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A Korean Poly-Herbal Medicine, SMO16, Regulates the RANKL/OPG Bone Remodeling in OVX Rat Model

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Abstract

Bone mass is regulated by bone resorption(osteoclast) and bone formation(osteoblast), and an imbalance between both processes results in osteoporosis. Osteoporosis is characterized by a loss of bone density and regulation of bone remodeling is crucial for the treatment of osteoporosis. In this study, we investigated whether SMO16, a Korean polyherbal medicine containing 5 herbal components, could regulate bone remodeling in an ovariectomized(OVX) rat model. To test the effect of SMO16 in the OVX model, the rats were divided into 3 groups: a control group, OVX group, and OVX + SMO16(2.25 g/kg) for 8 weeks(ST group). The expression of osteocalcin(OPC), receptor activator of nuclear factor kappa-B ligand(RANKL), and osteoprotegerin(OPG) were measured in the femur using specific antibodies. The bone mass was analyzed using dual-energy X-ray absorptiometry. In the OVX group, protein expression of OPC was decreased; however, in the ST group, OPC expression was significantly increased when compared with the OVX group. SMO16 significantly reduces RANKL-positive cells in the OVX rat femur. Additionally, SMO16 increased OPG expression levels. Importantly, SMO16 significantly increased bone density. These data suggest that SMO16 increases bone remodeling by regulating OPC, RANKL, and OPG signaling. SMO16 could be a potential alternative treatment for osteoporosis.

[Keywords] Osteoporosis, Osteocalcin, RANKL, Osteoprotegerin, Korea Herbal Medicine

1. Introduction

Osteoporosis is a disease characterized by loss of bone mass and weakened bone strength owing to qualitative changes in bones, classified into primary and secondary osteoporosis. Primary osteoporosis includes postmenopausal osteoporosis attributed to a decrease in female hormones, and senile osteoporosis occurs owing to a decrease in bone formation induced by hormonal, calcium, and dermal vitamin D deficiencies. Additionally, secondary osteoporosis can result in several diseases, including chronic kidney failure, thyroid-related diseases, rheumatoid arthritis, and diabetes, and can be induced by an overdose of steroids, anticonvulsants, or anticancer drugs[1][2].

For osteoporosis, Vitamin D and calcium intake are recommended as standard prevention and treatment strategies. Additionally, weight training, yoga, Pilates, jogging, sports dancing, and tennis have been recommended[3]. For the pharmaceutical management of osteoporosis, bone resorption inhibitors such as bisphosphonate and risedronic acid, or calcium and vitamin preparations, and hormone inhibitors are employed[4]. However, when osteoporosis progresses, bone formation accelerators are extremely crucial, and recombinant parathyroid hormone, teriparatide, has been utilized; however, this drug has is accompanied by severe side effects[5].

Furthermore, it is well known that osteoblasts play an important role in bone formation. In particular, osteoblasts express osteocalcin(OPC), receptor activator of nuclear factor kappa-B ligand(RANKL), and

osteoprotegerin(OPG). RANKL/RANK signal transduction regulates osteoclast formation, activation, and survival under diverse pathological conditions characterized by normal bone modeling and remodeling, as well as increased bone remodeling[6]. OPG binds to RANKL and prevents the bone from binding to RANK, thereby preventing excessive bone resorption[7]. Our previous study has demonstrated that SMO16(a Korean polyherbal medicine, including extract of *Carthamus tinctorius*, *Caragana sinica*, *Achyranthes aspera*, *Phlomis umbrosa*, *Eucommia ulmoides*, and *Drynaria fortunei*) regulates bone mass in ovariectomized(OVX) rat osteoporosis models[8]. However, the underlying mechanism by which SMO16 inhibits osteoporosis remains unclear. Thus, we investigated the molecular mechanism of SMO16 in an OVX rat model.

2. Materials

2.1. Chemicals

Vectastain ABC kits and DAB kit were obtained from Vector Laboratories(Burlingame, CA, USA). The antibodies used in this study included anti-OPC, anti-RANKL, and anti-OPG, purchased from Santa Cruz(Santa Cruz, MA, USA).

