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Cooperative effects of isoflavones and exercise on bone and lipid metabolism in postmenopausal Japanese women: a randomized placebo-controlled trial

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Abstract

Cooperative effects of isoflavones and exercise on bone and lipid metabolism have been exhibited in estrogen-deficient animals; however, results from clinical trials have not been published. In this study, we determined the effects of isoflavone intake and walking and their interaction on bone and lipid metabolism in postmenopausal women over 24 weeks. The bioavailability and metabolism of isoflavones (daidzein in particular) were also examined to clarify the mechanism of their bone-protective effects in humans. One hundred twenty-eight subjects were randomly assigned to 4 groups: placebo; placebo combined with walking (3 times per week); isoflavone intake (75 mg of isoflavones conjugates per day); and isoflavone combined with walking. The subjects were classified by equol status (producers or nonproducers) as identified using production of equol from daidzein in fecal culture. Bone mineral density (BMD), body composition, and serum concentrations of isoflavones were assessed. Serum high-density lipoprotein cholesterol concentration significantly increased (6.1%, $P = .03$), and fat mass in the whole body significantly decreased (−4.3%, $P = .0003$) from the baseline in the combined intervention group. There were no significant differences in BMD between baseline and postintervention in any of the treatment groups. However, the percent changes in BMD in equol producers were −0.53% and +0.13% in the sub–whole body and total hip, respectively. This was significantly different compared with −1.35 and −1.77 for the sub–whole body and total hip, respectively, in nonproducers in the isoflavone group ($P = .049$ and .040, respectively). The mean serum equol concentration was significantly higher in equol producers than in nonproducers in the isoflavone groups, but not in the placebo group. The combination of isoflavones and exercise exhibited favorable effects on serum lipid and body composition of postmenopausal women. The findings of this study suggest that the preventive effects of isoflavones on bone loss depend on the individual's intestinal flora for equol production.

1. Introduction

Menopause is often associated with the incidence of several chronic diseases including osteoporosis, cardiovascular disease, and obesity [1-4]. Hormone replacement therapy (HRT) is the effective regimen to prevent these diseases in postmenopausal women [5,6]; however, it is accompanied by an increased risk of unfavorable outcomes [7].

Recently, phytoestrogens have received a great deal of attention for their potential role in preventive osteoporosis and hypercholesterolemia because they are not as likely as steroid hormones to cause undesirable side effects in estrogen-deficient animals and postmenopausal women [8-11]. The predominant phytoestrogens found in plants are soybean isoflavones, including genistin, daidzin, and glycitin, which have structures similar to that of estrogen [12]. We previously reported that genistein dose-dependently inhibited bone loss in both female and male osteoporotic animal models without any adverse effects [13-15]. However, conflicting results have been reported

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in several observational clinical studies, even among Asians who consume 10 to 100 times more isoflavones than Westerners [16]. Setchell et al [17] have recently suggested that equol, a specific intestinal bacterial metabolite of the isoflavone daidzein, is the single most important factor influencing the clinical efficacy of soy isoflavones in preventing bone loss, and individual variation in production capability may explain the mixed results in many studies.

On the other hand, it is well established that exercise is also effective in preventing bone loss and hypercholesterolemia resulting from estrogen deficiency in both animal and human studies [18-20]. Although high-intensity exercise can be expected to increase bone mass in pre/postmenopausal women, it is also often associated with stress fractures, especially in fragile skeletons. Walking is a relatively safe and common exercise among elderly people. However, it has a relatively low impact on bones and is, therefore, insufficient for the prevention of bone loss in postmenopausal women [21]. Thus, in clinical research, it has been shown that a combination of estrogen with exercise is more effective in increasing trabecular bone mineral density (BMD) in older women as compared with either treatment alone [22]. In this context, we have recently demonstrated that, in the prevention of bone loss and fat gain in estrogen-deficient animals, a combined intervention of moderate-intensity exercise and isoflavone administration was more advantageous than either treatment alone [23-25]. To assess this issue in humans, we examined the cooperative effects of soy isoflavone intake and walking on bone and lipid metabolism in postmenopausal Japanese women. Furthermore, we stratified the subjects by equol status, which is dependent on the individual's intestinal flora, to determine the actual effects of soy isoflavone on bone loss in early postmenopausal women.

The following questions were addressed in the present study:

1. Are there any cooperative effects of isoflavones and walking on bone and lipid metabolism and the body composition of humans?
2. Is there a positive association between soy isoflavone intake and the concentrations of serum isoflavones, including daidzein and equol, based on equol status?
3. Is there any difference in the effect of soy isoflavone intervention on the change in BMD between equol producers and nonproducers among postmenopausal Japanese women?

2. Materials and methods

2.1. Subjects

Subjects were recruited for this study through advertisements in local newspapers, and those who met the following criteria were enrolled in the study. Healthy postmenopausal women aged 45 to 60 years who were within 5 years of natural menopause defined as at least 12 months since last menstrual cycle were enrolled for the study. The subjects had not previously used hormone therapy, lipid-lowering medications, antibiotics, or any other medication known to affect the skeleton. They provided written informed consent to participate in the study. The protocol was approved by the institutional review board of the National Institute of Health and Nutrition of Japan, and the study was carried out according to the guidelines of the Declaration of Helsinki.

One hundred forty-five potentially eligible women were invited to the screening examination. The criteria for the invitation were as follows: willingness to participate;

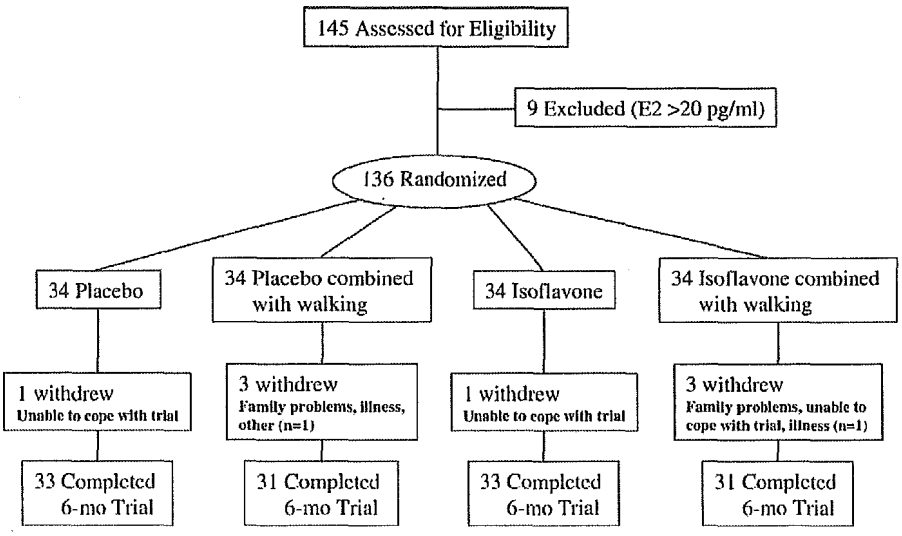

Fig. 1. Flow chart describing the progress of the participants during the trial.

clinically healthy (no cardiovascular, musculoskeletal, respiratory, or other chronic diseases that might limit walking exercise); sedentary (no regular sports activities for at least 2 years), nondieting, nonsmoking, and having no apparent occupational or leisure time responsibilities that might impede their participation. Nine participants were excluded at the medical screening because of their serum estradiol (E_2) concentrations (>20 pg/mL). Thus, 136 women were randomly assigned to 4 groups: (1) placebo; (2) placebo combined with walking; (3) isoflavone; and (4) isoflavone combined with walking. Eight women withdrew from the study because of illness, family problems, and feeling the intervention was a burden. The 128 subjects completed 6-month intervention and their data were included in the analysis (Fig. 1).

2.2. Intervention

Placebo or isoflavone capsules were blindly allocated to researchers and subjects throughout the study. Participants in the 2 groups, isoflavone and isoflavone combined with walking, received 2 capsules containing a total of 75 mg of isoflavone conjugates (47 mg as aglycone form, Fujiflavone P40, Fujicco, Kobe, Japan) with dextrin, daily in the morning. The 75 mg of isoflavone conjugate contained daidzin (38.3 mg), malonyldaidzin (0.2 mg), acetyldaidzin (2.1 mg), daidzein (0.6 mg), genistin (8.6 mg), acetylgenistin (0.6 mg), genistein (0.2 mg), and glycitin (23.4 mg) with glycitein (1.0 mg). The remaining subjects were assigned to receive 2 placebo capsules containing only dextrin, daily in the morning.

