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Effects of silk fibroin hydrolysate on bone metabolism in ovariectomized rats

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Abstract

This study aimed to investigate the effects of silk fibroin on bone metabolism in ovariectomized rats. A total of 30 Sprague-Dawley rats were randomized into sham-operated (SHAM), ovariectomized control (OVX), alendronate (OVX+ALEN, 10 mg/kg body weight/d), low silk fibroin (OVX+SF100, 100 mg/kg body weight/d), and high silk fibroin (OVX+SF300, 300 mg/kg body weight/d) groups. All the rats were fed by gavage for 12 wk. At the end of 12 wk, blood and urine were collected for analysis of bone turnover markers, and bone mineral density (BMD) was measured by micro-computed tomography. The results show that the OVX group ($p < 0.05$) displayed the highest mean body weight gain. Among the five groups, serum levels of bone alkaline phosphatase (ALP) and urine levels of deoxypyridinoline (DPD) were highest in the OVX group ($p < 0.05$). Bone ALP levels in the ALEN group were significantly lower than that of the silk-treated groups. On the other hand, DPD levels were not significantly different between the ALEN and silk-fibroin-treated groups ($p < 0.05$). The trabecular BMD was significantly higher in the ALEN and silk-treated groups compared to the OVX group ($p < 0.05$). In conclusion, this study showed that silk fibroin has similar effects as alendronate, which is used in osteoporosis medication. Therefore silk fibroin might be a new candidate for the prevention and treatment of osteoporosis in patients.

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Introduction

Bone remodeling involves sequential bone formation and bone resorption in order to maintain bone homeostasis (Kim *et al.,* 2007a). Osteoclasts are the cells responsible for bone resorption, which is a process that includes demineralization and osteolysis. Osteoblasts are involved in bone formation, which is a process that includes osteogenesis and calcification (Suda *et al.,* 1997). The activities of both these types of cells are understood to be regulated by systemic hormones and other factors (Cnalis *et al.,* 1988). Imbalance between bone formation and bone resorption leads to bone loss and causes bone diseases, such as osteoporosis (Rhee *et al.,* 1983).

Rapid bone loss in menopausal women is caused mainly by

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estrogen deficiency, which is one of the factors responsible for regulating the production and activity of cytokines to increase bone remodeling (Recker *et al.,* 2004; Bilezikian 1998). Estrogen deficiency promotes osteoclast cell differentiation and activity by increasing the production of several pro-inflammatory cytokines, such as interleukin-6 (IL-6) and interleukin-1β (IL-1β) (Jilka 1998), and it also reduces the production of osteoprotegerin (OPG). On the other hand, estrogen deficiency increases the production of the receptor activator of nuclear factor kappa-B ligand (RANKL). Consequently, the increased RANKL/OPG ratio stimulates the formation, maturation, and activity of osteoclasts (To *et al.,* 2012).

The long-term use of current anti-osteoporotic drugs to treat bone disease produces side effects such as nausea, headache, weight gain, breast pain, and uterine bleeding. In addition, their long-term use has contributed to increased incidence of endometrial cancer and breast cancer among patients (Andersen *et al.,* 1999). Accordingly, there is a pressing need for the development of effective new therapeutic agents that function similar to estrogen, with low toxicity and fewer side effects (Kim *et al.,* 2007b). A recent study showed that flavonoid compounds in green tea improve bone density by increasing the activity of osteoblast cells. However, research concerning the tea's composition and the optimal amount of consumption for achieving the bone-related health benefits is lacking (Chen *et al.,* 2005). Additionally, a number of studies have shown that isoflavones, one of the phytoestrogens found in soybeans and soybean products, improve bone health by maintaining bone density and inhibiting bone resorption (Washbum *et al.,* 1999; Song *et al.,* 1999; Arjmandi *et al.,* 1996; Lee *et al.,* 2002; Cassidy 1999). The mechanism for the effects of soy protein on bone health, however, is still being debated (Choi and Jung, 2005).

