Estrogen deficiency is associated with increased bone turnover and acceleration of bone loss, which lead to an increased susceptibility to bone fracture. A number of studies have previously reported that soybean isoflavones, genistein and daidzein, dose-dependently inhibited bone loss in both female and male osteoporotic animal models without causing notable effects on the reproductive organs [16]. Ishimi et al. [17] also determined the selective effects of genistein on B-lymphopoiesis and bone loss caused by estrogen deficiency in ovariectomized (OVX) mice. In this study, genistein (0.7 mg/day s.c.) inhibited bone loss due to OVX without any uterine hypertrophy, which has been thought to be a side effect of estrogen administration. The dose which induced uterine hypertrophy was 10 times higher than that which inhibited bone loss. This suggests that genistein may be a natural selective estrogen receptor modulator (SERM). Interestingly, isoflavones inhibited bone loss in androgen-deficient male mice, indicating that soybean isoflavones prevent bone loss due to androgen deficiency in males.

Similarly, equol could also inhibit bone loss due to OVX. Fujioka et al. [18] reported that administration of equol (0.5 mg/day s.c.) inhibited bone loss of the whole body and femur in OVX mice. Although E_2 administration (0.03 μg/day s.c.) prevented OVX-induced bone loss from all regions, uterine hypertrophy occurred in E_2-administered OVX mice [18]. These results suggest that similar to SERMs, isoflavones including equol inhibit bone loss apparently without estrogenic activity in the reproductive organs in estrogen-deficient animals.

It is now recognized that one of the mechanisms by which estrogen deficiency causes bone loss is the stimulation of osteoclast formation, a process enhanced by several inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β. Furthermore, Nakamura et al. [19] recently reported a critical role for the osteoclastic estrogen receptor-α (ERα) in mediating estrogen-dependent bone maintenance in female mice. They selectively ablated ERα in differentiated osteoclasts [ERα(DeltaOc/DeltaOc)] and found that ERα(DeltaOc/DeltaOc) females, but not males, exhibited trabecular bone loss, similar to the osteoporotic bone phenotype in postmenopausal women. Furthermore, estrogen induced apoptosis and upregulation of Fas ligand expression in osteoclasts of the trabecular bones of wild type but not ERα(DeltaOc/DeltaOc) mice. The expression of ERα was also required for the induction of apoptosis by tamoxifen and estrogen in cultured osteoclasts. These results support a model in which estrogen regulates the life span of mature osteoclasts via the induction of the Fas/Fas ligand system, thereby providing an explanation for the osteoprotective function of estrogen as well as SERMs including isoflavones. On the other hand, isoflavones have been reported to inhibit bone resorption via other nonhormonal effects, including antioxidant activity, inhibition of tyrosine kinase and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). In fact, statins, cholesterol-lowering agents that inhibit the activity of HMG-CoA reductase, induce bone formation and inhibit bone resorption both in vitro and in vivo.

