Isoflavones Reduce Arterial Stiffness
A Placebo-Controlled Study in Men and Postmenopausal Women
Helena J. Teede, Barry P. McGrath, Lakmini DeSilva, Marja Cehun, Andriana Fassoulakis, Paul J. Nestel

Objective—This study was undertaken to address the vascular effects of isolated isoflavones as potential contributors to their cardioprotective properties, focusing on biochanin and formononetin.

Methods and Results—In a randomized, double-blind trial, 80 healthy subjects, 46 men and 34 women, 45 to 75 years of age, received isoflavones enriched in either biochanin or formononetin (precursors of genistein and daidzein; 80 mg/d) crossed over randomly with placebo in two 6-week periods. The end points were measured at baseline and after each intervention and included large artery stiffness (systemic arterial compliance and pulse wave velocity), endothelial function in conduit arteries (flow-mediated vasodilation), 24-hour ambulatory blood pressure, and total peripheral resistance. Isoflavone intervention significantly reduced arterial stiffness with improved systemic arterial compliance ($P = 0.04$; repeated-measures ANOVA, Bonferroni correction) attributable to a reduction in total peripheral resistance ($P = 0.03$) and a corresponding reduction in central pulse wave velocity ($P = 0.02$) compared with placebo. Isoflavones did not affect blood pressure ($P = 0.5$) or flow-mediated vasodilation ($P = 0.44$). Improvements seemed limited to formononetin-enriched isoflavones (adjusted $P = 0.06$). Formononetin treatment also reduced circulating vascular adhesion cell molecule-1 ($P = 0.01$).

Conclusions—In normotensive men and postmenopausal women, red clover isoflavones enriched in formononetin reduced arterial stiffness and total vascular resistance but had no effect on blood pressure. These effects may partly explain the lower cardiovascular risk in populations eating isoflavone-rich diets. (Arterioscler Thromb Vasc Biol. 2003;23:1066-1071.)

Key Words: isoflavones ■ arterial stiffness ■ endothelial function ■ blood pressure ■ vascular cellular adhesion molecule-1

Estrogens have been shown to exert beneficial cardiovascular effects; however, the vascular benefits of estrogens may be negated by their procoagulant effects. Phytoestrogens are ubiquitous, nonsteroidal, plant-derived compounds with structural similarity to estrogen that are ligands for the estrogen receptors ER$\alpha$ and ER$\beta$. Soybean and red clover are rich sources of isoflavones, a subgroup of phytoestrogens that includes daidzein, formononetin (daidzein precursor), genistein, and biochanin (genistein precursor).

Epidemiological studies have suggested reduced cardiovascular risk in populations consuming high-isoflavone diets. Investigations in animals and in humans have suggested that dietary soy protein containing isoflavones may reduce blood pressure, improve lipid profiles, and improve vascular function. The effects of isolated isoflavones on vascular function in humans have been less consistent. In 2 interventional studies using isoflavones, arterial stiffness has improved whereas FMD has been reported as unaffected and improved. A dietary survey of 180 women who were at least 20 years postmenopausal showed that one index of arterial stiffness, central pulse wave velocity (PWV), deteriorated as consumption of isoflavones fell.

Recent prospective observational studies have demonstrated that arterial stiffness is predictive of future cardiovascular events and that progression of arterial stiffness is significantly associated with increased cardiovascular mortality. Dietary interventions that lead to improvement in arterial stiffness might therefore be expected to reduce the risk of future cardiovascular events.

We have therefore investigated the effects of isolated isoflavones on a comprehensive set of vascular functions. In this double-blind, randomized crossover, placebo-controlled study in healthy normotensive men and postmenopausal women, we have investigated the vascular effects of 2 isolated red clover isoflavone tablets containing predominantly formononetin (precursor of daidzein) or predominantly biochanin (precursor of genistein). End points included pa-
rameters of large artery stiffness (systemic arterial compliance [SAC] and central PWV [PWV A-F], peripheral PWV [F-D]), endothelial function (flow-mediated vasodilation [FMD]), and total peripheral resistance (TPR), all measured by noninvasive Doppler ultrasound and tonometry techniques as well as 24-hour blood pressure. Urinary isoflavone excretion was measured to monitor background diet and compliance with treatment.