Participants who were randomized into the walking groups were expected to attend three 1-hour long exercise classes each week. The exercise program consisted of a 10-minute warm-up period, a 45-minute supervised walking exercise session, and a 5-minute cooldown period. Participants were carefully instructed on the proper manner of walking to eliminate possible injury. The participants were instructed to maintain the speed of walking at 5 to 6 km/h, and this was monitored with a pedometer.

Nonwalking group participants did not engage in sports training and were asked to continue their customary activity levels. All participants were instructed to record their daily physical activity level that was continuously monitored by the pedometer, and their diaries were obtained and checked for completeness once a month.

2.3. Questionnaire interview

Individual information was collected by trained interviewers in face-to-face interviews based on a structured and previously validated questionnaire, and included sociodemographic data; years since menopause; physical activities, including hours spent sitting, standing, walking, sports, and leisure activities; medications; smoking and alcohol drinking; and other factors that may have possible confounding effects on the relation between dietary isoflavone consumption and metabolism of bone and lipid. The dietary assessment of intakes of soy isoflavones, calcium, vitamin D, total energy, and protein was based on 3-day diet records obtained at baseline and at 6 months.

2.4. Blood and urine samples

Fasting (>12 hours) blood samples were collected before BMD measurement by venipuncture in EDTA-containing tubes, refrigerated immediately, and within 2 hours centrifuged at 1500 rpm for 30 minutes at 4°C. Serum samples from each participant were stored frozen at −20°C. Serum concentrations of total cholesterol and triacylglycerol (TG) were determined using commercial kits (Cholesterol C-Test and Triglyceride G-Test, Wako Pure Chemical, Osaka, Japan). Serum high-density lipoprotein cholesterol (HDL-C) in the serum was measured by an enzymatic method (HDL-Cholesterol Test, Wako Pure Chemical). Estradiol was assessed by radioimmunoassay (Amersham Biosciences, Piscataway, NJ). A serum bone-specific alkaline phosphatase (BALP) (Alkphase-B; Metra Biosystems Ink, Mountain View, CA) was measured by using a microplate coated with an anti-BALP monoclonal antibody. Serum intact osteocalcin was measured using sandwich enzyme immunoassay that uses polyclonal antibodies against 20 N-terminal residues (amino acids 1-20) and against 7 C-terminal residues (amino acids 43-49) (Biomedical Technology, Stoughton, MA). Urine samples were collected from a second voiding at the same time as serum extraction and they were stored at −20°C. Urinary deoxypyridinoline (DPD) was measured using sandwich enzyme immunoassay (PYRILINKS-D Assay, Metra Biosystems Ink).

2.5. Measurement of serum isoflavones

Serum concentrations of isoflavones were determined in each subject's sample by reversed-phase high-performance liquid chromatography (HPLC). Duplicate samples of serum were incubated with sulfatase (EC 3.1.6.1; Sigma Chemical, St Louis, MO) and β-glucuronidase (EC 3.2.1.31; Waco Pure Chemical Industries, Osaka, Japan) at 37°C for 2 hours to release the aglycones of the isoflavones; this was followed by purification of reactants using a Sep-Pak C18 cartridge (Waters, Milford, MA). Isoflavones were separated at 35°C by reversed-phase HPLC on a 4.6 × 250 mm Capcell Pak C18 column (Shiseido, Tokyo, Japan) using a Tosoh CCP & 8020 system with a diode array detector PD8020 (Tosoh, Tokyo, Japan). Elution was performed at a flow rate of 1 mL/min with a linear gradient of acetonitrile solution (10%-35%) containing a constant 0.1% acetic acid. Data were simultaneously acquired at 254 nm (daidzein, genistein, glycitein) and 280 nm (equol).

2.6. Identification of equol production in feces

Human feces collected from 122 subjects at baseline were stored at −80°C until use. Frozen fecal samples were thawed at room temperature, and 1 g was diluted in 9 mL Dulbecco phosphate-buffered saline (−) buffer (Nissui, Tokyo Japan)

and suspended. Fecal suspensions (0.5 mL) were incubated with 4.5-mL brain heart infusion medium (Difco Laboratories, Tokyo, Japan) containing 10 μg/mL daidzein at 37°C for 96 hours in anaerobic grove box (Hirasawa, Tokyo, Japan) of $CO_2/H_2/N_2$ (10:10:80, vol/vol). The fecal cultures (0.5 mL) were harvested and extracted with ethyl acetate, and then the ethyl acetate (isoflavone fraction) was evaporated. The residues were redissolved in 1 mL of HPLC solvent. Isoflavones were separated at 40°C by reversed-phase HPLC on a 4.5 × 250 mm Capcell Pak C18 column (Shiseido). The mobile phase consisted of 17% methanol and 3% ethyl acetate in 0.05% phosphate (A) and 2% ethyl acetate in methanol (B) with a linear gradient of 0% to 40% B. The flow rate was 1 mL/min, and data were acquired at 280 nm. Daidzein and equol were obtained from Extra Synthese, Genay, France. Equol producer status was determined by production of equol from daidzein in fecal culture after 96 hours' incubation. The average conversion rate from daidzein to equol in equol producers and non-producers was 87.4% and 0%, respectively.

2.7. Bone mineral density and body composition

Bone mineral density, including the lumbar spine (L2-L4), left hip, and sub–whole body (excluding head region), and body composition were assessed by dual energy x-ray absorptiometry at baseline and after 6 months with the use of Hologic QDR-4500A scanner (Hologic, Waltham, MA). The same staff conducted the scans and analysis. The short-term within-subject in vivo precision error in our laboratory for BMD was 0.5% for the spine, 1.5% for the total hip, and 0.8% for the whole body. Long-term precision was 0.35% by daily testing the spine phantom over the previous 1 year.

2.8. Statistical analysis

All values are expressed as means and SDs. Differences in baseline characteristics between the different groups were tested by 1-factor analysis of covariance (ANCOVA). Paired t test with Bonferroni adjustment was performed to determine whether change over the course of intervention

Table 1
Characteristics of subjects by study groups at baseline and at 6 months of intervention

	Placebo (n = 33)	Walking (n = 31)	Isoflavone (n = 33)	Isoflavone + walking (n = 31)
Age (y)	54.9 (2.9)	55.2 (2.8)	53.8 (2.9)	54.4 (2.9)
Years since menopause	3.7 (2.1)	3.6 (1.8)	2.7 (1.4)	3.2 (1.4)
Height (cm)				
Baseline	156.7 (6.3)	155.3 (6.3)	155.8 (4.3)	154.8 (5.5)
After 6 mo	156.4 (6.1)	155.1 (6.3)	155.6 (4.3)	154.6 (5.3)
Weight (kg/m^2)				
Baseline	51.4 (7.1)	54.1 (7.3)	51.5 (5.4)	52.9 (5.3)
After 6 mo	51.0 (7.3)	53.1 (7.3)	50.9 (5.7)	52.1 (5.5)
BMI (kg/m^2)				
Baseline	20.9 (2.2)	22.4 (2.9)	21.3 (2.5)	22.1 (2.0)
After 6 mo	20.8 (2.3)	22.1 (2.9)	21.1 (2.6)	21.8 (2.0)
Daily intake				
Isoflavone (mg)[a]				
Baseline	48.1 (30.6)	47.7 (25.0)	44.4 (26.9)	49.4 (25.0)
After 6 mo	45.6 (26.8)	44.1 (24.2)	40.8 (25.5)	36.9 (27.1)
Calcium (mg)				
Baseline	671.5 (190.9)	723.8 (221.5)	695.8 (253.5)	691.5 (213.3)
After 6 mo	625.4 (190.0)	693.0 (220.3)	621.7 (219.1)	722.9 (220.0)
Vitamin D (μg)				
Baseline	9.2 (5.9)	12.3 (13.1)	9.8 (5.9)	9.2 (5.0)
After 6 mo	7.0 (3.9)	6.3 (3.4)	6.8 (3.5)	7.2 (4.1)
Vitamin K (μg)				
Baseline	429.8 (172.2)	463.6 (207.0)	376.3 (211.0)	438.3 (181.9)
After 6 mo	389.5 (145.5)	471.5 (226.6)	383.0 (235.6)	434.6 (206.2)
Protein (g)				
Baseline	75.0 (13.5)	79.5 (17.4)	72.4 (14.3)	73.5 (13.5)
After 6 mo	73.3 (16.2)	69.4 (13.3)	72.5 (18.2)	74.0 (10.9)
Total energy (kJ)				
Baseline	8287.7 (1370.3)	8337.9 (1368.2)	8045.9 (1510.4)	8350.8 (1421.3)
After 6 mo	7951.7 (1743.9)	8044.2 (1440.0)	8049.2 (1638.9)	8155.0 (1271.5)
No. of walking ($\times 10^4$)				
During 6 mo	109.3 (40.3)	144.4 (61.9)*	108.8 (49.4)	159.1 (52.2)*

Values are expressed as mean (SD). There were no significant differences among the 4 groups for any of these characteristics at baseline and at 6 months. There were no significant differences between the baseline and after 6 months of intervention in each group.

