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NOTE

Evidence of Estrogenic Effect by the Three-Month-Intervention of Isoflavone on Vaginal Maturation and Bone Metabolism in Early Postmenopausal Women

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Abstract. Objective of the present study is to determine the estrogenic effect of isoflavone on vaginal epithelia and bone metabolism in early postmenopausal women. Twenty-two postmenopausal women were randomly assigned to either a group that was given isoflavone extract (61.8 mg) for three months or a control group that was given placebo. We measured the L2-4 bone mineral density (BMD) before and 3 months after treatment by dual X-ray absorptiometry (DXA). Blood and urine samples were obtained from the women before and 3 months after treatment. We measured FSH using radioimmunoassay and, urinary pyridinoline and deoxypyridinoline levels by HPLC. For endocrine cytology, vaginal smears were collected before and 3 months after the treatment. Three months after the treatment, the serum FSH levels and the BMD values did not significantly differ between the two groups. Urinary excretion of isoflavone was significantly higher in the group given isoflavone compared with that given placebo ($p<0.03$). Numbers of parabasal and intermediate types of cells were significantly decreased ($58.2 \pm 12.4\%$ to $25.0 \pm 10.7\%$; $p<0.05$) and increased ($24.1 \pm 8.7\%$ to $63.7 \pm 10.7\%$; $p<0.05$), respectively in the isoflavone group, but remained unchanged in the control group. Urinary pyridinoline excretion was significantly decreased (49.6% vs. before, $p<0.01$ by paired t-test) in the isoflavone group. The intake of 60 mg of isoflavone daily for 3 months produced maturational changes of vaginal epithelia without affecting serum FSH levels, and could possibly slow down bone turnover rates as judged by decreased urinary pyridinoline excretion.

Key words: Postmenopausal women, Isoflavone, Vaginal differentiation, Bone metabolism

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AN estrogen deficiency causes several climacteric symptoms, vaginal epithelial atrophy and bone loss. Hormone replacement therapy (HRT) is believed to be a superior strategy with which to decrease the frequency of these symptoms [1, 2]. However, the reported compliance with HRT is as low as 30%, because of atypical breakthrough bleeding and an increased incidence of breast cancer [3].

Soy has estrogenic activity even when it is ingested as foodstuffs, such as Tofu, Miso, or Natto. The major source of estrogenic activity in soy is isoflavones. Among soy isoflavones, daidzein and genistein and their glycoside compounds of daidzin and genistin possess estrogenic [4] and anticarcinogenic activities [5, 6], respectively. A study using ovariectomized rats has shown that daidzin and genistin reduce bone loss and bone strength reduction [7], or decrease elevated serum triglyceride levels [8] after an estrogen deficiency.

Natural healing or naturopathy is a currently accepted means of improving the quality of life for climacteric or menopausal women. However, such strategies are still classified as an alternative to HRT. Healthy climacteric women need to know through empirical testing whether a natural estrogen mimic could be an effective replacement treatment [9]. Isoflavone is one of the best and widespread potential

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HRT alternatives [10], because soy contains very high isoflavone concentration [11], and soy flour supplementation reduces hot flushes and menopausal symptom scores [12] and positively affects vaginal epithelial maturation in postmenopausal women [13].

The aim of this study is to investigate the estrogenic effect of three months isoflavone administration on vaginal epithelia and bone metabolism.

Materials and Methods

Isoflavone source

Isoflavone samples were prepared by extracting soybean hypocotyls (100 kg) in boiling water (1000 L) for 30 min.

The extract was poured into synthetic resins (SP-850, Mitsubishi Chemical Industry Ltd., 70 L) for absorption by batch processing and then washed three times with 140 L of water. After adding 70 L of 60% ethanol, the resins were stirred thoroughly for 30 min to elute isoflavones.

The elution process was repeated three times and the entire eluate was concentrated and dried to obtain crude isoflavone extract. The contents of isoflavones are listed in Table 1 (30.9 mg/capsule).

