

Fermented soybeans by *Rhizopus oligosporus* reduce femoral bone loss in ovariectomized rats

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BACKGROUND/OBJECTIVES: Soy isoflavones are structurally similar to estrogen and bind to estrogen receptors, suggesting that they exhibit estrogenic activities; therefore, they are referred to as phytoestrogens. Fermentation may affect the bioavailability of isoflavones altering soy isoflavone glycosides in the form of aglycones. Thus, this study investigated the effects of fermented soybeans by *Rhizopus oligosporus* on bone metabolism in both young rats as a pilot test and in ovariectomized (ovx) old rats as a model of menopause.

MATERIALS/METHODS: In the pilot test, a total of 24 seven-week-old female Sprague-Dawley (SD) rats were fed one of three diets for a period of four weeks: casein, unfermented soybean product, or fermented soybean product by *R. oligosporus*. In the ovx rat model, 20-week-old SD rats weighing 260-290 g underwent either sham-operation (n = 10) or bilateral ovariectomy (n = 30) and were then fed the AIN-93M diet for one week. Thereafter, rats were fed sham-casein, ovx-casein, ovx-soybean, or ovx-fermented soybean diet for five weeks. After decapitation, femoral bones were isolated and preserved in 9% formalin for assessment of bone mineral density (BMD), bone mineral content (BMC), and bone-breaking strength (BBS).

RESULTS: OvX rats showed significantly increased weight gain and decreased uterine wet weight. Of particular interest, ovx rats fed fermented soybeans showed increased uterine wet weights compared to control rats. Fermented soybean diet caused a significant increase in plasma 17- β estradiol concentrations in young rats, and 17- β estradiol levels were enhanced in ovx rats to match those of sham-operated ones. Significantly lower femoral BMD and BMC were observed in ovx rats compared to sham-operated controls, whereas bone areas did not differ statistically among the groups. In addition, BBS tended to be increased in ovx rats fed soybeans and fermented soybeans.

CONCLUSIONS: Supplementation of fermented soybeans could have preventive and therapeutic effects against osteoporosis in postmenopausal women.

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INTRODUCTION

Among the various physiologically active substances in soybeans, isoflavones are known to possess many pharmacological properties, including anti-cancer effects, reduction of low-density lipoprotein (LDL) cholesterol, and prevention and suppression of osteoporosis [1-3]. Isoflavones are typically found as glycosides in soybeans at concentrations between 0.1-3% [4,5]. Soy isoflavone glycosides become physiologically more active in humans or animals once hydrolyzed to aglycone substances. Fermentation using *Rhizopus oligosporus* may affect the bioavailability of isoflavones by altering the structures of isoflavone glycosides in the form of aglycones, thereby improving the rates of digestion and utilization of isoflavones [6,7]. Among traditional fermented soybean products, Japanese natto or Korean cheongkukjang, fermented by *Bacillus subtilis*, has an unfavorable smell, whereas Indonesian tempeh, fermented

by *R. oligosporus*, is odorless [8]. Spores of *R. oligosporus* produce white fluffy mycelia and bind beans together for creation of an edible patty of fermented soybeans.

Soy isoflavones are structurally similar to estrogen and bind to estrogen receptors, suggesting that they exhibit estrogenic activities; therefore, they are referred to as phytoestrogens [9]. Phytoestrogen concentrations in urine and plasma of Japanese women consuming a traditional diet were high, as were those of vegetarians; the incidence of breast and ovarian cancers in these groups was low [10]. A case-control study also confirmed that a phytoestrogen-rich diet may offer protective benefits against breast cancer [11]. However, the biological activities of phytoestrogens depend on many factors, including receptor numbers, types of receptors (ER- β and ER- α), and the relative ligand-binding affinities of receptors, which may be partially estrogen agonists and antagonists [12]. These effects of isoflavones appear to be tissue-specific. Isoflavones are thought