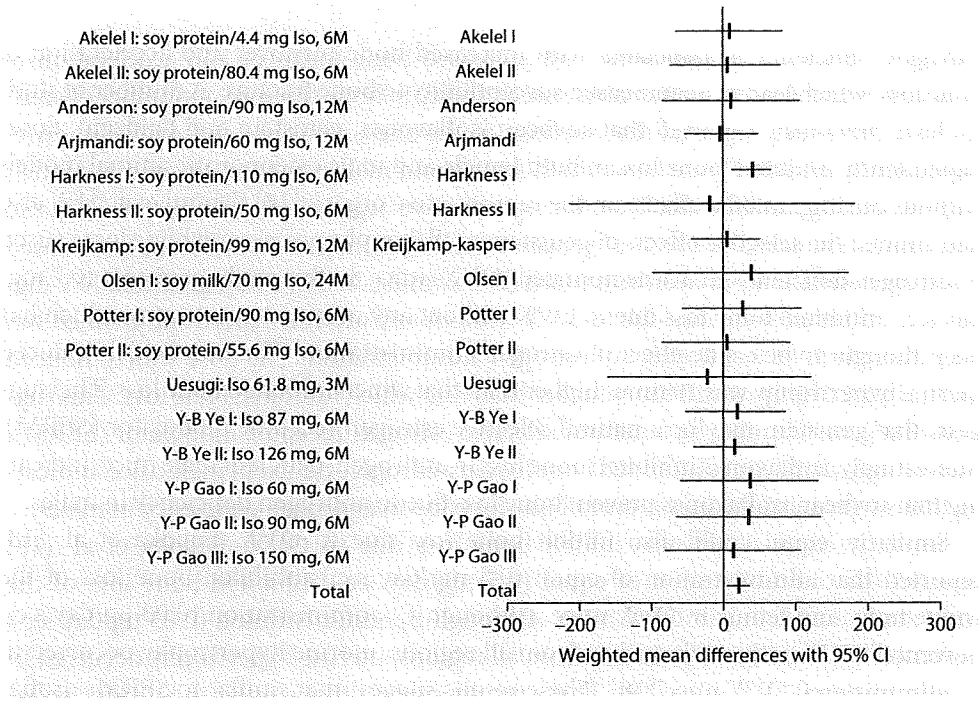

Fig. 2. Meta-analysis of 10 RCTs of isolated isoflavone intervention for BMD of lumbar spine in postmenopausal women [9].

Effects of Isoflavone on Bone Metabolism: Observational and Intervention Studies

Observational studies indicate that women who have high soy intake have less risk for osteoporosis than women who consume a typical Western diet [8, 16]. Recently, increasing numbers of RCTs have tested the effects of soy isoflavones on bone mineral density (BMD) in postmenopausal women. So far, one meta-analysis of RCTs has evaluated the effect on spine BMD of soy isoflavones (fig. 2). This included 10 trials testing both extracted soy isoflavones and isolated soy protein containing isoflavones, and revealed a significantly beneficial effect of soy isoflavone intake on BMD in lumbar spine at more than 90 mg/day [9]. However, the results from human studies are still controversial. Recently, Brink et al. [10] reported that consumption of isoflavone-enriched products (110 mg/day of isoflavone aglycone equivalents) for 1 year did not affect BMD and bone turnover in apparently healthy early postmenopausal white women. Potential reasons for these inconsistencies could include different races, the timing of exposure to isoflavone, duration of intervention, and difference in diet and isoflavone intake from foods between the isoflavone and placebo groups.

Alternatively, interindividual differences in isoflavone metabolism could be a contributing factor. Recent studies suggest that the clinical effectiveness of isoflavones on bone metabolism might be due to their ability to produce the metabolite equol in the intestine [11].

Combined Effects of Isoflavones and Exercise on Bone Metabolism in Estrogen-Deficient Status

It has been shown that running exercise partially prevented bone loss induced by estrogen deficiency. Frost [20] showed that estrogen deficiency increased the 'set point' for the skeleton to respond to loading, causing the skeleton to be less sensitive to mechanical force and decreasing its bone mass. In this regard, it is likely that phytoestrogens can influence the set point of the mechanical loading that affects bone mass. Wu et al. [21] assessed the combined effects of isoflavones and exercise on BMD in postmenopausal Japanese women as well as in OVX mice. The combined intervention of moderate exercise and the submaximal dose of genistein administration showed a cooperative effect in preventing bone loss in OVX mice. Furthermore, they recruited 136 subjects (average age was 55 years), who were postmenopausal within 5 years of natural menopause, and randomly assigned them to four groups: placebo; walking combined with placebo (3 times/week, 6 km/h); isoflavone intake (75 mg conjugates/day; equivalent to 47 mg of aglycone; Fujicco Co. Ltd., Kobe, Japan) in addition to the normal diet, and isoflavone combined with walking exercise [21]. After 1-year intervention, 108 subjects completed the study. BMD of the lumber spine, left hip and subwhole body was assessed by DXA using Hologic QDR-4500 (Hologic Inc., Waltham, Mass., USA) at baseline and after 1 year.

Average daily intake of isoflavone aglycone from soy foods was around 28 mg per day in the subjects in 4 groups at baseline and after 1 year. There were no significant differences in daily intake of isoflavones and other nutrients among the groups at baseline, and between baseline and after 1 year in each group. Of the percent change in BMD, walking showed significant main effects on the preservation of BMD in the total hip region after 1 year. Interventions with isoflavones or walking showed significant main effects on the preservation of BMD at Ward's triangle in the hip after 1 year. Combined intervention of isoflavone intake and walking exercise for 1 year showed a trend for a greater effect on BMD at total hip and Ward's triangle regions than either intervention alone [21].

Since the effect of isoflavone alone was modest compared with those in the Westerners [21], the subjects in the placebo and isoflavone intervention groups were stratified based on their fecal equol production, and serum equol concentrations were measured in order to investigate whether any difference exists in the effects of isoflavone on BMD between equol producers and nonproducers in postmenopausal Japanese women [22].

