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Title: Combined effects of soy isoflavone and fish oil on ovariectomy-induced bone loss in mice

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Abstract: Both soy isoflavone and n-3 polyunsaturated fatty acids are known to reduce the levels of bone-resorbing cytokines; however the synergistic effects of these food ingredients have not been examined yet. The current study was performed to elucidate the effect of concomitant intake of soy isoflavone and fish oil on bone mass in ovariectomized mice. Eight-week-old ddY female mice were subjected to ovariectomy (OVX) or sham surgery and then fed an AIN-93G with safflower oil (So) as a control lipid source, isoflavone-supplemented safflower oil (So+I), fish oil instead of safflower oil (Fo) or isoflavone-supplemented fish oil (Fo+I) for 4 weeks. Femoral bone mineral density was significantly decreased by OVX; however, this decrease was inhibited by the intake of isoflavone and/or fish oil. Histomorphometric analyses showed that bone volume and trabecular thickness in the distal femoral trabecular bone were significantly lower in the So group than in the sham group, but those were restored in the Fo+I groups. The number of osteoclasts was significantly decreased by isoflavone intake. The increased rate of bone resorption after OVX was inhibited by isoflavone and/or fish oil. The serum concentration of tumor necrosis factor alpha was increased after OVX, but was significantly lower by the combination of isoflavone with fish oil than isoflavone or fish oil alone. The results of this study indicated that the intakes of soy isoflavone and/or fish oil might have the ameliorating effects on bone loss due to OVX. Further, the concomitant intake of soy isoflavone and fish oil at a low dose showed better effects on cytokines related with bone resorption.

To

The Editor

Journal of Bone and Mineral Metabolism

Subject: Submission of a revised manuscript entitled “Combined effects of soy isoflavone and fish oil on ovariectomy-induced bone loss in mice”

Chief Editor

Dear Dr. Yoshiki Seino:

Thank you very much for your letter dated January 28, 2010 with the comments by the reviewer for our manuscript (#JBMM-D-09-00232) entitled “Combined effects of soy isoflavone and fish oil on ovariectomy-induced bone loss in mice”. We appreciate a number of valuable criticisms and constructive comments. We carefully considered the criticisms and comments raised by the reviewer, performed additional experiments, and revised the manuscript accordingly.

I hope that the revised version would satisfactorily meet the comments raised by the reviewer. I am looking forward to hearing from you at your earliest convenience.

Sincerely yours,

Hiroshige Chiba, PhD

Josai University

To reviewer 2

We greatly appreciate your valuable comments and critical suggestions. According to your suggestions, we revised the manuscript as follows.

- I agree that this would be a previously unpublished data, but I feel that it has only too narrow or limited scope. I think that the authors should have additionally done the experiments on bone strength.

Answer: We inserted new sentences in the Methods section in p 8, line 3-6 and the Results section in p.11, line 7-11. According to your suggestion, we discussed the bone strength indexes from calculating automatically by the software attaches to the device.

- Page 3, line 4: The authors' statements here are far from scientifically correct. They state that the incidence of fracture caused by osteoporosis in Asians is approximately half that in Europeans and Americans. Indeed, the incidence of hip fracture in Japan is lower than that in Europeans and Americans, but the incidence of vertebral fracture in Japan is rather higher than Europeans and Americans. They cite reference 1 as the supporting paper for their discussion. I cannot understand why they cite this paper here. This paper is not written by the epidemiological researchers and it is almost 20 years old. Regarding the incidence of osteoporotic fractures in Japan, it would be more appropriate to cite other papers such as the ones by Dr. Fujiwara or Dr. Hagino.

Answer: As you suggested, we cited more appropriate papers for incidence of osteoporosis in Japan, and added the three papers (No. 1-3) to the Reference sections.

- Page 3, line 6: The authors state that the lower fracture incidence in Japan may be due to the consumption of soybeans. As written above, the incidence of hip fracture in Japan is lower than that in Europeans and

Americans, but the incidence of vertebral fracture in Japan is rather higher than Europeans and Americans. Therefore, I will focus my comments here to hip fracture. As far as I know, there are some reports suggesting that higher "natto" intake is related to lower hip fracture incidence, but there have been no publications showing that higher soybean consumption is related to decreased incidence of hip fracture. Thus, the authors' introduction here has no scientific basis.

● Page 3, line 11: The authors state that the intake of soy proteins has been demonstrated to inhibit BMD decrease by clinical studies. However, the results from epidemiological and clinical studies are contradictory and many of the recent meta-analyses or large-scale studies do not seem to support the major role of soy proteins in preventing BMD loss and osteoporotic fracture. Some examples are shown below. Therefore, I think that citing only reference 5 and 6 is misleading to the reader.

Answer: As you suggested, the efficacy of soy protein and soy isoflavones on preventing of bone loss seems to be contradictory, especially in human study. So, we added references including some manuscripts as you indicated, and some sentences about the effective dose of isoflavones and consumption periods in P 3, line 4-19 in the text. Of course, scientific data and basis on the efficacy of soy isoflavones should be increased for human. However, we have done animal experiments by using a model of osteoporosis. So, the results are originally limited to discuss about preventing bone loss in postmenopausal women, but we probably could know the detail of bone metabolism in OVX mice fed a combination of soy isoflavone and fish diet.

● Page13, line 5 and line 8: In line 5, the authors used the term "cancellous, but they used "trabecular". Are there any special reasons to use these two different terms carrying almost identical meaning?

Answer: P 13, line 17 and 20 in the revised manuscript. According to your suggestion, the word “cancellous” was changed to “trabecular” throughout revised

manuscript.

● Page14, line8: In line 8, the authors state that the concomitant intake of fish oil and isoflavone enhanced endochondral ossification. Are they discussing the phenotypic changes in the bone or growth plate cartilage? Are they discussing modeling or remodeling? I could not exactly understand the discussion here.

Answer: These sentences p 15, line 1 in the revised manuscript were deleted because these sentences are irrelevant statement.

● Page19, line3: Reference #19 is adopted from an article from the so-called commercial journal in Japanese, not from the peer-reviewed academic journals. I think that this article is inappropriate as the reference to a scientific paper.

Answer: According to your suggestion, this reference was deleted.

● Throughout this paper, the authors expressed their results as mean +/- SE. For expressing the distribution of observed data, SD rather than SE should be used.

Answer: According to your suggestion, SE of results was changed to SD, throughout the revised manuscript.

