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## Effect of SMO16, a KOREAN Poly-Herbal Medicine, on Bone Mass in Ovariectomized Rat

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### Abstract

*The objective of this study was to explore the effect of SMO16, a Korean poly-herbal medicine, on bone mass in ovariectomized (OVX) rats. The loss of bone mass is known to be associated with hormone deficiency in OVX rats. In the present study, we successfully produced the osteoporosis animal-model by dissecting the ovary. To determine the protective effect of SMO16 in OVX rat, SMO16 was administered at a dose of 2.25 g/kg in saline solution for 8 weeks after OVX. Furthermore, we evaluated the direct effect of SMO16 by using a soft X-ray system and Masson's trichrome staining, phloxine-tartrazine staining, and immunohistochemistry in vivo. SMO16 significantly regulated the expression of proteoglycan and osteopontin. These data suggest that SMO16 exerts a protective effect against osteoporosis via the activation of proteoglycan and osteopontin that are closely associated with bone balance. Moreover, these findings suggest that SMO16 can act as a potential therapeutic agent for the treatment of osteoporosis.*

**[Keywords]** Ovariectomized Rat, Osteoporosis, Osteopontin, Osteoblast, Korean Medicine

### 1. Introduction

The skeletal system is a complex structure composed of bones and plays an important role in maintaining weight and stability in the body. Although bones have the ability to remodel during an organism's lifetime, bone homeostasis is weakened by various risk factors such as genetic disorders, nutritional imbalance, and aging[1]. Osteoporosis is one of the most common problems affecting bones and is characterized by a decrease in osteoblast. The loss of bone mass is also influenced by a deficiency of osteopontin(OPN) from osteoblasts. Consequently, clinical manifestations of osteoporosis include reduced proteoglycan and extracellular matrix in bone[2].

Osteoporosis is associated with the female hormone estrogen, and women are more likely to be affected than men. Furthermore, according to a WHO survey, the incidence of

osteoporosis and the increase in the elderly population are closely related[3]. Currently, FDA-approved drugs such as alendronate, ibandronate, risedronate, zoledronic acid, calcitonin, raloxifene, denosumab, and estrogen are prescribed for the treatment and prevention of osteoporosis, and all have been reported to cause side effects, including gastric problems[4]. Therefore, alternative treatment strategies are required to improve gastric problems in patients with osteoporosis. Recently, an interest in natural products to treat osteoporosis has been growing.

In the present study, we examined the anti-osteoporosis effects of a natural product decoction containing *Carthamus tinctorius*, *Caragana sinica*, *Achyranthes aspera*, *Phlomis umbrosa*, *Eucommia ulmoides*, and *Drynaria fortunei*. We demonstrated that the decoction extract inhibits osteoporosis by upregulating the expression of OPN. These findings may

provide an alternative strategy for the treatment of osteoporosis without causing gastric problems.

## 2. Methods

### 2.1. Chemicals

The decalcification solution, trichrome stain, tartrazine, bouins, and phloxine B were purchased from Sigma(St. Louis, MO, USA). The Vectastain ABC kits were obtained from Vector Laboratories(Burlingame, CA, USA). The following antibody used in this study anti-OPN purchased from Santa Cruz(Santa Cruz, MA, USA).

### 2.2. Design of experiments

To explore the effect of SMO16, we performed X-ray analysis, Masson's trichrome staining, phloxine-tartrazine staining, and immunohistochemistry on the femur of an ovariectomized rat.

### 2.3. Procedure

#### 2.3.1. Plant materials and water extraction of SMO16

The *C. tinctorius*(30 g), *C. sinica*(30 g), *A. aspera*(30 g), *P. umbrosa*(30 g), *E. ulmoides* (30 g), and *D. fortune* (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 an extract (yield: 15%). The dose was determined to be 2.25 g/kg.

#### 2.3.2. Preparation of osteoporosis model

The rat ovariectomy procedure was similar to that in previous studies by Liu M et al[5]. Briefly, 94 virgin Wistar female rats (weight  $250 \pm 20.0$  g) were obtained from Orient Bio, Inc.(Seoul, Korea). The rats were maintained at 22°C with a 12 h light/dark cycle. All experiments and animal care were in conformity with institutional guidelines(SEMCARE 16-06-01). The rats were divided into three groups: a normal control group, ovariectomized(OVX)

group, and the group treated with SMO16 after OVX(ST). The SMO16, in 50% ethanol, was orally administered, every day for 8 weeks at a dose of 2.25 g/kg.

