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Anti-osteoporotic effects of *Boswellia serrata* gum resin extract *in vitro* and *in vivo*

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ABSTRACT

BACKGROUND/OBJECTIVES: This study evaluated the beneficial effects of an ethanol extract of *Boswellia serrata* gum resin (FJH-UBS) in osteoporosis.
MATERIALS/METHODS: MC3T3-E1 osteoblastic cells and RAW 264.7 osteoclastic cells were treated with FJH-UBS. The alkaline phosphatase (ALP) activity, mineralization, collagen synthesis, osteocalcin content, and Runt-related transcription factor 2 (RUNX2) and Osterix expression were measured in MC3T3-E1 cells. The actin ring structures, tartrate-resistant acid phosphatase (TRAP) activity, and the nuclear factor of activator T-cells, cytoplasm 1 (NFATc1) expression were evaluated in RAW 264.7 cells. Ovariectomized ICR mice were orally administered FJH-UBS for eight weeks. The bone mineral density (BMD) and the serum levels of osteocalcin, procollagen 1 N-terminal propeptide (P1NP), osteoprotegerin, and TRAP 5b were analyzed.
RESULTS: FJH-UBS increased the ALP activity, collagen, osteocalcin, mineralization, and RUNX2 and osterix expression in MC3T3-E1 osteoblastic cells, whereas it decreased the TRAP activity, actin ring structures, and NFATc1 expression in RAW 264.7 osteoclastic cells. In ovariectomy-induced osteoporosis mice, FJH-UBS positively restored all of the changes in the bone metabolism biomarkers (BMD, osteocalcin, P1NP, osteoprotegerin, and TRAP 5b) caused by the ovariectomy.
CONCLUSION: FJH-UBS has anti-osteoporotic activity by promoting osteoblast activity and inhibiting osteoclast activity *in vitro* and *in vivo*, suggesting that FJH-UBS is a potential functional food ingredient for osteoporosis.

Keywords: Boswellia; osteoblasts; osteoclast; ovariectomy; osteoporosis

INTRODUCTION

Osteoporosis is a systemic disease of the skeleton characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to increased bone fragility and fracture risk with serious complications. Osteoporosis significantly reduces morbidity, mortality, and the quality of life [1].

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Conflict of Interest
The authors declare no potential conflicts of interest.

Author Contributions
Conceptualization: Lee HS, Hong IK, Jang Y, Kim EJ; Formal analysis: Jung JI, Lee HS; Data analysis: Jung JI, Lee HS; Data visualization: Jung JI, Kim HB; Writing - original draft: Lee HS, Jung JI, Kim EJ; Writing - review & editing: Lee HS, Kim EJ.

The leading cause of primary osteoporosis is an estrogen deficiency in menopause or aging. A balance between bone formation by osteoblasts and bone resorption by osteoclasts is crucial for maintaining bone mass. On the other hand, osteoporosis occurs when the rate of bone resorption exceeds bone formation. Therefore, anti-resorptive and pro-formative drugs can be used to treat osteoporosis. Most osteoporosis drugs currently available interfere with bone resorption. While bone-forming drugs have only been studied recently [2], issues of cost-effectiveness, practical function in cortical bone, and possible side effects must be considered [3]. Anti-resorptive drugs, which interfere with the function of osteoclasts, include bisphosphonates, estrogens, selective estrogen receptor modulators (SERMs), and antibodies against receptor activator of nuclear factor κB ligand (RANKL) [4,5]. Long-term osteoporosis medications are necessary because osteoporosis cannot be cured quickly. Nevertheless, these drugs can cause severe side effects. Hormone therapy increases the risk of breast cancer, epithelial cell carcinoma, myocardial infarction, blood clots, and stroke. Bisphosphonates increase the risk of atrial fibrillation, atypical femur fractures, and osteonecrosis of the jaw, and SERMs increase the risk of thrombosis. In addition, parathyroid hormone drugs increase the risk of vomiting, headache, dizziness, hypercalcemia, and hypercalciuria, and calcitonin drugs increase the risk of cancer [6,7]. Currently, there is no safe and effective method to restore lost bone mass. Therefore, a safe therapeutic agent with fewer side effects is needed for treating or preventing osteoporosis.

Boswellia serrata is a tree in the Burseraceae family that grows wild in the alpine regions of India and Africa. When the trunk of the Boswellia tree is wounded, the sap exudes and hardens into a gum resin called Indian frankincense. This resin has long been used in traditional medicine to treat chronic inflammatory diseases, including osteoarthritis [8-11]. Therefore, it is used as a functional health material to improve joint and cartilage health. *B. serrata* gum resin contains terpene, tetracyclic triterpenic acid, and triterpenic acid (boswellic acid). There are six major boswellic acids: keto-β-boswellic acid (KBA), 3-O-acetyl-11-keto-β-boswellic acid (AKBA), α-boswellic acid, β-boswellic acid, 3-O-acetyl-α-boswellic acid, and 3-O-acetyl-β-boswellic acid [10]. All six boswellic acids have anti-inflammatory effects [12,13]. Among them, KBA and AKBA have the strongest antioxidant and anti-inflammatory effects [14]. In addition to their efficacy in arthritis, KBA and AKBA were reported to play important roles in the bone metabolism, such as reducing bone loss, strengthening bone, and increasing bone formation, as well as in arthritis [15]. Boswellic acid inhibits osteoclastogenesis [16,17]. In particular, AKBA has beneficial effects in treating and preventing periprosthetic osteolysis [18]. Despite this, few studies have examined the anti-osteoporotic effects of *B. serrata* gum resin extract and its mechanism.

A previous study reported the beneficial effects of a *B. serrata* gum resin extract (FJH-UBS) enriched with KBA and AKBA in an in vitro osteoarthritis-like model [19] and in a monosodium iodoacetate-induced osteoarthritis animal model [20]. This study examined the anti-osteoporotic activity and underlying mechanisms of FJH-UBS *in vitro* and *in vivo*.

MATERIALS AND METHODS

Preparation of extracts

The *B. serrata* gum resin extract (FJH-UBS) was supplied by Frombio Co. Ltd. (Yongin, Korea). FJH-UBS was prepared using a previously described method [19]. Briefly, *B. serrata* gum resin originating from India was extracted with 95% ethanol at 50–60°C for 6 h under reflux.