Methods

Study Design

Eighty healthy participants, 46 men and 34 postmenopausal women, completed a 15-week randomized, double-blind, crossover placebo-controlled trial. Participants aged 45 to 75 years, mean±SEM 54±0.7 years, were recruited from community advertisements. They had not consumed antibiotics, isoflavone products, or supplements for 3 months, nor had they taken estrogen therapy for 12 months before entry. Individuals received dietary counseling (verbal and written) on exclusion of all dietary sources rich in phytoestrogens for 3 months before and during the study. Dietary habits were stable throughout the study based on a 3-day food frequency diary completed during both stages of the study and demonstrating excellent compliance in avoiding isoflavone-containing foods.

Postmenopausal status was defined as 12 months of amenorrhea and follicle-stimulating hormone (FSH) >20 IUL. Exclusion criteria included smoking (last 5 years), diabetes, alcohol consumption >30 g/day, hypertension, abnormal uterine bleeding, cervical cytology or mammogram, and coexistent major illness. The study was approved by the two institutional human research and ethics committees (Southern Health and Alfred Hospitals, Melbourne, Australia). All participants gave written informed consent.

After a 2-week run-in phase, a baseline medical assessment was conducted, and height, weight, waist-hip ratio, and heart rate were measured. The subjects were then randomized to placebo or isoflavone treatment for 6 weeks. After this, a 1-week washout (placebo) was given before crossover to alternate therapy for 6 weeks. The total study duration was 15 weeks. Randomization was performed independently using computer-generated random numbers, with 40 participants allocated to each of the biochanin/placebo and formononetin/placebo arms, respectively.

The isoflavone tablets were prepared by Novogen Ltd (North Ryde, NSW, Australia) and were identical in appearance, bottled with the same labels. The 500-mg tablets, extracted from 10 g of red clover, contained 40 mg of isoflavones with 2 tablets daily with a dose of 80 mg. The product was tested to ensure content uniformity of isoflavone content and was provided in a single batch by Novogen Ltd (North Ryde). The two isoflavone preparations contained predominantly either biochanin (B) or formononetin (F). In B, the B:F ratio was 3.5, with 4% genistein and <1% daidzein. In F, the ratio F:B was 4.9, with <1% genistein and daidzein.

Isoflavone Excretion

Compliance with medication was assessed using urinary isoflavone excretion analyzed using high-performance liquid chromatography (HPLC) on an aliquot of urine from a 24-hour collection, correcting for urine volume to obtain the total excretion volume. This was performed at baseline and after each intervention. The assays were completed by Novogen using HPLC. The principal analytes were formononetin, daidzein, biochanin, genistein, and, in approximately 25% of subjects, the metabolites equol and O-desmethylyangiolensin. Aliquots (10 mL) of urine were mixed with 100 mL glacial acetic acid, and the mixture was incubated for 24 hours at 37°C. Isoflavones were eluted with 3 mL methanol from a C-18 solid-phase extraction column (Waters Pty Ltd) and separated by high-performance liquid chromatography. The HPLC system consisted of a 25-cm, 5-Nm C-18 stationary phase column (Symmetry, Waters Pty Ltd) and a gradient acetonitrile/water mobile phase. The limit of detection of the assay for each isoflavone was 5 ng/mL. The interassay coefficient of variation was <15%. Samples for all subjects were processed in one batch.

Vascular Parameters

The same experienced research assistants measured all vascular parameters. Studies were performed after an 8-hour fast (including avoidance of caffeine-containing drinks) in a darkened, quiet, air-conditioned clinical laboratory after 10 minutes of rest in the supine position.