#### 2.3.3. X-ray analysis

The radiographic analysis of the femora was performed as previously described[6], using a soft X-ray system(model SRO-M50; SOFRON, Tokyo, Japan).

#### 2.3.4. Histochemistry and immunohistochemistry

The femurs were treated with decalcification solution for 12 h, and then embedded in paraffin. Then, 5- $\mu$ M-thick sections were cut and stained with Masson's trichrome and phloxine-tartrazine. To detect OPN, the tissue sections were treated with blocking serum, 10% normal goat serum, for 4 h at room temperature(RT). Then, the sections were reacted in a humidified chamber at 72 ° C for 72 h with mouse anti-OPN.

The sections were incubated with a secondary antibody: biotinylated goat anti-mouse IgG1 for 24 h at RT. Next, they were incubated with an avidin biotin complex kit. After color development in 0.05 M tris-HCl buffer(pH 7.4) containing 0.05% 3,3'-diaminobenzidine and 0.01% HCl, the cells were stained with hematoxylin.

#### 2.3.5. Data analysis

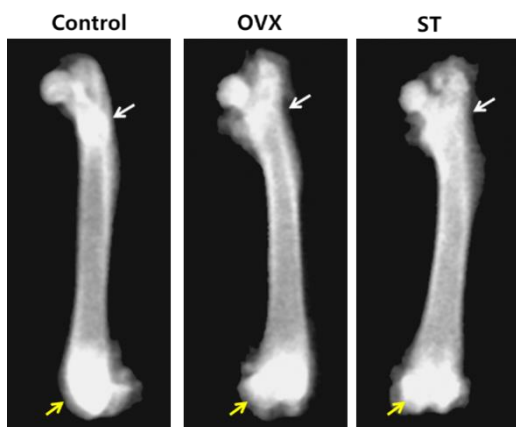
Immunohistochemical results were quantified(means  $\pm$  standard error) by image analysis using image Pro Plus(Media Cybernetics, USA). The mucosa, randomly selected from each group, was imaged at 400x magnification and at positive pixels / 50,000,000 pixels. Statistical analysis was performed using SPSS software(SPSS 23, SPSS Inc., USA). One-way ANOVA was performed to verify significance ( $P < 0.05$ ) and post-test with least significant difference(LSD).

## 3. Results

### 3.1. Effect of SMO16 assessed through X-ray analysis in OVX rats

To investigate whether SMO16 can protect the loss of femur structure, we analyzed the bone using a soft X-ray system. As shown in <Figure 1>, the mass of bone decreased in the OVX group. In contrast, bone loss was inhibited in the ST group than in the OVX group.

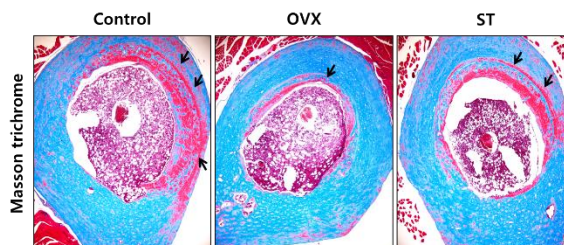
**Figure 1.** The therapeutic effect of SMO16 on ovariectomy-induced bone loss was analyzed by X-ray. Representative image of X-ray in the femur of control group, ovariectomized(OVX) group, and ST(SMO16 treated after OVX for 8 weeks) group. The white arrow points to the greater trochanter of proximal. The yellow arrows point to the epicondyle of distal.



### 3.2. Effect of SMO16 on proteoglycan expression in OVX

To determine whether SMO16 can regulate the formation of bone matrix, we examined proteoglycan expression using Masson's trichrome staining. OVX mice were treated with 2.25 g/kg SMO16 for 8 weeks. After 8 weeks, the untreated OVX group showed a decrease in proteoglycan than the control group did. However, the ST group showed a dramatic improvement in proteoglycan expression. The expression of proteoglycan extended from the spongy bone to compact bone <Figure 2>.

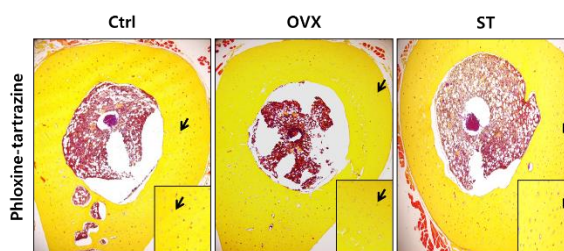
**Figure 2.** The effect of SMO16 on proteoglycan in ovariectomized rat. The panels represent the control group, ovariectomized group(OVX), and treated with SMO16 after ovariectomy(ST) group. The femur tissues were stained with Masson's trichrome. The black arrows indicate proteoglycan(4x magnification).