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The extract was filtered and concentrated. The concentrate was added to the same volume of 100% hexane, mixed, allowed to stand for 10 min at room temperature, and filtered to remove lipids. The filtrate was dried at 70°C to a moisture content of 5% or less and then mixed with maltodextrin (10%, w/w). The resulting powder was used as FJH-UBS. The KBA and AKBA content in FJH-UBS analyzed by high-performance liquid chromatography was 81.96 ± 0.77 mg/g FJH-UBS.

Cell culture and differentiation induction

MC3T3-E1 murine osteoblastic cells and RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (Manassas, VA, USA). The MC3T3-E1 cells were cultured in α-minimal essential medium (α-MEM), and RAW 264.7 cells were cultured in Dulbecco's modified eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100,000 U/L penicillin, and 100 mg/L streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air.

MC3T3-E1 cells at 90% confluence were incubated in osteoblast differentiation-inducing medium (ObDIM; α-MEM with 10% FBS, 10 mmol/L β-glycerophosphate, and 50 mg/L ascorbic acid) to induce osteoblast differentiation. The medium was replaced with fresh ObDIM every three days.

RAW 264.7 cells were incubated in an osteoclast differentiation-inducing medium (OcDIM; DMEM with 10% FBS, 50 μg/L RANKL, and 10 μmol/L PD98059) for five days to induce the differentiation of RAW 264.7 cells into osteoclasts. The medium was replaced with fresh OcDIM every two days.

Cell viability assay

The MC3T3-E1 cells and RAW 264.7 cells were seeded in 24-well plates at a density of 5×10^4 cells per well. After 24 h of incubation, the cells were treated with FJH-UBS at concentrations ranging from 0 to 200 μg/mL. The cells were then incubated for 72 h, and the cell viability was assessed using an MTT assay, as described elsewhere [21,22].

Measurement of alkaline phosphatase activity in osteoblasts

MC3T3-E1 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h. After incubation for 24 h, the cells were incubated in ObDIM containing FJH-UBS at different concentrations for five days. After five days of incubation, the alkaline phosphatase (ALP) activity of the cells treated with different FJH-UBS concentrations was assayed using an ALP Assay Kit (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions.

Determination of collagen synthesis in osteoblasts

Collagen synthesis was determined by staining cells with Sirius Red, an anionic dye with a selective and strong affinity for collagen. MC3T3-E1 cells were seeded in 24-well plates at a density of 5×10^4 cells per well and treated with FJH-UBS for eight days, as described above. Collagen synthesis was determined using a Sirius Red Collagen Detection Kit (Chondrex Inc., Redmond, WA, USA) according to the manufacturer's instructions.

Determination of mineralization in osteoblasts

Mineralization was determined by staining the cells with Alizarin Red S, an anthraquinonoid derivative dye with a high affinity for calcium. The MC3T3-E1 cells were seeded in 24-well

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plates at a density of 5×10^4 cells per well and treated with FJH-UBS for 18 days, as described above. The calcium deposition levels were measured using an Alizarin Red S Staining Quantification Assay Kit (ScienCell Research Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.

Measurement of osteoblast-secreted osteocalcin production in osteoblasts

MC3T3-E1 cells were seeded in 24-well plates at a density of 5×10^4 cells per well and treated with FJH-UBS for 18 days, as described above. The 24 h-conditioned cell culture media was collected on day 18. Osteoblast-secreted osteocalcin in the 24 h-conditioned cell culture media was quantified using an osteocalcin enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.

Measurement of tartrate-resistant acid phosphatase activity in osteoclasts

The RAW 264.7 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 24 h. After 24 h incubation, the cells were incubated in OcDIM containing FJH-UBS at different concentrations for five days. After five days of incubation, the tartrate-resistant acid phosphatase (TRAP) activity in the cells treated with different FJH-UBS concentrations was assayed using a TRAP assay Kit (Takara Bio Inc.) according to the manufacturer's instructions.

Morphological observation of actin ring in osteoclasts

The RAW 264.7 cells were seeded on cover glasses in 24-well plates at a density of 1.5×10^4 cells per well and treated with FJH-UBS as described above. After five days of incubation, the cells were rinsed with phosphate-buffered saline and fixed using a 4% paraformaldehyde solution containing 0.1% Triton X-100. The cells were then stained with Alexa Fluor 594 Phalloidin (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; Sigma-Aldrich Co., St. Louis, MO, USA) for actin and nuclei staining, respectively. The morphological changes in the actin ring of the cells were observed under a microscope (AxioImager, Carl Zeiss, Jena, Germany).

Real-time reverse transcription-polymerase chain reaction

MC3T3-E1 cells and RAW 264.7 cells were plated in multi-well plates and treated with FJH-UBS for three days, as described above. The total RNA was extracted from the cells using the RNeasy® Plus Mini Kit (Qiagen, Valencia, CA, USA). A real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using a HyperScript™ RT Master Mix Kit (GeneAll Biotechnology, Seoul, Korea), a QuantiNova SYBR Green PCR Kit (Qiagen), and a Rotor-Gene 3000 PCR Cycler (Corbett Research, Mortlake, Australia), as described elsewhere [23]. **Table 1** lists the primer sequences used in this study. The relative mRNA expression of the target genes was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*).

Table 1. Primer sequences used in this study

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Nfatc1	CAGTGTGACCGAAGATACCTGG	TCGAGACTTGATAGGGACCCC
Osterix	CGCTTTGTGCCTTTGAAAT	CCGTCAACGACGTTATGC
Runx2	AGGGACTATGGCGTCAAACA	GGCTCACGTCGCTCATCTT
Gapdh	TGGGTGTGAACCATGAGAAG	GCTAAGCAGTTGGTGGTGC

Nfatc1, the nuclear factor of activator T-cells, cytoplasm 1; *Runx2*, Runt-related transcription factor 2; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.

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Ethical statements and animal

The Institutional Animal Care and Use Committee of Hallym University (Hallym 2022-31) approved all animal experimental protocols. Animal care and experimentation followed the institutional guidelines for the care and use of laboratory animals to ensure their welfare and minimize distress.

Five-week-old female ICR mice were purchased from Doo Yeol Biotech Co., Ltd. (Seoul, Korea) and housed at the animal research facility at Hallym University. The mice were maintained under specific pathogen-free conditions with a temperature of 23 ± 3°C, relative humidity of 50 ± 10% and a 12-h light/dark cycle. The mice were given access to a commercial rodent chow and tap water *ad libitum*.

