

Effects of combined treatment with fermented soybean (natto) intake and exercise on bone metabolism in ovariectomized rats

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Abstract

Objectives: Using ovariectomized rats, we examined the influence of combined exercise tolerance and natto intake on the bone loss inhibitory effect.

Methods: We divided female Wistar rats into the following groups: Ovariectomy, Ovariectomy + Exercise, Ovariectomy + Natto Intake, Ovariectomy + Exercise + Natto Intake, and Pseudo-operative (Sham group). After conducting experiments on each group, we collected the tissues and performed morphological and molecular biological analyses.

Results: In comparison with the Ovariectomy group, only in the Ovariectomy + Exercise group was there a significant bone loss inhibitory effect in the femoral cancellous bone. Although there was a tendency toward this trend seen in the Natto Intake and Exercise + Natto Intake groups, these differences were not significant. The increase in messenger RNA expression levels of alkaline phosphatase (osteoblast marker) in the bone marrow caused by ovariectomy was suppressed by individual factors, and by those in combination. However, messenger RNA expression levels of estrogen receptor alpha in the bone marrow showed a decreasing tendency with each factor, and decreased significantly with the combination, similar to the Sham group.

Conclusion: This suggests that natto intake and exercise maintain bone mass by different molecular mechanisms and that these two factors do not simply act synergistically in combination to maintain bone mass.

Keywords

Fermented soybean (natto), isoflavone, exercise, bone density, ovariectomized rats

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Introduction

Postmenopausal osteoporosis resulting from estrogen deficiency leads to an increased risk of fracture. This has become a serious public health problem in an aging society. In recent years, it has become clear that the traditional Japanese food known as “natto” (fermented soybean) has a bone loss inhibitory effect. In an epidemiological survey, Kaneki et al.¹ indicated that there was a statistically significant correlation between the amount of natto intake and the frequency of bone fractures. Animal experiments using feed with natto indicated that the feed inhibited femur bone loss in ovariectomized rats, which were used as a model for postmenopausal women.^{2,3} Thereafter, studies^{4–6} were conducted on the bone loss inhibitory effect of vitamin K₂ (menaquinone-7), which is one of the main ingredients in natto; in fact, vitamin K₂-fortified natto has been made available on the retail market in recent years.⁷ In addition to

the vitamin K₂, natto also includes soybean isoflavone, which has a structure similar to that of estrogen. Studies

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exploring the relationship of soybean nutritional components (such as isoflavone and zinc) to bone metabolism have also been conducted.^{8–13}

Bone mass is mediated by appropriate weight-bearing activity and exercise through bone tissue sensors and adaptations to mechanical stress. Studies^{14–16} of the relationship between exercise and bone density suggest that four to five sessions per week of moderate exercise (such as treadmill running) can inhibit bone loss in the femur and tibia of ovariectomized rats. In addition, the role of estrogen receptor 1 (ER α) in the mechanical stress transduction pathways is becoming clear in the maintenance of osteoblasts that contribute to bone formation.^{17–19} Recent experiments investigating the bone loss inhibitory effect of combined exercise and soybean isoflavone use have been conducted. These studies have reported no synergistic effects between the two²⁰ and that exercise and isoflavone maintain bone mass through different mechanisms.²¹

Natto is a simple and convenient food that is familiar to the Japanese populations. Moreover, since the health food boom, it has become available worldwide. Since natto intake and exercise have been identified to be effective in preventing osteoporosis, consuming natto can be recommended as a simple way to help prevent osteoporosis in a large population. However, previous studies reveal inconsistencies in the influence of a combination of isoflavone intake and exercise on bone metabolism. Thus, in this study, we investigated the effect of natto intake and exercise on bone density. Furthermore, to determine the function of molecules affecting bone density we examined the expression of the alkaline phosphatase (ALP; a marker of the osteoblast) and ER1 messenger RNA (mRNA) in bone marrow, using rats as a preliminary model, before investigating the effect in humans.

