ORIGINAL ARTICLE

Effects of dietary supplementation with isoflavones from red clover on ambulatory blood pressure and endothelial function in postmenopausal type 2 diabetes

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Objective: The aim of this study was to determine whether dietary supplementation with isoflavones from red clover affected ambulatory blood pressure and forearm vascular endothelial function in postmenopausal type 2 diabetic women.

Design: Sixteen postmenopausal type 2 diabetics treated with diet or oral hypoglycaemic therapy completed a randomized double-blind crossover trial of dietary supplementation with isoflavones from red clover (approximately 50 mg/day) for 4 weeks compared to placebo. Twenty-four-hour ambulatory blood pressure recordings and forearm vascular responses to acetylcholine, nitroprusside and L-nitromonomethylarginine (L-NMMA) were measured at the end of each treatment period.

Results: Mean daytime systolic and diastolic blood pressures were significantly lower during isoflavone therapy compared to placebo (-8.0 ± 3.4 and -4.3 ± 1.9 mmHg respectively, $p < 0.05$). The increase in forearm vascular resistance following L-NMMA was significantly greater during isoflavone supplementation (20.9 ± 6.5) than placebo (3.7 ± 2.9 arbitrary units, $p < 0.05$), suggesting an improvement in basal endothelial function. Plasma lipoproteins, glycated haemoglobin and forearm vascular responses to acetylcholine and nitroprusside did not differ significantly between isoflavone and placebo therapy.

Conclusion: Isoflavone supplementation from red clover may favourably influence blood pressure and endothelial function in postmenopausal type 2 diabetic women.

Keywords: blood pressure, endothelial function, isoflavones, red clover, type 2 diabetes
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Introduction

Cardiovascular disease is a major cause of death in women with type 2 diabetes [1]. The prevalence of hypertension and dyslipidaemia in type 2 diabetic women is high [2,3] and contributes to the increased risk of cardiovascular events along with hyperglycaemia and insulin resistance [3]. Impaired endothelial function is associated with hypertension, dyslipidaemia, diabetes and menopause [4] and is a significant predictor of the risk of cardiovascular events [5,6]. Endothelial dysfunction is believed to be a key factor in the pathogenesis of atheroma and the precipitation of cardiovascular events [7].

Oestrogen has been demonstrated to improve basal endothelial function in perimenopausal women [8] as well as having beneficial effects on blood pressure [9] and lipoproteins [10]. However, prospective randomized trials of hormone replacement therapy (HRT) with oestrogen and progestogens have reported adverse

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cardiovascular outcomes [11,12]. Isoflavones have partial oestrogen agonist-like effects [13,14]. Soy protein, which is high in isoflavone content, has been demonstrated to have beneficial effects on endothelial function in animal models of atheroma [15]. The present study investigated the effects of dietary supplementation with isoflavones from red clover on 24-h ambulatory blood pressures and endothelial function measured by forearm vascular responses to acetylcholine and the nitric oxide synthase inhibitor L-NMMA and to the nonendothelial-dependent vasodilator nitroprusside in postmenopausal type 2 diabetics.

Methods

The study consisted of a randomized double-blind 4-week trial of therapy with isoflavones compared to 4 weeks of placebo. Each treatment phase was separated by a 4-week placebo wash out period, and the study was preceded by a 4-week single-blind placebo run in phase during which the subjects adhered to a low isoflavone diet. Any patients previously receiving antihypertensive or lipid-modifying therapy had these medications ceased at the start of the run in phase. A study population of 16 patients completing the trial was planned.

The inclusion criteria were postmenopausal status defined as amenorrhea for 12 months and a serum follicle-stimulating hormone level >40 U/l, type 2 diabetes diagnosed by WHO criteria for at least 6 months treated with either oral hypoglycaemic diet or a combination of these and a seated blood pressure of $<150/90$ mmHg on no antihypertensive therapy or antihypertensive monotherapy.