Total Systemic Arterial Compliance and Total Peripheral Resistance

Noninvasive determination of blood flow and pressure waveforms was applied to determine SAC and TPR as previously described. Aortic volumetric blood flow was measured from a handheld 3.5- or 4-MHz continuous-wave Doppler flow velocimeter (Multidoplex MD1, Huntleigh Technology) at the suprasternal notch. Simultaneous driving pressure was ascertained by application tonometry with a pressure transducer (Millar Mikro-tip, Millar Instruments) over the right carotid artery. Pressure waveforms were calibrated against Dina map brachial mean artery pressures by linear interpolation (CRITIKON 1846 SX). Compliance over the total systemic arterial tree was calculated by the following formula:

\[
SAC = \frac{Ad}{R(Ps-Pd)}
\]

where Ad is the area under the BP diastolic decay curve from end systole to end diastole, Ps is end-systolic BP, Pd is end-diastolic BP, and R is total peripheral resistance.

Calculated SAC values were derived from estimated aortic root diameter based on body surface area, as previously described. Estimated aortic root diameter correlates well with echocardiography measurements of aortic root diameter (r=0.91, n=56; P<0.001).

Pulse Wave Velocity

PWV was determined from recorded pressure waveforms over both the aortofemoral (A-F) and the femoro-dorsalis pedis (F-D) arterial segments. Pulse transit time was defined as the time between the foot of simultaneously recorded pressure waves (end of diastole and beginning of systole), averaged over 10 cardiac cycles. Velocity was derived from pulse transit times divided by measured distances between the 2 recording sites, as previously described.

Brachial Artery Flow-Mediated Vasodilation

Brachial artery diameter was measured from B-mode ultrasound images captured on a Diasonics DRF-400 machine using a 10-MHz transducer while an ECG trace was simultaneously recorded. Longitudinal scanning identified the clearest image of the brachial artery above the elbow, with continuous scanning held for 30 seconds before and 4 minutes after ischemia, induced via a pneumatic tourniquet inflated around the upper arm to 40 mm Hg above systolic pressure for 4 minutes. Vessel diameter was measured during systole and diastole and averaged over 5 cardiac cycles. FMD was determined as the percentage change from baseline to 60 seconds after ischemia, the point of maximal dilation.

Twenty-Four-Hour Ambulatory Blood Pressure Monitoring

The primary efficacy ambulatory blood pressure variables were the mean 24-hour ambulatory systolic pressure (ABSBP) and diastolic blood pressure (ADBP). ABPM was performed using a portable lightweight device (Accutracker, Suntech Medical Instruments, Model II). The accuracy of the ABPM was confirmed in each subject by simultaneous auscultation and sphygmomanometry. Patients wore the device for 26 hours with measurements every 30 minutes during the day and hourly overnight. Subjects received verbal and written instructions on the monitors and completed a diary to record sleep, medication, posture activity, and symptoms.
Total Peripheral Resistance

TPR was derived in the process of measuring SAC from the same pressure and flow waveforms.

Repeatability

We have previously reported satisfactory repeatability for SAC, PWV (A-F), and PWV (F-D) with repeatability coefficients of 9.2%, 3.2%, and 5.0%, respectively. FMD curves were also very similar between visits 2 to 4 weeks apart. A repeatability substudy was completed in 25 participants during the present trial, with 2 consecutive baseline evaluations, and similar results were obtained. Based on these results, this study was powered to detect changes of 3% to 4% in SAC, <2% in PWV(A-F), 2% to 3% in PWV(F-D), and 15% in FMD.

Vascular Cellular Adhesion Molecule-1

This assay was carried out in 33 available plasma samples from among the 40 subjects treated with F-rich isoflavones after noting that arterial stiffness was reduced in this group. Vascular cellular adhesion molecule-1 was assayed by ELISA (R&D Systems). CVs for the intrassay and interassay were 5.0% and 9.2%, respectively.

Statistical Analysis

There was no important heterogeneity of variance, and, therefore, data are summarized as arithmetic means±1 SEM. The primary end point was change in outcome variables over the 6-week time period for each intervention. The level of significance was accepted at P<0.05. Statistical calculations were performed using the SYSTAT version 10 (SPSS Inc).