Subjects

Twenty-two postmenopausal women visited JT Chen Clinic, Tokyo, Japan. The criteria for inclusion in the study were: 1) age range between 45 and 65 years, and between five to ten years after natural menopause; 2) body weight between 35 and 70 kg; 3) no evidence of disease associated with osteoporosis or other major medical problems; 4) no history of using drugs, including estrogen, known to affect bone metabolism and 5) submission of written, informed consent to participate in the study.

The women were divided equally and randomly into groups A or B and given isoflavone extract (containing 30.9 mg of isoflavones; Table 1) or placebo (containing only dextrin) twice each day for 3 months, respectively. Compliance was assessed in monthly interviews.

The subjects were given neither specific instructions regarding dietary phytoestrogen or calcium intake, nor a program of exercise. The soy and initial calcium intake from food consumed during an average day was estimated from a marking sheet questionnaire (Meiji Nutrition Center Tokyo, Japan). The estimated isoflavone and calcium intake was around 50 and 550 mg per day respectively, both in treated and control groups.

Table 1. Content of isoflavone extract

Isoflavone	Content (mg/capsule)
Daidzin	15.6
Genistin	3.4
Glycitin	10.6
6"-O-malonyldaidzin	0.3
6"-O-malonylgenistin	n.d.
6"-O-malonylglycitin	n.d.
6"-O-acetyldaidzin	0.5
6"-O-acetylgenistin	n.d.
6"-O-acetylglycitn	0.3
Daidzein	n.d.
Genistein	n.d.
Glycitein	0.2
Total isoflavones	30.9

n.d.:no detected

Bone analysis

L2-4 bone mineral density (BMD) was measured before and 3 months after the treatment, using dual X-ray absorptiometry (DXA: DPX-L, Lunar Inc., WI, USA) with an assay precision of ± 1.0%.

Biochemical analysis

Venous blood samples were collected from the subjects following an overnight fast. Serum samples were immediately centrifuged at 4°C and stored under –40°C within 1 h of centrifugation. Serum GOT, GPT, γ-GTP, LDH, UA, BUN, TP, albumin, triglyceride, total cholesterol, HDL-cholesterol, alkaline phosphatase, calcium, phosphorus, and creatinine (Cr) were measured using an autoanalyzer (Hitachi 736, Tokyo, Japan). Serum calcium levels were corrected for albumin levels. Serum follicular stimulation hormone (FSH) was measured by radioimmunoassay at Mitsubishi-BCL Laboratory (Tokyo, Japan).

Urinary isoflavone and pyridinoline excretion analysis

We assessed isoflavone and pyridinoline excretion in 24-hour urine specimens were collected before and

3 months after treatment. After measuring the volume, each sample (10 ml × 2 tubes) was stored at –30°C until analysis.

Urinary creatinine levels were determined by the Jaffe method, using commercial kits (Creatinine-test Wako, lot No. Dr370, Wako Pure Chemical Industry Ltd., Osaka, Japan). Then, the creatinine coefficient value (mg/day/body weight) was calculated, and the value within a range of 10.8–25.2 was considered to be appropriate for further analysis.

Urinary isoflavone levels were measured according to Yamori *et al*. [14]. Briefly, each urine sample (1 ml) was digested with sulfatase (EC 3.1.6.1 Type H-1, lot No. 66H33731, Sigma Chemical Co. Ltd.) and β-glucuronidase (EC 3.2.1.31, lot No. ESR7445, Wako Pure Chemical Industry Ltd.). Isoflavone aglycons were analyzed by HPLC.

The urinary excretion of pyridinoline and deoxypyridinoline was measured by HPLC according to the methods of Takahashi *et al*. [15].

The values of isoflavone and pyridinoline were corrected for urinary excretion of Cr.

Vaginal cytology

For endocrine cytology, pap smears from the lateral vaginal wall were collected before and 3 months after treatment. Cytology was assessed at the Department of Cytology, Cancer Institute Hospital, Tokyo. We determined the mean ratio of parabasal, intermediate and superficial type cells in five fields at 100 × magnification in every specimen.