[a] Except isoflavone capsules used for intervention.

* $P = .0004$; significant main effect of walking on the number of walking recorded by pedometer monitoring was analyzed using the 2-factor ANOVA model described in Materials and Methods.

Table 2
Serum E_2, lipid concentrations, and biomarkers of bone turnover, and their percent changes by study groups at baseline and at 6 months of intervention

	Placebo (n = 33)	Walking (n = 31)	Isoflavone (n = 33)	Isoflavone + walking (n = 31)	Main effects	
					Walking	Isoflavone
E_2 (pg/mL)						
Baseline	11.78 (2.64)	13.75 (6.55)	11.71 (3.48)	11.99 (3.08)		
After 6 mo	10.84 (2.00)	11.41 (4.02)	10.48 (1.19)	10.84 (1.67)	NS	NS
% Change	−5.77 (15.44)	−7.23 (41.88)	−6.08 (19.98)	−5.03 (23.51)	NS	NS
Osteocalcin (ng/mL)						
Baseline	10.51 (2.40)	10.47 (2.88)	9.23 (2.09)	9.50 (2.42)		
After 6 mo	9.89 (2.47)	9.21 (2.37)*	8.62 (2.20)	8.92 (2.19)*	NS	NS
% Change	−4.99 (15.92)	−9.91 (20.63)	−3.60 (19.14)	−5.85 (12.60)	NS	NS
BALP (U/L)						
Baseline	30.37 (11.61)	29.03 (6.65)	27.77 (8.63)	29.26 (8.26)		
After 6 mo	29.55 (9.59)	30.15 (6.43)	28.50 (6.08)**	29.47 (7.41)	NS	NS
% Change	−0.31 (15.40)	4.96 (15.55)	8.09 (19.24)	3.00 (13.94)	NS	NS
DPD (nmol/L per mmol/L creatinine)						
Baseline	7.76 (1.83)	7.65 (1.68)	7.30 (2.37)	6.88 (1.63)		
After 6 mo	7.35 (2.04)	7.21 (1.42)	6.98 (1.22)	6.89 (1.61)	NS	NS
% Change	−2.89 (27.47)	−1.06 (25.36)	−1.38 (23.87)	2.63 (20.81)	NS	NS
Total cholesterol (mg/dL)						
Baseline	227.4 (33.4)	232.8 (31.5)	227.9 (29.5)	230.7 (35.2)		
After 6 mo	223.0 (37.0)	236.2 (32.3)	230.3 (32.0)	232.0 (36.6)	NS	NS
% Change	−0.97 (15.19)	1.85 (10.29)	1.56 (12.65)	2.10 (13.13)	NS	NS
HDL-C (mg/dL)						
Baseline	71.7 (14.9)	71.0 (18.6)	74.2 (18.3)	66.2 (13.5)		
After 6 mo	71.5 (13.6)	71.7 (19.4)	73.5 (18.2)	69.0 (13.4)*	NS	NS
% Change	0.60 (10.85)	1.96 (10.61)	−0.97 (12.65)	6.14 (12.09)	$P = .04$†	NS
TG (mg/dL)						
Baseline	102.5 (49.0)	114.2 (70.2)	83.9 (38.5)	106.7 (55.1)		
After 6 mo	93.8 (41.8)	100.5 (32.4)	84.4 (34.9)	100.7 (61.7)	NS	NS
% Change	−3.66 (31.09)	−1.84 (30.64)	8.15 (35.96)	2.37 (62.04)	NS	NS

Values are expressed as mean (SD). NS indicates not significant.

* $P < .05$, significantly different from the baseline.

** $P < .01$, significantly different from the baseline.

† $P = .04$; significant main effect of walking on percent change of HDL-C was analyzed using the 2-factor ANCOVA model described in Materials and Methods.

was significantly different from baseline in each group. Percent change in BMD, body composition, serum lipid, and biomarkers of bone turnover was calculated {[(post-intervention − baseline values)/baseline values] ×100} for each group. Two-factor analysis of variance or ANCOVA was performed to determine the effect of isoflavone intake, walking, and their interactions after 6 months of intervention. When the subjects were stratified by equol status,

Table 3
Serum isoflavone concentrations by study groups at baseline and at 6 months of intervention

	Placebo (n = 33)	Walking (n = 31)	Isoflavone (n = 33)	Isoflavone + walking (n = 31)	Main effects	
					Walking	Isoflavone
Daidzein (nmol/L)						
Baseline	159.7 (143.0)	142.1 (146.0)	166.7 (128.7)	242.5 (360.0)		
After 6 mo	268.7 (276.2)	210.8 (199.9)	888.8 (841.7)**	899.7 (719.4)**	NS	$P < .0001$†
Genistein (nmol/L)						
Baseline	180.7 (136.1)	164.3 (183.8)	220.0 (199.9)	304.2 (371.9)		
After 6 mo	281.4 (457.0)	178.0 (178.6)	322.4 (275.7)	336.2 (301.0)	NS	NS
Glycitein (nmol/L)						
Baseline	63.8 (43.6)	54.3 (46.3)	66.8 (43.0)	65.5 (59.4)		
After 6 mo	65.3 (37.4)	71.7 (40.6)	194.0 (186.8)**	168.3 (155.2)**	NS	$P < .0001$†
Equol (nmol/L)						
Baseline	73.8 (201.7)	37.2 (79.4)	93.9 (196.1)	97.8 (232.3)		
After 6 mo	31.7 (131.9)	41.2 (131.1)	238.4 (348.6)*	228.4 (297.0)*	NS	$P < .0001$†

Values are expressed as mean (SD).

* $P < .05$, significantly different from the baseline.

** $P < .001$, significantly different from the baseline.

† Significant main effect of isoflavone ($P < .0001$) on serum daidzein, glycitein, and equol concentrations at 6 months was analyzed using the 2-factor ANCOVA model described in Materials and Methods.

3-factor ANCOVA was used to determine the effects of isoflavone, walking, equol status, and their interactions. Body weight, height, and daily intake including calcium, vitamin D, protein, and total energy were used as covariates in the analyses of body composition, BMD, and serum biomarkers to adjust for possible confounding. The significant differences in serum isoflavone concentrations and percent change in BMD between equol producers and nonproducers in each group were examined by using Student t test. Statistical analyses were performed using the PC SAS program, version 6.12 (SAS Institute, Cary, NC), and statistical significance was set at less than .05.

3. Results

3.1. General

The physical characteristics, daily intake of nutrients, and activity levels of the subjects at baseline and at 6 months of intervention are shown in Table 1. There were no significant differences in age, years since menopause, height, weight, BMI, and daily intake of isoflavones, calcium, vitamin D, and total protein among the different treatments groups at baseline. Average daily intake of isoflavone from soy foods (except isoflavone capsules) in each group at baseline was 44.4 to 49.4 mg. Six months of intervention of walking and isoflavones did not affect these parameters. The number of steps recorded by pedometer monitoring during the 6 months of intervention was significantly higher in the 2 walking groups as compared with the nonwalking groups.

3.2. Classification of equol producers and nonproducers

Fresh fecal samples were collected from 122 subjects to classify equol producers and nonproducers. Sixty-eight subjects (55.7%) were classified as equol producers because their fecal bacteria were able to convert daidzein to equol.

