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Original Research Article

# Safflower Bud Dietary Prevents Ovariectomy-induced Osteoporosis in Rats

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**Abstract** - Safflower (*Carthamus tinctorius* L.) seeds have long been clinically used in Korea to promote bone formation and prevent osteoporosis. In addition, the safflower buds (SB) were found to have more useful functional ingredients than safflower seed. Thus, we investigated the preventive effects of SB diet in ovariectomized (OVX) rats. The rats were divided into five groups; sham operated group, OVX alone group, OVX plus 17β-estradiol ($E_2$ 10 ㎍/㎏, *i.p.*) and OVX plus SB diet feeding group (0.3% or 1%). Feeding of SB diet (0.3% or 3%) to OVX rats markedly increased trabecular formation in femur compared to OVX rats. Feeding of SB diet (0.3% or 3%) to OVX rats also decreased TRAP activity compared to OVX rats. These results suggest that SB diets have bone sparing effects by the decrease of osteoclast activity. We also observed that OVX rats fed with SB diet (0.3% or 3%) exhibited the decrease of calcium and phosphorus in serum compared to OVX-induced rats. Therefore, SB may be beneficial for the patients of osteoporosis, especially in postmenopausal women.

**Key words** - Ovariectomized rats, Safflower bud, Osteoporosis, Osteoclast

## Introduction

It has been well documented that osteoporosis is characterized by the decrease of mineral density in bone, which is induced by the decrease of osteoblastogenesis and the increase of osteoclastogenesis (Cauley *et al.,* 2000; Cummings and Melton, 2002; Kim *et al.,* 2014). Estrogen deficiency in postmenopause woman is a major risk factor for osteoporosis (Brennan *et al.,* 2012). OVX rats are good model of postmenopausal model (Lelovas *et al.,* 2008). Osteoporosis risks can be reduced with lifestyle changes such as diet and exercise and sometimes medication. Antiosteoporotic medication on fracture healing includes calcium, vitamin D, and several others (Wensel *et al.,* 2011).

It has been reported that the Safflower (*Carthamus tinctorius* L.) seed is a good traditional herbal medicine in Korea and other Asian countries (Bae *et al.,* 2002). In addition, bone healing was significantly repaired in rats supplemented with safflower seed powder compared to control rats (Seo *et al.,* 2000). Safflower seeds have been documented to exhibit bone protecting effect in OVX rats (Kim *et al.,* 2002; Alam *et al.,* 2006). Recently, the safflower bud (SB) retains contents of useful functional ingredients such as flavonoids and polyphenol contents, compared to safflower leaf, stem and root (Hiramatsu *et al.,* 2009). Thus, in this experiment, we investigated the preventive effects of SB in osteoporosis using OVX rat models.

## Materials and Methods

### Animals and treatments

The 8-week-old female Sprague-Dawley strain rats were purchased from Sam tacos (Oh San, Korea) and were housed for a week to obtain the adaptation period. The rats underwent either a sham-operation or were OVX (n = 8-10 for each group). Sham rats were fed a AIN-76 diet, whereas OVX rats were divided into 4 groups that were fed a AIN-76 diet and AIN-76 diet containing SB (0.3% and1 %) for 16 weeks. Rats were housed in clean environmental conditions with a temperature 23 ± 2℃, with a relative humidity of 55 ± 5%, and with a 12-h

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light/dark cycle during the 16-week intervention period. All rats had free access to distilled water and diet throughout the study. The food intake and body weight were measured at intervals of a week. Safflower buds (SB) used in this experiment are produced in Jangheung and were purchased from the market of Jangheung. SB were washed with water and air-dried SB were used. Safflower germinated sprouts are pulverized using a grinder to prepare powder of safflower germination. The diet for Ovx + SB rats contained 0.3% or 1% SB mixed with the standard rat chow. The diet was prepared by mixing 0.3 or 1% SB into 100 g standard rat chow. 17b-estradiol ($E_2$) was purchased from Sigma-Aldrich Chemical Company (MO, USA). Rats were subcutaneously injected 5-times per week with 100 μL of vehicle in the sham and OVX groups, and with 17β-estradiol (10 ㎍/㎏/day). For the experiments, rats were fed with 0.3% and 1% SB diets, as described above. The experimental protocol was approved by the Animal Care and Use Committee of Chonnam National University.

