Seoul, Korea) containing protease inhibitors (Sigma-Aldrich). The Bradford method was used for quantification of total protein. Subsequently, 20 µg of each sample was resolved using 10% SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% bovine serum albumin (Sigma-Aldrich) for 1 h at RT and then incubated with primary antibodies against runt-related transcription factor 2 (Runx2) and osteocalcin (Ocn) overnight at 4°C. After washing, membranes were incubated with secondary antibodies for 1 h at RT. The membranes were washed again and probed with enhanced chemiluminescence (ECL) Western blotting reagents (Merck KGaA, Darmstadt, Germany). The bands were visualized and densitometric analysis was performed using Image J software.
factor 2 (Runx2; 1:700 dilution; cat. no. 12556; Cell Signaling Technology, Inc., Danvers, MA, USA), transcription factor Sp7 (osterix; 1:1,000 dilution; cat. no. ab22552; Abcam, Cambridge, MA, USA) and β-actin (1:1,000 dilution; cat. no. sc-69879; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted in TBS containing 0.1% Tween-20 (TBS-T) overnight at 4˚C. The membrane was washed and incubated with m-IgG BP-HRP (1:2,000 dilution; cat. no. sc-516102; Santa Cruz Biotechnology, Inc.) and mouse anti-rabbit immunoglobulin G-horseradish peroxidase (1:2,000 dilution; cat. no. sc-2357, Santa Cruz Biotechnology, Inc.) diluted in TBS-T for 2 h at RT. Following washing, the bands were visualized with enhanced chemiluminescence (ECL) reagent (Amersham; GE Healthcare, Chicago, IL, USA). β-actin was used as an internal loading control for Runx2 and osterix. The band intensity was quantified using ImageJ software version 1.38e (National Institutes of Health, Bethesda, MD, USA). All experiments were performed in triplicate.

Statistical analysis. Significance was determined by one-way analysis of variance and followed by Dunnett’s post-hoc test, using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference. All values are expressed as the mean ± standard error of the mean.

Results

Effect of PU on the growth plate thickness of femur. The thickness of the epiphyseal plate was significantly increased in the OVX group compared with the Sham group. Administration of E2 and Ca decreased the growth plate thickness compared with OVX group. Similarly, PU-treated mice (1, 10 and 100 mg/kg) exhibited an amelioration of growth plate hyperplasia compared with the OVX-induced osteoporotic mice (Fig. 1).

Effects of PU on BMD and BMC. The BMD level of the OVX group (0.115±0.004 g/cm²) was significantly decreased by 0.025 g/cm² compared with the Sham group (0.140±0.006 g/cm²). E2 injection and Ca administration as positive controls significantly increased the level of BMD.
AMELIORATES OSTEOPOROSIS

was identified that the longitudinal bone plate and the loss of bone mass. In addition, serum calcium and administration of PU recovered the hyperplasia of growth BMD and BMC were decreased in OVX mice, as expected, increased in OVX mice. Also, the bone fragility parameters study, the thickness of epiphyseal growth plate was markedly risk factors, including loss of bone mass, deterioration of bone structure and hyperplasia of epiphyseal growth plate (12,13).

Osteoporotic bone exhibits high incidence rates of fracture and maintenance of bone integrity (15). The results of the present study demonstrated that treatment with PU significantly increased serum calcium levels. Therefore, it appears that PU treatment ameliorates the destruction of bone structure and bone minerals in osteoporosis.

Imbalance between bone resorption and bone deposition is a crucial pathogenic event in osteoporosis (16), as development and maintenance of bone tissue requires a continuous process of bone resorption and bone deposition (3). Osteoblasts, differentiated from bone marrow mesenchymal stem cells, are responsible for bone formation (17). As calcium deposition is accompanied by bone mineralization in the process of bone formation, calcium content in mature osteoblasts differentiated from osteoblast like Saos-2 cells was observed in the present study. PU treatment notably increased the calcium content of the mineralized matrix. Therefore, these results demonstrate that PU treatments have the capability to promote bone matrix mineralization in osteoblasts.

To clarify this osteogenic effect of PU, the expression of bone differentiation-associated markers including Runx2 and osterix were analyzed in Saos-2 cells. The mineralization of osteoblasts is regulated by several osteogenic factors including osteoblast differentiation and mineralization by stimulating expression of Runx2 during osteogenic differentiation was improved by PU treatment in Saos-2 cells, while osterix expression was not increased in PU-treated cells.

Discussion

Osteoporotic bone exhibits high incidence rates of fracture risk factors, including loss of bone mass, deterioration of bone structure and hyperplasia of epiphyseal growth plate (12,13). BMD and BMC measurements are the primary parameters used for the diagnosis of osteoporosis (14). In the present study, the thickness of epiphyseal growth plate was markedly increased in OVX mice. Also, the bone fragility parameters BMD and BMC were decreased in OVX mice, as expected, and administration of PU recovered the hyperplasia of growth plate and the loss of bone mass. In addition, serum calcium level is positively associated with activity of bone formation and maintenance of bone integrity (15). The results of the present study demonstrated that treatment with PU significantly increased serum calcium levels. Therefore, it appears that PU treatment ameliorates the destruction of bone structure and bone minerals in osteoporosis.