Induction of ovariectomy-induced osteoporosis and treatment in mice

After one week of acclimation, each mouse underwent either a sham operation (n = 10) or ovariectomy (n = 30), according to the method described by Souza *et al.* [24]. The mice in the OVX group underwent a bilateral ovariectomy. A sham operation was conducted on the Sham group mice, in which the ovaries were only exteriorized but not resected. The mice were maintained for six weeks without administering FJH-UBS to induce osteoporosis. After six weeks, the mice were assigned randomly to four experimental groups (n = 10 per group): (i) sham-operated group (Sham), (ii) ovariectomized group (OVX), (iii) ovariectomized and 80 mg/kg body weight (BW) FJH-UBS treated group (OVX+FU), and (iv) ovariectomized and 5 mg/kg BW soy isoflavones (a positive control)-treated group (OVX+SI) group. Soy isoflavones were selected as the positive control for the study owing to their established effectiveness in osteoporosis. This choice enables a pertinent comparison with FJH-USB because the recognized role of soy isoflavones in osteoporosis research is a valuable benchmark for evaluating the therapeutic potential of FJH-USB. The mice in the OVX+FU and OVX+SI groups were given FJH-UBS or soy isoflavones dissolved in sterile water orally once a day for eight weeks. The mice in the Sham and OVX groups received an equal volume of sterile water as the vehicle by gavage. The mice were weighed once a week during the FJH-UBS treatment period. At the end of the experimental period, the mice were anesthetized using tribromoethanol diluted in tertiary amyl alcohol. Blood was then collected from the orbital vein to prepare serum. The mice were euthanized by cervical dislocation, after which the livers, uteri, and femurs were excised.

Measurement of femoral weight and bone mineral density

The femurs were excised, dissected to remove soft tissue, and weighed. The femoral bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (PIXImus™, GE Lunar, Madison, WI, USA).

Measurement of bone metabolism-related indicator levels in serum

The serum levels of osteocalcin (MyBiosource, SanDiego, CA, USA), procollagen 1 N-terminal propeptide (P1NP, MyBiosource), osteoprotegerin (R&D Systems, Minneapolis, MN, USA), and TRAP5b (MyBiosource) were measured using the relevant ELISA kits.

Statistical analysis

Statistical analyses were performed using the Statistical Analysis System for Windows version 9.4 (SAS Institute, Cary, NC, USA). The data are presented as the mean ± standard errors (SEM). A student's *t*-test was used to analyze the differences between the Sham and OVX groups. The means of the three groups (OVX, OVX+FU, and OVX+SI groups) were compared using an

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analysis of the variance followed by Duncan's multiple comparison test. A *P*-value < 0.05 was considered significant.

RESULTS

Effects of FJH-UBS on the viability of MC3T3-E1 and RAW 264.7 cells

The cells were incubated for 72 h in a culture media containing 0–200 μg/mL FJH-UBS and assayed using the MTT assay to determine the FJH-UBS concentration that could affect the viability of MC3T3-E1 and RAW 264.7 cells. No significant cytotoxicity was observed when the MC3T3-E1 cells were treated with FJH-UBS at up to 20 μg/mL for 72 h. In RAW 264.7 cells, no significant cytotoxicity was observed after the FJH-UBS treatment at concentrations ranging from 0 to 50 μg/mL (**Fig. 1**). Therefore, FJH-UBS concentrations below 20 μg/mL were used in the following experiments to exclude cytotoxic effects.

FJH-UBS enhances osteoblast activity in MC3T3-E1 cells

The ALP activity, an early-stage marker of osteoblast differentiation, in MC3T3-E1 cells was first measured. ObDIM significantly increased the ALP activity. The ALP activity in cells treated with 5 μg/mL FJH-UBS was similar to that of ObDIM-induced cells, but a treatment with 10 or 20 μg/mL FJH-UBS increased the ALP activity in a dose-dependent manner (**Fig. 2A**).

Collagen is another early-stage marker of osteoblast differentiation. Therefore, the effects of the FJH-UBS treatment on collagen synthesis were measured. ObDIM increased the collagen levels significantly. On the other hand, treatment with 5–20 μg/mL FJH-UBS increased the collagen level more dose-dependent than ObDIM (**Fig. 2B**).

The degree of mineralization and osteocalcin production were measured to determine if FJH-UBS affected the late-stage differentiation of osteoblasts. The levels of mineralization and osteocalcin were increased significantly by ObDIM in MC3T3-E1 cells and were increased further by the FJH-UBS treatment in a dose-dependent manner (**Fig. 2C and D**).

The MC3T3-E1 cells were then cultured at FJH-UBS concentrations ranging from 0 to 20 μg/mL and analyzed by RT-PCR to determine if FJH-UBS affects the expression of key

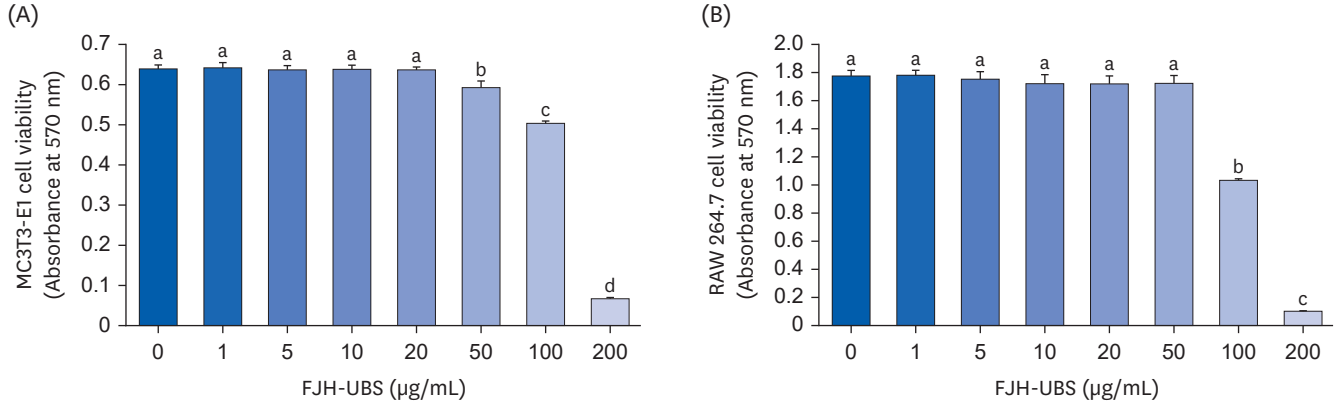

Fig. 1. Effects of FJH-UBS on the viability of MC3T3-E1 cells and RAW 264.7 cells.
MC3T3-E1 cells (A) and RAW 264.7 cells (B) were plated in 24-well plates. After incubation for 24 h, the cells were incubated for 72 h in media containing 0–200 μg/mL of FJH-UBS. The cell viability was measured using the MTT assay. Each bar represents the mean ± SE (n = 4). Means without a common letter are significantly different at $P < 0.05$.