**Sample preparation and storage**

The bone sample that was frozen at -80℃ was ground in liquid nitrogen. Trizol reagent (1 ㎖) was added and oscillated. Chloroform (0.2 ㎖) was added and oscillated vigorously. The mixture was allowed to stand at room temperature for 5 min and centrifuged at 12,000 × g at 4℃ for 15 min. The upper aqueous phase was transferred into an Eppendorf tube. An equal volume of isopropanol was added, oscillated, and then allowed to stand at room temperature for 10 min. The solution was centrifuged at 12,000 × g at 4℃ for 10 min. The supernatant was then discarded. Afterward, the precipitates were washed once with 1 ㎖ 75% ethanol [prepared with diethylpyrocarbonate (DEPC) water], centrifuged at 7500 × g, and dried in air. The sample was diluted with DEPC water. The absorbance (A) was determined using an ultraviolet spectrophotometer. The mRNA concentration and the purity of the sample were calculated according to the ratio of A260/A280. The sample was stored at -20℃.

**Detection of cathepsin K expression**

The total RNA was extracted using the Trizol method and reverse transcribed into cDNA. The PCR reaction system, with a total volume of 50 μl, contained 4.0 μl cDNA, 5.0 μl 10X PCR buffer, 3.0 μl 25 mM $MgCl_2$, 1.0 μl 10 mM dNTPs, 1.0 μl 10 μM upstream primers, 1.0 μl 10 μM downstream primers, and 5 μl β-actin primer mixture (with 28.5 μl ddH2O and 0.5 μl 5 U/μl *Taq*).

In the amplification, a pre-denaturation step was performed at 50℃ for 2 min, followed by 40 cycles of 95℃ for 10 min, 94℃ for 30 s, and 61℃ for 1 min. The primer design and synthesis template had GenBank accession No. NM_031560.2. Upstream and downstream primer sequences used in this study were 5'-GGGAGACATGACCAGCGAAG-3' and 5'-CTGAAAGCCCAACAGGAACC-3'. β-actin was used as an internal standard. Upstream and downstream primers were 5'-CCGTCTTCCCCTCCATCG-3' and 5'-GTCCCAGTTGG TGACGATGC-3'. The PCR product length was 195 bp. The application amount of each sample was 1 μl. Considering that errors in RNA concentration quantification and RNA reverse transcription efficiency may lead to differences in cDNA contents among samples with the same volume, β-actin was used as the internal standard. The relative content of the target gene was calculated according to the ratio between the value of the target gene and that of the internal standard.

**H & E staining & TRAP staining**

The femur was decalcified in 10% EDTA in 0.01 M phosphate buffer (pH 7.4) at a temperature of 4℃ for 1 week. The femur was then dehydrated in a graded series of ethanol solutions at 4℃, embedded in paraffin and sectioned at a thickness of 5 ㎛. The serial histological sections were cut longitudinally (4 ㎛) and then stained by TRAP kit. Images of the growth plate and proximal tibia were photographed by using a CX31 microscope (Olympus, Tokyo, Japan). Measurement of the ratio between osteoclasts and the perimeter of trabecular bone was performed on the primary and secondary spongiosa. Bone perimeter was calculated using Image Pro Plus 3.5 (Media Cybernetics, MD, USA).

**MicroCT analysis**

The femur was dissected from the femoral region and fixed with 4% paraformaldehyde fixed storage system and a high-resolution micro-computerized tomography (Three-dimensional micro focus computed tomography; micro-CT, Sky-Scan 1172TM, Skyscan, Kontich, Belgium) using a micro video

image was obtained. Fine image was obtained from Nercon Ver 1.3 (Skyscan) and was rebuilt as the gray scale level and a two-dimensional image reconstruction of the CTAn (SkyScan) software was used to reconstruct a three-dimensional model.

**Serum analysis**

After 16 weeks of experiment time, the experimental rats were fasted for 24 h and anesthetized.

Blood samples were collected from the heart by cardiac puncture. Bloods were centrifuged at 6000 × g for 10 min; then, serum samples were stored at －80℃ for biochemical determinations. The analysis of phosphorus and Ca2+ was measured using blood analyzer.

**Statistical methods**

Statistical analyses were performed using GraphPad Prism software version 4.0 (GraghPad Software, San Diego, CA, USA). The data for all of the measurements were analyzed using a one-way analysis of variance (ANOVA) with subsequent post hoc multiple comparison by Dunnett's test. Statistically significant values were defined as $P < 0.05$.