Statistical analyses

StatView-J version 4. 11 (Abacus Concepts, Inc., Berkeley, CA) performed all statistical calculations.

The significance of differences between the mean values of two groups was estimated by Student's t-test and the significance of changes was estimated by a paired t-test; p values below 0.05 were considered to indicate statistically significant differences.

Results

Age, postmenopausal duration, height, weight or BMI, L2-4 BMD values between the two groups did not significantly differ (Table 2). All of the isoflavone group and 10 of 11 in the control group completed the study.

Changes in urinary excretion of isoflavone

Urinary daidzein and genistein excretion did not significantly differ between the two groups before treatment. However, 3 months after treatment, the isoflavone group excreted significantly more isoflavones than the control group ($p<0.03$) (Table 3).

Changes in vaginal cytology

The ratio (mean ± SE) of parabasal type cells after ingesting isoflavone was significantly decreased compared with before (25.0 ± 10.7% vs 58.2 ± 12.4%; $p<0.05$ by the paired t-test), and that of intermediate

Table 2. Baseline characteristics of isoflavone and placebo groups

	Isoflavone	Placebo	Total
Number of participants	11	10	21
Age	54.9 ± 7.5	52.5 ± 6.8	53.7 ± 6.9
Postmenopausal duration	6.3 ± 5.3	5.7 ± 6.6	6.0 ± 6.0
Height (cm)	152.6 ± 7.0	157.5 ± 5.0	155.1 ± 6.4
Weight (kg)	50.8 ± 5.2	53.2 ± 3.6	52.0 ± 4.6
BMI	22.3 ± 2.7	22.8 ± 2.8	22.6 ± 2.8
BMD (L2–4)	1.00 ± 0.27	1.01 ± 0.19	1.01 ± 0.23

a)Values are means ± S.D.

Table 3. Urinary excretion of isoflavone metabolic products

	Isoflavone		Placebo		p-Value
	Baseline	3 Months	Baseline	3 Months	
Daidzein (mg/24H)	13.29 ± 5.69	50.78 ± 9.33*	4.74 ± 1.08	7.90 ± 2.63	0.006
Genistein (mg/24H)	6.09 ± 2.48	17.69 ± 7.40	1.50 ± 0.36	3.32 ± 1.14	0.214

a) Values are expressed means ± S.D. b) Data were compared using two-way ANOVA with repeated measures. (p-Value)
c) Values with superscript are significantly different from baseline. (*$p<0.01$ Analysed by paired t-test)

type cells significantly increased (24.1 ± 8.7% to 63.7 ± 10.7%; p<0.05 by the paired t-test). However, the ratios of these cells did not significantly change in controls (Table 4).

Changes in biochemical parameters

Serum GOT, GPT, g-GTP, LDH, UA, BUN, TP, albumin, triglyceride, total cholesterol, HDL-cholesterol, alkaline phosphatase, calcium, phosphorus levels within or between the isoflavone and placebo group did not significantly change (Table 5 and 6).

The FSH levels also did not significantly change within or between the isoflavone and placebo groups.

Changes in urinary pyridinoline excretion

We found a significant difference in urinary pyridinoline excretion between the isoflavone and placebo groups (p = 0.01 by repeated ANOVA), and 3 months after the isoflavone treatment the level significantly decreased (49.6% vs before, p<0.01 by the paired t-test) (Table 6).

