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Isoflavones from Red Clover Improve Systemic Arterial Compliance But Not Plasma Lipids in Menopausal Women*

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ABSTRACT

The possibility that the heightened cardiovascular risk associated with the menopause can be reduced by increasing dietary isoflavone intake was tested in 17 women by measuring arterial compliance, an index of the elasticity of large arteries such as the thoracic aorta. Compliance diminishes with age and menopause.

An initial 3- to 4-week run-in period and a 5-week placebo period were followed by two 5-week periods of active treatment with 40 mg and then 80 mg isoflavones derived from red clover containing genistein, daidzein, biochanin, and formononetin in 14 and 13 women, respectively, with 3 others serving as placebo controls throughout. Arterial compliance, measured by ultrasound as a pressure (carotid artery) and volume (outflow into aorta) relationship, was determined after each period; plasma lipids were measured twice during each period. Urinary output of isoflavones was also determined.

Arterial compliance rose by 23% relative to that during the placebo period with the 80-mg isoflavone dose and slightly less with the 40-mg dose (mean ± SEM: placebo, 19.7 ± 1.5; 40 mg, 23.7 ± 0.7; 80 mg, 24.4 ± 1.4). In the three women receiving continuous placebo, compliance was 16 ± 2.2, similar to that during the run-in period for the remaining subjects (17 ± 2.1). ANOVA showed a significant ($P < 0.001$) difference between treatments; by Bonferroni multiple comparisons and by paired t test, differences were significant between placebo and 40- and 80-mg isoflavone doses (by paired t test: $P = 0.039$ for placebo *vs.* 40 mg; $P = 0.018$ for placebo *vs.* 80 mg). Plasma lipids were not significantly affected.

An important cardiovascular risk factor, arterial compliance, which diminishes with menopause, was significantly improved with red clover isoflavones. As diminished compliance leads to systolic hypertension and may increase left ventricular work, the findings indicate a potential new therapeutic approach for improved cardiovascular function after menopause. (*J Clin Endocrinol Metab* **84:** 895–898, 1999)

THE HIGH consumption of legume-derived isoflavones has been credited with the relative protection that Japanese women enjoy from complications of the menopause, including less cardiovascular disease, resembling the probable benefits of hormone replacement therapy in Western menopausal women (1). The mechanisms have not been clearly defined. The lipoprotein profile is more clearly improved with estrogen (2), and a direct beneficial effect on arterial vasculature has also been established for estrogen (3, 4). We reported recently that isoflavones from soybeans in amounts resembling consumption among Japanese improved arterial compliance in menopausal and perimenopausal women (5). This occurred in the absence of any reduction in plasma lipids. A recent study in monkeys has shown a marked reduction in coronary atherosclerosis with soy protein, but not with soy protein depleted of isoflavones (6). These effects have been attributed to the weakly estrogenic effects of these isoflavones, which also posses antioxidant properties (7).

Similar isoflavones are present in other plants, and red clover contains, in addition to genistein and diadzein, which are present in soybean, the methylated forms biochanin and formononetin. We tested an isolated, purified extract of red clover isoflavones on arterial compliance in 17 menopausal women. As impaired compliance has been shown to be associated with aging, menopause, hypertension, and possibly hyperlipidemia (5, 8) and to probably be partly responsible for systolic hypertension in elderly people (9), supplementation with red clover isoflavones was investigated as a possible therapeutic intervention.

Subjects and Methods

Test subjects

Women were invited through newspaper advertisements to take part in a clinical trial of a purified red clover isoflavone extract (Novogen Pty. Ltd., North Ryde, Australia) that might reduce measurable cardiovascular risk. Respondents had been clearly postmenopausal for at least the preceding year. Objective measurements of FSH confirmed menopausal status.

Exclusion criteria were age more than 70 yr; hormone replacement therapy, which several women discontinued for at least 6 weeks beforehand; and supplements such as evening primrose oil and vitamin E, which were also stopped 4–6 weeks before the study. Inclusion criteria also required a FSH level above 40 and a plasma cholesterol level at recruitment between 5–7 mmol/L. No subject took any regular medication that might have affected plasma lipids or cardiovascular function. Smoking and drinking more than 14 standard alcoholic drinks weekly and body mass index greater than 32 were other exclusion criteria. Physical examination showed that they were free of apparent cardiovascular disease. Relevant details are shown in Table 1. The trial was approved by Alfred Hospital human ethics committee and was carefully explained to obtain informed consent.