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to exert their estrogenic effects by acting as ER agonists in non-reproductive tissues, such as bone and vascular epithelia [13]. The binding affinity of genistein, a typical soybean isoflavone, to ER- β is 20- to 30-fold higher in comparison with that of ER- α [14]. Humans can synthesize S-equol from soy isoflavone daidzein, and it is significant that, unlike R-equol, this enantiomer has a relatively high affinity for ER- β [9]. ER- β is found in tissues that are responsive to hormone replacement therapy, reducing the risk of osteoporosis and cardiovascular disease [15,16]. Therefore, intake of isoflavones has been suggested as a replacement for estrogen therapy in postmenopausal woman.

Estrogen plays an important role in growth and metabolism of bone tissue, and ovarian hormone deficiency is one of the most important risk factors for osteoporosis. Data from animal experiments provided evidence indicating that soy protein can attenuate menopausal bone loss [17,18], and it was suggested that isoflavones from soy might have protective effects on bone [19]. In humans, cross-sectional studies conducted in Asian populations have reported significant positive associations between soy protein or isoflavone intake and bone mineral density (BMD) [20-24]. In postmenopausal women, consumption of soy isoflavones has been shown to cause a significant increase in lumbar spine bone mineral content (BMC) and BMD [25].

Thus, the aim of this study was to investigate the effects of fermented soybeans by *R. oligosporus* on blood estradiol concentration as well as postmenopausal bone loss using an ovariectomized (ovx) rat model. We first examined the effects of fermented soybeans by *R. oligosporus* in young female rats as a pilot study and in ovx old rats as a model of menopause.

MATERIAL AND METHODS

Experimental animals and treatment protocols

As a pilot test to investigate the estrogenic effects of fermentation, a total of 24 seven-week-old female Sprague-Dawley (SD) rats were randomly assigned to three diets: casein, unfermented soy product, or fermented soy product by *Rhizopus oligosporus* and fed for a period of four weeks. Forty 20-week-old female rats weighing 260-290 g were obtained from Daehan Biolink (Eumsung, Korea) and housed individually in raised wire cages at $23 \pm 1^\circ\text{C}$ with a 12 h light/12 h dark cycle. After one week of acclimation, animals were divided by body weight into four blocks of 10 rats each, using a randomized complete block design. The rats were anesthetized with ketamine (100 mg/kg BW, Yuhan Co., Seoul, Korea) and subjected to sham-operation or bilateral ovariectomy at the start of the study. They were then allowed to recover in the same laboratory environment for the next seven days and provided rat chow and water *ad libitum*. After the recovery period, the rats were provided with diets containing milk casein, soybeans, or fermented soybeans by *R. oligosporus*. Weekly body weight gain and food consumption were recorded. The rats were decapitated after food deprivation for 12 h after the fifth week of the experiment. The uteri and femoral bones were removed and their blood was collected. All aspects of this study were conducted according to the standards of Kookmin University Institutional Animal Care and Use Committee (KMUIACUC-2009-004).

Table 1. Composition of experimental diets

Ingredients (g/kg)	Control, casein	Soybean	Fermented soy
Corn starch	529.486	452.486	452.486
Sucrose	100	100	100
Cellulose	50	7	7
Casein	200	0	0
Soybean	0	385	0
Fermented soybean	0	0	385
L-cystine	3	3	3
Corn oil	70	5	5
Mineral mixture	35	35	35
Vitamin mixture	10	10	10
Choline	2.5	2.5	2.5
T-butylhydroquinone	0.014	0.014	0.014
Total	1000	1000	1000

Experimental diets

Rats were fed diets based on AIN-93M containing milk casein, soybeans, or fermented soybeans by *R. oligosporus* (kindly provided by Bifido Co., Hongchun, Korea), shown in Table 1. The diets were iso-caloric (3.95 kcal/g) and equally contained 20% kcal from protein. The soybean diets were composed of 385 g of powdered soybeans/kg total ingredients, 7 g of cellulose, and 5 g of corn oil, whereas the control casein diet contained 200 g of casein, 50 g of cellulose, and 70 g of corn oil. The vitamin and mineral mixtures were purchased from Harlan Teklad (Madison, USA). Cellulose, DL-methionine, and choline chloride were purchased from Sigma (St. Louis, USA). The source of casein was Junsei Chemical (Tokyo, Japan). Cornstarch and corn oil were purchased from Daesang (Seoul, Korea).