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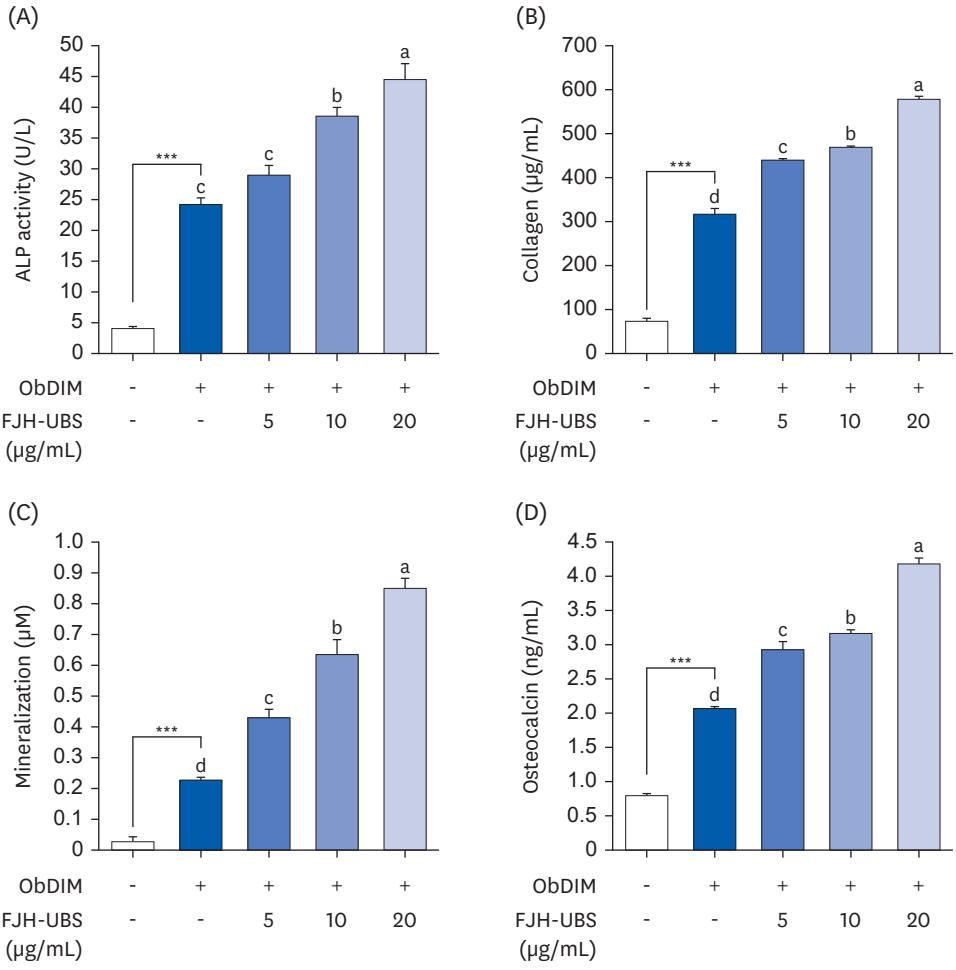

Fig. 2. Effects of FJH-UBS on the ALP activity, collagen synthesis, mineralization, and osteocalcin production in MC3T3-E1 cells.
MC3T3-E1 cells were plated in multi-well plates. After incubation for 24 h, the cells were incubated in ObDIM containing various concentrations of FJH-UBS for five days (ALP activity), eight days (collagen synthesis), or 18 days (mineralization and osteocalcin production). (A) ALP activity was measured using TRACP and ALP assay kits. (B) Collagen synthesis was measured by Sirius Red staining. (C) Mineralization was measured by Alizarin Red S staining. (D) Twenty-four hour-conditioned media were collected. The contents of osteocalcin in the 24 h-conditioned media were measured using an osteocalcin enzyme-linked immunosorbent assay kit. Each bar represents the mean ± SE (n = 6).
ObDIM, osteoblast differentiation-inducing medium; ALP, alkaline phosphatase.
$^{***}P < 0.001$ significantly different from the ObDIM-untreated group. Means without a common letter are significantly different at $P < 0.05$.

transcription factors involved in osteoblast differentiation. The levels of Runt-related transcription factor 2 (Runx2) and osterix mRNA expression were significantly higher in the ObDIM-treated (differentiated) cells than in the non-ObDIM-treated (undifferentiated) cells. They were significantly higher after the FJH-UBS treatment. Compared to the ObDIM group, the mRNA expression of Runx2 was increased significantly by a treatment with 20 μg/mL FJH-UBS. Finally, osterix mRNA expression was increased dose-dependently by the FJH-UBS treatment (**Fig. 3**).

FJH-UBS suppresses osteoclast activity in RAW 264.7 cells

RAW 264.7 cells were cultured with OcDIM and different FJH-UBS concentrations, and the TRAP activity was measured to determine the effect of FJH-UBS on osteoclast differentiation.

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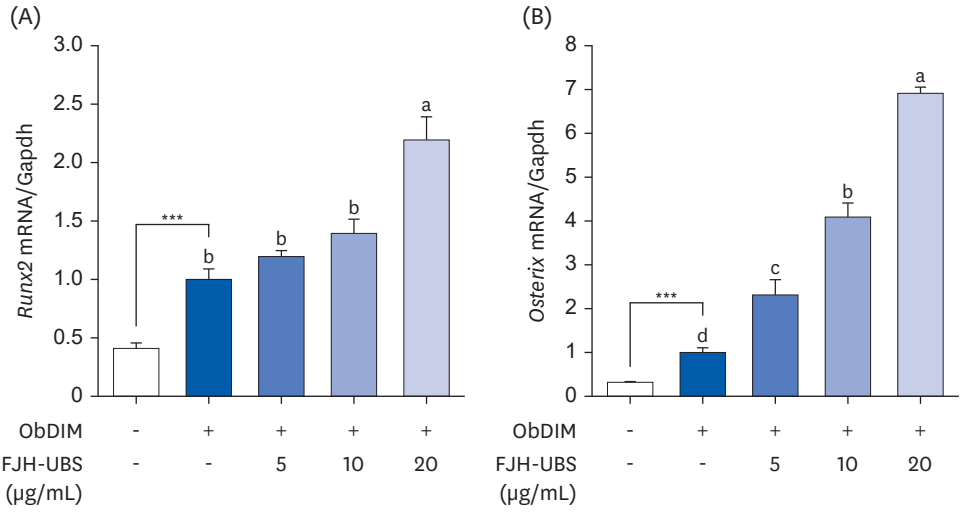