Exclusion criteria included significant concomitant disease other than previous cardiovascular disease or cardiovascular disease which would interfere with the successful completion of the study (including heart failure), regular alcohol consumption >40 g/day, smoking, the use of major tranquillisers or tricyclic antidepressant drugs, inability to adhere to a low isoflavone diet, a glycated haemoglobin of $>10\%$ and age >75 years.

Two tablets of isoflavones or an identical placebo were taken with the morning meal throughout the study. A low isoflavone diet was continued throughout the entire study, and dietary compliance was enquired about at each visit. Each isoflavone tablet contained 25 mg of formononetin, 2.5 mg of biochanin and <1 mg of genistein and daidzein. Formononetin and biochanin have been demonstrated to be extensively converted to daidzein and genistein, respectively, in humans [16]. During the placebo phases of the study, the subjects took two tablets which were identical in appearance and taste to the active medication but which contained no isoflavones. The subjects returned the bottles containing their study medication including any remaining tablets at the end of each study period for tablet count to assess compliance. The bottles of tablets supplied to the patients for each study period contained an excess of tablets above that needed. At the end of the run in and on treatment periods, blood was taken for serum lipids, glycated haemoglobin estimations and coagulation studies (plasma D-dimer and fibrinogen levels). All biochemical and haematologial tests were performed using standard commercial assays by the South-eastern Sydney Area Laboratory Service. The South-eastern Sydney Laboratory Service holds current accreditation with the National Analytical Testing Authority (NATA), an authority which regulates the approval of laboratories within Australia and ensures that strict quality assurance is maintained.

At the end of each 4-week treatment period, the subjects attended at 0900 hours, having abstained from caffeine for 12 h and alcohol for 24 h and having fasted from 2400 hours the preceding night. Measurements of forearm vascular responses to acetylcholine, nitroprusside and L-NMMA were then taken. The subjects were then fitted with a SpaceLabs 90207 ambulatory blood pressure monitor (SpaceLabs, Redmond, WA, USA) for the measurement of 24-h ambulatory blood pressure profiles. The SpaceLabs 90207 monitor has been previously validated [17]. The monitors were calibrated against a standard mercury sphygmomanometer, prior to each study.

Measurement of Forearm Vascular Resistance Responses

Forearm vascular resistance responses were measured using a Hokanson strain gauge plethysmograph (Hokanson, Bellevue, WA, USA). A 27.5-gauge needle was inserted in the brachial artery and a paediatric cuff placed around the wrist and inflated to 200 mmHg to exclude the hand from the circulation. A blood pressure cuff placed around the upper arm was inflated intermittently to a pressure of 60 mmHg to exclude venous outflow from the forearm using 30-s cycles of inflation and deflation. The rate of change of volume in the forearm was detected using a mercury strain gauge wrapped around the widest point of the forearm. Five or six cycles were repeated for each infusion rate of each drug and 15 min of rest was allowed between the infusion of each drug, by which time baseline forearm vascular resistance had returned to normal.

The drugs infused were acetylcholine 3, 6, 12, 24 μg/min, nitroprusside 2, 8 and 16 μg/min and L-NMMA 8 mmol/min. Dose–responses were studied firstly for acetylcholine, then for nitroprusside following which L-NMMA was infused for 10 min. A further dose–response study was then performed for acetylcholine following nitric oxide synthase inhibition with L-NMMA. Blood pressure was monitored at 1-min intervals using a SpaceLabs 90207 ambulatory blood pressure monitor, with the cuff wrapped around the arm contralateral to that used for plethysmography studies. Forearm vascular resistance was calculated as mean arterial pressure measured from the SpaceLabs monitor divided by forearm blood flow. L-NMMA was purchased from the Sigma Chemical Company (St Louis, MO, USA) and prepared for human use by the St George Hospital, Department of Nuclear Medicine. The acetylcholine used was Michol (Ciba Vision, Castle Hill, NSW, Australia) and sodium nitroprusside was obtained from David Bull Laboratories (Parkville, Victoria, Australia).