Possible Role of Equol Status in the Effects of Isoflavones on Bone Health

Equol is one of the main metabolites of the isoflavone daidzein. Equol production depends on the individual's intestinal flora, and research has shown that approximately 30–50 % of individuals in the population studied are capable of producing equol from daidzein [11]. Setchell et al. [11] reported that compared with the control group, equol producers showed a 2.4% increase in their lumbar spine BMD, whereas no significant change in this BMD was observed in the nonproducers after a 2-year isoflavone intervention.

Wu et al. [22] assessed the effects of equol-producing activity on BMD in postmenopausal Japanese women. Fifty-four women (29 in the placebo, 25 in the isoflavone group) completed the 1-year intervention, and their data were used for equol analysis.

The percentage of equol producers in our subjects was 55% by assessment of equol production in fecal suspension incubated with daidzein under anaerobic condition. The number of equol producers and nonproducers was 15 and 14 in the placebo group, and 15 and 10 in the isoflavone group, respectively. Serum daidzein, which was determined by reverse-phase high-performance liquid chromatography, dramatically increased in the isoflavone group after 1 year. However, there was no difference in serum daidzein between equol producers and nonproducers. Conversely, serum equol was increased only in equol producers in the isoflavone group after 1 year. Serum equol did not change in equol producers in the placebo group. The percent change in bone loss at total hip and intertrochanter of the hip of equol producers was significantly lower than that of equol nonproducers in the isoflavone group, as assessed by Student's t test (fig. 3). However, none of the differences between producers and nonproducers was observed in the placebo group. From these results, the effects of isoflavone on bone mass might depend on the equol-producing activity in postmenopausal Japanese women [22].

Isolation of Equol-Producing Bacteria from Human Feces

Intestinal bacteria play a key role in isoflavone metabolism; young infants with undeveloped gut microflora do not produce equol, while germ-free animals also do not produce equol or O-DMA. A number of strains involved in daidzein metabolism were identified. However, the identification of equol-producing bacteria is complicated. Several candidate bacteria responsible for daidzein metabolism have been suggested; for example, a *Clostridium* sp. and *Eubacterium ramulus* metabolized daidzein to O-DMA in vitro, and equol was found in soymilk fermented with some strains of *Bifidobacterium*. Uchiyama et al. [15] and Ishimi et al. [23] were the first to find, mainly four, equol-producing bacteria in human feces; they concluded that the *Lactococcus* 20-92 strain (*Lc.* 20-92) as homologous to *L. garvieae* is the most appropriate bacteria for food usage because we have dietary habit of *L. garvieae*.

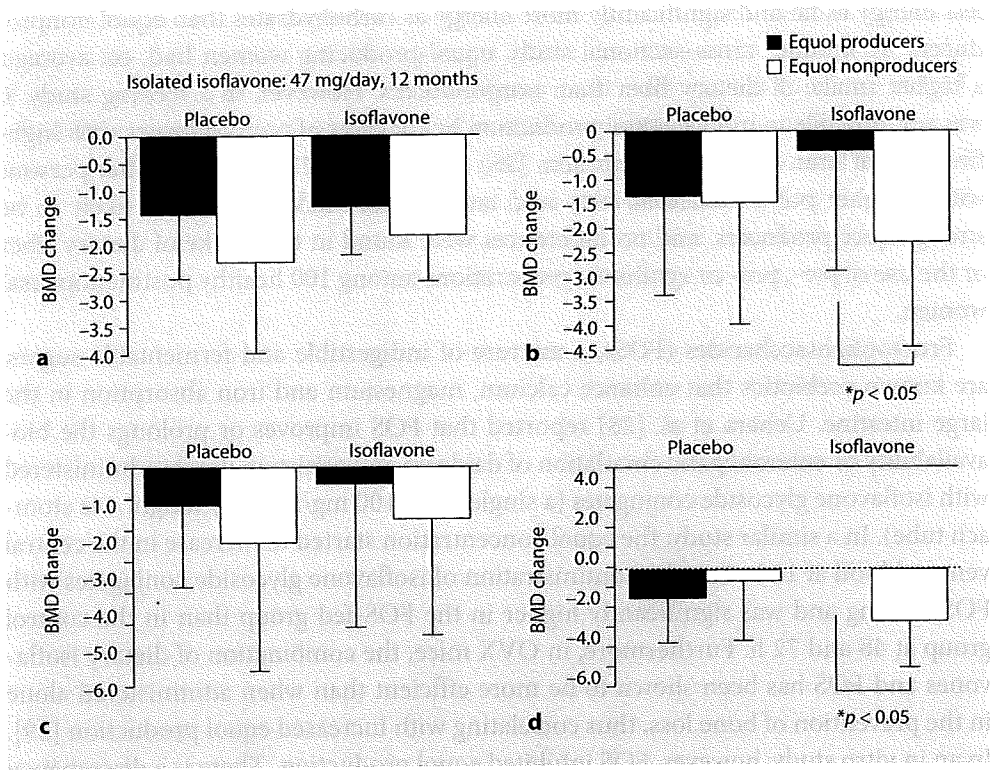

Fig. 3. Percent change in BMD of the whole body and hip analyzed by equol status in postmenopausal Japanese women [22]. **a** Whole body. **b** Total hip. **c** Femoral neck. **d** Intertrochanter. Statistical differences as assessed by Student's t test.