2.2. Plant material and water extraction of SMO16

The SMO16 extract was prepared according to the methods described in our previous study[8]. Briefly, *C. tinctorius*(30 g), *C. sinica*(30 g), *A. aspera*(30 g), *P. umbrosa*(30 g), *E. ulmoides*(30 g), and *D. fortunei*(30 g) used in the experiment were placed in 2,000 mL of distilled water, preheated for 3 h, and then filtered. The filtrate was reduced to 50 mL using a rotary evaporator, then concentrated and lyophilized to obtain 27 g of extract(yield: 15%). The dose was determined as 2.25 g/kg.

2.3. Preparation of osteoporosis model

Female Wistar rats(Orient bio Inc., Seongnam, Korea) were maintained at 22°C with a 12 h light/dark cycle. All experiments and animal care were performed in accordance with institutional guidelines(SEMCARE 16-06-01). The experimental animals were divided into 3 groups: untreated group, ovariectomized(OVX) group, and OVX with SMO16(2.25g/kg) treatment for 8 weeks. According to the procedure established by Cao H et al[9], the rats(n=15) were anesthetized using isoflurane, and bilateral ovaries were removed.

2.4. Histochemistry and immunohistochemistry

The femurs were fixed using the 10% formalin and treated with a decalcification solution for 12 h. Next, the bones were embedded in paraffin and sectioned. The sections were treated with xylene and a series of ethanol concentrations(100 % to 60%). The tissues were incubated in 5% bovine serum albumin(BSA) in phosphate-buffered saline for 1 h and stained with specific antibodies such as anti-OPC, anti-RANKL, and anti-OPG. The representative images were obtained using microscopy(K1-fluo, Nanoscope system Deajeon, Korea).

2.5. Dual-energy x-ray absorptiometry analysis

After 8 weeks, the rats were sacrificed and the bone mass was measured. The bone mass was analyzed using dual-energy x-ray absorptiometry(DXA, Medikors Inc., Seoul, Korea).

2.6. Data analysis

Immunohistochemical results were quantified(means \pm standard deviation) by image analysis using Image-Pro Plus(Media Cybernetics, USA). The mucosa, randomly selected from each group, was imaged at a 400 \times magnification and at positive pixels/50,000,000 pixels. Statistical analysis

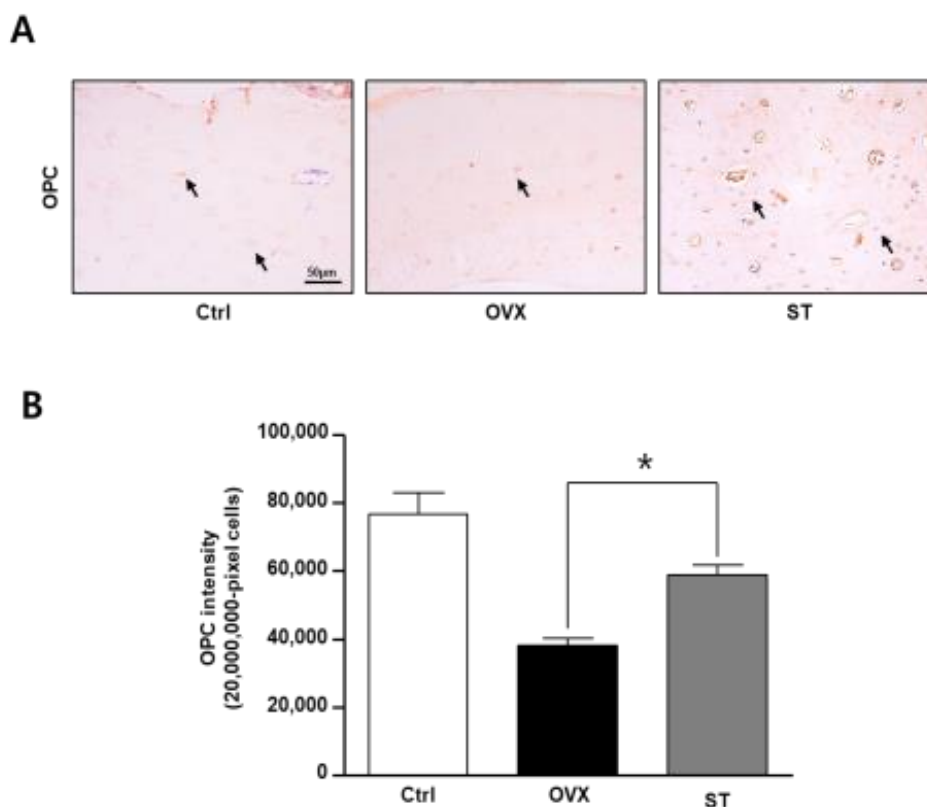
was performed using SPSS ver. 23.0(IBM Corp., Armonk, NY, USA). One-way ANOVA was performed to verify significance($P < 0.05$), followed by the least significant difference(LSD) test.