● There are numerous typographical and grammatical errors. Examples are written below.

1) Page2, line10: "analysis" should be "analyses. Have the authors performed only a single analysis?

Answer: P 2, line 10 in the revised manuscript. The word "analysis" was changed to "analyses".

2) Page2, line17: "may" should be "might".

Answer: P 2, line 18 in revised manuscript. According to your suggestion, the ward

“may” was changed to “might”.

3) Page2, line18: "ameliorate" should be "ameliorating"

Answer: P 2, line 18 in revised manuscript. According to your suggestion, the ward “ameliorate” was changed to “ameliorating”.

4) Pag3, line12: "inhibits" should be "inhibited"

Answer: This word was deleted because sentences were modified in P3 line 4-19 revised manuscript.

5) Page 8, line 18: "BER/BV" should be "BFR/BV".

Answer: P 9, line 7 in revised manuscript. “E” was changed to “F”.

6) Page10, line16: "were" should be inserted before "fed".

Answer: P 11, line 5 in revised manuscript. According to your suggestion, the ward “were” was inserted before “fed”.

7) Page10, line 18: "Regarding trabecular BMD was higher" makes no sense.

Answer: According to your suggestion, the sentences in p 10, line 18 in the original manuscript, “Regarding trabecular BMD was higher” was deleted because this sentence was irrelevant.

8) Page11, line 11: "was" should be inserted before "markedly".

Answer: According to your suggestion, the word “was” was inserted before “markedly” P 12, line 3 in the revised manuscript.

9) Page13, line13: "do" should be "does".

Answer: P 14, line 9 in the revised manuscript. The word was changed as you suggested.

10) Page 14, line 1: "increase" should be "increased"

Answer: P 14, line 17 in the revised manuscript. The ward “increase” was changed to “increased”.

11) Page 14, line 20: "may be to" makes no sense.

Answer: P 15, line 13. According to your suggestion, the sentence “may be to” was eliminated before “promote”.

TITLE:

Combined effects of soy isoflavone and fish oil on ovariectomy-induced bone loss in mice

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Running head:

Effects of soy isoflavone and fish oil on bone mass

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1 Japanese Government.

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Abstract

Both soy isoflavone and n-3 polyunsaturated fatty acids are known to reduce the levels of bone-resorbing cytokines; however the synergistic effects of these food ingredients have not been examined yet. The current study was performed to elucidate the effect of concomitant intake of soy isoflavone and fish oil on bone mass in ovariectomized mice. Eight-week-old ddY female mice were subjected to ovariectomy (OVX) or sham surgery and then fed an AIN-93G with safflower oil (So) as a control lipid source, isoflavone-supplemented safflower oil (So+I), fish oil instead of safflower oil (Fo) or isoflavone-supplemented fish oil (Fo+I) for 4 weeks. Femoral bone mineral density was significantly decreased by OVX; however, this decrease was inhibited by the intake of isoflavone and/or fish oil. Histomorphometric analyses showed that bone volume and trabecular thickness in the distal femoral trabecular bone were significantly lower in the So group than in the sham group, but those were restored in the Fo+I groups. The number of osteoclasts was significantly decreased by isoflavone intake. The increased rate of bone resorption after OVX was inhibited by isoflavone and/or fish oil. The serum concentration of tumor necrosis factor alpha was increased after OVX, but was significantly lower by the combination of isoflavone with fish oil than isoflavone or fish oil alone. The results of this study indicated that the intakes of soy isoflavone and/or fish oil might have the ameliorating effects on bone loss due to OVX. Further, the concomitant intake of soy isoflavone and fish oil at a low dose showed better effects on cytokines related with bone resorption.

Keywords: bone-resorbing cytokines; flavonoid; polyunsaturated fatty acids; osteoporosis

Introduction

The incidence of hip fracture caused by osteoporosis in Asians is lower than that in Europeans and Americans, despite the facts that the prevalence of vertebral fracture is higher in Asian populations [1-3].

On the other hand, epidemiological studies indicate that women, who have high soy intake, have a lower risk of osteoporosis than women who consume a typical Western diet [4-6]. Consequently, many menopausal women use phytoestrogens to maintain their bone mineral density (BMD) because they are unlikely to cause the undesirable effects associated with steroid hormones [7, 8]. A meta-analysis of randomized controlled trials (RCTs) has estimated the effect of ingesting soy isoflavones on lumbar spine BMD [9]. However, soy isoflavone or protein intervention trials in postmenopausal women indicated modest or no effects of soy isoflavones on preserving BMD in the hip or spine [10-15]. Thus, the efficacy of soy isoflavones on preventing bone loss seems to be still contradictory. One of solutions might be effective daily intake of isoflavones and consuming periods. Ma et al. [9] suggested that the favorable effect became more significant when >90 mg/day of isoflavones were consumed and soy isoflavone consumption for 6 months could be enough to exert beneficial effects on bone in menopausal women.

Asian diets also include large amounts of fishes such as sardines and tuna. These fishes

contain oil that is abundant in n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have well-known anti-inflammatory and immunomodulatory effects [16, 17]. Fish oil increases the ratio of n-3/n-6 fatty acids and competitively inhibits prostaglandin E₂ (PGE₂) production from arachidonic acid, which is a metabolite of the n-6 PUFA, linoleic acid [18]. PGE₂ enhances production of the bone-resorbing cytokines, including IL-1, IL-6, and TNF- α , and promotes bone resorption by induction of the differentiation of osteoprecursors to osteoclasts [19]. Intake of oil with a high content of n-3 PUFAs also decreases the amount of PGE₂ in bones [20], and Sun et al [21] demonstrated that fish oil had a greater effect than corn oil in inhibiting bone loss after ovariectomy. These studies suggest that n-3 PUFAs regulate the production of bone-resorbing cytokines and thereby maintain bone mass.

Nutritional factors are important in the primary prevention of osteoporosis, and intake of either soy isoflavone or fish oil alone has been shown to inhibit bone loss. Ward et al [22] reported that the combination intakes of genistein, daidzein and polyunsaturated fatty acid (PUFA) showed an additive effect in preventing bone loss at the lumbar spine. Another report [23] indicated a complementary action of the dietary combination soy isoflavone and 25% n-3 PUFA for attenuating bone mineral reduction in OVX rats at tibia. However, these reports did not clarify the inhibitory effect of combination intakes of isoflavone glycoside and low dose of n-3 PUFA on bone loss in OVX animals. Therefore, the current study was performed in order to assess the inhibitory effect of soy isoflavone as glycoside and/or fish oil as PUFA on

bone loss in ovariectomized mice, which have been used as a model of osteoporosis.