### 3.3. Effect of SMO16 on bone remodeling in OVX

To evaluate whether SMO16 can influence new bone formation, we performed phloxine-tartrazine(PT) staining on the bone from the experimental groups(control group, OVX group, and ST group). Both the control and ST groups had similar patterns, and showed the presence of Haversian canals, whereas in the femur from OVX group, the Haversian canals showed a decrease in staining <Figure 3>.

**Figure 3.** The effect of SMO16 on Haversian canals in ovariectomy rat. The photomicrographs showed Haversian canals in femur tissue. The tissue stained with phloxine-tartrazine. The black arrow indicates Haversian canals(4x and 200x magnification).

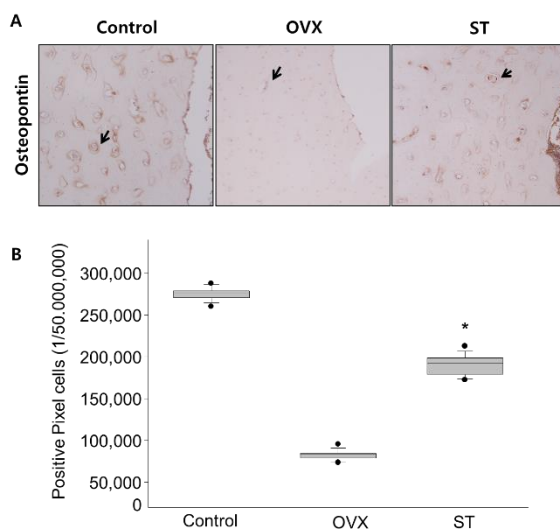


### 3.4. Effect of SMO16 on OPN expression in OVX rats

In order to understand the effect of SMO16 against osteoporosis, we performed immunohistochemistry with an antibody to OPN. The expression of OPN in the OVX group decreased compared to that in the control

group. In contrast, in the ST group, SMO16 significantly promoted the expression of OPN in the femur of the OVX rat <Figure 4>.

**Figure 4.** The histological analysis of the ovariectomized rat. The histological analysis of femur tissue. The femur tissue of control group, ovariectomy (OVX) group, and treated with SMO16 after OVX (ST group) was stained with specific antibody, anti-osteopontin(1:100). The black arrow indicates osteopontin-positive osteoclast cells (400 × magnification). The bar graphs were obtained from panel A. \*, p < 0.05 versus the OVX group.



#### 4. Discussion and Conclusion

Until now, all the available medicines for the treatment of osteoporosis have various side effects and are not suitable for long-term use. In the present study, we explored a medicinal product that combines not only the potential for long-term use, but also is a natural substance effective for osteoporosis prevention. We prepared and tested SMO16, a Korean poly-herbal medicine containing six natural compounds, including *C. tinctorius*, *C. sinica*, *A. aspera*, *P. umbrosa*, *E. ulmoides*, and *D. fortune*. Interestingly, all the formulations showed anti-osteoporosis property. *C. tinctorius* and *A. aspera* showed a protective effect on gastric ulcer[7].

Osteoporosis is commonly characterized by reduced bone mass, proteoglycan, and ex-

tracellular matrix. Furthermore, the Haversian canals, which consist of capillaries and nerve fibers in the bone, play a critical role in new bone formation by supplying nourishment to osteocytes. Our data show that SMO16 significantly regulates bone mass in OVX rats. In the histological analysis, SMO16 induced OPN expression. This suggests that SMO16 may have a potential effect of inducing the differentiation of osteoblasts, because OPN is an osteoblast marker.

In sports science, exercise exerts a sustained stress on the bones, increasing bone density. Regular exercise such as dancing, walking, and swimming is recommended for people with osteoporosis[8][9]. However, osteoblast damage with hormone abnormalities should be treated by medication[10]. Hence, we suggest exercise along with a balanced diet and health supplements for the prevention of osteoporosis.

Until now, all the available medicines for the treatment of osteoporosis have various side effects of SMO16 against osteoporosis in vivo. Our results suggest that SMO16 upregulates proteoglycan, the presence of Haversian canals, and OPN that is attributable not only to bone remodeling, but also to the regulation of osteoblasts. Therefore, SMO16 is a promising therapeutic agent against osteoporosis induced by ovariectomy.

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