Materials and methods

Preprocessing and rearing environment of experimental animals

A total of 25 six-week-old female Wistar rats (Japan SLC) were purchased and bred preliminarily for 1 week. Prior studies employing 4–6 sample subjects^{2,3,9,11,16} have established that exercise and the nutrient included in natto influence bone metabolism. Therefore, we set 5 as our minimal sample size. The animal room was maintained at a temperature of 23°C \pm 1°C and humidity of 60% \pm 5%, lighting followed a 12-h light/dark cycle (turned on at 6:00, turned off at 18:00), and water and feed MF (Oriental Yeast Co., Ltd.; Table 1) were freely available. After the preliminary period, 20 of the rats underwent ovariectomy (OVX) according to the following procedure: after being anesthetized with Nembutal (pentobarbital sodium) (dose: 35 mg/kg of weight, Dainabot Co., Ltd), their abdomens were shaved, an incision was made through the skin and muscularis, and the adipose tissue, ovaries, and oviducts were exposed. After ligating the oviducts,

the ovaries were removed, and the muscularis and skin were sutured. Sham operations were performed in which the ovaries were not removed from the remaining 5 rats. The rats were then divided into five groups of five rats each: OVX, OVX+Exercise, OVX+Natto Intake, OVX+Exercise+Natto Intake, and Sham operation (Sham). After surgery, the rats were allowed to heal and recover for 2 weeks. They were then given 1 week to become accustomed to the powdered feed and treadmill exercise. The dry, powdered natto was provided by the Domestic Animal Product Research Laboratory, College of Agriculture, Ibaraki University. It was mixed into the powdered feed and fed to the rats. The content ration of dry powdered natto in the powdered feed was 5% based on the amount of menaquinone-7 (at least 9.4 μ g per 100 g of rat feed)^{2,3} that affects rat bone metabolism (Table 1).

Running exercise training and the natto intake program

The experimental period was 3 months. A rat treadmill (Natsume Seisakusho Co., Ltd) was used with rats in the exercise groups. The running exercises were moderate and designed to raise blood pressure and heart rate but not cause extreme stress. Running speed was 10 m/min for the first 5 min and 20 m/min for the following 25 min; the incline was set at 0°. Each rat ran a total of 30 min/day, 5 days/week (Monday through Saturday). During the experimental period, the rats were weighed prior to exercising, with measurement of feed intake occurring 3 days/week (Tuesday, Thursday, and Saturday).

Blood component and bone density analysis

Post-experimentation, the rats were anesthetized with Nembutal (30 mg/kg, intraperitoneal administration) and were euthanized by exsanguination. Blood, bone marrow, and femur bone samples were then taken. After the blood samples were taken, the serum was separated and refrigerated. A quantification of 17 β -estradiol (E2) was performed through radioimmunoassay (RIA, solid-phase with tube, Mitsubishi Chemical Medience). Femur bone density measurements were performed using the DHF-155H X-ray device (Hitachi Medical Corporation). The images thus obtained were analyzed using NIH imaging software according to the following method. Since the metaphyseal cortical femur bone of Wistar rats has a thickness of around 0.4–0.6 mm, we set the regions of interest (ROI) as those within 0.3 mm of the cortical bone surface and \geq 0.8 mm from the cancellous bone surface, and calculated the average pixel values. We then created an analytical curve indicating the relationship between thickness of a control sample and pixel value, and estimated bone density based on the approximation thus obtained. We used an aluminum step wedge (8 steps of 2 mm each) as the sample. Statistical analysis was one-way analysis of variance (ANOVA) with significance set at 0.05%.

Table 1. Components of the rat feed and dry powdered natto.

Analytical test item	Result	Method
Components of the rat feed		
Water	7.9 g/100 g	Dry method by heating at atmospheric pressure
Protein	23.1 g/100 g	Kjeldahl method
Fat	5.1 g/100 g	Soxhlet extraction method
Minerals	5.8 g/100 g	Direct incineration method
Carbohydrates	58.1 g/100 g	Deduction method
Calories	359 kcal/100 g	Calorie conversion factor
Components of dry powdered natto		
Water	7.1 g/100 g	Dry method by heating at atmospheric pressure
Protein	38.0 g/100 g	Kjeldahl method
Fat	20.1 g/100 g	Soxhlet extraction method
Minerals	4.8 g/100 g	Direct incineration method
Carbohydrates	30.0 g/100 g	Deduction method
Calories	453 kcal/100 g	Calorie conversion factor
Phosphorus	685 mg/100 g	ICP atomic emission spectroscopy
Calcium	189 mg/100 g	ICP atomic emission spectroscopy
Soybean isoflavone	73.5 mg/100 g	High-performance liquid chromatography
Menaquinone-7 (MK7)	3030 µg/100 g	High-performance liquid chromatography

ICP: inductively coupled plasma.