Materials and Methods

Determination of isoflavone and fish oil contents in diets

Ohta et al. [24] and Hidaka et al. [25] have shown that isoflavone glycoside (Fujiflavone P-40) mixed with diets at concentrations of 0.5% and 0.25%, respectively, maintains the bone mass in ovariectomized mice with no effect on the uterus. Uesugi et al. [26] confirmed that daidzein or genistein administration at concentrations of 50 mg·kg⁻¹·d⁻¹ or more improved bone turnover in a similar manner to estrogen, and Picherit et al. [27] found that administration of isoflavone aglycone (genistein: 159 mg/g, daidzein: 156 mg/g, glycitein: 33 mg/g) at doses of 40 mg·kg⁻¹·d⁻¹ or more decreased urinary deoxypyridinoline, a bone resorption marker, in rats after removal of mature ovaries. An isoflavone aglycone dose of 40 mg·kg⁻¹·d⁻¹ corresponds to an isoflavone glycoside dose of approximately 60 mg·kg⁻¹·d⁻¹ (genistein: 30 mg·kg⁻¹·d⁻¹, daidzein: 29 mg·kg⁻¹·d⁻¹, glycitin: 6 mg·kg⁻¹·d⁻¹). On the basis of this conversion, the amount of diet required was calculated to be 4 g per day per 40 g body weight, (Table 3) and the diet was prepared containing 0.25% P-40 (genistein: 13 mg·kg⁻¹·d⁻¹, daidzein: 56 mg·kg⁻¹·d⁻¹).

Then, we also examined whether the dose of how much fish oil affects on BMD in intact female mice for 4 weeks. Female C57BL/6J strain mice were obtained from Tokyo Laboratory Animals Science Co. (Tokyo, Japan) at 7 weeks of age and fed an AIN-93G diet for 1 week to

1 stabilize the metabolic conditions. Mice were housed in a 12-h light/12-h dark cycle at
2 constant temperature of 22 ± 2 °C and humidity of $55 \pm 5\%$. The amount of fish oil increased
3 from 0 to 50% lipid energy with concomitant decrease of safflower oil from 50 to 0% lipid
4 energy, maintaining the total amount of fat constant at 50% lipid energy. BMD of the femur
5 were measured by dual-energy X-ray absorptiometry (DXA) by using a bone densitometer
6 adapted for small animal research (model DCS-600R; Aloka, Tokyo, Japan). Whole femoral
7 BMD was higher in the mice fed the diet containing 30 and 40 % fish oil compared with that
8 of 0% fish oil and 50% safflower oil groups. On the other hand, BMD tended to be higher ($P =$
9 0.07) in the mice fed the diet containing 20% fish oil group than that of 0% fish oil (Fig. 1).
10 Based on these results, we decided that submaximal dose of fish oil, 20 %, was adopted for the
11 dose for the combination study with isoflavone.

12 Therefore, this study was performed using 0.12% isoflavone P-40 and 8.0 w/w % fish oil
13 (20% of lipid energy for total energy).

15 *Experimental design*

16 Eight-week-old female ddY strain mice were purchased from Japan SLC (Shizuoka,
17 Japan). The mice were housed in individual cages in a temperature- and humidity-controlled
18 room, and were given free access to food and distilled water. The mice underwent either a
19 sham- operation or were ovariectomized (OVX) ($n = 7$ for each group). Sham mice were fed a
20 So diet, whereas OVX mice were divided into 4 groups that were fed a diet containing 8%

safflower oil (So), So with 0.25% Fujiflavone P-40 (So+I), 8% fish oil (Fo), or Fo with I (Fo+I) for 4 weeks. Of the total fatty acid content of safflower oil, oleic acid (18:1n-9) and linoleic acid (18:2n-6) comprise approximately 45%, respectively. Similarly, the fish oil contained 7% EPA (20:5n-3) and 23% DHA (22:6n-3) (Table 1). Fujiflavone P-40 (isoflavone content, 46.6%; Fujicco, Kobe, Japan) was supplemented to the diets (0.25% in the diets). Fujiflavone P-40 contained daidzin (22.3%), glycitin (15.0%), genistin (5.4%), daidzein (0.2%), glycitein (0.4%), genistein (0.06%) and others isoflavone (3.2%). The dose of isoflavone conjugates in this study was approximately 0.12% in the So+I and Fo+I diets. The experimental diets were based on the AIN-93G diet with each oil as shown above replacing soybean oil [28] (Table 2). At the end of the experiment, the mice were killed with pentobarbital sodium (Nembutal; Dainippon Pharmaceutical Co., Osaka, Japan). In each experiment, body and uterine weights were measured, and the right and left femora were removed for the measurement of BMD and histomorphometric analysis.

The study was approved by the Josai University Animal Use Committee, and the mice were maintained in accordance with the university guidelines for the care and use of laboratory animals.

Femoral BMD and bone structure index

The femoral BMD of mice was examined radiographically using computed tomography (CT) (LCT-100; LaTheta, ALOKA, Tokyo, Japan) according to the manufacturer's protocol.

CT scanning was performed at 2-mm intervals from the diaphragm to the bottom of the abdominal cavity.

The minimum moment of inertia of cross sectional areas and the polar moment of inertia of cross sectional areas that represent the flexural rigidity and torsional rigidity, respectively, were also calculated automatically by the software attached to the device. According to the manufacture, the precision error (as % CV) is within 2% range for all measurements.

Femoral calcium contents

Femurs were dried overnight at 100°C, weighed, and then converted to ash by heating for 48 h at 550°C. The ashed samples were extracted for analysis using 1 M hydrochloric acid. The amount of calcium (Ca) was determined by atomic absorption spectrophotometry (Spectr AA220FS; Varian, Australia) according to the method of Gimblet et al. [29].