Silk is a natural protein that is used commercially in textile fiber and biomedical sutures owing to its excellent mechanical strength and biological stability. Silk from the silkworm *Bombyx mori* mainly consists of two kinds of proteins, fibroin and sericin. Silk fibroin (SF) is composed of light and heavy chain polypeptides of 25 and 325 kDa, respectively. Fibroin is encased in a sericin coat, which is a glue-like protein that holds two fibroin filaments together (Alman *et al.,* 2003). SF is used in a various fields owing to its unique physical and chemical properties (Kweon *et al.,* 2014; Kim *et al.,* 2014; Chon *et al.,* 2013; Seok *et al.,* 2013; Yun and Lee, 2013; Yoo and Um, 2013; Cho *et al.,* 2013; Yeo *et al.,* 1999). The hydrolysates of SF, which are obtained by the hydrolysis of acids or enzymes, are water-soluble peptides with current applications in functional foods, cosmetics, drugs, and so on. Research has revealed various benefits of SF hydrolysates, including hypoglycemic effects in patients with type 2 diabetes (Nam and Oh, 1995), inhibitory effects on the absorption of alcohol (Kim *et al.,* 1996), and the promotion of collagen synthesis and re-epithelialization (Roh *et al.,* 2006). Furthermore, recent studies have reported the use of SF as a supplement for wound healing and bone and cartilage regeneration (Kim *et al.,* 2010). Previous experiments have shown indirect effects of SF on the inhibition of osteoclastogenesis in RAW 264.7 cells (Yeo *et al.,* 2008) and direct effects of SF on anti-resorptive activities through both inhibition of osteoclastogenesis and the induction of osteoclast apoptosis (Chon *et al.,* 2012). So far, however no existing studies have been performed on animals to evaluate the effects of SF on bone metabolism.

Based on the results of previous studies, the present study administered SF to ovariectomized rats in order to closely analyze the effects of SF on menopausal bone metabolism by evaluating morphological, biochemical, and immunological markers.

Materials and Methods

Preparation of SF

Raw silk (*Bombyx mori L.*) cocoons were obtained from the Rural Development Administration of Korea. The raw materials were degummed twice with 0.5% on-the weight-of–fiber (OWF) Marseilles soap and a 0.3% OWF sodium carbonate solution at 100˚C for one h. The resulting silk was then washed with distilled water. Subsequently, SF was dissolved in 6N hydrochloric acid (HCl) for five h. The acid degradation was stopped and then neutralized by adding sodium hydroxide. Sephadex G-25 gel filtration chromatography (Pharmacia GradiFrac, UV-1 detection, Sweden) was used to remove the salt residue. The SF solution and 3% (v/v) proteases were mixed under nitrogen gas and hydrolyzed at 55˚C for 24 h. Subsequently, the solution was heated in a bath of boiling water to stop the enzyme reaction. Then SF powder was obtained using a freezing drier.

Animals and diet

The Animal Care and Use Review Committee (IACUC) of Kyung Hee University approved the protocol of our experiments. A total of 30 five-wk-old female *Sprague-Dawley* rats (140-150 g) were purchased from SLC, Inc. (Shizuoka, Japan). The rats were housed in polycarbonate cages in temperature-controlled rooms (22 ± 2°C), with a relative humidity of 55 ± 5% and a 12-h light/dark cycle. The rats were fed a pellet chow diet and were provided water *ad libitum* for an adaption period of two wk. After the rats were adapted, sham operations (n = 6) were performed by exposing but not excising their ovaries. In contrast, ovariectomies (n = 24) were performed by ligating and excising the ovaries of the rats while they were maintained under anesthesia using isoflurane inhalation (3% dissolved in oxygen). Postoperative rats were kept for eight wk until osteoporosis was confirmed by examining bone mineral density (BMD) using micro-computed tomography (μ-CT). Subsequently, the animals were randomized into five groups of six animals each. The SF groups received oral administration of SF in doses of either 100 mg or 300 mg SF/kg body weight/d. As a positive control, six rats orally received 10 mg alendronate/kg body weight/d (i.e., the ALEN group). The SHAM-operated and untreated OVX (negative control) groups were administered distilled water. All the rats were fed the same experimental diet (AIN93G pellets, USA) and were received an additional 12 wk of treatment. After 12 wk of treatment, the animals were sacrificed and various tissues were collected, weighed, and frozen in liquid nitrogen prior to storage at -70°C.

Body weight and food consumption

The body weights of the animals were measured twice per wk, and food consumption was measured daily. The food efficiency ratio (FER) was calculated using the following formula:

[weight gain(g)/d]/[amount of food consumed(g)/d].