Calorie conversion factor (protein \times 3.47 + fat \times 8.37 + carbohydrates \times 4.12). Deduction method: 100 g - (water + protein + fat + minerals).

Processing was done using the SPSS v16.0 (SPSS Japan) and Excel 2007 (Microsoft). The Tukey's honest significant difference (HSD) test (significance set at 0.05%) was applied to tests of each group combination.

Genetic analysis

Total RNA (50 µl) was extracted from the femoral bone marrow of each rat using a nucleic acid extraction kit (RNeasy Mini Kit, Qiagen) and then purified. We calculated the optical density (OD) 260/280 nm ratio using a microspectrophotometer (Nanodrop, Scrum). We determined that a ratio of ≥ 1.9 indicated satisfactory purification. Next, we performed RNA reverse transcription (SYBR PrimeScript RT-PCR Kit II, Takara Bio, Inc.) using a random primer and oligo (dT) primer and synthesized complementary DNA (cDNA). We detected mRNA by real-time quantification via polymerase chain reaction (PCR) using an Mx3000P system (Agilent Technologies). The target genes were *Rattus norvegicus* ER α mRNA and *Rattus norvegicus* ALP, liver/bone/kidney ALP mRNA. We measured the cycle threshold (Ct) value of each type of mRNA via the comparative Ct method (delta-delta cycle threshold) using beta-2-microglobulin (B2M) mRNA as the endogenous control. The PCR conditions were one cycle at 95°C for 5 s and 50 cycles at 60°C for 20 s, using the reaction system for a total of 25 µL. ER α mRNA and ALP1 mRNA from all samples were calibrated for B2M mRNA using the control. All data are expressed as relative values with the Sham group being 1. To determine significant differences between the groups, ANOVA with 0.05% significance was used;

Tukey's HSD test (0.05% significance) was used on the tests of all group combinations.

Ethical considerations

This study was performed with the approval of the Animal Experiment Committee of Ibaraki Prefectural University of Health Sciences (Ethics Approval Number 023) and in accordance with its policies for animal experimentation.

Results

Intake and weight variation

The daily average intake of animals in all groups during the entire experiment period ranged from 12.3 \pm 2.1 g/day, for which there were no significant differences between the groups. There were likewise no significant between-group differences in terms of weight variance throughout the experimental period. The weight average of animals in all groups was 204.8 \pm 10.4 g at the final experimental period.

Effect of OVX

Our analysis of blood E2 concentrations indicated that the all OVX groups (OVX, OVX+Exercise, OVX+Natto, OVX+Exercise+Natto) were below the 10 pg/mL detection limit. However, the average value in the Sham group was 18.3 \pm 3.6 pg/mL, significantly higher than that of the OVX groups. Our measurements of uterine weight (Figure 1) indicated that the OVX groups (average: 96.1 \pm 25.8 mg) had

significantly lower values than the Sham group (average: 618.1 ± 110.4 mg) ($p < 0.001$). This, considered together with the blood E2 concentrations, indicates the effect of OVX.

Influence on femoral bone density

Our analysis of the density of femoral cancellous and cortical bones is shown in Figure 2. Cancellous bone was significantly less dense only in the OVX group (0.97 ± 0.06 AI equivalent) in comparison to the Sham group (1.13 ± 0.06 AI equivalent) ($p < 0.01$), but there were no significant differences between the Sham group and the other four groups. There was also a significant difference between the OVX+Exercise group

(1.12 ± 0.05 AI equivalent) and the OVX group (0.97 ± 0.06 AI equivalent) ($p < 0.05$), indicating that exercise inhibited the loss of bone density due to OVX. However, there were no significant differences between the groups for cortical bone.

Expression levels of ALP mRNA in bone marrow

The OVX group (4.26 ± 3.07 relative values with the Sham group being 1) had significantly higher ALP mRNA expression levels than the Sham group ($p < 0.05$), but there were no significant differences between the other groups (Figure 3).

Expression levels of ER α mRNA in bone marrow

In comparison to the Sham group, the OVX (6.99 ± 4.53 relative values with the Sham group being 1), OVX+Exercise (5.56 ± 3.23), and the OVX+Natto groups (4.17 ± 2.11) had significantly higher ESR1 mRNA expression levels ($p < 0.05$), but the OVX+Exercise+Natto group (1.41 ± 0.71) did not show a significant difference (Figure 4).