The outcome variables were divided into 2 sets. The first set included the primary end points, the descriptors of vascular function [SAC, PWV(A-F), PWV(F-D), FMD], and the second set included blood pressure (24-hour ambulatory SBP and DBP) and TPR as potential mechanisms for any observed changes in vascular function. Repeated-measures ANOVA (RM-ANOVA) was completed for each of the independent variables, which involved testing multiple hypotheses, thus increasing the risk of type I error (false-positive inference). This was controlled for by using the Ryan-Holm Stepdown Bonferroni procedure. Furthermore, for the outcomes of the RM-ANOVA, in which P<0.05, the analyses were repeated with the inclusion of intervention order as a second independent variable to assess if order of treatment was a confounding variable. The effect of sex was analyzed by adding sex as an independent variable. The initial analyses were carried out for the entire group of 80 subjects, comparing isoflavones from both sources against placebo. When a significant difference was found, additional analyses were carried out for the B-rich and F-rich isoflavones. For VCAM-1, RM ANOVA with appropriate adjustments was carried out.

Results

The baseline characteristics of the group were as follows: body mass index (25±1 kg/m²), heart rate (59±1 bpm), SAC (0.53±0.02 compliance units), PWV(A-F) (8.7±0.2 m/sec), PWV(F-D) (9.6±0.2 m/sec), FMD (9.0±0.6%), 24-hour ASBP (128±2 mm Hg), 24-hour ADBP (73±0.7 mm Hg), and TPR (16.6±0.7 resistance units). The two treatment arms (B and F) did not differ significantly in any of these parameters at baseline.

Dietary phytoestrogen intake was low both at baseline and throughout the study, based on food frequency questionnaires and confirmed by urinary isoflavone excretion (<0.5 mg was excreted per 24 hours). Likewise, after placebo, on average <0.5 mg of total isoflavones were excreted per 24 hours. After active therapy, isoflavone excretion rose significantly. On B, total isoflavone excretion increased >14 fold (P<0.005). On F, total isoflavone excretion increased >32 fold (P<0.005). No adverse effects of therapy were noted during the trial.

Vascular Compliance and Endothelial Function

There was no effect of time or order. Table 1 demonstrates mean (±SEM) systemic arterial compliance, pulse wave velocity, and flow-mediated vasodilation. The Figure shows the percentage changes in SAC and PWV(A-F) with isoflavone compared with placebo therapy. Isoflavone

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**TABLE 1. Isoflavones Versus Placebo: Effects on Vascular Function in All Subjects (n=80)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline Mean (±SEM)</th>
<th>After Intervention Mean (±SEM)</th>
<th>Change Mean (±SEM)</th>
<th>P Value</th>
<th>P’ (Adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemic arterial compliance, CU</td>
<td>0.52±0.02</td>
<td>0.57±0.02</td>
<td>0.05±0.02</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>PWV (A-F), m/s</td>
<td>8.69±0.2</td>
<td>8.39±0.2</td>
<td>-0.30±0.10</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>PWV (F-D), m/s</td>
<td>9.69±0.2</td>
<td>9.93±0.2</td>
<td>0.22±0.20</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Flow mediated dilatation, %</td>
<td>8.3±0.7</td>
<td>9.7±0.7</td>
<td>1.4±0.9</td>
<td>0.05</td>
<td>0.33</td>
</tr>
<tr>
<td>Total peripheral resistance, RU</td>
<td>17.0±0.7</td>
<td>16.0±0.6</td>
<td>-1.1±0.5</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>24-hour systolic blood pressure, mm Hg</td>
<td>126±2</td>
<td>125±1</td>
<td>-1.2±1.2</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>24-hour diastolic blood pressure, mm Hg</td>
<td>72±1</td>
<td>72±1</td>
<td>-0.3±0.5</td>
<td>0.01</td>
<td>0.04</td>
</tr>
</tbody>
</table>

P values refer to the analysis of change in each variable during isoflavone versus change during placebo therapy. P value is after adjustment for treatment order; P’ is after adjustment via the Ryan-Holm Stepdown Bonferroni procedure.
supplementation (combined data for both isoflavone preparations) reduced arterial stiffness as shown by improved SAC and PWV(A-F) compared with placebo. There were no significant effects on PWV(F-D) or brachial artery FMD.