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TABLE 1. Entry characteristics of 17 women who completed all phases (14 active to end of 40 mg phase and 3 placebo)

No.	Age (yr)	Wt (kg) start	Wt (kg) end	BMI end
1	53	72.2	71.8	25.9
2	62	69.6	71.3	25.4
3	51	60.5	60.3	22.0
4	41	60.0	60.4	22.4
5	55	79.2	79.5	28.9
6	64	73.0	72.4	24.9
7	50	77.6	79.1	29.6
8	57	66.6	65.7	23.1
9	49	67.6	68.2	25.5
10	59	69.0	69.0	23.7
11	55	59.8	57.4	20.0
12	49	63.0	63.9	25.7
13	58	76.8	74.4	30.0
14	56	74.6	72.7	27.0
15	71	69.0	68.0	24.2
16	51	59.5	59.9	24.9
17	65	71.0	71.5	24.7
Mean ± SD	55.7	68.8	68.5	25.2

Weights at the start represent postscreening, before run-in phase at randomization.

Experimental design

The design comprised a run-in period that averaged 3 weeks, during which the women became familiar with dietary principles, including avoidance of legumes, identifying fat content of foods, and maintaining a regular food pattern with a target of no more than 30% energy from fat. The women received placebo tablets during this phase.

The women were then randomized into an active intervention group or a placebo group (although only every fifth woman was randomized to take placebo throughout as an index of changes over time). The remaining women were assigned to take 2 placebo tablets/day for 5 weeks and then 1 Promensil (Novogen; 40 mg isoflavone) and 1 placebo tablet daily for a further 5 weeks and then 2 80-mg isoflavone tablets daily for a final 5 weeks. Of 26 women enrolled, only 19 completed the study with respect to the plasma lipid investigations, and only 17 underwent measurements of arterial compliance to the end of the first intervention period. The high dropout rate was due to intercurrent illnesses and changed geographic circumstances and was higher during the placebo phases than during the intervention phases due to intolerable menopausal symptoms requiring hormone replacement treatment. Thus, only 3 women instead of 5 originally randomized to placebo throughout the study completed the trial. The study was carried out double blind, with an external monitor supervising the trial.

The background diet constraints were applied throughout and were supervised closely by a dietitian. Subjects were encouraged to compose their diets of whole grain cereal foods, fruit and vegetables, low fat dairy products, fish, lean and skinless poultry, as well as lean meat. Soy-based food products and leguminous vegetables were omitted. Subjects kept 3-day food records during each phase of the trial. A normal exercise routine was encouraged. The subjects attended the clinic every 2 weeks, when either tablets were dispensed or compliance with diet and medication was checked. Twenty-four-hour urine samples were collected after the designated active and placebo periods for measurements of isoflavonoid excretion to monitor absorption.

Isoflavone supplements

The standardized isoflavone supplement used was a proprietary product called Promensil (Novogen), prepared from red clover extract containing 40 mg/tablet total isoflavones comprising four primary isoflavones: genistein, 4 mg; daidzein, 3.5 mg; and their methylated precursors biochanin, 24.5 mg; and formononetin, 8.0 mg, present as hydrolyzed aglycones. The placebo tablet contained excipients without isoflavones and was similar in taste and appearance.

Laboratory measurements

Measurements were made at the end of each period, *i.e.* run-in (baseline diet only), placebo, and two active periods. Blood for plasma lipid measurements was collected on 2 consecutive days. The women taking placebo throughout were tested at equivalent time points.

The determination of systemic arterial compliance, which measures the elasticity of the main conduit arteries and which included frequent automated arterial pressure measurements, was carried out near the end of each period.

Systemic arterial compliance

Systemic arterial compliance (SAC) was estimated using the area method of Liu *et al.* (10), which requires measurement of volumetric blood flow and associated driving pressure to derive an estimated compliance over the total arterial system according to the formula: $SAC = A_d[R(P_s - P_d)]$, where A_d is the area under the blood pressure diastolic decay curve from end systole to end diastole, R is the total peripheral resistance, Ps is the end-systolic blood pressure, and Pd is the end-diastolic blood pressure. Both aortic flow, measured with a flow velocimeter over the suprasternal notch, and pressure signals, measured by applanation tonometry over the right carotid artery, were digitized at 4 MHz using a analog to digital conversion board. Data were acquired and analyzed with purpose-written software (J. D. Cameron). The computation of compliance proceeds automatically; the observer is required only to ensure stable baselines and consistently reproducible pressure-flow traces. The methodology has been described fully in our previous publications (5, 11).

Plasma lipids

Plasma was separated from chilled blood samples and frozen at −80 C. Measurements were carried out in batches for plasma glucose, cholesterol, and triglyceride by enzymatic kits on a Cobas-Bio automated analyzer (Roche, Basel, Switzerland). High density lipoprotein (HDL) cholesterol was separated from plasma by selective precipitation of other lipoproteins.

Isoflavone content

Isoflavone excretion in urine was estimated by assay of the total isoflavone content using high performance liquid chromatographic analysis (performed by Novogen) of an aliquot of a 24-h urine collection and correcting for urine volume to obtain the total excretion value.