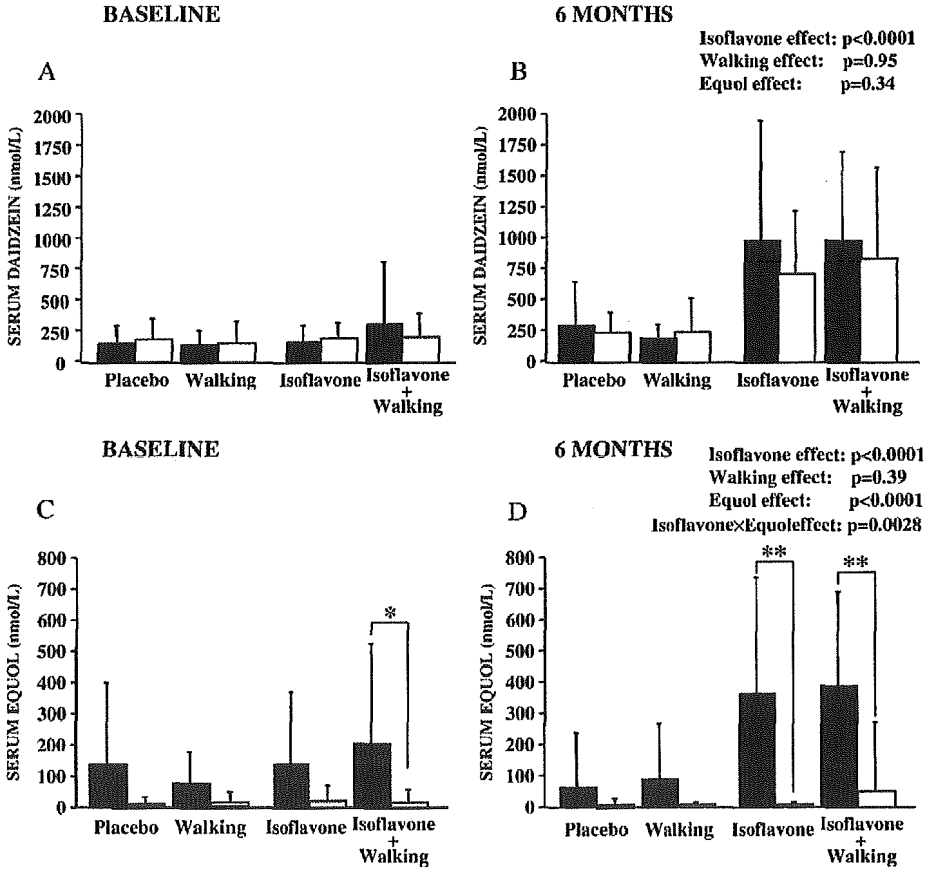

Fig. 2. Mean (SD) serum daidzein and equol concentrations in the study groups at baseline (A, C) and at 6 months of intervention (B, D). The subjects were stratified by equol status in each study group, and referred to as equol producers (■) and nonproducers (□). Differences in daidzein and equol concentrations among the study groups were nonsignificant at baseline (A, C). Differences in daidzein concentration between equol producers and nonproducers were not significant at baseline (A). Differences in equol concentration between equol producers and nonproducers in the isoflavone combined with walking group were significant (Student t test; $^{*}P < .05$) at baseline (C). The main effects of isoflavone, walking, and equol status and their interaction on serum daidzein and equol concentrations at 6 months were analyzed using 3-factor ANCOVA model described in Materials and Methods (B, D). Differences in daidzein concentration between the equol producers and nonproducers in each group were nonsignificant at 6 months (B). Differences in equol concentration between equol producers and nonproducers in the 2 isoflavone intervention groups (isoflavone and isoflavone combined with walking) were significant (Student t test; $^{**}P < .01$) at 6 months of intervention (D).

Fifty-two subjects (42.6%) were classified as nonproducers. Two subjects (1.6%) could not be classified as either equol producers or nonproducers. The number of equol producers and nonproducers in each group is as follows: the number of equol producers was 17, 15, 22, and 14 in the placebo, walking, isoflavone, and isoflavone combined with walking groups, respectively; the number of nonproducers was 11, 13, 11, and 17 in the placebo, walking, isoflavone, and isoflavone combined with walking groups, respectively. The equol producers defined from fecal analysis were almost the same as those who had high concentration of serum equol.

Serum concentrations of E_2, lipids, and biomarkers of bone turnover at both baseline and at 6 months of intervention are shown in Table 2. Statistically significant differences in serum concentrations of E_2, lipids, and biomarkers of bone turnover at baseline were not observed among the different groups. When these indices at 6 months were compared with those at baseline, the serum E_2, total cholesterol, TG concentrations, and the urinary biomarker of bone resorption (DPD) were not changed by walking, isoflavone, or their combination. High-density lipoprotein cholesterol concentration significantly increased from baseline by 6.1% ($P = .03$) in the combination of walking and isoflavone group, but did not significantly change in the other groups. Intact osteocalcin significantly decreased from baseline by 9.9% and 5.9% in walking and combined groups ($P = .01$ and .01, respectively). BALP significantly increased from baseline by 8.1% in the isoflavone group.

Table 4
Body composition, BMD, and their percent changes by study groups at baseline and at 6 months of intervention

	Placebo (n = 33)	Walking (n = 31)	Isoflavone (n = 33)	Isoflavone + walking (n = 31)	Main effects	
					Walking	Isoflavone
Sub–whole-body BMD (g/cm^2)						
Baseline	1.002 (0.096)	0.979 (0.100)	1.003 (0.108)	1.008 (0.070)		
After 6 mo	0.994 (0.089)	0.976 (0.095)	0.996 (0.105)	1.002 (0.069)	NS	NS
% Change	−0.74 (1.55)	−0.26 (1.58)	−0.69 (1.19)	−0.88 (1.57)	NS	NS
Whole-body lean mass (kg)						
Baseline	36.9 (3.7)	37.9 (4.2)	37.1 (3.2)	37.5 (3.2)		
After 6 mo	37.2 (3.9)	38.0 (4.3)	37.5 (3.5)	37.9 (3.2)	NS	NS
% Change	0.77 (2.39)	0.23 (2.27)	0.82 (1.75)	0.79 (1.84)	NS	NS
Whole-body fat mass (kg)						
Baseline	15.1 (4.5)	16.8 (4.3)	15.0 (4.1)	16.1 (3.5)		
After 6 mo	15.0 (4.5)	16.2 (4.2)*	14.5 (4.0)*	15.4 (3.7)*	NS	NS
% Change	0.17 (6.75)	−3.37 (6.35)	−2.92 (8.82)	−4.33 (6.03)	$P = .04$‡	NS
Lumbar spine BMD (g/cm^2)						
Baseline	0.907 (0.130)	0.879 (0.122)	0.891 (0.123)	0.909 (0.097)		
After 6 mo	0.904 (0.129)	0.866 (0.113)	0.884 (0.121)	0.901 (0.099)	NS	NS
% Change	−0.27 (2.64)	−1.30 (2.26)	−0.73 (2.44)	−0.90 (2.28)	NS	NS
Total hip (g/cm^2)						
Baseline	0.787 (0.126)	0.780 (0.114)	0.777 (0.125)	0.807 (0.089)		
After 6 mo	0.781 (0.121)	0.775 (0.109)	0.773 (0.126)	0.803 (0.086)	NS	NS
% Change	−0.66 (1.99)	−0.60 (2.31)	−0.50 (2.54)	−0.41 (1.86)	NS	NS
Femoral neck BMD (g/cm^2)						
Baseline	0.676 (0.114)	0.671 (0.116)	0.668 (0.106)	0.699 (0.094)		
After 6 mo	0.672 (0.104)	0.667 (0.110)	0.665 (0.094)	0.696 (0.093)	NS	NS
% Change	−0.25 (3.74)	−0.39 (4.09)	−0.04 (4.15)	−0.35 (3.68)	NS	NS
Trochanter BMD (g/cm^2)						
Baseline	0.599 (0.122)	0.592 (0.097)	0.591 (0.089)	0.600 (0.078)		
After 6 mo	0.594 (0.115)	0.589 (0.094)	0.593 (0.092)	0.597 (0.075)	NS	NS
% Change	−0.69 (2.80)	−0.31 (3.44)	0.29 (3.19)	−0.37 (2.49)	NS	NS
Trunk fat mass (kg)						
Baseline	6.5 (2.5)	7.9 (2.6)	6.8 (2.4)	7.7 (2.3)		
After 6 mo	6.1 (2.3)*	7.4 (2.5)*	6.4 (2.4)*	7.0 (2.2)*	NS	NS
% Change	−2.94 (9.84)	−6.62 (9.46)	−6.18 (14.23)	−7.56 (8.55)	NS	NS
Legs fat mass (kg)						
Baseline	2.9 (0.9)	2.9 (0.7)	2.7 (0.7)	2.7 (0.6)		
After 6 mo	3.0 (0.9)†	2.8 (0.7)*	2.7 (0.7)	2.6 (0.6)*	NS	NS
% Change	2.41 (7.22)	−1.45 (7.09)	0.91 (6.56)	−2.09 (6.86)	$P = .009$#	NS

Values are expressed as mean (SD).

* $P < .05$, significantly decreased as compared with the baseline.

† $P < .05$, significantly increased as compared with the baseline.

‡ $P = .04$; significant main effect of walking on percent change of whole-body fat mass was analyzed using the 2-factor ANCOVA model described in Materials and Methods.