Discussion

Similar to those of previous studies,^{14,15} the results of the experiments conducted in this study indicate that moderate exercise inhibits the femoral cancellous bone density loss caused by OVX in rats. However, while the groups that combined natto intake and exercise did show a tendency toward inhibition of bone loss, the differences were not significant. Previous studies^{2,3} have indicated that the intake of natto inhibits loss of calcium content as well as the dry weight of the femur. This, then, indicates the possibility that the combination of natto with exercise somehow weakens the inhibitory effect. An additional possibility is that, in this

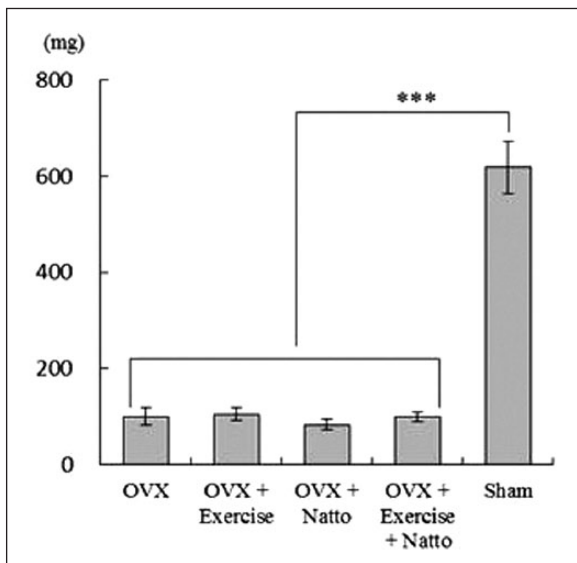


Figure 1. Change in uterus weight due to ovariectomy (OVX).

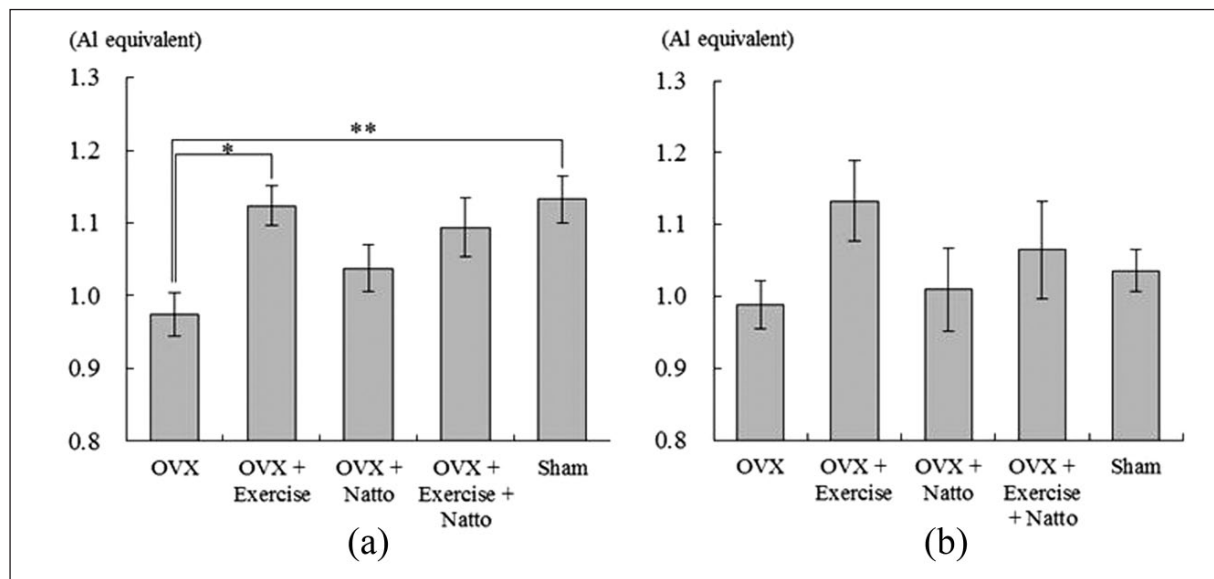


Figure 2. Comparison of femoral bone density.