Serum estradiol assay and tissue collection

The rats were decapitated after food deprivation for 12 h. Trunk blood was collected immediately in heparinized tubes and then centrifuged at 3,000 rpm for 20 minutes. Plasma was collected and kept at -40°C until enzyme immunoassay for determination of plasma concentrations of 17β -estradiol using an Estradiol EIA kit (Cayman Chemical Co., Ann Arbor, USA). After blood collection, uteri were harvested and weighed.

Determination of femur BMD (bone mineral density), BMC (bone mineral content), and BBS (bone-breaking strength)

After rats were sacrificed, their left femurs were excised, cleaned of adhering soft tissues, and preserved in 0.1 mol/L of phosphate-buffered 9% formalin for determination of BMD and BMC. BMD and BMC were measured using a dual energy X-ray densitometer (Model pDEXA Sabre, Norland Co., Dayton, USA) designed especially for small animals. Right femurs were dried overnight at $55 \pm 5^\circ\text{C}$ for determination of BBS using a Texture Analyzer (Model TA-HDi, MHK Trading Co., Boochun, Korea) under the following conditions: pre-speed of 2 mm/s, test-speed of 0.05 mm/s, and distance of 1.0 mm.

Statistical analysis

Data are presented as the mean and standard error of the groups. Analyses were performed using one-way analysis of

variance (ANOVA) in SPSS software (version 18.0, Chicago, Ill, USA). When ANOVA indicated statistical significance, differences among the groups were analyzed using Duncan's multiple range test. The significance level was set at $P < 0.05$.

RESULTS

Feed intakes and weight gains

Data on feed intake and weight gain of young rats are shown in Table 2. No significant differences in feed intake, weight gain, or feed efficiency ratio (FER) were observed among the groups. Therefore, feeding of casein, soybeans, or fermented soybeans as protein sources had no effect. As shown in Table 3, significantly higher weight gains and FER were observed in the ovx groups than in the sham-operated group. Ovariectomy induced weight gain by enhancing feed efficiency.

Table 2. Total feed intake, weight gain, and feed efficiency ratio (FER) in young rats¹⁾

Group	Initial body weight (g)	Weight gain (g)	Feed intake (g)	FER
Casein	181.4 ± 6.2 ^{NS}	62.2 ± 4.8 ^{NS}	578.6 ± 13.5 ^{NS}	0.107 ± 0.006 ^{NS}
Soybean	180.8 ± 5.4	47.8 ± 6.1	497.4 ± 43.1	0.100 ± 0.013
Fermented soy	180.3 ± 5.4	61.5 ± 11.0	555.1 ± 14.0	0.108 ± 0.017

¹⁾ Values are expressed as mean ± SE, n = 8.
NS; not significant.

Table 3. Total feed intake, weight gain, and feed efficiency ratio (FER) in old rats¹⁾

Group	Initial body weight (g)	Weight gain (g)	Feed intake (g)	FER
Sham-casein	262.3 ± 6.6 ^{NS}	26.5 ± 4.1 ^b	637.8 ± 19.2 ^{NS}	0.041 ± 0.006 ^b
Ovx-casein	274.3 ± 5.7	67.4 ± 11.8 ^a	680.8 ± 22.8	0.096 ± 0.014 ^a
Ovx-soybean	273.4 ± 5.1	57.5 ± 7.1 ^a	632.3 ± 15.9	0.090 ± 0.011 ^a
Ovx-fermented soy	273.7 ± 4.1	62.0 ± 12.0 ^a	666.4 ± 26.4	0.090 ± 0.017 ^a

¹⁾ Values are expressed as mean ± SE, n = 10.
Values in a column with different superscripts are significantly different ($P < 0.05$) as assessed by one-way ANOVA and Duncan's multiple range test.
NS; not significant.