$P = .009$; significant main effect of walking on percent change of the leg fat mass at 6 months was analyzed using the 2-factor ANCOVA model described in Materials and Methods.

By the 2-factor ANCOVA analysis, there was a significant main effect of walking (P = .04), but not isoflavone (P = .53) on the percent change in HDL-C.

3.3. Serum isoflavone concentrations

Table 3 shows the serum concentration of isoflavones at baseline and at 6 months. At baseline, there were no significant differences in the concentrations of isoflavones, except genistein (P = .041), among the 4 groups. The administration of isoflavones resulted in a marked increase in the serum concentrations of daidzein (P < .001), glycitein (P < .001), and equol (P < .05), but not that of genistein from baseline. In contrast, the placebo treatment did not modify the circulating concentrations of isoflavones. When using the 2-factor ANCOVA model, we found a highly significant effect of isoflavone (P < .0001).

Since it has been reported that the production of equol depends on the individual's intestinal flora, the subjects were stratified into 2 subgroups, referred to as equol producers and nonproducers. The serum concentrations of daidzein and equol are shown in Fig. 2. The production of equol in individuals was confirmed by measuring their ability to produce equol in feces. Differences in daidzein concentration between equol producers and nonproducers in each group were nonsignificant at baseline (Fig. 2A). However, the serum equol concentration was significantly higher in equol producers than in nonproducers in the isoflavone combined with walking group at baseline (P < .05) (Fig. 2C). After 6 months of intervention, the serum daidzein concentration markedly increased in the 2 isoflavone-administered groups, but not in the 2 placebo groups, regardless of whether they were equol producers or nonproducers (Fig. 2B). When using the 3-factor ANCOVA model, we found a significant main effect of isoflavone (P < .0001). On the other hand, serum equol at 6 months significantly increased from baseline (P < .05) in equol producers in the 2 isoflavone groups (Fig. 2D). In contrast, serum equol remained at baseline levels in nonproducers, which were significantly lower than those of equol producers (P < .01). In placebo and walking groups, serum equol remained at baseline levels in both equol producers and nonproducers (Fig. 2D). Again, using the 3-factor ANCOVA model, we found 2 significant main effects of isoflavone and equol status (both were P < .0001) and their interaction (P = .0028) on equol concentrations at 6 months (Fig. 2D).

3.4. Body composition and BMD

There was no significant difference among the 4 groups at baseline with respect to body composition, fat and lean mass in the whole body, and fat mass of the trunk and legs (Table 4). Fat mass in the whole body significantly decreased from baseline in the isoflavone (−2.9%, P = .006), walking (−3.4%, P = .004), and combined intervention (−4.3%, P = .0003) groups, but slightly increased in the placebo group (0.2%, P = .56). Fat mass in the legs significantly decreased in walking (−1.45, P = .04) and combined interventions (−2.09%, P = .01), but was not significantly changed in the isoflavone group (0.91%, P = .41). On the contrary, the fat mass in the legs significantly increased in the placebo group (+2.41%, P = .04) compared with baseline. Using the 2-factor ANCOVA model, we found significant main effects of walking on whole-body fat mass (P = .04) and legs fat mass (P = .009), but a nonsignificant effect of isoflavone (P = .12 and .41, respectively). There were no significant differences in BMD among the different groups in the sub–whole body, lumbar spine, and hip regions at baseline (Table 4). The percent changes in BMD in all the regions after 6 months showed similar trends in each group, but statistically significant main effect and interactions were not detected by the 2-factor ANCOVA model. When the subjects were stratified according to their equol-producing status, no statistically significant main effects or interactions were observed by 3-factor ANCOVA. However, the percent changes in BMD in equol producers were −0.53% and +0.13% in the sub–whole body and total hip, respectively, which were significantly different as compared with the percent changes of −1.35% and −1.77% in nonproducers in the isoflavone group (P = .049 and .040, respectively) (Fig. 3). In contrast, there were no significant differences in the percent changes in BMD between the equol producers and nonproducers in the placebo, walking, and isoflavone combined with walking groups.

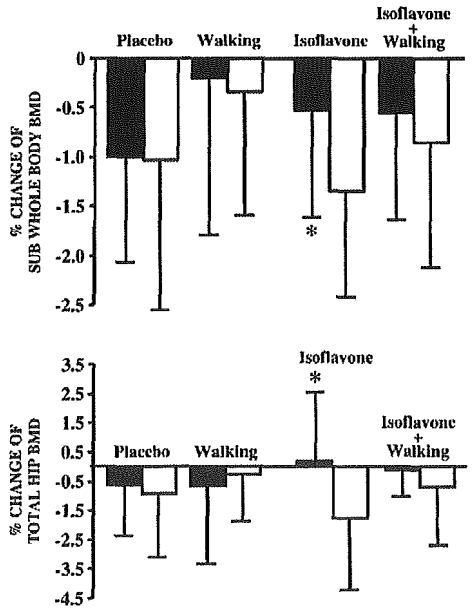

Fig. 3. Mean (SD) percent changes in BMD in the whole body and total hip at 6 months of intervention in the study groups. The subjects were stratified by equol status in both the study groups, referred to as equol producers (■) and nonproducers (□). Differences between equol producers and nonproducers in the isoflavone group were significant in the sub–whole body and total hip (Student t test; *P < .05).

4. Discussion

This randomized placebo-controlled study shows that the combined intervention of soy isoflavone intake and walking is most effective in decreasing the body fat and increasing the serum HDL-C concentration in early postmenopausal women, although the significant main effect was found for walking alone. Although isoflavone intervention for 6 months did not show a significant bone-protective effect, there was a significant difference in the percent change in BMD in the sub–whole body and hip regions between subjects stratified by their equol status.

It has been reported that soy isoflavone administration effectively lowered the serum cholesterol level in ovariectomized animals [25,26]. However, it is unclear whether isoflavones have clinically relevant and beneficial effects on lipid metabolism in humans [27]. In this study, it appeared that isoflavone intake for 6 months did not affect the blood lipid concentrations in healthy postmenopausal women. This result is similar to the reports from several clinical trials, which did not observe a hypocholesterolemic effect of isoflavones in postmenopausal women [27,28].

On the other hand, it has been reported that physical activity, such as brisk walking, significantly improved lipid metabolism and reduced body fat among overweight and obese postmenopausal women [29]. In a previous study, we reported that the combined intervention of exercise and isoflavone intake increased the HDL-C and decreased the body fat mass in ovariectomized mice [25]. In this study, we also observed an increase in HDL-C and a decrease in fat mass in postmenopausal women in the isoflavone combined with walking group. These results suggest that a combination of these 2 interventions may be a useful regimen for the management of serum lipids and body composition in postmenopausal women.

The effects of dietary soy isoflavones on biomarkers of bone turnover have been investigated in a few human trials. In this study, we found that serum BALP significantly increased from baseline in the isoflavone group. This result is similar to that of Morabito et al [30] as well as that of Arjmandi et al [31] who reported that the intervention of isoflavones increased serum BALP in postmenopausal women. The beneficial effect of soy isoflavones on BMD is still controversial, although impressive data from many studies on animal models of postmenopausal osteoporosis support a significant bone-protective effect of genistein and daidzein [32]. Nagata et al [33] reported that neither soy product and isoflavone intake nor serum isoflavone concentrations were associated with BMD. However, Somekawa et al [34] reported a significantly positive correlation between isoflavone intake and BMD at the lumbar spine in postmenopausal Japanese women. Several previous studies on dietary intervention have examined the effect of soy isoflavone on bone loss in postmenopausal women. In a study reported by Potter et al [35], diets containing 90, 56, and 0 mg of soy isoflavones per day over 6 months affected the BMD of the lumbar spine by 2.2%, −0.2%, and −0.6%, respectively. Chen et al [36] reported that soy isoflavone aglycone supplementation of 80 mg/d resulted in mild, but statistically significant and favorable percent changes in hip BMC (but not BMD) compared with placebo in Chinese postmenopausal women who had lower baseline BMC values. In Western women, the loss of BMD and BMC in the lumbar spine, but not in the hip, was significantly lower in women taking 45 mg/d of a red clover–derived phytoestrogen supplement for 1 year than in those taking a placebo [37]. Furthermore, Morabito et al [30] reported that genistein treatment (54 mg/d) for 1 year significantly increased the BMD of the lumbar spine and femoral neck in Italian women. This improvement in BMD was similar or slightly greater than that observed with HRT treatment. In this study, we did not find an effect of soy isoflavone on BMD even in the combined group after 24 weeks of intervention. The most possible explanation for the discrepancy between the promising findings and our results is that the duration of the intervention in our 6-month trial has been too short. Other considerable factors are the dose of isoflavones and daily intake of isoflavones from the diet of the subjects. However, the dose of isoflavones used in this study, 47 mg as aglycone form, was not low compared with those in the previous studies [30,36,37]; the Japanese diet contains higher soy products and more readily absorbed forms of isoflavones compared with Western and Chinese diets [38]. In our trial, the subjects were not restricted intake of soy products during the intervention (average isoflavone intake of each group was from 44.4 to 49.4 mg/d). The duration of the supplementation and these dietary differences in soy foods may be major reasons for the lack of isoflavone's effects on BMD in this study. These conditions need to be elucidated in further research.