3. Results

3.1. SMO16 increased the protein expression of osteocalcin in the OVX model

To investigate the effect of SMO16 on bone loss in the *in vivo* model, we analyzed OPC expression in the femur. As shown in <Figure 1>, the level of OPC in the control group was $76,667 \pm 6,110$ pixel. In the ovariectomized rat(OVX group), the OPC level was, on average, $38,333 \pm 2,081$ pixel lower than the control group. However, OPC levels in the ST group were $58,933 \pm 2,685$ pixel higher than in the OVX group.

Figure 1. Effect of SMO16 on femur histomorphometry.

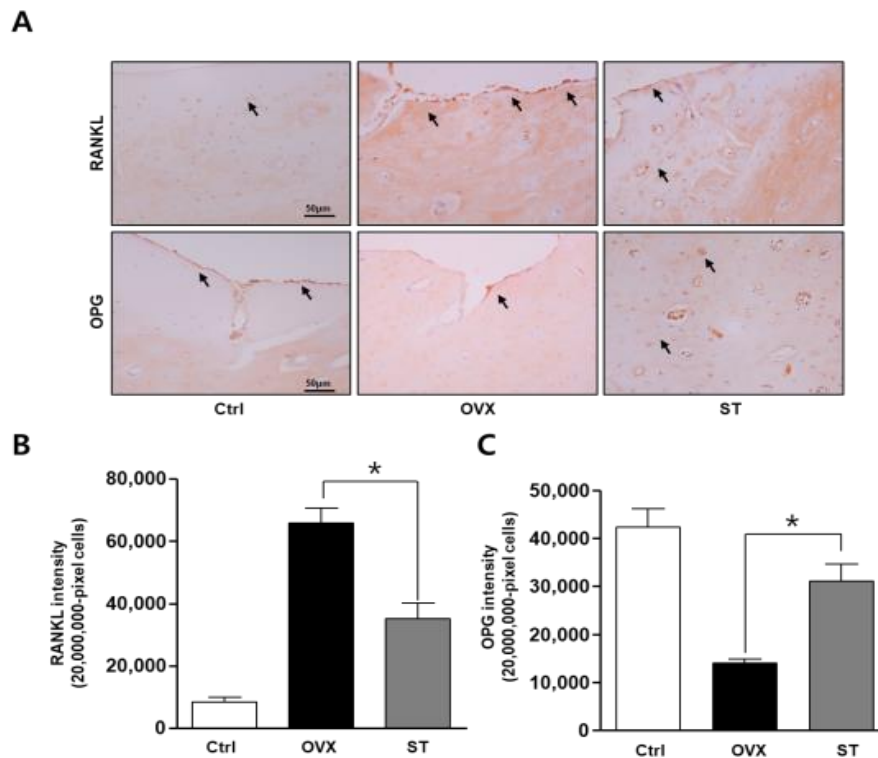


Note: (A) Representative photographs of osteocalcin(OPC) immunohistochemical staining in the rat femur. The arrow indicates OPC positive. (B) Bar graphs indicate the brown intensity of each photograph in a panel(A). Data are expressed as means \pm standard deviation. * $p < 0.05$ vs. the OVX group. Ctrl; control group, OVX; ovariectomized group, ST; SMO16 treated group.

3.2. Effect of SMO16 on protein expression levels of RANK and OPG

To determine whether SMO16 can regulate osteoclastogenesis, we performed immunohistochemistry using specific antibodies. As shown in <Figure 2>, RANKL expression in the OVX group increased by $65,666 \pm 4,932$ pixel when compared with the control group. SMO16 significantly decreased RANKL expression in the OVX group. Regarding OPG expression, protein levels in the OVX group were decreased by $28,399 \pm 2,883$ pixel; in the ST group, OPG expression was increased by $11,333 \pm 180$ pixel.

Figure 2. Immunohistochemistry analysis for RANKL-OPG.