Bone histomorphometry

In order to measure bone histomorphometric parameters, 12-week-old female mice were double-labeled with subcutaneous injections of 20 mg/kg tetracycline hydrochloride (Sigma, St. Louis, MO, USA) 72 h before sacrifice, and 10 mg/kg calcein (Dojindo, Kumamoto, Japan) 30 h before sacrifice. Femurs were removed from each mouse, and fixed with 70% ethanol. They were trimmed to remove the muscle, stained with Villanueva bone stain for 5 d, dehydrated in graded concentrations of ethanol, and then embedded in methyl methacrylate

(Wako Chemicals, Kanagawa, Japan) without decalcification. Sagittal plane sections (5 μm) of the lumbar region were cut using a microtome (Lieca, Germany). The cancellous bone was measured in the secondary spongiosa. Parameters of bone structure included bone volume per tissue volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular separation (Tb.Sp, μm), number of osteoclasts per surface (N.Oc/BS, %), and osteoblast surface (Ob.S/BS, %). The dynamic parameters assessed were bone resorption rate (BRs.R, $\mu\text{m}^3 \cdot \mu\text{m}^{-2} \cdot \text{d}^{-1}$), bone formation rate/bone volume (BFR/BV, $\% \cdot \text{y}^{-1}$), mineral appositional rate (MAR, $\mu\text{m}/\text{d}$), bone volume (BV, mm^2), osteoid volume/bone volume (OV/BV, %), osteoid surface/bone surface (OS/BS, %), and osteoid thickness (O.Th, μm).

Analysis of serum samples

Two serum bone metabolism markers, osteocalcin (OC) and the C-terminal telopeptide of type I collagen (CTX) were measured using a Mouse Osteocalcin EIA kit (Biomedical Technologies, Stoughton, MA, USA) and a RatLaps ELISA kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark), respectively. Serum, TNF- α , IL-1, and PGE₂ were measured using Mouse TNF- α , IL-1 ELISA kits (Endogen, Rockford, IL, USA), and a PGE₂ ELISA kit (Cayman, MI, USA), respectively.

Time-resolved fluoroimmunoassay (TR-FIA) for serum genistein and daidzein

The TR-FIA technique has previously been used to measure serum genistein and daidzein

[30] and also serum equol [31]. After enzymatic hydrolysis and extraction by diethyl ether, serum genistein and daidzein concentrations were determined fluorometrically using a DELFIA Victor 1420 multilabel counter (Perkin Elmer, Wellesley, MA, USA) and are expressed as nmol/L.

Statistical analysis

Results are expressed as the means \pm SE for each group. After one-way analysis of variance (ANOVA), Fisher's protected least significant difference (PLSD) test was used to determine significant differences among the groups. Differences were considered to be significant when the P value was less than 0.05.

Results

Body weight, food intake, and uterine weight

As shown in Table 3, the body weight was increased by OVX, but food intake of the experimental animals did not differ among the 5 groups. Isoflavone intakes were also similar both the groups administered isoflavone. The uterine weight was significantly decreased in OVX mice, and the intake of isoflavone and fish oil had no effect on uterine weight in these animals.

Femoral bone mineral density, bone metabolism markers, strength indexes, and calcium

contents

The whole, cortical and trabecular BMD was significantly lower in the So group than in the sham group (Table 4). Administration of isoflavone and/or fish oil significantly inhibited the bone loss in the whole, cortical, and trabecular bone, but the BMD in these groups which were fed experimental diet was not recovered to the sham level (Table 4). Whole and cortical BMD was higher in the Fo+I group than in the Fo group. Further, there was no significant difference in the cortical BMD between the sham and Fo+I group. The mean values of minimum moment of inertia of cross-sectional areas and the polar moment of inertia of cross-sectional areas are shown in Table 5. The former value represents the flexural rigidity, and the latter represents the torsional rigidity. These parameters were decreased by OVX, but the intake of isoflavone and/or fish oil significantly inhibited decreases.

Serum CTx, a bone resorption marker, and OC, a bone formation marker, were increased in the So OVX group and the increase was suppressed by the intake of isoflavone or fish oil or the combined intake of these two ingredients (Table 6). The Ca content was significantly lower in the So OVX group than in the sham group, and administration of fish oil or the concomitant intake of isoflavone and fish oil inhibited the extent of this decrease (Table 5). High Ca levels were also observed in femora of mice that received isoflavone alone.

Bone histomorphometric analysis

Figure 2 shows the histological analysis of trabecular bone collected from mice in each

group. Many trabecular connectivities were observed in the cancellous bone area beneath the growth plate cartilage of the distal femur in sham mice, whereas most of connectivities in lower region were disappeared in OVX mice. The bone loss in OVX+Fo+I group was markedly inhibited, compared with the OVX+So group, however it was not restored to that in the sham group (Fig. 2A, B).

In order to determine the effects of isoflavone administration on trabecular bone, histological sections of the distal femoral metaphysis were prepared, and BV/TV, and Tb.Th were evaluated (Fig. 2C). BV/TV and Tb.Th were markedly decreased by OVX; however, these parameters were significantly inhibited to decrease by the intake of isoflavone and/or fish oil. N.Oc/BS and, BRs.R, the parameters of bone resorption, were significantly increased by OVX, but these were decreased by isoflavone intake, and, and normalized by the combination of isoflavone and fish oil to the sham level. BFR/BV, the parameter of bone formation, was significantly increased by OVX, and the increase was inhibited by the intake of isoflavone and/or fish oil alone. Ob.S/BS (Fig. 2C), BV, OV/BV, OS/BS, and O.Th were significantly higher in So+I group compared with the So group in OVX mice (Fig. 2D).

Serum biochemical parameters

Serum TNF- α concentration was increased by OVX and the increase was inhibited by the intake of isoflavone and/or fish oil. The serum IL-1 concentration was also increased by OVX, and the increase was normalized by the isoflavone intake. Further, the IL-1 concentration was

lower in OVX mice fed the diets fish oil with isoflavone than in that in safflower oil with isoflavone or fish oil (Table 6). A negative correlation was found between serum TNF- α concentrations and femoral BMD (Fig. 3). The serum PGE₂ concentration was significantly higher in the So OVX group than in the sham group and this was substantially reduced by the concomitant intake of isoflavone and fish oil (Table 6).

Serum isoflavone concentrations

By treatment with soy isoflavones, daidzein, genistein, and equol were detected in serum. No significant differences were observed in the serum concentrations of these compounds between So+I and Fo+I groups (daidzein, So+I group: 330 \pm 71; Fo+I group: 280 \pm 51; genistein, So+I group: 122 \pm 22; Fo+I group: 102 \pm 30 nmol/L serum). However, serum equol level was 1.8-fold greater in the So+I group than in the Fo+I group (So+I group: 1002 \pm 56; Fo+I group: 563 \pm 77 nmol/L serum).