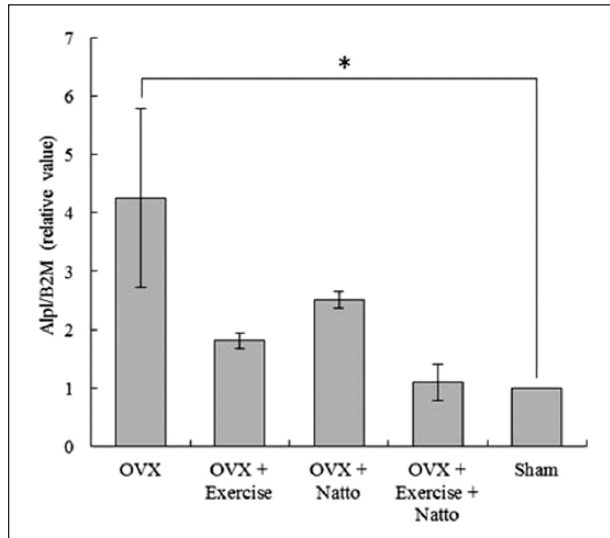


Figure 3. Alpl mRNA expression levels in bone marrow.

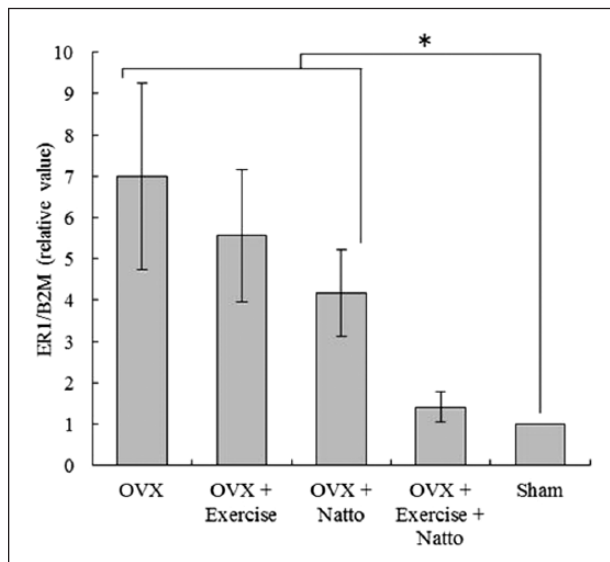


Figure 4. ERI mRNA expression levels in bone marrow.

study, the inhibition could not be detected on the radiology analysis. Moreover, it is also necessary to improve accuracy by analyzing bone through use of micro computed tomography (CT).

In bones, ALP is present in the cell membrane of osteoblasts and is one of the indicative markers of osteogenesis activity. The maintenance of bone mass is achieved through a balance between the osteogenesis action of osteoblasts and the bone resorption action of osteoclasts.²² After menopause, bone resorption occurs faster than osteogenesis, which causes loss of bone mass. The increase in the expression levels of ALP mRNA in OVX rats is thought to be due to a compensatory effect in response to increased bone resorption metabolic turnover.²³ The results of this study indicate the same levels

of ALP mRNA expression in groups with two factors, and two factors combined as the Sham group, which suggests that the resultant bone resorption metabolic turnover was inhibited to the same degree in the Sham group. Since there have been previous reports of blood, ALP activation increases that were inhibited in soybean isoflavone-administered OVX rats,²⁰ and osteogenesis improvement was noted in the mice that received the vitamin K₂ (200 mg/kg diet) for 12 weeks,²⁴ we assume that the same mechanism was at work when natto was administered to rats in this study. Our results are also in accordance with previous findings¹⁴ indicating that serum ALP activity was at the same level in OVX rats that engaged in regular running exercise training as in those of the Sham group. In addition, the results of this study indicate that when natto intake and exercise are combined, the same degree of inhibition of ALP mRNA expression is observed as when the two are undertaken separately. This suggests that under the conditions of this study, there is no synergistic or damping effect on ALP mRNA expression when the two factors are combined and that bone metabolic turnover is maintained at the same level as when the two factors are undertaken separately.