Blood sample analysis

At the end of the study, the animals were anesthetized with ethyl ether and their blood was collected by cardiac puncture. Serum was separated by centrifugation at 3,000 rpm for 15 min at 4°C, and stored immediately at -70°C until analyzed. Serum osteocalcin was measured in duplicate using MILLOPLEX bone hormone panel (Millipore, Billerica, MA, USA). Bone alkaline phosphatase (ALP) (IDS Ltd., Boldon, UK) was measured using commercially available ELISA (enzyme-linked immunosorbent assay) kits. Pro-inflammatory cytokines (IL-1β and IL-6) were measured using Millipore's MILLIPLEX rat cytokine panel (Millipore). Measures of OPG (IDS Ltd.) and RANKL (IDS Ltd.) were obtained with Millipore's MILLIPLEX rat bone panel 2 (Millipore).

Urine sample analysis

Each animal was housed individually in metabolic cage. At 12 wk, urine was collected for 24 h from each cage. During the urine collection period, the rats were restricted from their diet to avoid urine contamination. The rats were allowed free access to water. The instruments used for urine collection were washed with 0.1 N HCl. Urine samples were centrifuged at 2,000 rpm for 15 min at room temperature, and the top layer was collected and stored at -70°C until analysis. Urinary deoxypyridinoline (DPD), which is a useful marker of bone resorption, was measured by an ELISA kit using collagen cross-links™ (Metra Biosystems Inc., Mountain view, CA, USA).

Morphological analysis by μ-CT

μ-CT was performed on the left tibiae of the animals using the SkyScan 1076 (SkyScan, Kontich, Belgium) system at the beginning and the end of the experimental period. In brief, the trabecular microarchitecture together with three-dimensional (3D) images was scanned at 50 kV and 200 μA at a rotation step of 0.4°. Analyses of the reconstructed scans were also performed using NRecon cone-beam algorithm software (SkyScan), and the files were imported into CTan software (SkyScan) for 3D analysis and image generation. On the original 3D images, morphometric indices, including bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular pattern factor (Tb.Pf), structure model index (SMI), and BMD were measured.

Statistical analysis

Statistical analysis was performed using SPSS software (Version 20.0, SPSS Inc., Chicago, USA). The results were expressed as the means ± standard deviation (S.D.). One-way ANOVA was performed to determine the individual group

Table 1. Weight gain, dietary intakes, and food efficiency ratio (FER)

	SHAM	OVX	ALEN	SF100	SF300
Weight gain (g/d)	1.20 ± 0.13[b]	1.68 ± 0.22[a]	1.61 ± 0.19[a]	1.59 ± 0.26[a]	1.51 ± 0.20[a]
Dietary intake (g/d)	10.59 ± 1.64	11.07 ± 1.50	10.99 ± 1.36	10.66 ± 2.01	10.19 ± 1.48
FER (%)	1.03 ± 0.09[b]	1.38 ± 0.12[a]	1.33 ± 0.12[a]	1.35 ± 0.10[a]	1.35 ± 0.09[a]

Data are expressed mean ± S.D. Different superscript letters within a row indicate significant differences by Duncan's multiple range test at $p < 0.05$.
Food efficiency ratio (FER): [weight gain(g)/d]/[amount of food consumed(g)/d].

differences. Significant differences ($p < 0.05$) among the means were determined by Duncan's multiple range tests.

Results

Body weights and organ weights

The weight gain of the ovariectomized rats was significantly greater than the weight gain of the SHAM group ($p < 0.05$). The dietary intakes of all groups were not significantly different. The FERs were also higher in the ovariectomized rats in compared to the SHAM group ($p < 0.05$). The values of body weight, weight gain, and FERs in the SF300 group were lower than the corresponding values in the SF100 group, but there were no significant differences between the groups (Table 1). Liver, spleen, and kidney weights were not significantly different among the five experimental groups. Uterine weights of the ovariectomized rats were significantly lower than the uterine weights in the SHAM group ($p < 0.05$) (data not shown).

3D images using μ -CT and BMD

The μ-CT images showed that the microarchitecture of the animals' trabecular bones markedly improved after the administration of SF and alendronate after ovariectomy. The BMD levels in the trabecular bones of the OVX group were significantly lower than those of the ALEN and SF (i.e., both SF100 and SF300) groups ($p < 0.05$). The BMD levels in the SF-treated groups (SF100 and SF300) were lower compared to the ALEN group, but no significant difference was observed between groups (Fig. 1).

Trabecular bone histomorphometric parameters

Both the BV/TV and Tb.N of the animal trabecular bones in the SHAM group were the highest among the five groups ($p < 0.05$).