24-h Ambulatory Blood Pressure Monitoring

The SpaceLabs 90207 ambulatory blood pressure recorder was programmed to record blood pressure and heart rate every 30 min between 0800 and 2200 hours and every 60 min for the remaining period of the 24 h. The subjects were instructed to perform their normal daily activities. Hourly blood pressure measurements were calculated from the mean of half-hourly recording between 0800 and 2200 hours. Measurements between 0600 and 2200 hours were defined as daytime readings while measurements between 2200 and 0600 hours were considered to be nighttime readings. Mean daytime and nighttime blood pressure values were calculated from these two periods. Ambulatory blood pressure recordings were accepted if $> 80\%$ of the programmed readings were obtained and no editing of blood pressures was performed.

Statistical Analysis

The data were analysed by repeated measure analysis of variance or Student's paired *t*-test using the Statistica 6.0 software (Statsoft, Tulsa, OK, USA). Treatment order was included in the analysis as an independent variable to test for treatment-order or carry-over effect. The sample size was based on subject between-day variability from previous studies in the same research unit and had an 80% power to detect a 15% difference in forearm vascular responses between isoflavone and placebo therapy. Results are expressed as mean ± s.e.m. except where otherwise indicated.

Ethical Approval

The study was approved by the South-east Sydney Area Health Service Ethics Committee (Southern Section) as all subjects provided written informed consent.

Results

Nineteen women entered the study, of whom successful data were obtained for forearm vascular resistance studies on 16 and ambulatory blood pressure data on 18. There were equal numbers of subjects in each treatment order. One subject had an unsatisfactory ambulatory blood pressure recording on one study day and three subjects (including a subject with an unsatisfactory ambulatory blood pressure recording) had unsatisfactory forearm vascular resistance studies on at least one study day because of difficulty maintaining intrabrachial artery infusions. The mean age (±s.e.m.) of the women was 62 ± 2 years (range 53–74) and their duration of type 2 diabetes was 7.4 ± 1.3 years (0.5–20.0). Their average time since menopause was 11.2 ± 1.9 years (3.0–35.0) and their body mass index at baseline was 29.6 ± 1.2 kg/m^2 (21.2–39.8). Their biochemical results at the end of the run in period were fasting plasma glucose 7.4 ± 0.65 mmol/l (3.70–10.30), glycated haemoglobin 7.16 ± 0.21% (5.80–9.10), cholesterol 5.49 ± 0.33 (3.70–10.30), triglycerides 1.77 ± 0.24 (0.70–4.50), high-density lipoprotein (HDL) cholesterol 1.25 ± 0.06 mmol/l (0.80–1.80), low-density lipoprotein (LDL) cholesterol 3.31 ± 0.27 (0.90–6.60) (all mmol/l).

Six subjects had previously been receiving antihypertensive monotherapy [angiotensin converting enzyme (ACE) inhibitors], while seven had previously been receiving lipid-lowering therapy. These treatments were stopped prior to the 1-month run in phase. Concomitant medication, including oral hypoglycaemic therapy, was not altered in any of the subjects during the course of the study. Four of the subjects were receiving no diabetic drug therapy and were managed on diet alone. The remaining 15 subjects were receiving dietary therapy and drug therapy, which consisted of metformin plus sulphonylureas (11 subjects), metformin alone (three subjects) or sulphonylureas (one patient). In addition, one patient was receiving acarbose. Other concomitant medications included anticonvulsants (three subjects), non-steroidal anti-inflammatory drugs or analgesics (three subjects), bone sparing agents (vitamin D ± calcium) (four subjects), proton pump inhibitors (one subject) and low-dose aspirin (two subjects). None of the subjects consumed more than 20 g of alcohol per day.