Statistical analysis

The active treatment group was analyzed initially by one-way repeated measures ANOVA, corrected subsequently by Bonferroni correction for multiple comparisons, and analyzed finally by paired *t* test between the various interventions (run-in, placebo, and 40- and 80-mg doses). As only three women completed the placebo-only arm of the study, their data were not subjected to statistical analysis.

Results

General results

Four intervention periods comprised an initial run-in period during which placebo tablets were taken, averaging 3 weeks, and then sequentially placebo, 40 mg isoflavone, and 80 mg isoflavone periods, each of 5-week duration. Fourteen women completed the first 3 phases, and 13 women completed the entire study; 3 additional women took only placebo tablets throughout. The results include the run-in data as a reference point, but interpretation and conclusions will be limited to placebo *vs.* active groups. The data have been examined in the first instance for effects of time and order of treatment, including possible carry-over. None of these was found to be a confounder. The findings are summarized in Table 2.

TABLE 2. Values for arterial compliance, arterial pressure, and plasma lipids during the four periods for the active group
Arterial compliance values for the three women in the placebo-only group are shown in *parentheses.*

Period	Run-in	Placebo	40-mg dose	80-mg dose
Arterial compliance	18.5 ± 6.4	19.7 ± 5.7	23.7 ± 5.3	24.4 ± 4.9
(placebo-only group)	(17)	(16)	(16)	(16)
Mean arterial pressure (mm Hg)	81 ± 7	82 ± 11	81 ± 9	87 ± 11
Plasma cholesterol (mmol/L)	5.96 ± 0.98	6.11 ± 0.82	5.94 ± 0.93	5.91 ± 0.64
HDL cholesterol (mmol/L)	1.57 ± 0.25	1.60 ± 0.23	1.68 ± 0.27	1.67 ± 0.24
LDL cholesterol (mmol/L)	3.81 ± 0.89	4.00 ± 0.82	3.77 ± 0.94	3.76 ± 0.72
Plasma triglyceride (mmol/L)	1.22 ± 0.49	1.10 ± 0.41	1.09 ± 0.30	1.05 ± 0.36

Data for 13 women who completed all 4 phases.
[a] ANOVA showed significant difference between treatments ($P < 0.001$) attributable to differences between placebo and 40- and 80-mg doses (both $P < 0.05$; run-in period data excluded from post-ANOVA analysis, Bonferroni correction). Paired *t* test differences between placebo and 40-mg, $P < 0.04$; and between placebo and 80 mg, $P < 0.02$.

TABLE 3. Total urinary isoflavone excretion in subjects in the active stream and subjects on placebo throughout

Group	Start of run-in	End of run-in	End of placebo	End of 40 mg	End of 80 mg
Active	0.97 ± 0.20	0.67 ± 0.14	0.88 ± 0.18	11.16 ± 1.71	24.62 ± 2.62
Placebo	0.23 ± 0.19	0.25 ± 0.25	0.27 ± 0.17	0.35 ± 0.20	2.49 ± 1.77

Values are the mean ± SEM, expressed as milligrams per 24 h.

The mean values for arterial compliance for the four active treatment periods (that is excluding the subjects assigned to placebo throughout) were: run-in, 18.5 ± 6.41; placebo, 19.7 ± 5.70; 40 mg isoflavone, 23.7 ± 5.27; and 80 mg isoflavone, 24.4 ± 4.93 arbitrary units (pressure as millimeters of Hg per flow as milliliters per min). The corresponding values for the three individuals assigned placebo throughout were 17, 16, 16, and 16 U, respectively (SDs of 8–9 for this small group). The placebo group, although very small, served to show the minimal changes with time and the consistency of compliance values with the run-in and placebo periods in the active stream. The data from the placebo-only arm were not subjected to statistical analysis either within the group or between the two groups.

By one-way repeated measures of variance that had a power of 0.9 to detect a less than 5% difference, a significant difference between treatments in the active stream was demonstrated ($P < 0.001$); this was attributable to a significant increase in compliance with both the 40- and 80-mg isoflavone doses compared with placebo (both $P < 0.05$). By paired *t* test, for placebo *vs.* 40 mg, the difference was significant at $P = 0.039$; and for placebo *vs.* 80 mg, the difference was significant at $P = 0.018$. Arterial compliance between the two treatment phases was not significantly different, nor was the difference between placebo and run-in periods.

Importantly the arterial compliance values correlated for individuals across placebo and the two isoflavone periods (between placebo and 40 mg: r = 0.60; $P = 0.032$; between placebo and 80 mg: r = 0.63; $P = 0.021$). This supports the homogeneity of the values across time.