Ishimi et al. [23] detected *Lc.* 20-92 in the feces of 133 of postmenopausal Japanese women using real-time PCR using the particular primer for *L. garvieae.*

The bacteria were detected in 47 of 133 samples (35.3%) [23]. Interestingly, the people with *L. garvieae* were not always equol producers, suggesting that some other bacteria and several factors such as hydrogen gas and short-chain fatty acids, which can affect the environmental conditions in the colon, might be also important for equol production in humans.

Food Factors Affecting Equol Production

It has been suggested that food factors contribute to the ability to produce equol; however, contradictory results were obtained from association studies. For example, Adlercreutz et al. [24] reported a positive association between urinary equol concentration and intake of fat and meat in a Japanese population, whereas in a Western population, Rowland et al. [25] reported that equol producers consumed significantly

less energy as fat and significantly more energy as carbohydrates than equol nonproducers. In another cross-sectional study, equol-producing women had, on average, a higher intake of dietary fiber than nonproducers. However, in a feeding study, it was not possible to induce equol production by the diets of nonproducers with high-fiber wheat bran cereal or soy protein [26]. Bolca et al. [27] suggested that persons with a higher polyunsaturated fatty acid and alcohol intake were more likely to be strong equol producers, and no differences were found in the intake of dietary fiber or the use of pre-, pro- or symbiotic preparations among 100 healthy postmenopausal women.

Fructooligosaccharides (FOS), a mixture of indigestible and fermentable sugars, are known prebiotics that enhance calcium, magnesium and iron absorption in the large intestine. Uehara et al. [28] reported that FOS improves or prolongs the bioavailability or enterohepatic circulation of daidzein and genistein in rats administered with isoflavone glycoside conjugates (a single dose, 100 mg/kg body weight, via stomach tube). In a similar study, the equol concentration started to increase in the central venous blood at 12 h after the administration of isoflavone glycoside conjugates with FOS feeding and was significantly higher in the FOS-fed group than in the control group at 48 and 72 h. Furthermore, in OVX mice, the combination of dietary isoflavones and FOS has been shown to be more efficient than when administered alone in the prevention of bone loss, thus correlating with increased equol production [29]. In an in vitro study, however, FOS inhibited equol production. There is a discrepancy between the results of the in vivo and in vitro studies. In French postmenopausal women, FOS did not increase urinary equol production. However, racial differences might exist with regard to isoflavone metabolism. Therefore, further human studies involving Asian subjects are required. Several factors such as animal species, race, sex, age, and genetic background, including individual variation in intestinal microflora and diet, should be considered with regard to isoflavone metabolism and metabolite production.

Safety Evaluation of Isoflavones in Japan

Soybean isoflavone was approved in 2001 as the principle ingredient in Food for Specified Health Uses (FOSHU) by the Japanese Ministry of Health, Labour and Welfare and is aimed at individuals concerned about bone health. There are tea, soymilk and soft drinks containing 40 mg of isoflavone conjugates, which is equivalent to 25 mg of aglycone form. In 2004, applications of a tablet containing soy isoflavone aglycones as its principal ingredient, and a fermented food containing isoflavone aglycone in amounts exceeding the usual content in FOSHU were filed for approval. Foods with fortified or condensed isoflavones had not been consumed before. And there is a possibility that tablets and capsules would be excessively consumed. Accordingly, the Japanese Food Safety Commission of the Cabinet conducted

Table 1. Effects of 1 year isoflavone intake on serum sex and thyroid hormone concentrations in postmenopausal Japanese Women