Greater than 90% of the ambulatory blood pressure recordings were valid for each of the 18 subjects included in the analysis of ambulatory blood pressure recordings. The results for mean 24-h daytime and nighttime blood pressures are presented in table 1. Mean daytime systolic and diastolic blood pressures were significantly lower during isoflavone treatment, and this was associated with a non-significant trend towards a lower overall 24-h mean systolic blood pressure during isoflavone therapy. Nighttime systolic and diastolic blood pressures, and daytime, nighttime and 24-h mean heart rates did not differ between the isoflavone and placebo therapies. Twenty-four-hour blood pressure profiles are shown in figure 1. There was no overall statistically significant difference between isoflavone and placebo therapies in the 24-h blood pressure profiles when analysed by repeated measures analysis of variance.

Baseline forearm vascular resistance did not differ significantly between the two therapies (table 1). Forearm vascular responses to acetylcholine and nitroprusside

Table 1 Mean daytime and nighttime systolic and diastolic blood pressures (SBP and DBP) and heart rates (HRs) during isoflavone or placebo therapy and baseline forearm vascular resistance (VR) measured by strain gauge plethysmography

	Active	Placebo	Difference ± s.e.	p
Mean 24 h				
SBP (mmHg)	123.9 ± 5.3	130.1 ± 4.4	−6.1 ± 3.3	0.077
DBP (mmHg)	70.3 ± 2.5	73.2 ± 3.2	−2.9 ± 1.9	0.155
HR (bpm)	82.2 ± 2.2	80.2 ± 9.3	+1.8 ± 1.5	0.250
Mean daytime				
SBP (mmHg)	126.3 ± 4.9	134 ± 4.6	−8.0 ± 3.4	0.029*
DBP (mmHg)	72.7 ± 2.4	77.1 ± 2.4	−4.3 ± 1.9	0.037*
HR (bpm)	85.3 ± 2.2	83.0 ± 2.23	+2.2 ± 1.9	0.257
Mean nighttime				
SBP (mmHg)	116.8 ± 5.7	120.6 ± 5.0	−3.8 ± 3.6	0.294
DBP (mmHg)	62.3 ± 2.8	64.4 ± 2.7	−2.4 ± 2.2	0.356
HR (bpm)	71.8 ± 2.7	71.8 ± 2.7	−0.1 ± 1.6	0.0945
VR (arbitrary unit)	33.8 ± 4.4	37.2 ± 4.9	−3.3 ± 6.0	0.599

Values are mean ± s.e.m.
*Statistically significant difference.