Setchell et al [17] recently suggested that equol, a specific metabolite of daidzein produced by intestinal bacteria, may be the single most important factor that influences the clinical efficacy of soy isoflavone in preventing bone loss. This metabolite is not found in soy, but is formed by the intestinal flora in only 45% of the postmenopausal women studied. Setchell et al [17] reported that the lumbar spine BMD of equol producers increased by 2.4% ($P < .001$) as compared with the control group, whereas there was no significant change in BMD in the nonproducers after 2 years of intervention with isoflavones. In this study, we also stratified the subjects based on the equol-producing capacity of the individual's intestinal flora to investigate the actual effects of soy isoflavone on bone loss in early postmenopausal women. These results firstly showed that the loss of BMD in the sub–whole body and total hip were significantly lower in equol producers compared with nonproducers in Japanese treated with isoflavone. Furthermore, we demonstrated that the beneficial effect of isoflavones on bone could be attributed to the serum concentration of equol,

which was significantly higher in equol producers than in nonproducers in the isoflavone group. In contrast, because serum equol remained at baseline levels in both equol producers and nonproducers in the placebo groups, the loss of BMD did not differ between the producers and nonproducers. Our findings strongly support the hypothesis that the clinical effectiveness of soy products in bone health may be because of the ability of the subject to biotransform soy isoflavones to the more potent estrogenic metabolite, equol. Thus, the failure to distinguish subjects who are equol producers from those who are nonproducers in previous clinical studies could plausibly explain the variance in the reports on the benefits of soy intake [32]. Several specific intestinal bacteria capable of metabolizing soy isoflavone to equol have been identified from human feces [39,40]. Examination of equol production by the subjects who maintain the bacteria is now under investigation.

We are also interested in evaluating the effect of walking on the metabolism of isoflavones and bone loss in postmenopausal women because combined intervention of a submaximal dose of isoflavone and a moderate intensity of running exercise expressed more advantageous effects on the prevention of bone loss and fat gain in female and male osteoporotic model of mice than either treatment alone [23,24]. In this study, although the intervention of isoflavone combined with walking decreased fat mass in whole body and legs, we did not find any efficacy of the combined intervention on the change in BMD in postmenopausal women. These results suggest that the adjustment on bone metabolism needs longer term than lipid metabolism in humans. Bone remodeling is a relatively slow process, and the time required to complete a cycle may increase with age. Thus, longer-term trial would be required to evaluate the effects of isoflavone and isoflavone combined with walking on bone mass.

In conclusion, the combined intervention of soy isoflavone and walking for 6 months exhibited cooperative effects on modifying lipid metabolism and body composition in postmenopausal Japanese women. The beneficial bone effects of isoflavones depend on the equol-producing capability of an individual's intestinal flora in humans.

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Original article

Determination of reference intervals for vibratory perception thresholds of the lower extremities in normal subjects

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Abstract The assessment of vibratory perception thresholds (VPTs) is important for evaluating human somatosensory functions and provides new aspects in clinical practice and research. However, there has been little information concerning determinants of the VPT in normal individuals, and there has been no investigation to determine the reference intervals for the lower extremities by vibrometers using appropriate statistical analysis. We determined reference intervals for the VPT in lower extremities of 377 healthy subjects (241 males, 136 females; ages 11–74 years) using Suzuki-Matsuoka vibrometer-5 according to the National Committee for Clinical Laboratory Standards guidelines. The VPT was measured at 12 points: ulnar styloids, patellae, medial and lateral malleoli and the tip of the great toes, dorsal aspect of the distal phalanx of the fifth toes. The effects of age, sex, height, weight, alcohol consumption, and smoking on the VPTs were also investigated. The VPTs of normal subjects increased significantly with age. The difference between the two sides was negligible for determining reference intervals of the VPT. The average VPT was higher in men than in women at the lateral malleolus, the great toe, the patella, and the ulnar styloid but not significantly different at the fifth toe or the medial malleolus. There were no significant differences in the VPTs among the four sites of the foot. The reference interval for the VPT of the lower extremity is less than 13×10^{-2} G. The influence of body mass index, smoking, and alcohol consumption on the VPT was not significant. We provide the reference interval for the VPT of lower extremities in normal subjects. This information can serve as a basis for future clinical applications of VPT measurements.

Introduction

Quantitative assessment of somatosensory deficit and motor dysfunction is of considerable clinical significance for the diagnosis and treatment of spinal disorders. However, clinical evaluation for sensory perception has mostly been carried out qualitatively owing to technical limitations as well as time constraints.

In recent years, vibratory perception threshold (VPT) testing has been used to detect peripheral neuropathy in terms of amplitude.[1,2] It has been shown that the psychophysical responses to vibration are determined by activation of the Meissner and Pacinian corpuscles, which are rapidly adapting receptors.[3] It is, therefore, important to determine the VPT in terms of acceleration, as these mechanoreceptors respond to acceleration of stimulation.[4] The development of biothesiometers — simple, hand-held measuring devices for VPT — has enabled quantitative measurement of peripheral, sensory nerve function. The Suzuki-Matsuoka vibrometer-5 (SMV-5; Medience, Tokyo, Japan) (Fig. 1), which is one of such biothesiometers, was developed for the diagnosis of diabetic neuropathy. It is a fine instrument that can measure responses to acceleration of vibratory stimulation.

In Japan, the SMV-5 has gradually been used for several clinical studies.[5,6] Ohnishi et al. made a comparative study of the three vibrometers (SMV-5, Vibratron II, TM-31A) and reported that SMV-5 was the most reliable.[7] When diagnosing peripheral neuropathy based on measurement of the VPT, the determination of reference intervals for VPT data is epidemiologically and clinically important. However, there has been little information concerning determinants of the VPT in normal individuals, and there has been no investigation to determine the reference intervals for the lower extremities by the vibrometers using appropriate statistical analysis. In 1995 the National Committee for Clinical Laboratory Standards (NCCLS) proposed a guideline for terminology and procedures for determining reference intervals (C28-A).[8]

The purpose of the present study was to establish reference intervals for the VPT in the lower extremity of healthy Japanese subjects according to the NCCLS

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guidelines. The effects of age, sex, height, weight, alcohol consumption, and smoking on the VPT were also investigated.

Materials and methods

Subjects

The VPT was examined in 377 healthy Japanese volunteers (241 males, aged 11–74, mean 34 ± 11 years; and 136 females, aged 11–74, mean 31 ± 13 years). Subjects participated voluntarily, with informed consent obtained before testing. The age distribution and demographics of the subjects are presented in Tables 1 and 2, respectively.

A brief questionnaire that included information on smoking habits, alcohol consumption, and hand dominance was completed by each subject. Subjects who had a history of neurological diseases or medical conditions that might predispose the person to sensory abnormalities (e.g., diabetes mellitus, malignancy, alcohol abuse, neck or back disorders, kidney failure) and those who had undergone surgery within a year were excluded. Subjects who were taking medications that may cause peripheral neuropathy were also excluded. The body mass index (BMI) was calculated as by dividing the weight in kilograms by the height in meters squared (BMI = weight/height^2).

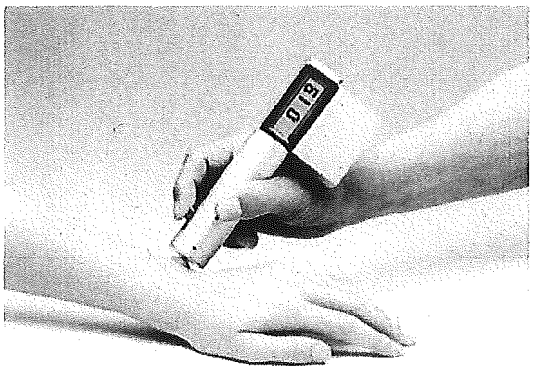

Fig. 1. Vibrometer used in present study: SMV-5 (Suzuki-Matsuoka vibrometer; Medience, Tokyo, Japan)

Stimulation and measurement procedures

Instrument

The VPT was determined by applying a stimulus from the electromagnetic vibrator, SMV-5, which is a hand-held instrument producing sine-wave vibration at a frequency of 220 Hz with an accelerometer recording the actual movements of a vibrating probe (between 0 and 150 × 10^{-2}G) by automatically controlling stimulatory strength. The probe is 15 mm in diameter with a flat contacting surface in a firm plastic cylinder. The real vibratory acceleration could be monitored directly on a digital display. The principle of the device action has been described by Suzuki et al.[9,10] Previous studies have already confirmed the validity and reliability of the VPT measurements by this equipment.[7] The coefficients of variation for the intra- and interobserver of the VPT measurements by the SMV-5 have been reported to be 15.2% and 18.5%, respectively.[5]

Testing procedure

The measurements were obtained in a silent, closed room with ambient temperature control (20°–24°C).