Table 6. Bone mineral density in young rats¹⁾

Group	Bone mineral density (g/cm ²)	Bone mineral content (g)	Area (cm ²)	Bone breaking strength ×10 dyne
Casein	0.179 ± 0.003 ^{NS}	0.303 ± 0.008 ^{NS}	1.70 ± 0.23 ^{NS}	10.06 ± 0.16 ^{NS}
Soybean	0.179 ± 0.003	0.313 ± 0.010	1.74 ± 0.21	10.16 ± 0.21
Fermented soy	0.177 ± 0.005	0.308 ± 0.011	1.74 ± 0.28	10.16 ± 0.43

¹⁾ Values are expressed as mean ± SE, n = 8.
NS; not significant.

Table 7. Bone mineral density in old rats¹⁾

Group	Bone mineral density (g/cm ²)	Bone mineral content (g)	Area (cm ²)	Bone breaking strength ×10 dyne
Sham-casein	0.202 ± 0.003 ^a	0.395 ± 0.004 ^a	1.96 ± 0.27 ^{NS}	11.01 ± 0.35 ^{NS}
Ovx-casein	0.193 ± 0.002 ^{ab}	0.382 ± 0.009 ^{ab}	1.96 ± 0.38	11.21 ± 0.58
Ovx-soybean	0.188 ± 0.003 ^b	0.365 ± 0.005 ^b	1.94 ± 0.31	11.94 ± 0.41
Ovx-fermented soy	0.195 ± 0.003 ^{ab}	0.386 ± 0.009 ^{ab}	1.98 ± 0.26	12.13 ± 0.60

¹⁾ Values are expressed as mean ± SE, n = 10.
Values in a column with different superscripts are significantly different ($P < 0.05$) as assessed by one-way ANOVA and Duncan's multiple range test.
NS; not significant.

Plasma 17β-estradiol concentration and uterine weights

Plasma concentrations of 17β-estradiol and uterine wet weights of young and old rats are shown in Tables 4 and 5, respectively. In young rats, uterine weights did not differ significantly among the groups, whereas significantly higher 17β-estradiol concentrations were observed in groups fed fermented soybeans than in those fed either casein or soybeans. The estradiol concentration of the fermented soybean group was twice as high as those of the casein and soybean groups. However, significantly lower uterine wet weights were observed in the ovx groups than in the sham-operated group. Ovariectomy reduced 17β-estradiol levels by almost half, whereas fermented soybeans increased levels up to that of the sham-operated group, indicating that fermentation tends to stimulate estrogenic production.

BMD, BMC, and BBS

BMD, BMC, bone area, and BBS of young and old rats are

Table 4. Plasma concentrations of 17β-estradiol and uterine weight in young rats¹⁾

Group	17β-estradiol (pg/mL)	Uterus weight (mg)
Casein	503 ± 53 ^b	576 ± 39 ^{NS}
Soybean	426 ± 137 ^b	454 ± 52
Fermented soy	1130 ± 181 ^a	476 ± 42

¹⁾ Values are expressed as mean ± SE, n = 8.
Values in a column with different superscripts are significantly different ($P < 0.05$) as assessed by one-way ANOVA and Duncan's multiple range test.
NS; not significant.