Fig. 3. Effects of FJH-UBS on mRNA expression of *Runx2* and *Osterix* in MC3T3-E1 cells.
MC3T3-E1 cells were plated in multi-well plates. After incubation for 24 h, the cells were incubated in ObDIM containing various concentrations of FJH-UBS for three days. The total RNA in cells was extracted, reverse transcribed, and real-time PCR was performed. The relative mRNA expression of *Runx2* (A) and *Osterix* (B) were analyzed. Targeted mRNA expression was normalized to that of *Gapdh* and is represented relative to the ObDIM-treated group. Each bar represents the mean ± SE (n = 6).
ObDIM, osteoblast differentiation-inducing medium; *Runx2*, Runt-related transcription factor 2; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.
$^{***}P < 0.001$ significantly different from the ObDIM-untreated group. Means without a common letter are significantly different at $P < 0.05$.

The TRAP activity was increased significantly in RAW 264.7 cells by OcDIM, which was decreased dose-dependently by the FJH-UBS treatment (**Fig. 4A**).

Experiments were then performed to determine the role of FJH-UBS in actin ring formation. The ability of cells to resorb bone can be indicated by the formation of actin rings in osteoclasts [25]. In the FJH-UBS untreated control group, the actin rings were the most vivid, and the cells were dense. These actin rings decreased significantly as the concentration of the FJH-UBS treatment increased (**Fig. 4B**). Hence, treatment with FJH-UBS could inhibit the differentiation of osteoclasts to a stage where bone resorption is possible.

The mRNA expression of the nuclear factor of activator T-cells, cytoplasm 1 (Nfatc1), was measured to determine if FJH-UBS affected transcription factors involved in osteoclast differentiation. Nfatc1 mRNA expression was increased significantly by OcDIM and decreased in proportion to the FJH-UBS treatment concentration (**Fig. 5**). Hence, the FJH-UBS treatment reduced the expression of transcription factors related to osteoclast differentiation, suppressing osteoclast differentiation.

FJH-UBS promotes bone metabolism in ovariectomized mice

The mice in the Sham (normal control group), OVX, OVX+FU (FJH-UBS treatment), and OVX+SI (soy isoflavone treatment, positive control) groups after inducing osteoporosis in ICR mice by an ovariectomy were compared to determine if FJH-UBS has anti-osteoporotic activity in an *in vivo* system. As shown in **Table 2**, the body weight gain was significantly higher in the OVX group than in the Sham group. This effect was reduced significantly by the FJH-UBS or soy isoflavone treatment to a level similar to that in the Sham group. The weight gain in the OVX+FU and OVX+SI groups was similar. The liver weight was significantly higher in the OVX group than in the Sham group and was reduced by the FJH-UBS or soy isoflavone treatment.

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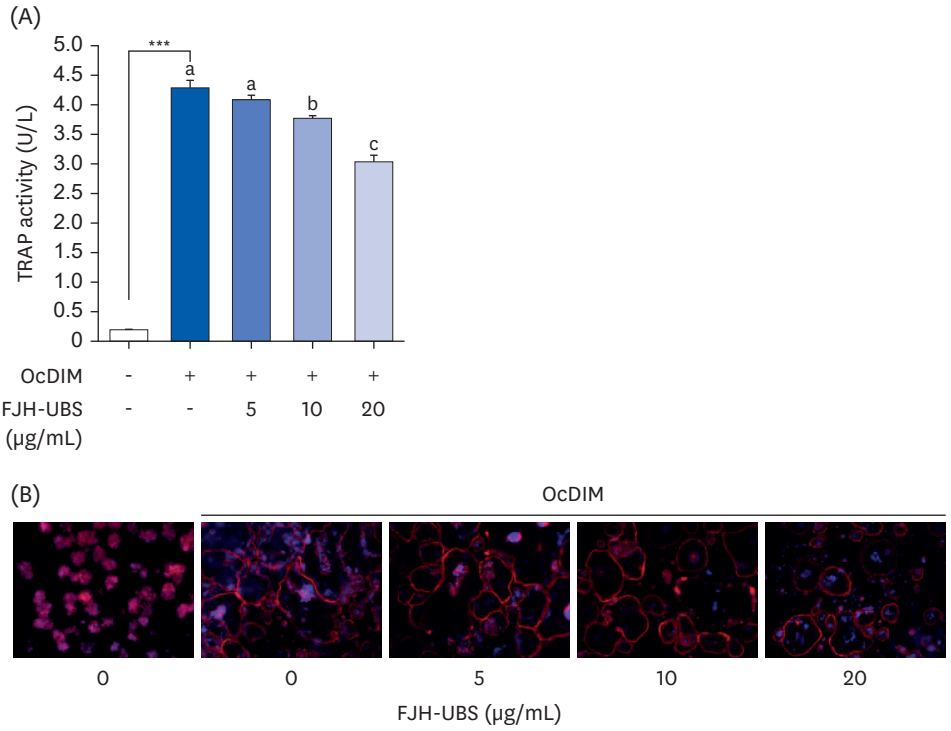

Fig. 4. Effects of FJH-UBS on TRAP activity and actin ring formation in RAW 264.7 cells.
RAW 264.7 cells were plated in multi-well plates. After incubation for 24 h, the cells were incubated in OcDIM containing various concentrations of FJH-UBS for five days. (A) TRAP activity was measured using TRACP and ALP assay kits. Each bar represents the mean ± SE (n = 6). (B) The cells were fixed with 4% paraformaldehyde containing 0.1% Triton X-100 and stained with Alexa Fluor 594 Phalloidin and DAPI to stain actin and nuclei, respectively. Morphological changes of actin ring in cells were observed under a microscope, Magnification, 200 x. Representative staining images are shown (n = 3).
OcDIM, osteoclast differentiation-inducing medium; TRAP, tartrate-resistant acid phosphatase; DAPI, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride.
$^{***}P < 0.001$ significantly different from the OcDIM-untreated group. Means without a common letter are significantly different at $P < 0.05$.