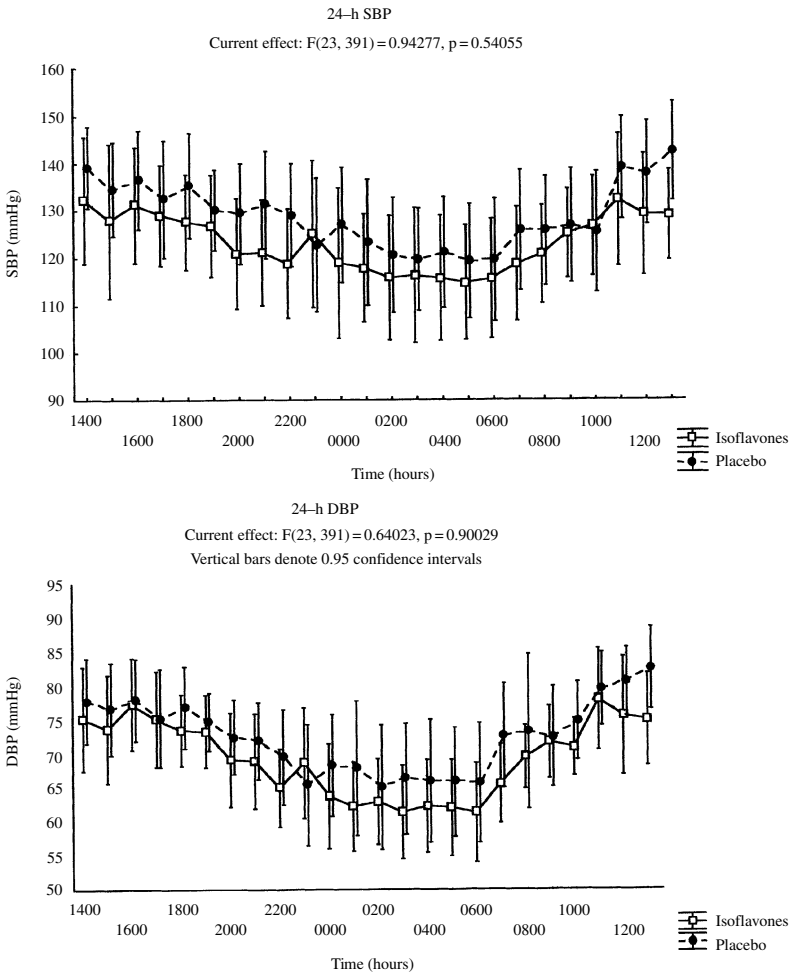

Fig. 1 Twenty-four-hour ambulatory blood pressure profiles during isoflavone and placebo therapy.

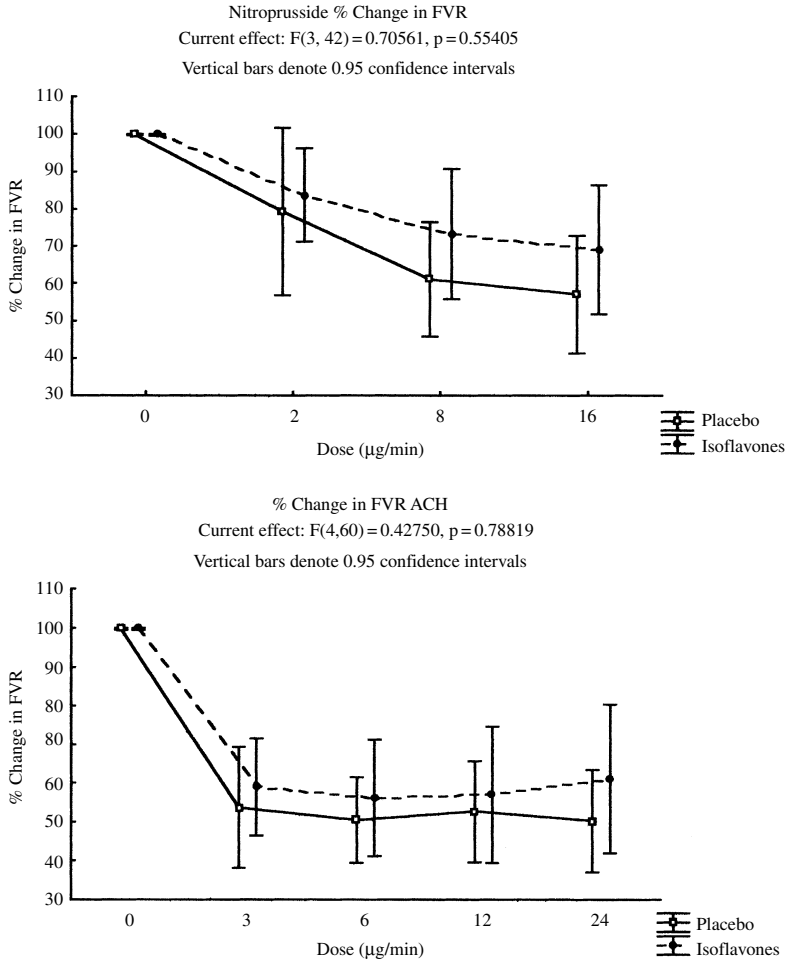

Fig. 2 Percentage changes in forearm vascular resistance (FVR) from baseline for acetylcholine and nitroprusside during isoflavone and placebo therapy.

are shown in figure 2. Both the change in forearm vascular resistance and the percentage change from baseline in forearm vascular resistance were similar during isoflavone therapy and placebo therapy for acetylcholine and nitroprusside; however, the increase in forearm vascular resistance in response to L-NMMA was significantly higher during isoflavone therapy and during placebo therapy (figure 3). Forearm vascular responses to acetylcholine following L-NMMA infusion did not differ significantly between isoflavone and placebo therapies (data not shown). Intrabrachial arterial infusion of acetylcholine, nitroprusside and L-NMMA did not measurably alter the systemic mean arterial pressure.