Discussion

This study clearly demonstrated that 60 mg of isoflavone ingested per day for 3 months could significantly improve the maturation of vaginal wall cells without affecting serum FSH levels. Isoflavone might

Table 4. Effect of isoflavones on vaginal pap smears

	Isoflavone		Placebo		p-Value
	Initial	3 Months	Initial	3 Months	
PBT (%)	58.2 ± 12.4	24.1 ± 8.7*	58.5 ± 15.5	56.9 ± 13.8	0.026
IMT (%)	25.0 ± 10.7	63.7 ± 10.7*	22.4 ± 10.3	30.8 ± 10.6	0.006
ST (%)	16.8 ± 9.6	12.2 ± 8.7	19.1 ± 11.4	12.3 ± 7.1	0.031

a) PBT, parabasal type cells; IMT, intermediate type cells; ST, superficial type cells. b) Values are expressed means ± S.D. c) Data compared using two-way ANOVA with repeated measures. (p-Value) Values with superscript are significantly different from baseline. (*p<0.01 Analysed by paired t-test)

Table 5. Changes in serum metabolic parameters after isoflavone ingestion

	Isoflavone		Placebo		p-Value
	Baseline	3 Months	Baseline	3 Months	
Tryglyceride (mg/dL)	126.5 ± 17.4	112.3 ± 16.0	117.7 ± 15.4	135.2 ± 9.9	0.22
Total cholesterol (mg/dL)	223.7 ± 14.7	218.7 ± 11.5	221.1 ± 6.3	219.9 ± 7.0	0.69
HDL-cholesterol (mg/dL)	65.2 ± 4.0	64.0 ± 4.1	70.6 ± 6.1	67.1 ± 5.5	0.28
FSH (IU/L)	56.5 ± 13.2	55.8 ± 10.8	73.5 ± 12.4	60.9 ± 13.3	0.89

a) Values are expressed means ± S.D. b) Data compared using two-way ANOVA with repeated measures (p-Value).

Table 6. Changes in bone metabolic parameters after isoflavone ingestion

	Isoflavone		Placebo		p-Value
	Baseline	3 Months	Baseline	3 Months	
Serum Ca (mg/dL)	9.6 ± 0.7	9.4 ± 0.3	9.3 ± 0.3	9.1 ± 0.3	0.75
Serum P (mg/dL)	3.6 ± 0.7	3.7 ± 0.7	3.5 ± 0.3	3.5 ± 0.3	0.26
Alp (IU/L)	150.3 ± 42.8	151.1 ± 61.4	177.4 ± 43.0	172.6 ± 46.8	0.64
Urinary Pyr/Cre(umol/mmol)	13.7 ± 5.0	6.8 ± 2.7*	12.9 ± 3.0	12.5 ± 4.4	0.01
BMD	1.04 ± 0.10	1.03 ± 0.10	1.04 ± 0.19	1.05 ± 0.22	0.39

Values are expressed as means ± S.D. b) Data compared using two-way ANOVA with repeated measures(p-Value). c) Values with superscript are significantly different from baseline. (*p<0.01 Analysed by paired t-test)

also slow the bone turnover rate as indicated by the decreased urinary pyridinoline excretion.

An animal study indicates that isoflavone has estrogenic properties [4], but its effect in postmenopausal women seems controversial. Murkies *et al*. [12] observed no maturation of vaginal cytology, nor serum FSH changes after the administration of 45 g of soy flour per day for 12 weeks. Conversely, Dalais *et al*. [16] identified a significant increase in the numbers of superficial cells caused by the same dose of a soy diet (52.6 mg/day isoflavones). Baird *et al*. [17] increased the daily dose to 177 g of soy protein (165 mg/day isoflavones) for 4 weeks and observed an increase in the number of superficial cells in vaginal cytology without serum FSH changes. Wilcox *et al*. [13] observed a significant increase in the number of superficial cells and a decrease in serum FSH levels after consuming 45 g of soy flour daily for 2 weeks, 10 g of red clover sprouts for 2 weeks and 25 g of linseed for 2 weeks. The difference in the estrogenic effect is likely due to the dose and the duration of the isoflavone intake.

The estrogen threshold hypothesis [18] is now widely accepted. Following this concept, the minimal blood estrogen level required to control bone turnover is as low as 30 pg/ml, and for vaginal maturation much less. To reach at least this level, between 0.15 to 0.3 mg/day of conjugated estrogen was required, which resulted in significant increases in sex hormone binding globulin or subsequent decreases in FSH [19]. Assuming that the estrogenic activity of phytoestrogens is about 0.1% of that of conjugated estrogen [17] and that the isoflavone intake from food among Japanese is approximately 50 mg/day [20], the daily 60 mg intake of isoflavone in this study should have been approximately equivalent on a molar basis to 0.3 mg/day of conjugated estrogen.