The uterus weight was significantly lower in the OVX group than in the Sham group. The uterine weight reduced by the ovariectomy was unaffected by the FJH-UBS or soy isoflavone treatment (**Table 2**). The food intake was similar in the experimental groups (data not shown).

The femur bone weight and BMD were significantly lower in the OVX group than in the Sham group, whereas they were increased to the same level as the Sham group by the FJH-UBS or soy isoflavone treatment. These results suggest that the FJH-UBS treatment restored the bone weight and bone density decreased by OVX (**Table 3**).

Table 2. Effect of FJH-UBS administration on the body weight, liver weight, and uterus weight in ovariectomized mice.

Variables	Sham	OVX	OVX+FU	OVX+SI
Initial body weight (g)	31.2 ± 1.0	39.7 ± 0.9***	39.8 ± 1.1	39.5 ± 0.5
Final body weight (g)	36.5 ± 1.9	48.1 ± 1.2***	44.3 ± 1.5	44.8 ± 0.9
Body weight gain (g)	5.3 ± 1.1	8.4 ± 0.4^{***,a}	4.5 ± 0.4^{b}	5.3 ± 0.6^{b}
Liver weight (g)	1.50 ± 0.07	1.95 ± 0.08^{***,a}	1.75 ± 0.06^{b}	1.65 ± 0.06^{b}
Uterus weight (g)	0.248 ± 0.021	0.062 ± 0.016***	0.087 ± 0.017	0.084 ± 0.016

Values are expressed as the mean ± SE (n = 10).
Sham, sham-operated group; OVX, ovariectomized group; OVX+FU, ovariectomized and 80 mg/kg body weight FJH-UBS-treated group; OVX+SI, ovariectomized and 5 mg/kg body weight soy isoflavones (a positive control)-treated group.
$^{***}P < 0.001$ significantly different from the Sham group. Mean without a common letter differs among the OVX, OVX+FU, and OVX+SI groups at $P < 0.05$.

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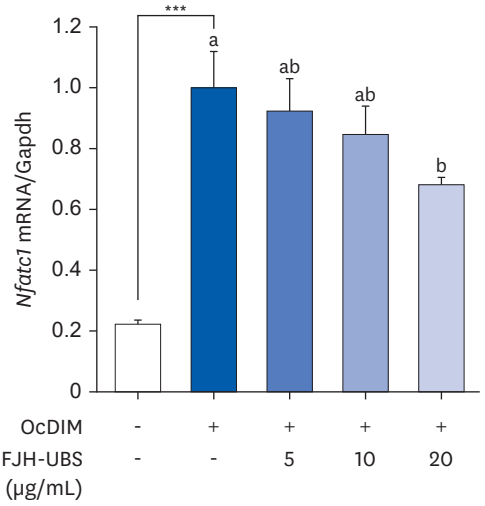

Fig. 5. Effects of FJH-UBS on *Nfatc1* mRNA expression in RAW 264.7 cells.
RAW 264.7 cells were plated in multi-well plates. After incubation for 24 h, the cells were incubated in OcDIM containing various concentrations of FJH-UBS for three days. The total RNA in cells was extracted, reverse transcribed, and real-time polymerase chain reaction was performed. The relative mRNA expression of *Nfatc1* was analyzed. Targeted mRNA expression was normalized to that of *Gapdh* and is represented relative to the OcDIM-treated group. Each bar represents the mean ± SE (n = 6).
OcDIM, osteoclast differentiation-inducing medium; *Nfatc1*, the nuclear factor of activator T-cells, cytoplasm 1; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.
$^{***}P < 0.001$ significantly different from the OcDIM-untreated group. Means without a common letter are significantly different at $P < 0.05$.

Table 3. Effect of FJH-UBS administration on weight and bone mineral density of femurs in ovariectomized mice

Variables	Sham	OVX	OVX+FU	OVX+SI
Femur weight (left + right, mg)	194.7 ± 6.8	184.9 ± 4.6^{b}	203.3 ± 5.1^{a}	198.4 ± 4.8^{a}
BMD (g/m^2)	0.093 ± 0.002	0.085 ± 0.002^{*,b}	0.091 ± 0.001^{a}	0.090 ± 0.001^{a}

Values are expressed as the mean ± SE (n = 10).
BMD, bone mineral density; Sham, sham-operated group; OVX, ovariectomized group; OVX+FU, ovariectomized and 80 mg/kg body weight FJH-UBS-treated group; OVX+SI, ovariectomized and 5 mg/kg body weight soy isoflavones (a positive control)-treated group.
$^{*}P < 0.01$ significantly different from the Sham group. Mean without a common letter differs among the OVX, OVX+FU, and OVX+SI groups at $P < 0.05$.

Fig. 6 shows the serum levels of bone turnover markers in the experimental groups. Osteocalcin, P1NP, and osteoprotegerin were measured as the markers of bone formation, and TRAP 5b was measured as a marker of bone resorption. The serum osteocalcin levels in the OVX group were significantly lower than in the Sham group. On the other hand, the levels recovered after the FJH-UBS treatment and were similar to those in the Sham group (**Fig. 6A**). The serum P1NP levels in the OVX group were significantly lower than those in the Sham group. These levels were increased significantly by the FJH-UBS treatment. The FJH-UBS treatment produced a more significant increase in the serum P1NP levels in the ovariectomized mice than the soy isoflavone treatment (**Fig. 6B**). The serum osteoprotegerin levels were significantly lower in the OVX group than the Sham group, but the levels in the FJH-UBS and soy isoflavone-treated groups were similar to those in the Sham group (**Fig. 6C**). As shown in **Fig. 6D**, the serum level of TRAP 5b was 400.6% higher in the OVX group than in the Sham group. This was reduced significantly in mice treated with FJH-UBS or soy isoflavone. These results confirm that the FJH-UBS treatment had a comparable or even superior effect on the bone formation and resorption markers in osteoporosis-induced animal models than soy isoflavones.