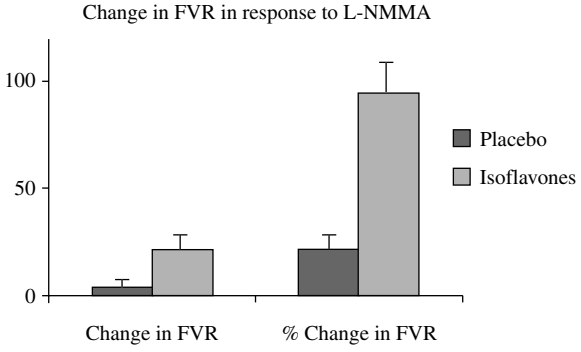

Fig. 3 Change in forearm vascular resistance (FVR) and percentage change in FVR in response to L-NMMA during isoflavone therapy. Differences between isoflavone and placebo therapy were statistically significant for both measurements ($p < 0.05$).

Body mass index, clinic blood pressures, heart rate, glycated haemoglobin, fasting blood glucose, serum cholesterol, triglycerides, HDL and LDL cholesterol did not differ significantly between the placebo and isoflavone periods, although there were non-significant trend towards a more favourable changes from baseline on active therapy than on placebo for total cholesterol and LDL cholesterol. Total cholesterol levels changed from baseline by $+1.16 \pm 1.79$ mmol/l (mean $\pm$ s.d.) on placebo compared to $+0.26 \pm 0.86$ mmol/l on active therapy ($p = 0.092$). The corresponding changes for LDL

cholesterol levels were $+0.62 \pm 0.89$ vs. -0.05 ± 0.99 mmol/l ($p = 0.061$), for triglycerides $+0.50 \pm 1.84$ vs. $+0.11 \pm 2.21$ mmol/l ($p = 0.543$), for HDL cholesterol $+0.06 \pm 0.23$ vs. $+0.05$ mmol/l ($p = 0.932$) and for glycated haemoglobin -0.05 ± 0.89 vs. $-0.04 \pm 0.99\%$ ($p = 0.963$). D-Dimer and plasma fibrinogen levels did not differ significantly between the placebo and active phases of the study (D-dimer 0.23 ± 0.05 and 0.20 ± 0.04 μg/ml, $p = 0.271$; fibrinogen 3.12 ± 0.22 and 3.13 ± 0.24 g/l, $p = 0.965$) (mean ± s.e.m.).

No adverse events were reported by the subjects and compliance with medication was 100%. There were no significant carry-over or treatment-order effects.

Discussion

The results of this study demonstrated significant improvement in daytime systolic and diastolic blood pressures in postmenopausal type 2 diabetics when receiving dietary isoflavone supplements from red clover. Nighttime blood pressures did not significantly change. It should be noted that the division of blood pressure readings into daytime and nighttime using arbitrary time points is not identical to measuring awake and sleeping blood pressures, as patients do not necessarily fall asleep and wake at the same times. Dietary supplementation with soy, which has a high content of isoflavones, has been demonstrated to reduce clinic blood pressures in men and in postmenopausal non-diabetic women [18,19], while one study found no effect on 24-h ambulatory blood pressures in postmenopausal type 2 diabetic women [20]. Soy and soy protein extracts contain substances in addition to isoflavones, and it is possible that other components contribute to a fall in blood pressure during soy supplementation. Dietary supplementation with isoflavones from red clover has been reported not to significantly alter ambulatory blood pressures in a study of 59 men and 20 women [21]. Differences in the proportions of isoflavones in the supplements and in the populations studied may explain the differences in results between the present study and theirs.