Table 5. Plasma concentration of 17β-estradiol and uterine weight in old rats¹⁾

Group	17β-estradiol (pg/mL)	Uterus weight (mg)
Sham-casein	426 ± 104 ^{NS}	603 ± 64 ^a
Ovx-casein	288 ± 95	227 ± 67 ^b
Ovx-soybean	211 ± 48	143 ± 9 ^b
Ovx-fermented soy	437 ± 123	329 ± 112 ^{ab}

¹⁾ Values are expressed as mean ± SE, n = 10.
Values in a column with different superscripts are significantly different ($P < 0.05$) as assessed by one-way ANOVA and Duncan's multiple range test.
NS; not significant.

shown in Tables 6 and 7. In young rats, no significant differences in BMD, BMC, bone area, and BBS were observed among the groups. In old rats, significantly lower BMD and BMC were observed in the ovx-soybean group than in the sham-operated group, whereas those of the ovx-fermented soybean group were not significantly different. This result confirms the beneficial effects of soy fermentation on bone metabolism. In addition, while no significant difference in BBS was observed among young rats, BBS showed an increase in ovx rats fed soybeans or fermented soybeans. In particular, mean BBS of the ovx-fermented soybean group was almost 10% higher than that of the sham-operated control.

DISCUSSION

This study evaluated the preventative effects of soybeans and fermented soybeans against loss of bone mass due to estrogen deficiency using ovx rats as an experimental animal model. Feed intake was unaffected by the type of dietary protein source, which included casein, soybeans, and fermented soybeans. However, significantly higher weight gain and food efficiency ratio were observed in the ovx groups than in the sham control group, suggesting that ovariectomy induced weight gain by enhancing feed efficiency. Our results are in agreement with those of previous studies in which body weights were significantly lower in sham-operated groups than in ovx groups [26, 27].

Regarding uterine wet weights, ovariectomy caused atrophy of uterine tissue. Significantly lower mean uterine weights were observed in the ovx groups (227 mg for casein, 143 mg for soybeans, 329 mg for fermented soybeans) than in the sham control (603 mg). This result is consistent with previous observations (660 mg for sham vs 160 mg for ovx) reported by Devareddy [26] and Cho *et al.* [28]. Deurveilher *et al.* [29] reported that hormonal treatment using estradiol and progesterone increased uterine weights in ovx middle-aged rats.

Treatment with fermented soybeans resulted in increased plasma concentrations of estradiol in young and ovx old animals, whereas unfermented soybeans had no effect. In addition, ferutinin, a phytoestrogenic compound extracted from *Ferula hermonis* roots, was shown to act on the uterus as a selective estrogen receptor modulator [30]. Although soy isoflavones work positively on plasma estradiol levels and stimulate the uterus, fermented soybeans appear to work more effectively by increasing the percentage of aglycones among total isoflavones.

In this study, ovx rats fed fermented soybeans showed increased BMD and BMC, whereas the diet type had no significant effect on BMD and BMC in young rats. Thus, estrogen deficiency after ovariectomy led to reduction in bone mass, while supplementation of fermented soybeans resulted in increased estrogen production and bone formation. Devareddy *et al.* [26] previously reported that soy treatment resulted in increased tibial BMC by 10.3% and BMD by 4.5%, which were significantly higher than those of ovx controls but still lower than those of sham animals. Ferretti *et al.* [31] suggested that ferutinin as estradiol benzoate could enhance recovery of bone loss due to severe

estrogen deficiency in ovx rats. However, an isoflavone-rich diet appeared to be effective in bone protection regardless of uterine wet weight [32]. Reduced bone mass is only one of many factors jeopardizing bone integrity, resulting in reduced bone strength and increased susceptibility to fractures. Soy isoflavones have been suggested to mimic the beneficial effects of estrogen, and their fermentation is known to exacerbate their effects. In menopausal women, rapid decrease in estrogen results in severe bone loss and incidence of skeletal fractures.

The above results confirm that fermented soybeans by *Rhizopus oligosporus* may alter estrogen utilization within the sex steroid pathway and reduce bone resorption. Therefore, consumption of fermented soybeans is suggested for treatment of menopausal women with osteoporosis.

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