Discussion

Our results showed that isoflavone and fish oil at low doses have an additive effect on inhibiting bone loss, especially in the femoral trabecular bone, by inhibiting bone resorption in ovariectomized mice. In the current study, the BMD in the femur of mice was markedly decreased after ovariectomy. Intake of soy isoflavone and/or fish oil inhibited the decrease in femoral whole, cortical and trabecular BMD. The concomitant intake of isoflavone and fish

oil had better effects in whole and cortical BMD than fish oil alone. Regarding trabecular BMD, the combination of fish oil and isoflavone showed a higher value than isoflavone alone. The changes in structural properties are associated with changes in both cross-sectional geometry and material properties [32]. The decreased minimum moment of inertia of cross sectional areas and the polar moment of inertia of cross sectional areas due to OVX, were inhibited by the intake of soy isoflavone and/or fish oil.

Furthermore, isoflavone and fish oil at concentrations that influenced BMD had no effect on uterine weight. It is suggested that the dose of isoflavone used in this study (approximately 120 mg/kg/d) does not have adverse effects. Ishida et al. [33] showed that administration of daidzin at 50 mg·kg⁻¹·d⁻¹ or genistein at 100 mg·kg⁻¹·d⁻¹ increased rat uterine weight, and Kanno et al. [34] found that administration of genistein at doses of 60 mg·kg⁻¹·d⁻¹ or more induced uterine hypertrophy. Similarly, Ishimi et al. [35] showed that genistein aglycone induced uterine hypertrophy at a dose 10-fold greater than that required to inhibit the decrease in bone mass (0.7 mg/d via skin).

The effect of n-3 PUFAs on bone metabolism has been investigated in numerous animal models including mice and rats [21, 36-39]. The results of these studies suggested that fish oil and n-3 PUFA increased calcium absorption [40, 41], increased bone formation [42, 43], decrease calcium excretion [43], and decrease bone resorption [21, 39, 44]. However, as these studies have not reported any histomorphometric analyses, it is not known whether the observed differences in BMD were due to alternations in bone resorption and/or in bone

formation. Bone mass is increased by endochondral and intramembranous ossification and by modeling and remodeling [45]. Our results showed that the concomitant intake of fish oil and isoflavone enhanced endochondral ossification (Fig 2A). We also observed various directions of osteoclast-induced bone resorption and maintenance of the trabecular thickness in mice with increased bone mass in the Fo+I group. Reduced differentiation or activation of osteoclasts may decrease bone resorption. OVX mice with isoflavone intake in the current study showed the significant reduction in the increase of the number of osteoclast, suggesting inhibition of osteoclast differentiation. On the other hand, osteoid is formed by osteoblasts and after brief interval, this was mineralized by also the cells [46-48]. The results from BV, OV/BV, MAR, and BFR/BV (Fig. 2C, D) indicate that the intake of isoflavone alone increased osteoid volume, and that of fish oil alone increased MAR and BFR/BS, indicators of calcification. These results raised the possibility that the combination of soy isoflavone and fish oil promote completeness of bone formation; that is, increased osteoid tissue (bone matrix) induced by the intake of isoflavone, and enhanced mineralization of osteoid tissue induced by the intake of fish oil. The interactions of fish oil and isoflavone were observed in Tb.Th, Ob.S/BS and O.Th. (Fig. 2C and 2D), suggesting that fish oil and isoflavone may increase bone mass synergistically in OVX mice.

Intake of fish oil also reduces PGE₂ production [18, 43] and PGE₂ enhances differentiation of osteoclast precursors and causes bone resorption [19]. Intake of fish oil was therefore expected to reduce osteoclast production. However, no reduction in the osteoclast

surface was observed in mice with the intake of fish oil alone compared with OVX+So group. These results indicate that although fish oil reduces the bone resorption activity of osteoclasts, it does not influence osteoclast differentiation. Serum cytokine concentrations were also significantly reduced by the concomitant intake of isoflavones and fish oil compared to the intake of either component alone, and a negative correlation was found between TNF- α concentrations and femoral BMD. Therefore, the concomitant intake of soy isoflavone and fish oil appears to reduce the production of bone-resorbing cytokines in an additive manner and leads to reduced bone resorption. However, as described above, the mechanism by which soy isoflavone decrease the reduction in bone resorption may differ from that of fish oil. Consequently, additional studies are required in order to elucidate the regulatory mechanisms underlying osteoclast differentiation and bone-resorbing activity.

Equol is produced from daidzein in the gastrointestinal tract; however, interindividual variation exists in its metabolism in humans [49]. Fujioka et al. [50] reported that administration of equol inhibited bone loss induced by estrogen deficiency. In this study, despite the result indicating that there was no difference in isoflavone intakes in the So+I and Fo+I groups, the serum equol level in the Fo+I group was lower than that in the So+I group. These results raise the possibility of a complementary effect on bone metabolism induced by the intake of fish oil, despite a decrease serum equol level in the Fo+I group, compared with that in the So+I group. However, the mechanism by which the combined administration of isoflavone and fish oil decreases serum equol levels remains unclear.

The results of this study indicated that the intakes of soy isoflavone and/or fish oil may have the ameliorating effects on bone loss due to OVX. Further, the concomitant intake of soy isoflavone and fish oil at a low dose showed better effects on cytokines related with bone resorption.

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- 8

Legends

FIGURE 1

The effect of different dietary fish oil (Fo) contents (10–50% total lipid content) on femoral bone mineral density (BMD) in intact female C57BL/6J mice. Mice were fed the diet for 4 weeks. A diet containing safflower oil (So; 50% total lipid content) was used as a control. Data are the means \pm SD for 6 mice. Means without a common letter are significantly different, $P < 0.05$.

FIGURE 2

Histological analysis of trabecular bone collected from sham-operated mice (Sham), ovariectomized (OVX) mice fed diets containing 8% safflower oil (So) with or without 0.12% soy isoflavone (I), and OVX mice fed diets containing fish oil (Fo) with or without soy isoflavones for 4 weeks. Femora were collected 4 weeks after the operation and sections were prepared from the distal metaphysis.