However, our analysis of the expression level of ER α mRNA in bone marrow after OVX indicate that there were lower expression levels in the groups that combined natto intake and exercise than in the groups that underwent only one of the two. Increased ER α mRNA expression after OVX is due to a compensatory effect in reaction to the decrease in blood E2 concentration. The intake of daidzein, a type of soybean isoflavone and E2 analog, inhibits the increase in ER α mRNA expression levels in OVX rats.²⁵ Based on this, we can assume that in this study, the intake of natto had the same inhibitory effect. In addition, this study elucidated the fact that exercise inhibits increases in ER α mRNA and that it, therefore, had significantly more effect when combined with natto intake. The mechanism by which exercise (i.e. mechanical stress) inhibits excessive ER α mRNA expression due to OVX is not currently known; as such, this issue requires further study in the future. ER α is known to be involved in osteoblasts and osteoclasts as a nuclear receptor in bone marrow and has been shown to be related to a large number of inhibitory functions, including the division and proliferation of cells in reaction to *in vivo* E2, and the induction of apoptosis.^{17,26,27} Furthermore, in recent years, ER α expression in osteoblasts has been shown to be related to cell responses to mechanical stress; it has also been elucidated that it is responsible for bone mass maintenance and partially for cell reinforcement¹⁷ and inhibition of apoptosis.¹⁸ The findings in this study revealed that ER α mRNA expression recovered to the same level as that of the Sham group through a combination of natto intake and exercise. This indicates that the condition for ER α to function normally is met. With a more detailed analysis of bone structure, in the future, it will be necessary to examine the activity of osteoblasts and the osteoclast.

Soybean isoflavone is bone-specific; it has no side effects on the female reproductive organs.²⁸ Moreover, since exercise is also essential for maintenance of bone mass, the combination of the two can be used as a bone mass maintenance program that does not require drugs. In addition to being a bone mass maintenance program that easily fits into daily life, it also has the merit of circumventing adverse reactions to osteoporosis drugs and contributing to the reduction of medical costs. From these standpoints, promoting the study of these mechanisms is extremely significant in our increasingly aging society. However, from an academic viewpoint, there are still a great many unresolved issues related to bone mass maintenance, including the response of osteoblasts and osteoclasts to isoflavone and other estrogen-like components, and the crosstalk in the mechanical stress transfer mechanism. The elucidation of these issues will be important subjects of future study.

Conclusion

This study highlights the effects of a combination of natto (the traditional Japanese fermented soybean food) intake and exercise on bone loss inhibition using ovariectomized rats (postmenopausal osteoporosis model). Our results revealed that natto intake and exercise maintain bone mass via different molecular mechanisms and that these two factors do not simply act synergistically in combination to maintain bone mass. The results of this study contribute toward elucidation of bone mass maintenance, including the response of osteoblasts and osteoclasts in the bone marrow to natto ingredients (vitamin K₂ and isoflavone, estrogen-like components), and the crosstalk in the mechanism of mechanical stress transfer. Further studies are recommended for a detailed analysis of bone metabolism related to bone mass maintenance, including an evaluation of the activity of osteoblasts and osteoclasts.

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Animal welfare: All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the US National Institutes of Health.

Declaration of conflicting interests

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Ethical approval

Not applicable. But, the present study was performed with the approval of the Animal Experiment Committee of Ibaraki Prefectural University of Health Sciences and in accordance with its policies for animal experimentation.

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References

1. Kaneki M, Hodges SJ, Hosoi T, et al. Japanese fermented soybean food as the major determinant of the large geographic difference in circulating levels of vitamin K₂: possible implications for hip-fracture risk. *Nutrition* 2001; 17: 315–321.
2. Yamaguchi M, Kakuda H, Gao YH, et al. Prolonged intake of fermented soybean (natto) diets containing vitamin K₂ (menaquinone-7) prevents bone loss in ovariectomized rats. *J Bone Miner Metab* 2000; 182: 71–76.
3. Yamaguchi M, Taguchi H, Gao YH, et al. Effect of vitamin K₂ (menaquinone-7) in fermented soybean (natto) on bone loss in ovariectomized rats. *J Bone Miner Metab* 1999; 17: 23–29.
4. Tsukamoto Y, Ichise H and Yamaguchi M. Prolonged intake of dietary fermented soybeans (natto) with the reinforced vitamin K₂ (menaquinone-7) enhances circulating. GAMMA-carboxylated osteocalcin concentration in normal individuals. *J Health Sci* 2000; 46: 317–321.
5. Tsukamoto Y, Kasai M and Kakuda H. Construction of a *Bacillus subtilis* (natto) with high productivity of vitamin K₂ (menaquinone-7) by analog resistance. *Biosci Biotechnol Biochem* 2001; 65: 2007–2015.
6. Knapen MHJ, Drummen NE, Smit E, et al. Three-year low-dose menaquinone-7 supplementation helps decrease bone loss in healthy postmenopausal women. *Osteoporos Int* 2013; 24: 2499–2507.
7. Takemura H. Prevention of osteoporosis by foods and dietary supplements. “Kinnotsubu honegenki”: a fermented soybean (natto) with reinforced vitamin K₂ (menaquinone-7). *Clin Calcium* 2006; 16: 1715–1722.
8. Devareddy L, Khalil DA, Smith BJ, et al. Soy moderately improves microstructural properties without affecting bone mass in an ovariectomized rat model of osteoporosis. *Bone* 2006; 38: 686–693.
9. Ishimi Y, Miyaura C, Ohmura M, et al. Selective effects of genistein, a soybean isoflavone, on B-lymphopoiesis and bone loss caused by estrogen deficiency. *Endocrinology* 1999; 140: 1893–1900.
10. Okabe Y and Tanimoto H. Effects of dietary intake of isoflavone aglycone-rich fermented soybeans on bone metabolism in ovariectomized rats. *J Health Sci* 2008; 54: 315–323.
11. Uesugi T, Toda T, Tsuji K, et al. Comparative study on reduction of bone loss and lipid metabolism abnormality in ovariectomized rats by soy isoflavones, daidzin, genistin, and glycitin. *Biol Pharm Bull* 2001; 24: 368–372.
12. Yamaguchi M, Igarashi A, Sakai M, et al. Prolonged intake of dietary fermented isoflavone-rich soybean reinforced with zinc affects circulating bone biochemical markers in aged individuals. *J Health Sci* 2005; 51: 191–196.
13. Yoo HW, Chang MJ and Kim SH. Fermented soybeans by *Rhizopus oligosporus* reduce femoral bone loss in ovariectomized rats. *Nutr Res Pract* 2014; 8: 539–543.
14. Barengolts EI, Curry DJ, Bapna MS, et al. Effects of endurance exercise on bone mass and mechanical properties in intact and ovariectomized rats. *J Bone Miner Res* 1993; 88: 937–942.