## Results and Discussion

**Effects of SB diets on serum biomarkers in OVX rats**

Because phosphate and $Ca^{2+}$ are implicated in dysfunction of bone disease (Yogesh *et al.,* 2011), we checked whether SB has effect on serum phosphate and $Ca^{2+}$ in OVX-induced rats. Serum phosphorus and calcium levels were decreased in OVX-induced rats and feeding of SB diets in OVX rats showed a significant protection effect on serum phosphorus and Ca2+ (Fig. 1). These results suggest that serum calcium and phosphorus levels are associated with osteoporosis and SB diets mediate bone protection by regulation of calcium and phosphorus levels. Qin *et al.* (2013) also reported similar result that the calcium and phosphorus levels are decreased in OVX-induced rats.

**Effects of SB diets on distal femoral bone histomorphometric parameters (BV/TV, Tb.N, and Tb.Th) in OVX rats**

OVX rats are classically used as an animal model for postmenopausal bone loss (Kalu, 1991). Currently, distal

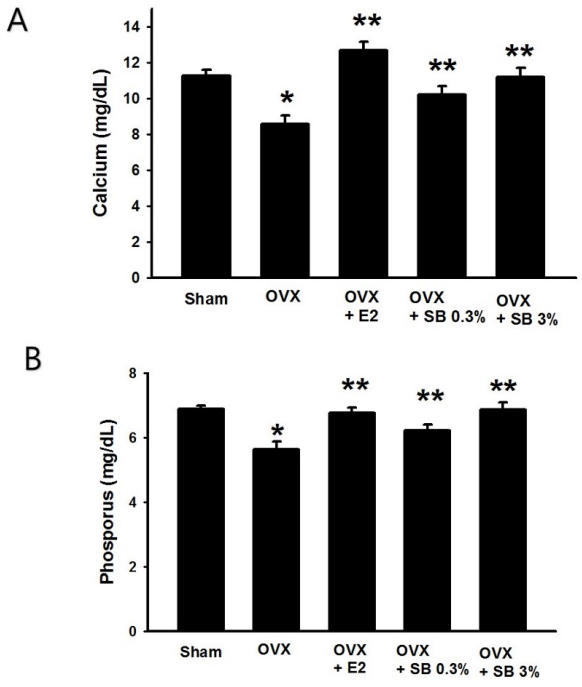


Fig. 1. Effects of safflower buds (SB) on serum calcium and phosphorus in ovariectomized (OVX) rats. OVX rats were treated with 17β-estradiol (E2, 10 μg/kg, i.p.) or fed with SB diet (0.3% or 3%). Each value is expressed as mean ± S.E., n = 8~10. *P < 0.05 compared with the sham group and **P < 0.05 compared with the OVX group.

femoral bone histomorphometric parameters such as bone volume/tissue volume (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th) are commonly used to measure bone diseases (Osterhoff *et al.,* 2012). So, we evaluated the effect of SB diets on BV/TV, Tb.N, and Tb.Th levels in OVX rats. In the present study, the histomorphometrical analysis of bone structure showed that BV/TV, Tb. N, and Tb. Th in OVX-induced rats were decreased compared Sham group (Fig. 2 and 3). In addition, feeding of SB diets in OVX rats showed a significant protection effect on BV/TV, Tb. N and Tb.Th analysis, compared to OVX-induced rats (Fig. 2 and 3). The changes in trabecular bone structure parameters in the OVX-induced rats were due to an imbalance in the normal remodeling process. We observed that SB diets prevented this change. The improvement observed in all structural parameters indicate that SB is effective in the prevention of OVX-induced bone loss due to bone resorption. Recently Rahman *et al.* (2014) reported similar result that the treatment of safflower oil prevents OVX-induced bone loss in mice.