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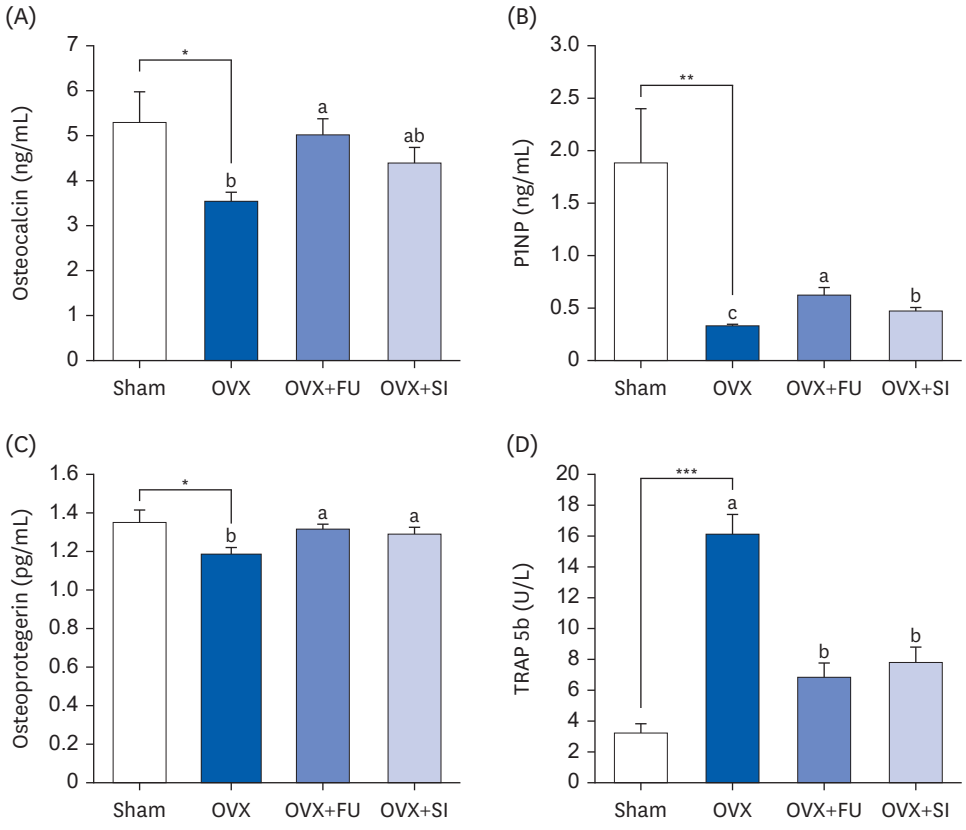

Fig. 6. Effect of FJH-UBS administration on the serum levels of bone turnover markers in ovariectomized mice.
Ovariectomized mice were orally administered either FJH-UBS (80 mg/kg body weight (BW)/day) or soy isoflavones (5 mg/kg BW/day) for eight weeks. Serum levels of osteocalcin (A), P1NP (B), osteoprotegerin (C), and TRAP 5b (D) were measured with the relevant ELISA kit. Each bar represents the mean ± SE (n = 10).
P1NP, procollagen 1 N-terminal propeptide; TRAP 5b, tartrate-resistant acid phosphatase 5b; Sham, sham-operated group; OVX, ovariectomized group; OVX+FU, ovariectomized and 80 mg/kg body weight FJH-UBS-treated group; OVX+SI, ovariectomized and 5 mg/kg body weight soy isoflavones (a positive control)-treated group.
$^{*}P < 0.01$, $^{**}P < 0.05$, $^{***}P < 0.001$ significantly different from the Sham group. Mean without a common letter differs among OVX, OVX + FU, and OVX + SI groups at $P < 0.05$.

DISCUSSION

Bone is a dynamic tissue in which continuous bone replacement occurs throughout life, and approximately 10% of bone is renewed each year [26]. The process involves osteocytes, osteoclasts, and osteoblasts. In normal bone remodeling, the balance between bone resorption and formation is controlled by cooperative cell signaling [26]. Thus, the bone mass is maintained when bone formation and bone resorption by osteoblasts and osteoclasts are balanced. Osteoporosis is a common disorder of bone remodeling characterized by the loss of bone microarchitecture, decreased bone strength, and decreased BMD, and is associated with an increased fracture risk. Primary osteoporosis is caused by an estrogen deficiency or postmenopausal aging. An estrogen deficiency causes a decrease in blood calcium, which increases bone resorption [26]. Hormone or estrogen replacement therapy has been used as an important measure to prevent and treat postmenopausal osteoporosis. On the other hand, it has been associated with side effects such as breast cancer, uterine cancer, and thromboembolic disease [27]. Therefore, efforts are being made to find inexpensive and safe natural products with anti-osteoporotic activity.

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B. serrata gum resin, or Indian frankincense, has long been used as a traditional medicine for various inflammatory diseases, including arthritis [9,11]. Recently, boswellic acid, a major pharmacological component of *B. serrata* gum resin extract, particularly AKBA, has been reported to have anti-osteoporotic activity. In ovariectomized Sprague-Dawley rats, supplementation with 35 mg/kg of AKBA for 42 days exhibited good anti-osteoporotic activity because the osteoclast activity was reduced by suppressing the nuclear factor (NF)-kB-induced tumor necrosis factor (TNF)-a signaling pathway [16]. Boswellic acid and *B. serrata* gum resin extract appeared safe because boswellic acid did not cause pathological changes in the animals up to 1,000 mg/kg BW [28]. Another study evaluating the safety of a novel water-soluble extract of *B. serrata* gum resin found no adverse effects in male and female Sprague-Dawley rats administered up to 500 mg/kg/day orally for 90 days [29]. On the other hand, just as other pentacyclic triterpenic acids have limited aqueous solubility and lipophilicity and reduced bioavailability and pharmacological activity, KBA and AKBA are extremely limited because of their low oral bioavailability [30]. In this study, the anti-osteoporotic effects of FJH-UBS were confirmed using in vitro and in vivo systems. FJH-UBS increased osteoblast differentiation and decreased osteoclast differentiation in vitro. The oral administration of 80 mg/kg/day of FJH-UBS to ovariectomized mice had significant osteoprotective effects on the blood-bone turnover markers and femur BMD. These results suggest that FJH-UBS has a beneficial effect in preventing and treating osteoporosis by directly increasing bone formation and inhibiting bone resorption.

The ALP activity, osteocalcin, collagen, and mineralization in MC3T3-E1 cells treated with FJH-UBS were increased significantly in a dose-dependent manner (**Fig. 2**). RUNX2 and Osterix are master transcription factors in osteoblast differentiation [31,32]. They are expressed in osteoblasts and play an essential role in osteoblast differentiation by regulating the expression of numerous osteoblastic genes, such as osteocalcin, bone sialoprotein, osteopontin, and collagen [32,33]. Osterix and Runx2 mRNA expression was increased significantly after the FJH-UBS treatment. In particular, the FJH-UBS treatment increased Osterix mRNA expression in a dose-dependent manner (**Fig. 3**). Osterix is an essential transcription factor for osteoblast differentiation and bone mineralization that is also expected to play an important role in the treatment of osteolytic diseases [32]. These results show that FJH-UBS increases bone formation by promoting osteoblast differentiation in MC3T3-E1 cells.