Arterial pressures did not change significantly over time; mean pressures during the four periods are shown in Table 2 (the higher mean value for the 80-mg dose is attributable to a single subject whose pressure rose by 15 mm Hg). Plasma lipoprotein levels did not change significantly with treatment (Table 2), although there was a downward trend in LDL cholesterol and an upward trend in HDL cholesterol, giving an approximately 10% reduction in the LDL/HDL cholesterol ratio between placebo and treatment values, which, however, failed to reach statistical significance. The mean body mass index remained virtually unchanged during the trial. Thus, some of the biological variables that might influence arterial compliance did not change, and this strengthens the conclusion that the improvement in compliance was due primarily to the isoflavone supplements.

Urinary isoflavones showed satisfactory absorption, with a dose-dependent increase in urinary isoflavone output (Table 3). Subjects receiving placebo throughout the study showed low isoflavone excretion, although there was a small increase at the end due to an elevated value in one subject. Absorption was at least 25% and was consistent across the 10-week intervention period.

Discussion

This study was undertaken to test the assumption that dietary isoflavones in quantities consumed by Asian women, afforded protection from coronary heart disease (CHD) through mechanisms that might also explain the reduced prevalence of CHD among menopausal women in western cultures who take estrogen replacement therapy (12). Soy protein (which contains two of the four isoflavones used in this trial), like estrogen, has been reported to lower low density lipoprotein (LDL) cholesterol (13) and to inhibit oxidizability of LDL (7). The menopause, untreated, leads to a rise in LDL cholesterol (12), endothelial dysfunction (14), and reduced carotid arterial pulsatility (15). We had previously reported that arterial compliance is significantly improved in menopausal women taking soybean isoflavones, although we could not demonstrate a reduction in plasma cholesterol (5). Red clover contains the isoflavones present in soybean (genistein and diadzein) as well as their methylated precursors (biochanin A and formononetin). The total isoflavone dosages used in the present study were similar to those taken during the soy isoflavone trial, and the results are very similar to those of the previous trial in terms of the magnitude of the rise in arterial compliance and the absence of significant effects on plasma lipids and arterial pressure. There

have been no other reports of isoflavone effects on this important parameter of arterial function.

The order of increase in arterial compliance resembled that observed in this institute with estrogen replacement therapy in menopausal women (16). We recently showed that 5-week consumption of flaxseed oil, rich in the n-3 fatty acid, α-linolenic acid, increased arterial compliance significantly over that measured when subjects ate a saturated fatty acid-rich diet (17) or when overweight women lost weight (18).

Thus, systemic arterial compliance is susceptible to significant improvement within weeks. As the increasing stiffness of the large conduit arteries, especially the descending aorta, is believed to contribute to cardiovascular disease, including systolic hypertension, coronary artery insufficiency, and left ventricular dysfunction (19), the demonstration of reversibility points to functional causal components. Because endothelial events influence the smooth muscle layer in the artery and because endothelial function is rapidly modifiable, we favor a mechanistic change, based on endothelium-related arterial relaxation. Estrogen supplementation has been reported to raise flow-mediated dilation in the brachial artery, a conduit artery, in menopausal women (20).

The effect of dietary legumes on plasma lipids has been controversial. Several well controlled comparisons of soy protein *vs.* animal protein (generally casein) have shown minimal if any differences (13), yet other studies have shown impressive cholesterol lowering (13), raising the question, as yet unanswered, of whether differences in some active components account for this discrepancy. The present study confirms our previous finding (5) of failure to reduce plasma LDL cholesterol with the isoflavones genistein and daidzein from soybeans. Nevertheless, the LDL/HDL cholesterol ratio declined by about 10%, as both lipids changed in opposing directions. Although not significant in the present small group of women, the change does suggest potential benefit, as demonstrated for whole soy protein.

Although we did not specifically document menopausal symptomatology, two of five women in the placebo-only stream withdrew, and several others withdrew early from the active stream before the treatment phases. Several women cited a return of intolerable hot flashing that required resumption of hormone replacement therapy. The study was placebo controlled and carried out double blind. However, it was not a cross-over design, so that placebo preceded treatment in the active stream. We had intended to monitor this through a smaller group of placebo-only women (every fifth subject randomized) in whom mean arterial compliance changed minimally and was similar to the mean values obtained in the active stream during their run-in and placebo periods. It is therefore most unlikely that the significant increments in arterial compliance that occurred during the 10 weeks on the two doses of isoflavone (the responses to the 40- and 80-mg doses were similar) were not due to the treatment. Interestingly, a recent report in menopausal women treated with long term conventional hormone replacement showed a 24% greater value in arterial compliance (using the technique employed in the present study) compared with women not taking replacement hormones (21), a change that is almost identical to that with isoflavones in the present study.

As decreasing arterial compliance is a common cause of systolic hypertension and may lead to left ventricular dysfunction (19), it is of interest that recent trials have shown decreased compliance in subjects with clinical coronary artery disease compared with controls (22, 23).

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