(A) Femoral distal trabecular bone stained with Villanueva bone stain (natural: $\times 100$). \circ , the intersection of more than three trabecular bone; \leftrightarrow , subchondral trabecular bone. (B) Panel B shows fluorescence microscopy images of the calcein and tetracycline layers in the trabecular bone, the distance between which reflects the mineral apposition rate. (fluorescence: $\times 100$) (C) Parameters of bone structure included bone volume per tissue volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular separation (Tb.Sp, μm), osteoclast number per surface (Oc.N/BS, %), osteoblast surface (Ob.S/BS, %). Dynamic

1 parameters were bone resorption rate (BRs.R, $\mu\text{m}^3 \cdot \mu\text{m}^{-2} \cdot \text{d}^{-1}$), bone formation rate/bone
2 surface (BFR/BS, $\mu\text{m}^3 \cdot \mu\text{m}^{-2} \cdot \text{y}^{-1}$), and mineral appositional rate (MAR, $\mu\text{m}/\text{d}$). (D) Bone
3 volume (BV, mm^2), ratios of osteoid volume to bone volume (OV/BV, %), and osteoid surface
4 to bone surface (OS/BS, %), and osteoid thickness (O.Th, μm). Microstructural parameters
5 were determined as described in the Materials and Methods. Data are the means \pm SD of 3
6 mice. Means without a common letter are significantly different, $P < 0.05$.

7 FIGURE 3

8 Correlation between whole femoral bone mineral density (BMD) and serum TNF- α levels,
9 in ovariectomized (OVX) mice treated for 4 weeks with 8% safflower oil (So, \square), So and
10 0.25% Fujiflavone P-40 (So+I, \blacksquare), 8% fish oil (Fo, \circ), Fo and I (Fo+I, \bullet). $R^2 = 0.51$, $P <$
11 0.0001.

TABLE 1			
Fatty acid composition of dietary lipids			
		Safflower oil	Fish oil
		<i>g/100g fatty acid</i>	
Ingredients			
14:0	MA		3.0
16:0	PA	5.6	18.2
16:1	POA	0.2	4.2
18:0	STE	2.2	4.9
18:1	OA	45.0	18.8
18:2 (n-6)	LA	45.3	1.3
18:3 (n-3)	ALA	0.8	0.8
20:4 (n-6)	AA	0.4	2.0
20:5 (n-3)	EPA		6.8
22:6 (n-3)	DHA		22.8
Others		0.5	17.2
Total		100	100
n-6/n-3		57.13	0.11

MA, myristic acid; PA, palmitic acid; POA, palmitoleic acid, STE, stearic acid; OA; oleic acid; LA, linoleic acid; ALA, α -linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

TABLE 2**Composition of experimental diets**

	So	So+I	Fo	Fo+I
<i>g/kg diet</i>				
Ingredients				
Casein Milk	200	200	200	200
β-Corn Starch	391.2	389.6	391.2	389.6
Corn Starch	129.9	129.4	129.9	129.4
Sucrose	98.4	98.0	98.4	98.0
Safflower Oil	80.0	80.0	–	–
Fish Oil	–	–	80.0	80.0
Cellulose	50	50	50	50
Mineral mixture ¹	35	35	35	35
Vitamin mixture ¹	10	10	10	10
Choline	2.5	2.5	2.5	2.5
L-Cystine	3.0	3.0	3.0	3.0
Isoflavone ²	–	2.5	–	2.5

1 Prepared according to the AIN-93G formulation

2 Isoflavone (Fujiflavone P-40; Fujicco, Tokyo, Japan.)

So, safflower oil containing diet (control diet); So+I, 0.12% isoflavone conjugates (I) containing diet; Fo, fish oil containing diet; Fo+I, Fo and I containing diet

TABLE 3

Final body weight, wet weight of the uterus and food intake in sham mice fed the diet containing safflower oil (So) without soy isoflavone (I), OVX mice fed the diets containing So with or without I and OVX mice fed the diets fish oil (Fo) with or without I for 4 weeks.¹

		Final Body Weight <i>g</i>	Uterus Weight <i>mg</i>	Food Intake <i>g/kg wt./d</i>	Isoflavone Intake <i>mg/kg wt/d</i>
Sham	So	34.1 ± 3.1 ^b	287 ± 33.4 ^a	120 ± 2.6	–
OVX	So	36.6 ± 1.5 ^a	21.4 ± 3.71 ^b	122 ± 4.3	–
	So+I	38.9 ± 2.8 ^a	22.4 ± 2.01 ^b	119 ± 3.4	124 ± 4.8
	Fo	37.3 ± 1.6 ^a	20.0 ± 1.48 ^b	123 ± 2.3	–
	Fo+I	37.8 ± 2.4 ^a	20.2 ± 3.11 ^b	122 ± 6.2	117 ± 3.6

¹ Values are means ± SD for 7 mice. ; means in a column with different superscript letters differ, $P < 0.05$.

TABLE 4

Femoral BMD using qCT of sham mice, fed the diet containing safflower oil (So) without soy isoflavone (I), OVX mice fed the diets containing So with or without I and OVX mice fed the diets fish oil (Fo) with or without I for 4 weeks.¹

		Whole	Cortical bone (mg/cm^3)	Trabecular bone
Sham	So	720 ± 40 ^a	893 ± 33 ^a	423 ± 42 ^a
	So	616 ± 17 ^d	805 ± 24 ^d	321 ± 23 ^d
	So+I	652 ± 39 ^{bc}	841 ± 32 ^{bc}	349 ± 13 ^c
	Fo	642 ± 16 ^c	823 ± 20 ^c	362 ± 42 ^{bc}
OVX	Fo+I	680 ± 32 ^b	866 ± 36 ^{ab}	389 ± 24 ^b

¹ Values are means ± SD for 7 mice. ; means in a column with different superscript letters differ, $P < 0.05$.

TABLE 5

Minimum moment of inertia of cross-sectional areas and polar moment of inertia of cross-sectional areas the femur in sham mice fed the diet containing safflower oil (So) without soy isoflavone (I), OVX mice fed the diets containing So with or without I and OVX mice fed the diets fish oil (Fo) with or without I for 4 weeks.¹

		Minimum moment of inertia of cross-sectional areas	Polar moment of inertia of cross-sectional areas
		<i>g.cm</i>	
Sham	So	56.0 ± 13.4 ^a	130 ± 15 ^a
	So	29.0 ± 3.25 ^c	94.0 ± 2.3 ^b
	So+I	39.4 ± 5.09 ^b	125 ± 5.8 ^a
	Fo	36.2 ± 4.63 ^b	119 ± 12 ^a
OVX	Fo+I	43.4 ± 8.85 ^b	131 ± 22 ^a

¹ Values are means ± SD for 7 mice. ; means in a column with different superscript letters differ, $P < 0.05$.