We examined whether a daily intake of 60 mg of isoflavone induces vaginal maturation with or without a decrease in the FSH level. As the beneficial effect of soy on menopausal symptomatology seems to require 12 weeks [21], we assessed FSH levels after 12 weeks of isoflavone intervention. We found that the FSH levels were not decreased, which seemed to be consistent with the result of Baird *et al*. [17].

Vaginal cytological findings suggest that soybean isoflavone stimulates vaginal epithelial maturation from the basal, to the intermediate type. This change was not consistent with the effects of conjugated estrogen that stimulates vaginal epithelial maturation from the basal to superficial type, indicating that the estrogenic effect of soybean isoflavone is very weak. This would explain the minimal changes in the FSH level.

A daily 60 mg intake of isoflavone seemed to exert a small estrogenic effect without affecting the hypothalamic FSH secretion profile, suggesting that isoflavone is expected to be a natural substitute for hormone replacement to relieve a chronic estrogen-deficient state in elderly women.

One of the largest concerns over weak hormone replacement in clinical practice is that endometrial proliferation tends to become simulated. Atypical breakthrough bleeding is a symptom of endometrial proliferation and often arises within two months of estrogen replacement, but no bleeding was observed in this study. The estrogenic activity of isoflavone is thought to be weaker than that of estriol, and Takahashi *et al*. reported that a daily oral dose of 2 mg of estriol reduces climacteric symptoms without affecting the endometrium [22]. These observations indicated that isoflavone does not stimulate endometrial proliferation.

Since we observed maturation in the vaginal cytology, isoflavone might reduce vaginal symptoms such as inflammation or dryness, atypical discharge or coital pain. Our study subjects were at a stage between 5 and 10 years after menopause and they did not complain of vaginal symptoms. Therefore, we could not assess the weak effect of isoflavones on maturation of the vaginal epithelium. Severe climacteric symptoms such as hot flushes, sweating, and palpitation often develop in the 2 to 3 years around menopause. Since our subjects did not complain of these symptoms, we could not substantiate the positive effect of isoflavone reported by others [12, 13, 17].

The excretion of urinary pyridinoline, a bone metabolic marker, was significantly decreased by 60 mg per day of isoflavone. This may due to the short observation period to assess the bone metabolism. The intake of 600 mg per day for 12 months of the isoflavone derivative, ipriflavone, increases bone density with a significant decrease in urinary pyridinoline excretion [23]. Conjugated estrogen at 0.3 mg/day, which we assumed to be approximately equivalent to 60 mg of isoflavone, significantly increases lumbar bone mineral density and decreases bone resorptive metabolic markers at least 6 months later [24]. As isoflavone has only weak estrogenic properties, a large

prospective study and at least one year of observation is needed to assess the effect of isoflavone on bone metabolism.

Menopause is related to an increased risk of coronary heart disease [25]. Postmenopausal women who take estrogens usually have lower rates of cardiovascular disease than women of a similar age who do not [26]. Walsh *et al*. reported that the oral conjugated estrogens at a dose of 0.625 mg/day favorably alter LDL and HDL levels in postmenopausal women [27]. However, since this dose is much higher than that required to influence bone metabolism [18], we could not determine the effect of isoflavone on serum lipoprotein.

Conclusions

Daily supplementation with soybean isoflavone (61.8 mg) for 4 weeks significantly decreased the ratio of parabasal type cells and increased that of intermediate type cells, and reduced bone resorption.

Daily isoflavone supplementation at this dose for 3 months did not affect liver function and serum FSH levels, indicating that this estrogen mimic can reduce the risk of osteoporosis and retard changes in vaginal differentiation without deleterious metabolic effects.

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