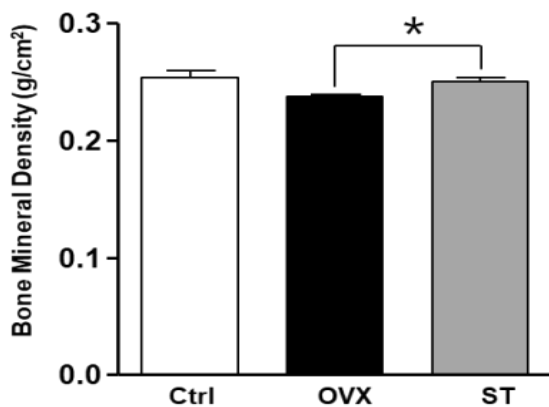


Note: (A) Representative photographs of immunohistochemical staining for RANKL and osteoprotegerin (OPG) in the rat femur. The arrow indicates RANKL and OPG positive, respectively. (B-C) Graphs indicate the brown intensity of each photograph in panel (A). Data are expressed as means \pm standard deviation. * $p < 0.05$ vs. the OVX group. Ctrl; control group, OVX; ovariectomized group, ST; SMO16 treated group.

3.2. Effect of SMO16 on bone density in the OVX rat model

To confirm whether SMO16 promotes bone density, we measured the femur density using the dual-energy X-ray absorptiometry. In the control group, bone density was 0.25 ± 0.05 g/cm²; in the OVX group, bone density was reduced to 0.23 ± 0.02 g/cm². SMO16 significantly increased the bone density to 0.25 ± 0.02 g/cm².

Figure 3. The bone mass density.



Note: The bone mass was measured using dual-energy X-ray absorptiometry. Data are expressed as means \pm standard deviation. * $p < 0.05$ vs. the OVX group. Ctrl; control group, OVX; ovariectomized group, ST; SMO16 treated group.

4. Discussion

It is well known that osteoporosis is associated with improper bone formation such as excessive osteoblast activation and inadequate osteoblast functions[10]. In this study, we demonstrated that treatment with SMO16 regulated bone remodeling in an OVX rat model. Furthermore, these results were confirmed by OPG/RANKL/OPC expression. Notably, SMO16 induced OPC expression. Furthermore, in the OVX rat model, SMO16 treatment significantly downregulated RANKL and upregulated OPG expression. RANKL is associated with osteoclast coupling[11]. Reportedly, RANKL-deficient mice exhibit osteoporosis as a phenotype of immature osteoclasts[12]. In osteoblasts, high levels of OPG expression have been documented[13]. Furthermore, OPG-deficient mice spontaneously develop severe osteoporosis[14]. These results indicated that osteoblastic activation was induced by SMO16. Therefore, we propose SMO16 as a candidate for osteoporosis therapy regulating bone remodeling.

Osteoporosis progresses without demonstrating specific symptoms and often remains asymptomatic until the appearance of fractures. Generally, osteoporosis results in fractures of the spine, wrist, and hip joints. In these patients, the RANKL /RANK factor, involved in the process of inducing bone regeneration, is altered. Furthermore, it has been reported that RANKL/RANK/OPG signaling could be a potential therapeutic target in bone cancer. As RANKL/RANK/OPG controls the abnormal response of bone remodeling and maintains bone homeostasis, it could play a key role in therapeutic approaches for bone cancer. Although our results have not been investigated in bone cancer models, key factors such as OPC, RANKL, and OPG were significantly altered by SMO16. Therefore, we speculate that SMO16 may have anticancer benefits against bone cancer.

Notably, unofficial statistics suggest that the bone density in adult Korean women is lower than that observed in women in Western countries, highlighted by the lack of physical activity. Some studies have reported that exercise and bone formation are closely related. We suggest SMO16 as a candidate prescription for promoting bone mass.