		Placebo	Isoflavone	Placebo vs Iso
		(n = 29)	(n = 25)	
Estradiol (pg/mL)	Baseline	12.66 (4.03)	12.32 (3.34)	NS
	After 1 year	12.98 (7.31)	11.98 (2.94)	NS
	% change	6.09 (57.71)	1.20 (23.80)	NS
FSH (U/L)	Baseline	70.36 (26.02)	68.19 (18.66)	NS
	After 1 year	60.00 (19.83)*	58.12 (17.86)*	NS
	% change	−12.36 (8.40)	−14.05 (9.01)	NS
LH (U/L)	Baseline	26.68 (13.87)	27.70 (9.32)	NS
	After 1 year	22.43 (11.12)*	22.33 (7.90)*	NS
	% change	−12.16 (15.98)	−19.10 (14.44)	NS
Progesterone (ng/mL)	Baseline	0.27 (0.11)	0.29 (0.16)	NS
	After 1 year	0.21 (0.10)*	0.24 (0.12)*	NS
	% change	−24.54 (29.47)	−14.07 (18.08)	NS
T3 (ng/ml)	Baseline	1.13 (0.16)	1.08 (0.13)	NS
	After 1 year	1.10 (0.16)	1.03 (0.16)	NS
	% change	−1.60 (60.11)	−3.92 (7.96)	NS
T4 (μg/dL)	Baseline	8.57 (1.12)	8.19 (1.28)	NS
	After 1 year	8.46 (1.13)	7.54 (1.42)*	NS
	% change	−1.85 (7.46)	−4.48 (6.23)	NS
TSH (mU/L)	Baseline	2.34 (1.10)	2.30 (0.74)	NS
	After 1 year	2.75 (2.81)	2.25 (1.10)	NS
	% change	12.71 (69.59)	−9.22 (32.74)	NS

* Significantly different from baseline by paired *t*-test, $p < 0.05$

a safety evaluation on soy isoflavones, and issued a Notice: *'Basic approaches to evaluating the safety of FOSHU containing soy isoflavones'* in 2006. The main contents of the report are summarized in 3 points. Firstly, the upper limit of isoflavone aglycone intake from FOSHU was set at 30 mg/day for additional consumption with a normal diet. This limit represented half of the 57.3 mg soy isoflavones (aglycone equivalent) in the soymilk that was given daily to the premenopausal Japanese women in whom estrogen level tended to decrease and menstrual cycles tended to be longer over 2–3 cycles. Secondly, the maximum recommended level for safe isoflavone aglycone intake in a daily diet is 70–75 mg/day at the present time. This limit was selected on the basis of two findings. One was the National Nutrition Survey in 2002 in Japan that showed

the 95th percentile intake of soy isoflavones at 64–76 mg/day. The other was the result of an experiment in which postmenopausal Italian women taking a 150-mg soy isoflavone aglycone tablet daily showed no effects after 3 years, but showed a significant increase in the occurrence of endometrial hyperplasia after 5 years as compared with a control group. One half of that amount was thus selected as the maximum recommended level, allowing for individual and experimental variations. Thirdly, it was not recommended that pregnant women, infants and children take soy isoflavone from FOSHU [30]. These criteria were also adapted to the so-called Health Foods fortified with isoflavones.

In order to examine the effects of isoflavone intake on hormone levels in postmenopausal women, Ishimi et al. [23] evaluated serum concentrations of estrogen, FSH, LH, progesterone and thyroid hormones in their participants. There were no significant differences in estrogenic hormone levels between the placebo and isoflavone treatment groups (table 1). The same was observed with thyroid hormone levels. These results suggest that additional isoflavone intake with a normal diet (total intake was about 75 mg of aglycone equivalent a day) for a year did not affect serum hormone levels in postmenopausal Japanese women [23]. Marini et al. [31] recently reported that 54 mg/day of genistein supplementation for 3 years exhibited a promising safety profile in breast and endometrium with positive effects on bone formation in a cohort of osteopenic postmenopausal women.

It is well known that consuming soy foods has many benefits. For example, they are a good source of protein, calcium; soy protein decreases serum cholesterol, and isoflavones maintain bone health in postmenopausal women. Therefore, the Japanese Ministry of Health, Labour and Welfare set the goal at 100 g/day of beans intake in the Health Japan 21 Program in 2000. Soybeans have been consumed since ancient times in Asia, and there has been no report of any problems even in cases of excessive consumption. Soy isoflavone intake from soy foods in a normal daily diet is therefore considered safe.

Conclusions

Firstly, several factors such as race, age, diet, time of exposure, and individual variations in genetics and intestinal microflora affect the effects of isoflavones on bone health. Secondly, preventive effects of daidzein on bone loss in postmenopausal women might depend on the capacity of an individual to produce equol. Thirdly, 47–54 mg/day of isoflavone supplementation with a normal diet for 1–3 years has not shown any adverse effects at least in postmenopausal women. Further studies are required to address the numerous questions regarding the potential benefits, mechanisms of action and safety of isoflavones.

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