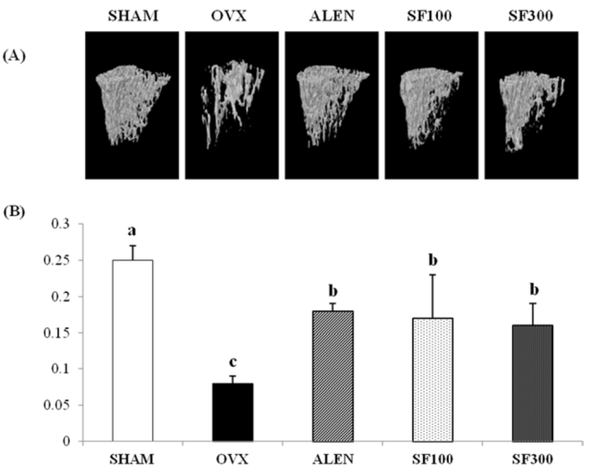

Fig. 1. 3D images (A) and bone mineral density (B) of trabecular bones
Data are expressed as the mean ± S.D. Different superscript letters within a row indicate significant differences by Duncan's multiple range test at $p < 0.05$.

The BV/TV and Tb.N of the animal trabecular bones in the ALEN and SF100 groups were significantly higher than those of the OVX and SF300 groups ($p < 0.05$). The Tb.Th values of the trabecular bones in the SHAM and OVX groups were the lowest ($p < 0.05$), and this value in the ALEN group was significantly lower than the values of the SF (SF100 and SF300) groups ($p < 0.05$). The Tb.Sp, Tb.Pf, and SMI of the animal trabecular bones of the OVX group were the highest ($p < 0.05$) among all the groups. Values for the Tb.Sp, Tb.Pf, and SMI of the trabecular bones in the ALEN and SF (SF100 and SF300) groups were not significantly different (Table 2).

Effects of SF on biochemical parameters

Serum levels of osteocalcin and bone ALP in the ALEN group were the lowest among the five groups ($p < 0.05$). Serum levels of osteocalcin and bone ALP in the SF (SF100 and SF300) groups were significantly lower than those in the OVX group (p

Table 2. Trabecular bone histomorphometric parameters

	SHAM	OVX	ALEN	SF100	SF300
BV/TV (%)	13.69 ± 1.24^{a}	5.90 ± 0.64^{c}	8.70 ± 3.19^{b}	8.76 ± 3.44^{b}	6.11 ± 3.37^{c}
Tb.Th (mm)	0.087 ± 0.005^{c}	0.092 ± 0.003^{c}	0.098 ± 0.002^{b}	0.105 ± 0.007^{a}	0.101 ± 0.003^{a}
Tb.N (mm^{-1})	1.58 ± 0.13^{a}	0.57 ± 0.10^{c}	0.94 ± 0.32^{b}	0.84 ± 0.34^{b}	0.60 ± 0.32^{c}
Tb.Sp (mm)	0.27 ± 0.04^{c}	0.59 ± 0.04^{a}	0.44 ± 0.06^{b}	0.49 ± 0.16^{b}	0.52 ± 0.13^{b}
Tb.Pf (mm^{-1})	15.34 ± 1.53^{b}	19.49 ± 1.85^{a}	16.39 ± 3.03^{b}	14.32 ± 0.58^{b}	15.39 ± 3.44^{b}
SMI	2.09 ± 0.21^{c}	2.82 ± 0.16^{a}	2.52 ± 0.20^{b}	2.51 ± 0.18^{b}	2.54 ± 0.20^{b}

Data are expressed mean ± S.D. Different superscript letters within a row indicate significant differences by Duncan's multiple range test at $p < 0.05$.
BV/TV = bone volume fraction; Tb.Th = trabecular thickness; Tb.N = trabecular number; Tb.Sp = trabecular separation; Tb.Pf = trabecular pattern factor; and SMI = structure model index.