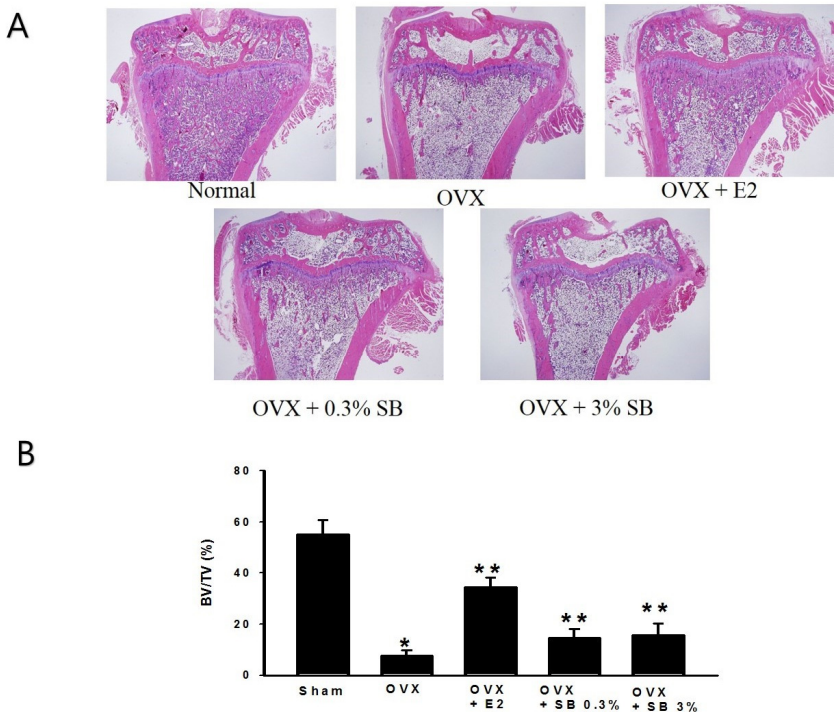


Fig. 2. Effects of safflower buds (SB) on femur H & E staining (A) and BV/TV (B) in ovariectomized (OVX) rats. OVX rats were treated with 17β-estradiol (E2, 10 $\mu$g/kg, i.p.) or fed with SB diet (0.3% or 3%). Each value is expressed as mean ± S.E., n = 8~10. *$P$ < 0.05 compared with the sham group and **$P$ < 0.05 compared with the OVX group.

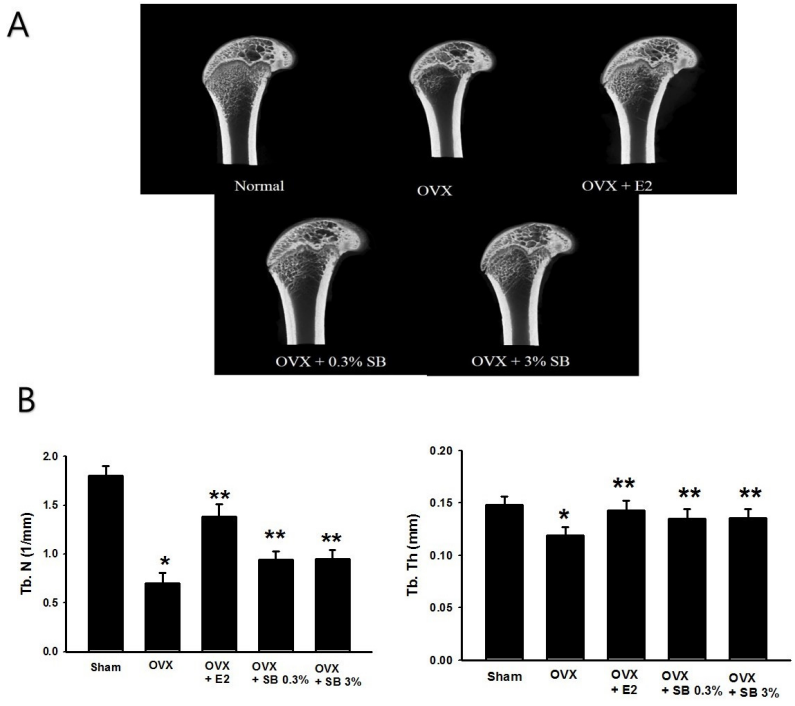


Fig. 3. Effects of safflower buds (SB) on femur microCT (A) and BV/TV (B) in ovariectomized (OVX) rats. OVX rats were treated with 17β-estradiol (E2, 10 $\mu$g/kg, i.p.) or fed with SB diet (0.3% or 3%). Each value is expressed as mean ± S.E., n = 8~10. *$P$ < 0.05 compared with the sham group and **$P$ < 0.05 compared with the OVX group.

Here we provided additional evidence that SB is also a therapeutic plant to inhibit bone loss.

**Effects of SB treatment on bone cathepsin K expression in OVX rats**

Cathepsin K is one of the most important endoproteinase that is expressed highly and selectively in osteoclasts (Michaela Kneissel *et al.,* 2004). However, it is not expressed in the osteoblasts or in the osteocytes. It is usually used as a marker for osteoclasts in bone. The overexpression of cathepsin K can increase cancellous bone turnover, suggesting that cathepsin K may be an important target and functions as a highly specific and sensitive biomarker in bone resorption treatment (Holzer *et al.,* 2005). In this study, TRAP expression was increased in the femur of OVX group (Fig. 4). The activity of TRAP in femur of OVX rats fed with SB diets was also significantly reduced compared with OVX alone group (Fig. 4). In addition, we also checked the TRAP mRNA expression in this study. The mRNA of TRAP in femur of OVX rats fed with SB diets was also significantly reduced compared with OVX alone group (Fig. 4). These results suggest that feeding of SB diets improved the decrease of osteoclast activity. This is the first report, to our knowledge, that SB diets have bone sparing effect by the decrease of osteoclast. The specific mechanism requires further study.