TABLE 6

Bone metabolism markers and Calcium content in the femur in sham mice fed the diet containing safflower oil (So) without soy isoflavone (I), OVX mice fed the diets containing So with or without I and OVX mice fed the diets fish oil (Fo) with or without I for 4 weeks.¹

		CTx	OC	Calcium
		<i>ng/mL serum</i>		<i>mmol/g dry bone</i>
Sham	So	9.45 ± 2.34 ^b	149 ± 46 ^b	6.93 ± 0.14 ^{ab}
	So	13.5 ± 1.32 ^a	219 ± 65 ^a	6.55 ± 0.56 ^c
OVX	So+I	11.2 ± 1.33 ^b	135 ± 26 ^b	7.10 ± 0.18 ^a
	Fo	11.8 ± 1.12 ^b	108 ± 12 ^b	6.83 ± 0.14 ^b
	Fo+I	10.9 ± 1.83 ^b	121 ± 40 ^b	7.19 ± 0.29 ^a

¹ Values are means ± SE for 7 mice. ; means in a column with different superscript letters differ, $P < 0.05$.

TABLE 7

Serum cytokines of sham mice fed the diet containing safflower oil (So) without soy isoflavone (I), OVX mice fed the diets containing So with or without I and OVX mice fed the diets fish oil (Fo) with or without I for 4 weeks.¹

		TNF- α	IL-1	PGE ₂
		(pg/mL serum)		(ng/mL serum)
Sham	So	26.5 \pm 4.90 ^b	30.8 \pm 18 ^{bc}	11.0 \pm 3.64 ^b
	So	38.5 \pm 9.71 ^a	62.8 \pm 27 ^a	15.2 \pm 1.69 ^a
	So+I	24.3 \pm 8.10 ^b	41.3 \pm 12 ^b	13.3 \pm 7.41 ^{ab}
	Fo	24.9 \pm 8.66 ^b	51.6 \pm 14 ^{ab}	8.11 \pm 9.47 ^{abc}
	Fo+I	16.4 \pm 3.10 ^c	23.4 \pm 15 ^c	4.72 \pm 3.91 ^c

¹ Values are means \pm SD for 7 mice. ; means in a column with different superscript letters differ, $P < 0.05$.