The TRAP activity and actin ring formation were analyzed in RAW 264,7 osteoclasts. TRAP is secreted from osteoclasts and reflects the number and activity of osteoclasts. Hence, the TRAP activity is used as a marker to confirm osteoclast differentiation [34]. The TRAP activity decreased dose-dependently after the FJH-UBS treatment (**Fig. 4A**). Bone resorption by osteoclasts occurs through a complex process involving bone attachment, cytoskeletal reorganization, and ruffled border formation. The actin ring is essential for bone resorption of osteoclasts. When osteoclasts attach to bone, actin rings are formed through multi-step cell signaling pathways by activating $\alpha_v\beta_3$ integrin. As osteoclasts differentiate, they form an actin ring structure in the bone, through which the bone matrix can be degraded and resorbed [25]. In this study, treating RAW 264.7 cells with FJH-UBS decreased the number of actin rings and induced their disorganization (**Fig. 4B**). Nfatc1 mRNA was analyzed as a transcription factor for osteoclast differentiation. NFATc1 promotes osteoclast fusion and activation by upregulating several genes involved in osteoclast adhesion, migration, acidification, and degradation in organic and inorganic bone matrices [35]. Osteoclasts can be inhibited by suppressing NFATc1. Hence, NFATc1 is expected to be a promising therapeutic target for

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treating osteoclast-related diseases [35]. FJH-UBS decreased Nfatc1 mRNA expression in RAW 264.7 cells in a dose-dependent manner (**Fig. 5**). Hence, FJH-UBS is effective in reducing bone resorption by inhibiting osteoclast differentiation in RAW 264.7 cells.

FJH-UBS affected osteoclasts and osteoblasts. FJH-UBS could promote bone formation by enhancing osteoblast differentiation because the treatment for osteoporosis is still biased towards anti-resorptive drugs, and there is no straightforward treatment to improve bone formation.

The bone turnover markers were measured after treating OVX-induced osteoporotic mice with FJH-UBS to determine if FJH-UBS has an anti-osteoporotic effect in the in vivo system. An ovariectomy is associated with weight gain and decreased bone formation [36]. In this study, the ovariectomized group gained 58.5% body weight, and their femur weight and BMD decreased by 5.3% and 9.4%, respectively, compared to the Sham group, despite no difference in food intake (**Tables 2** and **3**). Soy isoflavones were also administered to mice in the positive control group because soy isoflavones have been reported to have estrogen-like effects in estrogen-deficient animal models and postmenopausal women [37,38]. Furthermore, a previous study reported that supplementing ovariectomized mice with isoflavone-enriched whole soy milk inhibited BMD reductions [39]. In the present study, isoflavone supplementation suppressed OVX-induced weight gain and increased the femur weight and BMD to the level of the Sham group. Furthermore, the FJH-UBS treatment was as effective as isoflavone supplementation.

Bone loss due to an ovariectomy is determined by decreases in the length, weight, and thickness of the femur and lumbar vertebrae [40]. Because the bone turnover markers change faster than BMD or fracture risk, they are useful for an early assessment of the effectiveness of osteoporosis treatment [41], and the serum ALP, osteocalcin, and P1NP levels are biomarkers that reflect the osteoblast activity, including bone formation [42]. First, osteocalcin is produced by osteoblasts and is involved in bone mineralization. When the bone matrix is calcified by the deposition of newly formed osteocalcin, some is released into the blood. Therefore, the blood osteocalcin concentration indicates the osteoblast function related to bone formation [43]. Second, P1NP is a peptide produced by the post-translational cleavage of type I procollagen molecules by proteases during collagen accumulation by osteoblasts [43]. The Blood P1NP concentration is considered the most representative biomarker for measuring bone formation [44] and is used as an index to determine the effectiveness of an osteoporosis treatment [45]. Finally, osteoprotegerin is a type of osteoclastogenesis inhibitory factor. It is produced in osteoblasts and binds to and blocks the RANK receptor, inhibiting the osteoclast function and promoting bone formation. Therefore, a decrease in osteoprotegerin leads to osteoporosis [46]. The osteocalcin, P1NP, and osteoprotegerin levels were measured as markers of bone formation and TRAP 5b as a bone resorption marker in the blood of OVX mice. The serum osteocalcin, P1NP, and osteoprotegerin levels were significantly lower in the OVX group than in the Sham group ($P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively), and the serum TRAP 5b levels were significantly higher ($P < 0.001$). With FJH-UBS treatment, however, osteocalcin, and osteoprotegerin recovered to the same level as in the Sham group, and P1NP and TRAP 5b also increased significantly, albeit less than in the Sham group. In addition, the effects of FJH-UBS on P1NP exceeded that of the soy isoflavone treatment (**Fig. 6**). Thus, the FJH-UBS treatment is effective in the OVX-induced osteoporosis model by preventing bone resorption and promoting bone formation.

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This study investigated the effects of FJH-UBS on bone metabolism in osteoblast and osteoclast cell lines and ovariectomized mice. The FJH-UBS treatment increased the osteoblast differentiation markers (ALP activity, collagen synthesis, mineralization, osteocalcin, Runx2 mRNA, and osterix mRNA) in MC3T3-E1 osteoblastic cells and decreased the osteoclast differentiation markers (TRAP activity, actin ring formation, and Nfatc1 mRNA) in RAW 264.7 osteoclastic cells. The FJH-UBS treatment increased the BMD and blood markers of bone formation (osteocalcin, P1NP, and osteoprotegerin) and decreased the bone resorption marker (TRAP 5b) in OVX mice. The anti-osteoporotic effects of FJH-UBS were equal to or better than that in the soy isoflavone treatment group.

In conclusion, this study confirmed the anti-osteoporotic effects of FJH-UBS. These results show that FJH-UBS suppresses bone resorption and promotes bone formation. This is a very promising result, especially when there is a high demand for drugs that can activate the differentiation and function of osteoblasts for the treatment of osteoporosis. Nevertheless, a follow-up study on the bone formation-related functionality of FJH-UBS will be needed. In addition, further research on the appropriate concentration, availability, and mechanism of action for the clinical application of FJH-UBS is warranted.

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