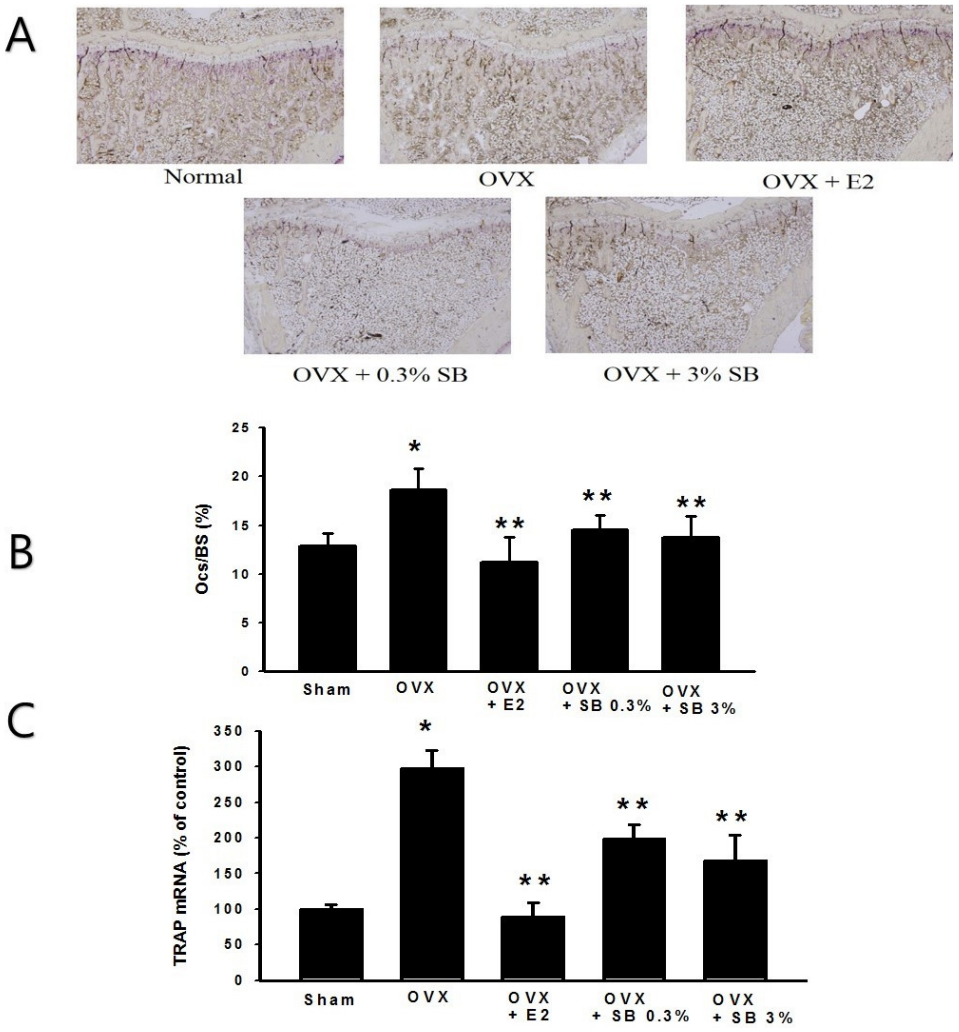


Fig. 4. Effects of safflower buds (SB) on femur TRAP immunohistochemistry (A), Ocs/BS (B) and TRAP mRNA (C) in ovariectomized (OVX) rats. OVX rats were treated with 17β-estradiol (E2, 10 $\mu$g/kg, i.p.) or fed with SB diet (0.3% or 3%). Each value is expressed as mean ± S.E., n = 8~10. *$P$ < 0.05 compared with the sham group and **$P$ < 0.05 compared with the OVX group.

The most significant finding of this study is that SB diets can effectively prevent OVX-induced osteopenia by regulating bone mineral structure and osteoclast activity. Taken together, our results suggest that SB is a potential candidate as new anti-osteoporosis components.

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