Table 1. Age distribution of subjects

	No. of patients, by decades of age						
Subjects	11–19	20–29	30–39	40–49	50–59	60–74	Total
Males	5	105	73	29	16	13	241
Females	10	74	22	14	13	3	136

Table 2. Demographics of subjects

Characteristic	Males (M = 241)	Females (M = 136)
Age	33.8 ± 11.2 (11–74)	30.8 ± 12.9 (11–74)
Height (cm)	171.6 ± 5.8 (151.0–185.0)	157.8 ± 6.0 (135.0–171.5)
Weight (kg)	68.0 ± 9.4 (41.5–110.0)	52.0 ± 7.4 (30.0–80.0)
BMI (kg/m^2)	23.1 ± 2.8 (15.9–34.7)	20.9 ± 2.7 (16.4–32.2)
Hand dominance	R:183, L:12, A:8, U38	R:100, L:5, A:2, U:29
Skin temperature (°C)	32.0 ± 1.6 (28.0–35.2)	31.7 ± 1.5 (28.7–35.3)

Results are means ± SD (range)
R, right dominance; L, left dominance; A, ambidextrous; U, unknown; BMI, body mass index

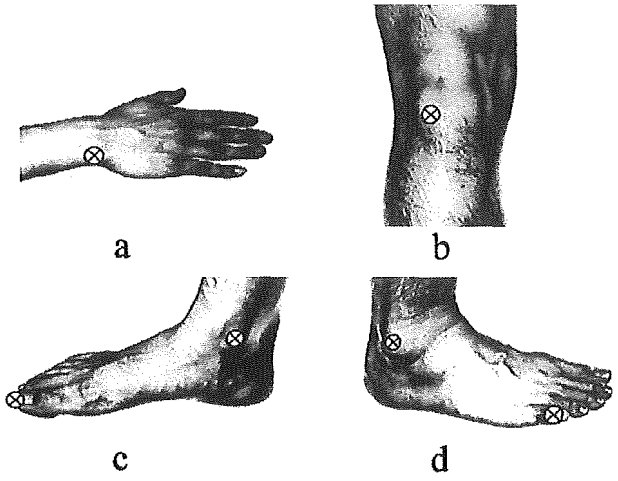

Fig. 2. Regions examined by SMV-5. **a** Ulnar styloid. **b** Patella. **c** Tip of great toe and medial malleolus of tibia. **d** Lateral malleolus of fibula and dorsal aspect of distal phalanx of fifth toe

The subject was in a supine or sitting position to provide optimal relaxation and concentration. The probe was placed at a right angle to the bare skin with gentle pressure to ensure full contact. The VPT was measured at 12 points (Fig. 2.): the ulnar styloids, the patellae, the medial malleoli and tip of the great toes, the dorsal aspect of the distal phalanx of the fifth toes, and the lateral malleoli. The stimulus was gradually increased from zero; and when the subject first perceived the stimulus, the value on the display was recorded. The measurements were repeated five times, and the median of five measurements was used to represent the VPT for each site. After completing the measurements, the peripheral temperature at the bilateral dorsal foot of each subject was measured by surface thermometer (Nihonkoden, Tokyo, Japan). Total time for the interview, trials, and actual measurements using SMV-5 was 20–25 min for each subject. The same apparatus was used throughout the whole study, and all measurements were carried out by the principal investigator.

To examine the effect of leg positions during measurements, the VPT values were compared between two age- and sex-matched groups of 76 subjects each who were tested with their knees bent or extended. To investigate the effect of the dominant side of the hand on the VPT value, the subjects were divided into groups based on their dominant hand. The differences in VPT values were assessed for all the combinations of dominant/nondominant hand and the measurement sites. To examine the effect of obesity, the VPT values at the six measurement sites were compared between 59 subjects with a BMI of 25 or more and 315 subjects with a BMI of less than 25. The subjects were also divided by the amount of alcohol consumed (i.e., those with none and intake of less than twice per week and those with intake of more than twice per week), and the VPT between the two groups were compared. The VPTs of the nonsmokers and habitual smokers were also compared. Heavy smokers who consume more than two packs per day and occasional smokers were excluded for this study.

Data analysis

In subjects who had a higher than maximum intensity of vibration (>150 × 10^{-2}G), 150 × 10^{-2}G was used as the VPT value. Although the VPT of the whole sample group (377 subjects) did not show a normal distribution, normality could be demonstrated by the Smirnov-Kolgomonov test when VPT values underwent logarithmic transformation ($\log_{10}$). However, because VPTs in each of the stratified groups did not show a normal distribution, nonparametric tests were used to compare VPTs among groups. Wilcoxon tests were used to determine statistically significant differences in VPTs between left and right sides of the body. The Mann-Whitney U-test was used to compare the results in different sex and age groups. The Wilcoxon signed rank test was used to assess differences among measured sites. The correlation between age and VPT measurements at each site was estimated by Spearman's rank correlation coefficients. $P < 0.05$ was considered significant. All statistical analyses were performed on a Macintosh computer (Apple, Cupertino, CA, USA) using Statview (version 5.0; SAS Institute, Cary, NC, USA).

Results

Males were significantly taller than females (171 ± 6 vs. 157 ± 6 cm, $P < 0.001$). The mean skin temperature of the dorsum of the foot in whole subjects was 31.9°C ± 1.6°C (range 28.0°–35.4°C). There were no significant differences in VPTs at any site between the two groups with measurements in different leg positions. Both the average and standard deviation of the VPTs showed a tendency to increase with age. There was a highly significant ($P < 0.001$) correlation between the age of the subjects and the VPT (Fig. 3), and the age-related increase in VPT was significant at all sites: The correlation between VPTs and age was strongest at the fifth toe ($r = 0.519$) followed by the great toe ($r = 0.443$), the lateral malleolus ($r = 0.391$), the medial malleolus ($r = 0.368$), the ulnar styloid ($r = 0.303$), and the patella ($r = 0.285$).

Table 3 shows means (±SD) of the vibratory perception thresholds at the six measurement sites for the left and right sides. Among all tested sites, the medial

Table 3. Vibration perception thresholds for tested sites

	Right	Left	*P*
Head of the ulna	1.8 ± 1.0	1.6 ± 0.7	**0.044**
Lateral malleolus	5.0 ± 5.3	5.0 ± 5.4	0.916
Fifth toe	5.1 ± 6.0	4.8 ± 5.1	0.916
Great toe	4.8 ± 5.7	5.1 ± 6.4	**0.047**
Medial malleolus	4.8 ± 5.1	4.1 ± 4.4	**0.004**
Foot	5.0 ± 5.0	4.9 ± 4.9	0.52
Patella	22.5 ± 40.1	25.0 ± 44.4	0.578

[$\times 10^{-2}$G]

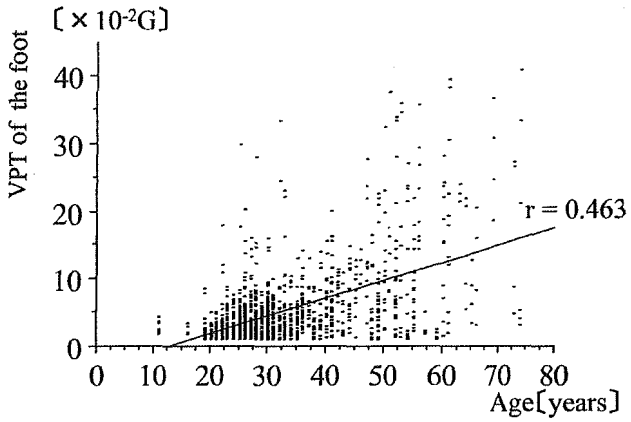

Fig. 3. Scatter-plots of vibration perception thresholds (*VPT*) of the foot in 377 normal individuals. Mean values of tested sides for two sides were plotted

malleolus, the great toe, and the ulnar styloid showed significant differences in VPT between the left and right sides. The VPT values were not significantly different between the dominant and nondominant sides.