FIGURE 1

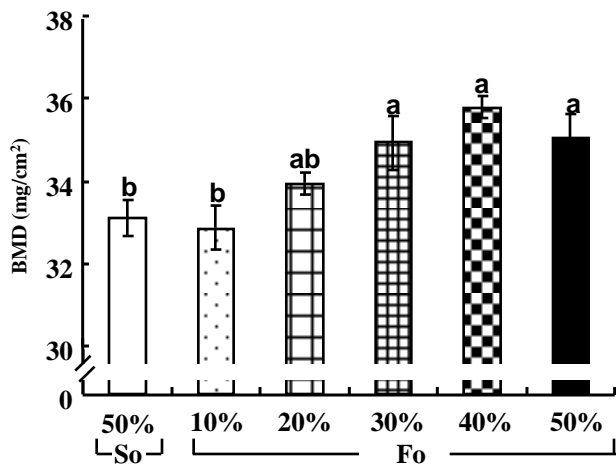


FIGURE 2A, B

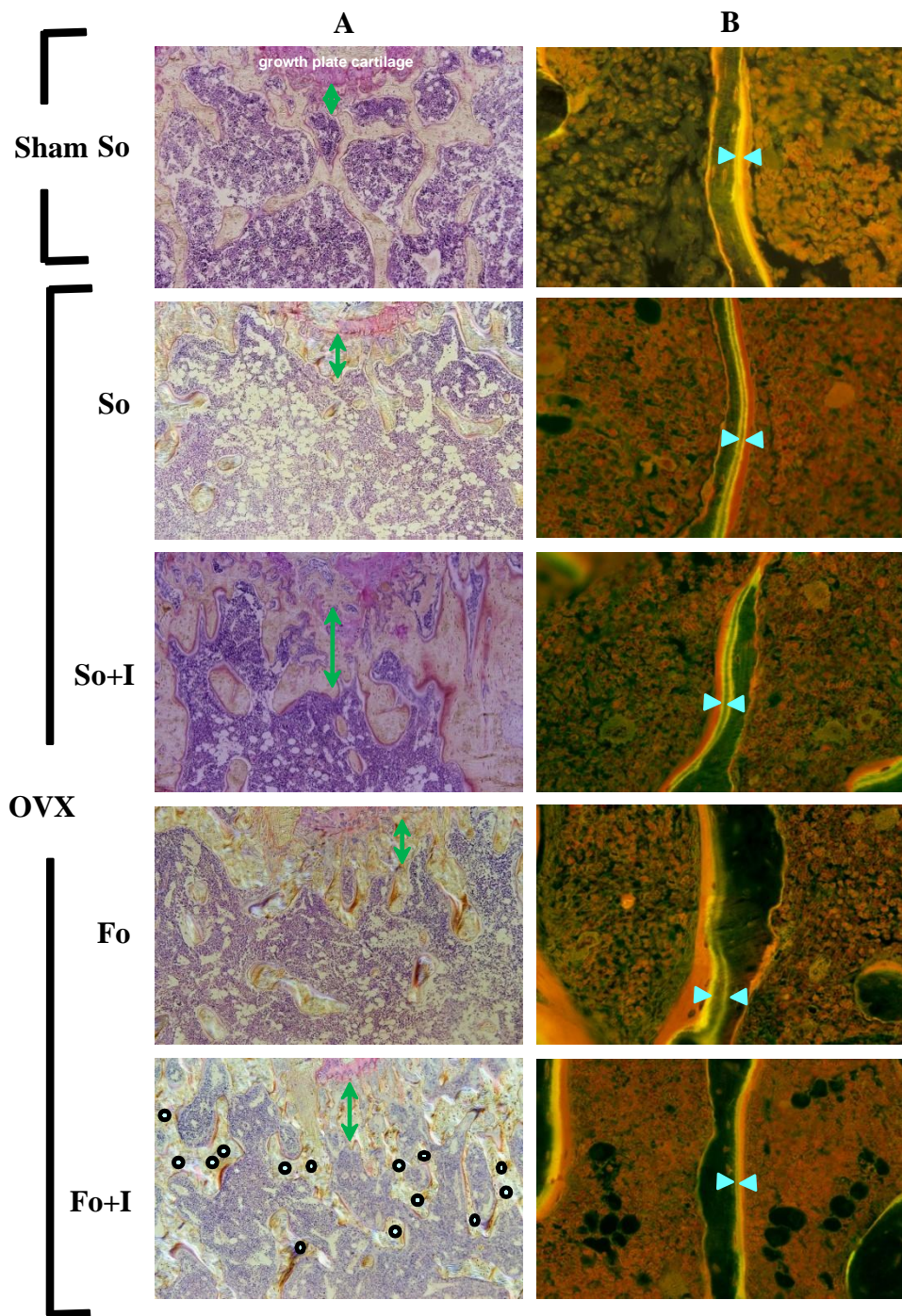


FIGURE 2C

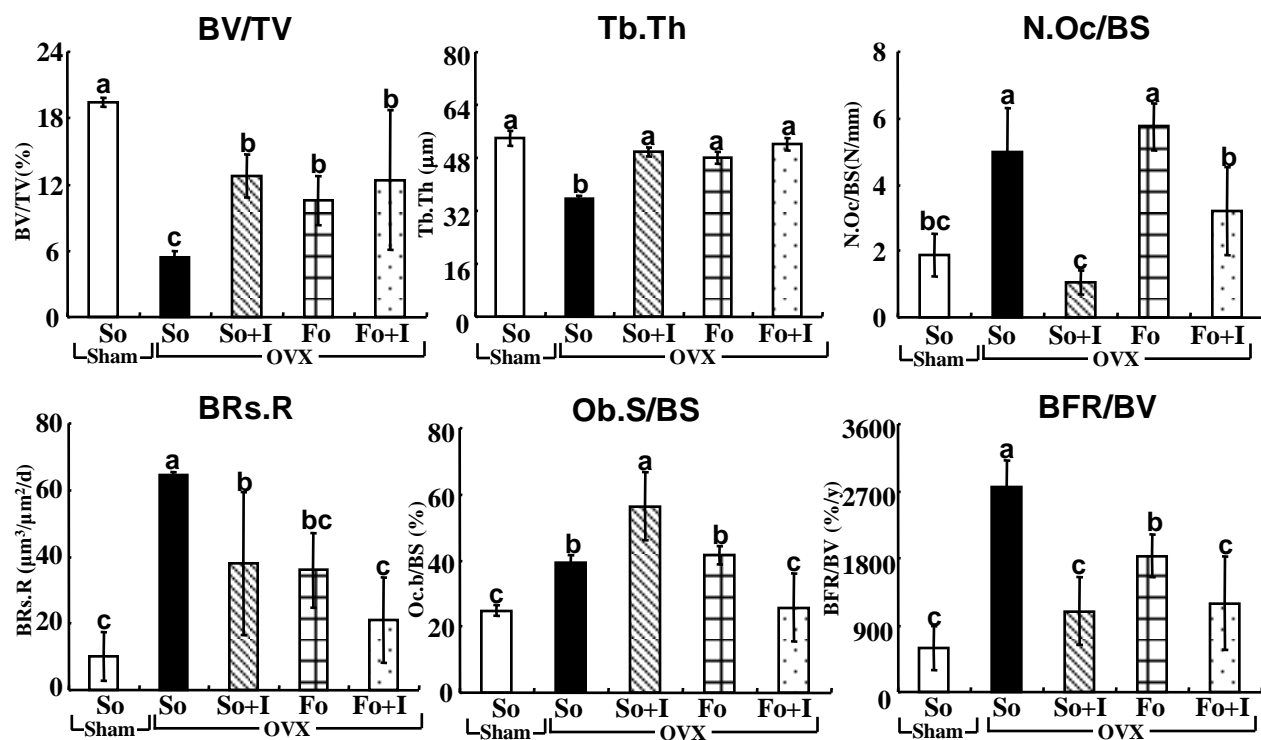


FIGURE 2D

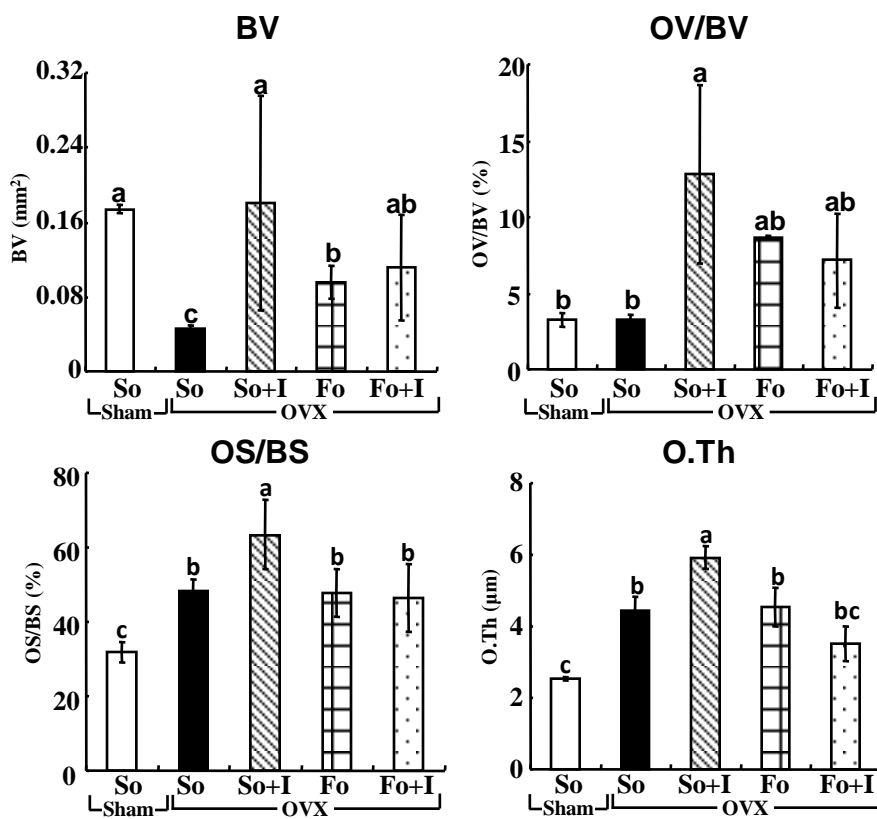


FIGURE 3