Table 3. Effects of silk fibroin on biochemical parameters

	SHAM	OVX	ALEN	SF100	SF300
Serum					
Osteocalcin (ng/mL)	17.53 ± 1.99^{c}	24.10 ± 3.04^{a}	11.78 ± 1.22^{d}	20.25 ± 2.33^{bc}	21.04 ± 2.71^{b}
Bone ALP (μg/L)	50.55 ± 5.72^{b}	57.01 ± 3.00^{a}	43.76 ± 2.11^{c}	51.09 ± 4.94^{b}	53.32 ± 2.34^{b}
Urine					
DPD (nM)	44.49 ± 8.98^{b}	75.78 ± 8.91^{a}	44.98 ± 12.69^{b}	45.69 ± 18.38^{b}	47.93 ± 12.50^{b}

Data are expressed mean ± S.D. Different superscript letters within a row indicate significant differences by Duncan's multiple range test at $p < 0.05$.
ALP = alkaline phosphatase; DPD = deoxypyridinoline.

Table 4. Effects of silk fibroin on immunological parameters

	SHAM	OVX	ALEN	SF100	SF300
RANKL (pg/mL)	2.44 ± 0.56^{c}	4.98 ± 1.02^{a}	4.09 ± 0.65^{b}	3.94 ± 0.63^{b}	3.32 ± 0.97^{b}
OPG (pg/mL)	38.52 ± 9.60^{a}	30.22 ± 6.57^{b}	34.82 ± 4.26^{a}	32.44 ± 3.73^{a}	33.36 ± 8.75^{a}
RANKL/OPG	0.07 ± 0.02^{c}	0.17 ± 0.03^{a}	0.12 ± 0.01^{b}	0.12 ± 0.03^{b}	0.11 ± 0.04^{b}
IL-1β (pg/mL)	13.27 ± 1.93^{b}	15.76 ± 1.21^{a}	13.62 ± 1.61^{b}	13.62 ± 1.37^{b}	14.72 ± 0.33^{b}
IL-6 (pg/mL)	30.50 ± 15.87^{b}	55.10 ± 10.32^{a}	15.30 ± 2.17^{c}	15.00 ± 0.94^{c}	18.30 ± 3.87^{c}

Data are expressed mean ± S.D. Different superscript letters within a row indicate significant differences by Duncan's multiple range test at $p < 0.05$.
RANKL = receptor activator of nuclear factor kappa-B ligand; OPG = osteoprotegrin; IL-1β = interleukin-1β; IL-6 = interleukin-6.

< 0.05). Urinary levels of DPD in the OVX group were higher than those of the SHAM, ALEN, and SF (SF100 and SF300) groups ($p < 0.05$). Urinary levels of DPD increased in the SF (SF100 and SF300) groups compared to the ALEN group, but the difference was not statistically different ($p < 0.05$) (Table 3).

Effects of SF on immunological parameters

Serum levels of RANKL were significantly higher, whereas serum levels of OPG were lower, in the OVX group than the levels in the SHAM, ALEN, and SF (SF100 and SHF) groups ($p < 0.05$). Consequently, the RANKL/OPG ratios of the ALEN and SF (SF100 and SF300) groups were significantly lower than the RANKL/OPG ratio of the OVX group ($p < 0.05$). The serum levels of IL-1β and IL-6 in the group treated with OVX were significantly higher than those of in all the other groups ($p < 0.05$). Serum levels of IL-6 in the SHAM group were higher than those of the ALEN and SF (SF100 and SF300) groups ($p < 0.05$).

Serum levels of RANKL, OPG, IL-1β and IL-6 in the ALEN and SF (SF100 and SF300) groups did not show any significant differences (Table 4).

Discussion and conclusion

Menopausal osteoporosis is a bone metabolic disease characterized by low bone mass and the structural deterioration of bone tissue, which is associated with estrogen deficiency (National Osteoporosis Foundation, 2010). Because medications currently used for its treatment have various side effects, natural compounds with beneficial effects on bone have been proposed as an alternative strategy for the prevention and treatment of osteoporosis (Banu *et al.,* 2012). The purpose of the present study was to investigate whether hydrolyzed SF is beneficial against menopausal bone metabolism in ovariectomized rats.

Morphological markers of trabecular bone are helpful tools to confirm bone metabolism. Trabecular bone, one of two types of osseous bone tissue, is typically found at the end ofs of long bones, proximal to joints and within the interior of vertebrae. In many previous studies in which BMD levels were measured after ovariectomies in subjects, significant decreases were found in trabecular bone volume, whereas no significant differences were observed in cortical bone volume (Cao *et al.,* 2001; Iwata *et al.,* 2005; Jiang *et al.,* 2003). The results of the present study also confirmed that ovariectomy leads to considerable loss of trabecular BMD. However, supplementation with ALEN and both doses of SF significantly increased the mean trabecular BMD in ovariectomized rats.