Table 2 compares B and F individually with corresponding placebo periods and shows that arterial stiffness parameters were reduced only with F ($P=0.02$ for both SAC and PWV A-F; after adjustment for multiple comparisons, $P=0.06$ and $P=0.07$, respectively). There was a trend toward improvement in FMD with B treatment, although this was not seen after adjustment for multiple comparisons. There were no significant differences in any of the vascular functional responses to isoflavones between men and women.

**Blood Pressure and Peripheral Vascular Resistance**

Table 1 demonstrates mean (±SEM) blood pressures, including 24-hour ASBP and ADBP, as well as TPR. Blood pressure did not change significantly in either group, whereas TPR fell in the isoflavone compared with the placebo groups ($P$ adjusted=0.03) (Figure). There were no significant changes in heart rate in any of the groups.

**VCAM-1 Concentration in Plasma**

Circulating VCAM-1 concentrations in plasma, expressed as median (25% to 75% CIs), were 11% lower after formononetin therapy (330 [287 to 409] ng/mL), compared with (361 [288 to 450] ng/mL) after placebo therapy ($P=0.009$).

**Discussion**

In this crossover study, 6 weeks of consumption of combined isoflavones, derived from red clover, by 80 healthy normotensive men and postmenopausal women significantly reduced arterial stiffness as measured by improvement in large artery biomechanics, including systemic arterial compliance and central pulse wave velocity (Table 1). Ambulatory blood pressure did not change; however, total peripheral resistance improved. Endothelial function, as reflected by mean brachial artery flow-mediated vasodilation, did not change with isoflavone therapy, although circulating VCAM-1 levels, an index of endothelial dysfunction, were lowered in the group treated with formononetin-enriched isoflavones (F). On additional analysis of the effects of specific isoflavones (Table 2), formononetin (F) and not biochanin (B) appeared largely responsible for the improvement in arterial stiffness, with the differences between biochanin and formononetin approaching significance after statistical adjustment (adjusted $P=0.06$). There were no differences in the vascular functional responses between men and women.

In the present study, although levels of isoflavone excretion were similarly low in both groups at baseline and during placebo therapy, 6 weeks of isoflavone therapy resulted in significant increases in urinary isoflavones, with greater increases in the F compared with the B group. This is consistent with previous pharmacokinetic studies, where the excretion of daidzein and formononetin have been greater than those of genistein and biochanin. However, pharmacokinetic studies have also suggested that the volume of distribution for B is greater than F and that B is more bioavailable than F. To date, these issues remain unresolved; however, it is generally agreed that the greater urinary excretion of F does not indicate greater absorption and bioavailability.

In the present study, the vascular effects of isoflavones included an increase in SAC, which was partly attributable to a reduction in TPR. At a physiological level, the increase in SAC is likely to have been associated with an increase in cardiac output, because blood pressure and heart rate did not change. Improved left ventricular function is one important consequence of reduced arterial stiffness. This increase in SAC is consistent with our previous work, where a 23% increase was noted during isolated mixed isoflavone treatment from red clover (40 to 80 mg including F, B, G, and D) as well as with isolated soy isoflavones. In a large placebo-controlled trial in 210 men and women consuming soy protein containing genistein and daidzein, we have demonstrated a nonsignificant trend toward improvement in vascular function encompassing SAC and central PWV. It should be noted that in the present study, SAC rose significantly only in those subjects who were taking formononetin, an isoflavone not found in soy. Although conversion from formononetin to daidzein occurred, 25% of formononetin was excreted intact, suggesting that formononetin may also exert a direct effect on the vasculature.