This study is the first to report improvements in blood pressure during dietary supplementation with isoflavones from red clover and the first to report improvements in the blood pressure of postmenopausal type 2 diabetic women during dietary supplementation with extracts that are rich in isoflavones. The results of our study are similar to those previously described during HRT in postmenopausal women [9,22,23], suggesting that the effect may be due to oestrogen-like activity of isoflavones. Oestrogens may lower the blood pressure by improving endothelial function [8] via a calcium antagonist effect [24] or by reducing sympathetic activity [25]. Dietary supplementation with the isoflavone genistein has been demonstrated to improve endothelial function in ovariectomized rats [15] and to directly relax rat mesenteric arteries *in vitro* [26], possibly via a calcium antagonist effect [27]. However, in contrast to a previous study of the effects of oestrogen replacement therapy on ambulatory blood pressure on postmenopausal women [9], the fall in blood pressure associated with isoflavone supplementation in the present study was not accompanied by a reduction in heart rate or basal forearm vascular resistance. It was not possible to accurately determine the mechanism of blood pressure reduction associated with isoflavone supplementation in the present study. Further studies are required to clarify this.

Isoflavone therapy significantly increased the forearm vascular response to nitric oxide synthase inhibitor L-NMMA but did not influence forearm vascular vasodilator response to acetylcholine. This suggests that isoflavone supplementation in postmenopausal type 2 diabetics improves basal, but not stimulated, forearm endothelial function in resistance vessels. This should not be confused with a change in actual basal forearm vascular resistance, which was not significantly altered. This is similar to the effect on endothelial function described, following chronic oestrogen replacement therapy in premenopausal non-diabetic women [8]. It is possible that the improvement in blood pressure and endothelial function during isoflavone therapy was the result of oestrogen-like activity of isoflavone supplementation.

Improvements in endothelial function and blood pressure could result because of beneficial effects of isoflavone supplementation on plasma lipids or insulin sensitivity. We found no significant change in plasma cholesterol, LDL, HDL, triglyceride or glycated haemoglobin levels; however, glycated haemoglobin levels are not a very sensitive measure of insulin resistance, particularly after only 4 weeks of therapy. A previous study using the same formulation of isoflavones from red clover found no significant effects on lipoproteins in postmenopausal women [28]. However, a recent study of dietary supplementation with soy protein in postmenopausal type 2 diabetics found small, but statistically significant, reductions in insulin resistance, total cholesterol and LDL cholesterol levels. No significant changes in clinic blood pressures were observed [29]. No significant changes in plasma lipids or glycated haemoglobin levels between placebo and active therapy were observed in the present study, although a trend towards more beneficial changes in total and LDL cholesterol

were noted on active isoflavone treatment. The study was not powered to detect relatively small changes in plasma lipids or glycated haemoglobin levels. It is possible that small, but beneficial, changes in plasma lipoproteins and insulin sensitivity occurred but were undetected in the present study and contributed in part to the improvements in blood pressure and basal endothelial function.

The study that we report has a number of potential limitations. Urinary isoflavones were not measured during the run in or treatment periods. It is therefore possible that the consumption of isoflavone-containing foods by subjects during the placebo phases of the trial has weakened the power of the study. This would have been of greater concern had no significant effects of treatment been found. The subjects were questioned about their dietary compliance at each visit and all reported good compliance. Furthermore, the high level of compliance found on tablet counts supports the proposition that the study population adhered to the protocol. The study was of relatively short duration and it is possible that longer term interventions may have different effects. The beneficial effects of isoflavone supplementation on blood pressure and endothelial function in the present study occurred in women who were not receiving ACE inhibitors or HMGCoA reductase inhibitors which are widely used in type 2 diabetics. It is unknown whether these beneficial effects would be observed in the presence of ACE inhibitor or HMGCoA reductase inhibitor therapy. Finally, the study was performed in subjects with a wide range of glycaemic control. It is possible that the observed effects may not be apparent in patients with tight glycaemic control.

In conclusion, the study has demonstrated for the first time that dietary supplementation with isoflavones from red clover improves blood pressure and basal endothelial function in postmenopausal type 2 diabetic women. Isoflavone supplementation may be of value in the management of postmenopausal type 2 diabetics, particularly those who are not receiving oestrogen therapy.

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