Exacerbation of osteoporosis decreases BV/TV, Tb.Th, and Tb.N parameter that are directly proportional to bone mass. On the other hand, values those are inversely proportional to bone mass, including Tb.Sp, Tb.pf, and SMI increase. Thus research using μ -CT bone structure is actively underway (Lee *et al.,* 2008). Moreover, studies report that bone structure parameters are correlated with BMD (Siu *et al.,* 2004; Song *et al.,* 2007). In the present study, the BV/TV, Tb.Th, and Tb.N values of both the SF-treated groups and the ALEN group increased, whereas the values of Tb.Sp, Tb.pf, and SMI decreased in these groups in ovariectomized rats. These results are consistent with many previous studies utilizing experimental osteoporotic models (Iwata *et al.,* 2005; Ko *et al.,* 2007; Peng *et al.,* 2008). Together with the findings of the current study, SF is implicated to alleviate osteoporosis by inhibiting bone loss. Additionally, the 3D images in this study suggest that bone loss in the OVX group is dramatically inhibited by treatment with SF.

The above results may be attributed to changes in immunological and biochemical markers. Estrogen deficiency promotes the synthesis of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. These cytokines impact osteoclast cell differentiation and activity. In particular, IL-1 and IL-6 play important roles in mediating bone loss caused by estrogen deficiency (Canalis *et al.,* 1988; Spelsberg et *al.,* 1999). As mentioned, our previous *in vitro* study showed that RAW264.7 cells treated with SF inhibit IL-1β expression (Yeo *et al.,* 2008). In addition, it has been reported that patients with osteoporosis treated with alendronate for 360 d show significantly reduced IL-1β levels (Papadaki *et al.,* 2004). In accordance with previous studies, this study also demonstrated that serum levels of IL-1β and IL-6 decreased in the SF groups as well as in the ALEN group.

Recent work has shown that RANKL, its receptor (RANK), and OPG are central players in both osteoclast development and activity. RANKL is the key regulatory factor for the differentiation and activation of osteoclasts through RANK. The biological activities of RANKL are regulated by OPG, a soluble decoy receptor for RANKL. On binding to RANKL, OPG blocks the RANKL/RANK interaction, inhibits the maturation of the osteoclasts, and consequently, interferes with bone resorption. In many situations, bone resorption is stimulated by both increased RANKL and decreased OPG, which can amplify pro-resorptive signals. Thus, it has been suggested that the RANKL/OPG ratio is the ultimate determinant of bone resorption (Liang *et al.,* 2011; Cheung *et al.,* 2003). In this study, the RANKL/OPG ratio decreased in the SF-treated groups suggesting a protective effect. Taken together, it is clear that SF treatment has anti-resorptive effects through a reduction in pro-inflammatory cytokines and RANKL/OPG ratio in ovariectomized rats. As a result, SF treatment significantly reduces the number of osteoclasts.

Serum levels of osteocalcin and bone-specific ALP are two major bone formation markers. Urinary DPD is a major bone resorption marker. Osteocalcin, an important non-collagen calcium-binding protein of the bone matrix, and ALP are synthesized by osteoblasts, bind to the extracellular matrix, and are released into the blood stream (Johnell *et al.,* 2002). DPD, which is destroyed by osteoclasts, is secreted from the bone matrix and excreted into the urine when it is not

metabolized in the body (Yang *et al.,* 2000). A study by Xie *et al*. (2005) reported that ovariectomy increases serum levels of ALP, osteocalcin, and urinary DPD. These results indicate that ovariectomy causes a significant increase in the rate of bone turnover. In our experiment, the SF-treated groups displayed suppressed serum ALP and osteocalcin levels as well as reduced urinary DPD excretion relative to the OVX group. Based on these observations, we speculate that SF plays a role in reducing the high turnover rate of bone in ovariectomized rats.

In conclusion, ovariectomy induces a significant increase in bone turnover, which results in dramatic trabecular bone loss. Treatment with SF, however, prevents trabecular bone loss at the tibia. Taking all the results into account, the effects of SF (not necessarily dose-dependent) were found to be similar to the effects of alendronate. That is, SF has a positive effect on menopausal bone metabolism, as shown in this study by the analysis of morphological, immunological, and biochemical markers in ovariectomized rats. Accordingly, our results strongly suggest that SF is a promising alternative for the management of postmenopausal osteoporosis in patients.

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