PWV, arguably the most robust of the noninvasive markers of vascular function in humans, is an index of arterial stiffness and is inversely related to arterial compliance. PWV correlates with cardiovascular risk factors and has been shown to predict cardiovascular morbidity and mortality in chronic renal failure and in essential hypertension, where a 1 m/sec increase in PWV equated to a 39% increase in all-cause
mortality. In the Rotterdam study, arterial stiffness was associated significantly with the degree of atherosclerosis, and Meaume et al showed that abnormal aortic PWV predicted cardiovascular morbidity in elderly people. Central PWV (A-F) reflects the function of the large central arteries, similar to SAC. PWV(F-D) that was not changed by isoflavone treatment in this study reflects peripheral arterial stiffness across the femoro-dorsalis vascular bed and is influenced by other factors, including sympathetic tone. Because of the short duration of the study, it is likely that the observed changes in central PWV are related to altered vascular tone rather than to structural changes.

The effects of isoflavones on vascular function and structure resemble those of estrogen. In animal studies, coronary vasodilatation improves with estrogen and isoflavone administration. Isoflavones are vasodilatory, inhibit responses to vasoconstrictors, and protect the endothelium from oxidative damage, whereas estrogen and isoflavones induce similar dose-dependent vasculoprotective effects after carotid injury. The effects after soy protein are mediated partly by isoflavones in soy, because studies in monkeys have shown a 90% reduction in atherosclerosis comparing soy/isoflavones with casein, whereas soy protein depleted of isoflavones was intermediate in its effect. Although putative substances in soy protein may also be responsible for some of the benefit, it is probable that the isoflavone content is of most relevance. Some of the discrepant results in the literature may therefore reflect differences between soy protein and isolated isoflavones.

In the present study, we have noted no change in FMD in men or postmenopausal women with isolated isoflavones. This is consistent with a previous study by Simons et al in 20 women where no improvement in FMD was noted with the same dose of isolated isoflavones (80 mg), although Squadrito et al have recently reported improved FMD after 6 months of supplementation with genistein. Genistein, infused directly into the brachial artery, has also been shown to cause significant nitric oxide–related vasodilation in the human forearm. Thus, the effect of isoflavones on endothelial function is conflicting, although the evidence of a direct vasodilatory response to infused genistein does indicate such an effect. Vasodilatory effects equivalent to that of 17β-estradiol have been demonstrated with several metabolic derivatives of daidzein in rat aortic ring preparations.

Estrogen, along with improving arterial function, has also been shown to suppress vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 is one of a group of molecules expressed in endothelium that is involved in atherosclerosis. It binds circulating monocytes to endothelium and is induced by proinflammatory macromolecules and cytokines. Increased expression of VCAM-1 in endothelium is a manifestation of endothelial dysfunction, and circulating VCAM-1 concentrations correlate with the extent of human atherosclerosis evaluated by carotid intima-media thickness and with future cardiovascular death. The reduction in circulating VCAM-1 noted in the present study suggests an effect of isoflavones on endothelial function. The discrepancy between circulating VCAM-1 and FMD effects may relate to the fact that these markers reflect differential functions of the endothelium; however, this requires additional research.

In this study, no effects of isoflavones were noted on ABP readings. This finding is consistent with previous work by Hodgson et al, who showed that 8 weeks of isolated isoflavones (55 mg/d) did not alter ABP in normotensive subjects. In contrast, the findings from our dietary soy/isoflavone study in normotensive subjects noted a significant reduction in blood pressure. Given these discrepancies and the fact that estrogen does not reduce blood pressure, the hypotensive effects of soy may not be mediated by estrogenic mechanisms and be unrelated to isoflavones.

The mechanisms of action of estrogenic compounds, including isoflavones, are varied and complex. At ERα and ERβ, isoflavones can act as agonists or antagonists or have selective agonist/antagonist activity, additionally modified by variable affinity for the ER subtypes and by tissue specificity based on coactivators and corepressors. Furthermore, each specific isoflavone has different characteristics as a ligand for the receptors, especially for ERβ, which is expressed more in vasculature than ERα. Indeed, it has been suggested that individual effects need to be characterized for each estrogenic compound on each specific estrogenic end point.

In conclusion, isolated formononetin-rich isoflavones (daidzein precursor) significantly improved arterial stiffness in healthy men and postmenopausal women without improving flow-mediated dilation or blood pressure. This reduction in arterial stiffness may be an important factor in the apparent reduction in cardiovascular risk demonstrated in epidemiological studies.

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References


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