15. Barengolts EI, Lathon PV, Curry DJ, et al. Effects of endurance exercise on bone histomorphometric parameters in intact and ovariectomized rats. *Bone Miner* 1994; 26: 133–140.
16. Shide N, Sakakura Y, Tsuruga E, et al. Influence of treadmill running exercise on tibia and gastrocnemius muscle in ovariectomized mature rats. *Nihon Undo Seirigaku Zasshi* 2001; 8: 85–92.
17. Lee K, Jessop H, Suswillo R, et al. Endocrinology: bone adaptation requires oestrogen receptor-alpha. *Nature* 2003; 424: 389.
18. Aguirre JI, Plotkin LI, Gortazar AR, et al. A novel ligand-independent function of the estrogen receptor is essential for osteocyte and osteoblast mechanotransduction. *J Biol Chem* 2007; 282: 25501–25508.
19. Armstrong VJ, Muzylak M, Sunter A, et al. Wnt/beta-catenin signaling is a component of osteoblastic bone cell early responses to load-bearing and requires estrogen receptor alpha. *J Biol Chem* 2007; 282: 20715–20727.
20. Figard H, Mouglin F, Gaume V, et al. Combined intervention of dietary soybean proteins and swim training: effects on bone metabolism in ovariectomized rats. *J Bone Miner Metab* 2006; 24: 206–212.
21. Liu K, Ma G, Lv G, et al. Effects of soybean isoflavone dosage and exercise on the serum markers of bone metabolism in ovariectomized rats. *Asia Pac J Clin Nutr* 2007; 16(Suppl. 1): 193–195.
22. Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *J Clin Invest* 2005; 115: 3318–3325.
23. Davey RA, Hahn CN, May BK, et al. Osteoblast gene expression in rat long bones: effects of ovariectomy and dihydrotestosterone on mRNA levels. *Calcif Tissue Int* 2000; 67: 75–79.
24. Kim M, Na W and Sohn C. Vitamin K1 (phylloquinone) and K2 (menaquinone-4) supplementation improves bone formation in a high-fat diet-induced obese mice. *J Clin Biochem Nutr* 2013; 53: 108–113.
25. Ebihara H and Koshihara Y. Study of daidzein on bone metabolism: individual variability in aging rats. *Soy Protein Res* 2003; 6: 116–121.
26. Krum SA, Miranda-Carboni GA, Hauschka PV, et al. Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. *EMBO J* 2008; 27: 535–545.
27. Nakamura T, Imai Y, Matsumoto T, et al. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. *Cell* 2007; 130: 811–823.
28. Miksicek RJ. Estrogenic flavonoids: structural requirements for biological activity. *Proc Soc Exp Biol Med* 1995; 208: 44–50.