Imbalance between bone resorption and bone deposition is a crucial pathogenic event in osteoporosis (16), as development and maintenance of bone tissue requires a continuous process of bone resorption and bone deposition (3). Osteoblasts, differentiated from bone marrow mesenchymal stem cells, are responsible for bone formation (17). As calcium deposition is accompanied by bone mineralization in the process of bone formation, calcium content in mature osteoblasts differentiated from osteoblast like Saos-2 cells was observed in the present study. PU treatment notably increased the calcium content of the mineralized matrix. Therefore, these results demonstrate that PU treatments have the capability to promote bone matrix mineralization in osteoblasts.

To clarify this osteogenic effect of PU, the expression of bone differentiation-associated markers including Runx2 and osterix were analyzed in Saos-2 cells. The mineralization of osteoblasts is regulated by several osteogenic factors including Runx2, osterix, bone morphogenic protein (BMP), mothers against decapentaplegic homolog 1, insulin-like growth factor (IGF)-1, β-catenin and transforming growth factor-β (18,19). In particular, Runx2 and osterix serve key roles in the differentiation and proliferation of the osteoblast lineage (20,21). Osteoblast-specific transcription factors are also involved in the process of newly-formed matrix mineralization, which leads to osteogenesis (22). In the present study, the expression of Runx2 during osteogenic differentiation was improved by PU treatment in Saos-2 cells, while osterix expression was not increased by PU. These data suggest that PU may induce osteoblast differentiation and mineralization by stimulating Runx2.

Lee et al (11) identified that the longitudinal bone growth rate of adolescent rats was increased by P. umbrosa administration via upregulation of IGF-1 and BMP-2. Also, a herbal-based formula including P. umbrosa was demonstrated to exhibit ameliorative effects on pre-, peri and
Figure 4. Effect of PU on matrix mineralization in Saos-2 osteoblast cells. (A) Differentiation of the Saos-2 cells was induced by AA+β-GP in the presence or absence of PU for 10 days. The mineralized matrix was stained with alizarin red and observed under a microscope. Magnification, x40. (B) Following staining, the dye was extracted and quantified. ###P<0.001 vs. non-treated cells. ***P<0.001 vs. AA+β-GP-induced differentiated cells. PU, *Phlomis umbrosa* powder; AA, L-ascorbic acid; β-GP, β-glycerophosphate.

Figure 5. Effects of PU on Runx2 and osterix expression levels in differentiated Saos-2 cells and undifferentiated cells. (A) Differentiation of Saos-2 cells was induced by AA+β-GP treatment in the presence or absence of PU for 10 days. (B) Undifferentiated Saos-2 cells were treated with PU for 10 days in the absence of AA+β-GP. Results are presented as mean ± standard error of the mean. **P<0.01 and ***P<0.001 vs. non-treated cells. PU, *Phlomis umbrosa* powder; Runx2, runt-related transcription factor 2; osterix, transcription factor Sp7; AA, L-ascorbic acid; β-GP, β-glycerophosphate.
post-menopausal symptoms in a randomized, double-blind, placebo-controlled trial involving 72 subjects (ISRCTN 95953434) (23). In the present study, PU exhibited osteogenic effects in OVX-induced osteoporosis mice, which was consistent with data from previous studies (11,23). Considering the data from previous studies and the experimental results of the present study, P. umbrosa may possess the potential to be used in post-menopausal osteoporosis.

P. umbrosa ameliorates osteoporosis through its osteogenic effects. P. umbrosa recovered bone mineral loss and the structure of osteoporotic bone. P. umbrosa promoted matrix formation in osteoblasts by regulating Runx2. Accordingly, P. umbrosa may represent a novel anti-osteoporotic herbal candidate for the treatment of osteoporosis as a bone-forming agent.

Acknowledgements

Not applicable.

Funding

The present study was supported by Basic Science Research Program through the National Research Foundation of Korea Grant funded by the Korean Government (grant no. NRF-2016R1D1A2B0393568).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contribution

All authors participated in the study design, interpretation and analysis of the data and review of the manuscript; JEL, MHK and WMY contributed to the analysis design, JEL, HL and MHK analyzed the data; JEL and WMY drafted the manuscript; and WMY provided supervision of the study.