5. References

- [1] Osterhoff G & Morgan EF & Shefelbine SJ & Karim L & McNamara LM & Augat P. Bone Mechanical Properties and Changes With Osteoporosis. *Injury*, 47(2), 11-20 (2106).
- [2] Mirza F & Canalis E. Management of Endocrine Disease: Secondary Osteoporosis: Pathophysiology and Management. *European Journal of Endocrinology*, 173(3), 131-151 (2015).
- [3] Howe TE & Shea B & Dawson LJ & Downie F & Murray A & Ross C & Harbour RT & Caldwell LM & Creed G. Exercise for Preventing and Treating Osteoporosis in Postmenopausal Women. *Cochrane Database System Review*, 6(7), CD000333 (2011).
- [4] Sunyecz JA. The Use of Calcium and Vitamin D in the Management of Osteoporosis. *Therapeutics and Clinical Risk Management*, 4(4), 827-836 (2008).
- [5] Bodenner D & Redman C & Riggs A. Teriparatide in the Management of Osteoporosis. *Clinical Interventions in Aging*, 2(4), 499-507 (2007).
- [6] Kearns AE & Khosla S & Kostenuik PJ. Receptor Activator of Nuclear Factor Kappa B Ligand and Osteoprotegerin Regulation of Bone Remodeling in Health and Disease. *Endocrine Review*, 29(2), 155-92 (2008).
- [7] Kohli SS & Kohli VS. Role of RANKL-RANK/Osteoprotegerin Molecular Complex in Bone Remodeling and its Immunopathologic Implications. *Indian Journal of Endocrinology Metabolism*, 15(3), 175-181 (2011).
- [8] Seo IB & Ahn SH. Effect of SMO16, a Korean Poly-herbal Medicine, on Bone Mass in Ovariectomized Rats. *International Journal of Sport*, 2(2), 18-22 (2017).
- [9] Cao H & Zhang Y & Qian W & Guo XP & Sun C & Zhang L & Cheng XH. Effect of Icaritin on Fracture Healing in an Ovariectomized Rat Model of Osteoporosis. *Experimental and Therapeutic Medicine*, 13(5), 2399-2404 (2017).

- [10] Bi H & Chen X & Gao S & Yu X & Xiao J & Zhang B & Liu X & Dai M. Key Triggers of Osteoclast-related Diseases and Available Strategies for Targeted Therapies: A Review. *Frontiers in Medicine*, 20(4), 234 (2017).
- [11] Ikebuchi Y & Aoki S & Honma M & Hayashi M & Sugamori Y & Khan M & Kariya Y & Kato G & Tabata Y & Penninger JM & Udagawa N & Aoki K & Suzuki H. Coupling of Bone Resorption and Formation by RANKL Reverse Signalling. *Nature*, 561(7722), 195-200 (2018).
- [12] Dougall WC & Glaccum M & Charrier K & Rohrbach K & Brasel K & De Smedt T & Daro E & Smith J & Tometsko ME & Maliszewski CR & Armstrong A & Shen V & Bain S & Cosman D & Anderson D & Morrissey PJ & Peschon JJ & Schuh J. Rank is Essential for Osteoclast and Lymph Node Development. *Genes & Development*, 13(18), 2412-2424 (1999).
- [13] Udagawa N & Takahashi N & Yasuda H & Mizuno A & Itoh K & Ueno Y & Shinki T & Gillespie MT & Martin TJ & Higashio K & Suda T. Osteoprotegerin Produced by Osteoblasts is an Important Regulator in Osteoclast Development and Function. *Journal of Endocrinology*, 141(9), 3478-3484 (2000).
- [14] Bucay N & Sarosi I & Dunstan CR & Morony S & Tarpley J & Capparelli C & Scully S & Tan HL & Xu W & Lacey DL & Boyle WJ & Simonet WS. Osteoprotegerin-deficient Mice Develop Early Onset Osteoporosis and Arterial Calcification. *Genes & Development*, 12(9), 1260-1268 (1998).

6. Contribution

6.1. Authors contribution

	Initial name	Contribution
Lead Author	SIB	-Set of concepts <input checked="" type="checkbox"/> -Design <input checked="" type="checkbox"/> -Getting results <input checked="" type="checkbox"/> -Analysis <input checked="" type="checkbox"/> -Make a significant contribution to collection <input checked="" type="checkbox"/> -Final approval of the paper <input checked="" type="checkbox"/> -Corresponding <input checked="" type="checkbox"/> -Play a decisive role in modification <input checked="" type="checkbox"/> -Significant contributions to concepts, designs, practices, analysis and interpretation of data <input checked="" type="checkbox"/>
Corresponding Author*	ASH	-Participants in Drafting and Revising Papers <input checked="" type="checkbox"/> -Someone who can explain all aspects of the paper <input checked="" type="checkbox"/>

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- Hataedock Treatment has Preventive Therapeutic Effects for Atopic Dermatitis through Skin Barrier Protection in Dermatophagoides Farina Induced NC/Nga Mice, Journal of Ethnopharmacology, 206 (2017).

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6.3. Funding agency

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