Because it was not feasible to compare VPT values directly between males and females owing to uneven age distribution, the same number (n = 96) of subjects adjusted for age were chosen from both groups and compared. As a result, the average VPT was higher in men than in women at the lateral malleolus, the great toe, the patella, and the ulnar styloid but not significantly different at the fifth toe or the medial malleolus.

The VPT values for the lower extremities were higher than those for the upper extremities in all decades. There was no significant difference in VPTs among the four sites of the foot. The VPT values measured at the foot were significantly lower than that at the patella. Wide variations of VPT were observed for the patella, and in 50 subjects (16%) the VPT at the patella exceeded the upper limit of measurement (150 $\times 10^{-2}$G).

There was no significant difference in VPTs with regard to different BMI groups at any measurement site. Similarly, no significant difference in VPT was observed at any site between 134 smokers and 229 nonsmokers. There were also no significant associations between the VPTs and alcohol consumption.

However, the difference in the VPT values between the two sides of the body was small ($<3 \times 10^{-2}$G) and negligible enough to determine the reference intervals. There was also no significant difference in the VPTs among the four sites of the foot. These eight values, therefore, were averaged for each subject. The mean and standard deviation were calculated for each sex and decade. As suggested by document C-28P (NCCLS), it is probably advisable to consider 95% range of the value of reference population as the reference intervals; which could be obtained by calculating the mean value ± 1.96× standard deviations of VPTs in our subjects. The upper limit alone was specified, as the lower limit has no clinical meaning. The reference intervals of the VPT for the foot of normal Japanese were defined by decades for each sex in Table 4. The reference interval for the VPT of the lower extremity is less than 13 $\times 10^{-2}$G for the total subjects.

Discussion

Although we cannot completely exclude abnormal values that may be obtained in normal subjects by chance, we set exclusion criteria (including metabolic disease, habitual drug use, and prior surgery) known to affect VPTs in the previous literature (a priori exclusion) when selecting the reference population. In addition, data obtained from these subjects (reference values) were subjected to rigorous statistical exclusion (a posteriori exclusion). As a result, the reference VPT values obtained in the present study from the population that included 377 subjects were confirmed to show a normal distribution by logarithmic transformation.

Several instruments have been developed to overcome lack of sensitivity and reproducibility in VPT

Table 4. Reference interval of VPT in the foot, by age group

Group	No.	Mean	SD	Minimum	Maximum	Reference interval
11–19 years						
Male	5	3.3	1.6	2.0	6.0	6.4
Female	10	2.0	1.2	1.0	5.0	4.4
20–29 years						
Male	105	3.3	2.1	1.0	12.0	7.4
Female	74	2.4	2.1	1.0	15.0	6.4
30–39 years						
Male	73	4.2	2.8	1.0	16.5	9.8
Female	22	3.9	2.4	1.0	10.0	8.7
40–49 years						
Male	29	6.8	4.8	2.0	21.0	16.1
Female	14	7.2	6.1	1.0	20.5	19.1
50–59 years						
Male	16	10.8	7.3	1.0	26.0	25.2
Female	13	4.9	5.2	1.0	20.0	15.1
60–74 years						
Male	13	13.3	10.1	3.0	32.0	33.1
Female	3	10.8	3.2	8.5	13.0	17.0
Totes						
Male	241	4.9	4.7	1.0	32.0	14.1
Female	136	3.5	3.6	1.0	20.5	10.5
All	377	4.4	4.4	1.0	32.0	12.9

[$\times 10^{-2}$G]

VPT, vibration perception threshold

measurements.[10–13] The reliability of VPT measurements seems to be dependent on the type of vibrometer used and on the observers who perform the procedure. Because of its high precision,[5] the SMV-5 vibrometer is sensitive enough to detect sensory impairment that cannot be detected by conventional neurological examinations.[9] In many studies, the VPT has been tested in normal subjects.[1,14–18] Hilz et al. reported the reference values for VPT in 530 healthy subjects using the Vibrameter. They presented the VPT in terms of amplitude, and only one site was studied in the lower extremity.[1] The Vibrameter requires a control unit in addition to the stimulating probe. The advantages of the SMV-5 vibrometer are that it is noninvasive, time-efficient, and easy to apply at various test sites and angles as it is small and portable. We applied it to five sites in the lower extremity on both sides and compared the VPT values of different anatomical sites.

The VPTs of normal subjects increased significantly with age in both the upper and lower extremities, as in earlier reports. Pearson was the first (1928) to study a large series with respect to the influence of aging on VPTs.[17] He found a slight decrease in vibratory sensitivity in different age groups, which became striking after age 50.[17] The present study yielded similar results. Usually, the vibration sense diminishes in the legs and feet with advancing age.[14,15,17,18] On the other hand, Laidlaw and Hamilton found no significant increase in VPTs in the hands and fingers with aging,[19] whereas a significant increase was obtained for the ulnar styloid in our study. The precise nature of the mechanism responsible for the age-related decrease in vibration sensitivity remains unknown. Among various hypotheses, one possibility is a diminution in the blood supply to the peripheral nerves,[20] as arteriosclerosis become prominent with aging.[21] It is generally accepted that VPTs determined at high frequencies (80–400 Hz) are the result of neural activity in Pacinian corpuscles and that the changes in the structures of the Pacinian corpuscle occur between birth and 93 years of age.[22] In addition to the changes in the structures, the population of Pacinian corpuscles decreases with age.[22] These changes, therefore, could be associated with the loss of vibratory sensitivity at high frequencies.[23] Moreover, there is progressive fiber loss and demyelinization in the peripheral nerve and the spinal nerve roots with aging.[24] Progressive changes such as loss of nerve cells also occur in the central nervous system, which may also contribute to the decreased vibration sense.[25] The spinal cord and spinal nerve roots may suffer significant compression in the elderly because of degenerative changes in the cervical and lumbar spine, even in the absence of symptoms and clinical findings. Indeed, we found a wide variation in the VPT values of elderly persons.

We found women to be more sensitive than age-matched men at several of the tested sites. The

difference between the sexes has been studied previously.[2,15,18,26] It has been suggested that the vascular disorders of the heart and the lower limbs occur more frequently and earlier in men than in women.[18] Therefore, separate reference intervals of VPT should be determined for each sex and decade.

Several authors have reported that sensitivity differs on the left and right sides of the body, with the limb on the left side reported to be more sensitive.[26] In the present study, the left side was more sensitive than the right side on the great toe, medial malleolus, and ulnar styloid. However, the difference in VPT values between the two sides of the body was small ($<3 \times 10^{-2}$G) and negligible enough for determining the reference intervals.

The VPT of the ulnar styloid was lower than those in the lower extremities, which is in accordance with previous reports. As early as 1897, Treitel showed that the VPT is most sensitive in the upper limbs, and that it is finest in the distal parts of the limbs and poorer on the trunk. One explanation may be the difference in the size of the projection field in the central nervous system. The hands have wide projection areas in the cerebral cortex. The difference in the areas of perception may be due to the difference in receptor density.[14]

The knees are not very sensitive to vibratory stimuli.[26,27] It was concluded that measuring the VPT at the patella with this instrument (SMV-5) is not feasible. No significant differences were found among the four foot regions (medial malleolus, lateral malleolus, great toe, fifth toe). Therefore, we provide average VPT values of the four sites as the reference interval of the foot.

The influence of BMI, smoking, and alcohol consumption on the VPT were not apparent in the present study. Previous studies have also revealed that neither habitual drinking nor smoking affected the VPT.

This study provides reference intervals of the VPT around the foot regions. The upper limit alone was specified, as the lower limit has no clinical meaning.

In the actual clinical applications there are several points to take into account. Measurements can be performed with the knees either bent (sitting position) or extended (supine position). Green[28] reported a U-shaped relation between skin temperature and the VPT in nondiabetic subjects, with 34°C being the nadir. He concluded that cooling might affect VPT by decreasing the sensitivity of Pacinian corpuscles; the reason for the decreased sensitivity due to warming is unclear. According to several other reports[15,29] the VPT may be stable between 28°C and 36°C skin temperature. It would be preferable to measure the VPT at four sites of the foot bilaterally for comparison.

The VPT assessment can be used in diagnostic procedures, research, and follow-up studies to investigate the course of the disease and the therapeutic effect. VPT measurements can be recommended as a complement to conventional neurological and neurophysiological evaluations. The present study is the first to define the reference interval of the VPT using a large population and taking into account the possible variables that may affect the measurement.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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