Ethics approval and consent to participate

Experimental protocols involving animals were approved by the Institutional Animal Ethics Committee of Kyung Hee University, Seoul, Korea [approval no. KHUASP (SE)-15-079].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Zhang W, Yang GJ, Wu SX, Li DQ, Xu YB, Ma CH, Wang JL and Chen WW: The guiding role of bone metabolism test in osteoporosis treatment. Am J Clin Exp Immunol 7: 40-49, 2018.
2. Das S and Crockett JC: Osteoporosis - a current view of pharmacological prevention and treatment. Drug Des Devel Ther 7: 435-448, 2013.
3. Sims NA and Martin TJ: Coupling the activities of bone formation and resorption: A multitude of signals within the basic multicellular unit. Bonekey Rep 3: 481, 2014.
4. Kanis JA and Register JY: European guidance for the diagnosis and management of osteoporosis in postmenopausal women - what is the current message for clinical practice? Pol Arch Med Wewn 118: 538-540, 2008.
5. Fujiwara S, Hamaya E, Sato M, Graham-Clarke P, Flynn JA and Burge R: Systematic review of raloxifene in postmenopausal Japanese women with osteoporosis or low bone mass (osteopenia). Clin Interv Aging 19: 1879-1893, 2014.
6. Sunyecz JA: The use of calcium and vitamin D in the management of osteoporosis. Ther Clin Risk Manag 4: 827-836, 2008.
7. Canderelli R, Leccesse LA, Miller NL and Unruh Davidson J: Benefits of hormone-replacement therapy in postmenopausal women. J Am Acad Nurse Pract 19: 635-641, 2007.
8. Xu Y, Ma X, An J, Ding J, Dai G, Liu Z, Song Z and Lin N: Treatment with QiBaoMeiRan, a Chinese herbal formula, prevents bone loss in ovariectomized rat. Climacteric 19: 98-106, 2016.
9. Shang X, Wang J, Li M, Miao X, Pan H, Yang Y and Wang Y: Antinociceptive and anti-inflammatory activities of Phlomis umbrosa Turcz extract. Fitoterapia 82: 716-721, 2011.
10. López V, Jäger AK, Akerrera S, Cavero RY and Calvo MI: Antioxidant activity and phenylpropanoids of Phlomis lychnitis L.: A traditional herbal tea. Plant Foods Hum Nutr 65: 179-185, 2010.
11. Lee D, Kim YS, Song J, Kim HS, Lee HJ, Guo H and Kim H: Effects of Phlomis umbrosa Root on Longitudinal Bone Growth Rate in Adolescent Female Rats. Molecules 21: 461, 2016.
12. Sandhu SK and Hampson G: The pathogenesis, diagnosis, investigation, and management of osteoporosis. J Clin Pathol 64: 1042-1050, 2011.
13. Kim MH, Choi YY, Han JM, Lee HS, Hong SB, Lee SG and Yang WM: Ameliorative effects of Schizandra chinensis on osteoporosis via activation of estrogen receptor (ER)-α/β. Food Funct 5: 1594-1601, 2014.
14. Soen S, Fukunaga M, Sugimoto T, Sone T, Fujiwara S, Endo N, Gori I, Shiraki M, Hagino H, Hosoi T et al.; Japanese Society for Bone and Mineral Research and Japan Osteoporosis Society Joint Review Committee for the Revision of the Diagnostic Criteria for Primary Osteoporosis: Diagnostic criteria for primary osteoporosis: Year 2012 revision. J Bone Miner Metab 31: 247-257, 2013.
15. Takayanagi H: Inflammatory bone destruction and osteoimmunology. J Periodontal Res 40: 287-293, 2005.
16. Rosen CJ and Boussein ML: Mechanisms of disease: Is osteoporosis the obesity of bone? Nat Clin Pract Rheumatol 2: 35-43, 2006.
17. Chen Q, Shou P, Zheng C, Jiang M, Cao G, Yang Q, Cao J, Xie N, Velletri T; Zhang X, et al.: Fate decision of mesenchymal stem cells: Adipocytes or osteoblasts? Cell Death Differ 23: 1128-1139, 2016.
18. Huang W, Yang S, Shao J and Li YP: Signaling and transcriptional regulation in osteoblast commitment and differentiation. Front Biosci 12: 3068-3092, 2007.
19. Wu M, Chen G and Li YP: TGF-β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. Bone Res 4: 1609, 2016.
20. Chen H, Ghorai-Javed FY, Rashid H, Adhami MD, Serra R, Gutierrez SE and Javed A: Runx2 regulates endochondral ossification through control of chondrocyte proliferation and differentiation. J Bone Miner Res 29: 2653-2669, 2014.
21. Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Fujiiwara S, Hamaya E, Sato M, Graham-Clarke P, Flynn JA and Burge R: Systematic review of raloxifene in postmenopausal Japanese women with osteoporosis or low bone mass (osteopenia). Clin Interv Aging 19: 1879-1893, 2014.
22. Byers BA and García AJ: Exogenous Runx2 expression enhances osteogenic effects in OVX-induced osteoporosis mice, which was consistent with data from previous studies (11,23). Considering the data from previous studies and the experimental results of the present study, P. umbrosa may possess the potential to be used in post-menopausal osteoporosis.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.