Soy isoflavones improve cardiovascular disease risk markers in women during the early menopause

[1]Thozhukat Sathyapalan, [1]Myint Aye, [2]Alan S Rigby, [3]Natalie J Thatcher, [4]Soha R Dargham, [5]Eric S Kilpatrick, [4]Stephen L Atkin

[1] Academic Diabetes, Endocrinology and Metabolism, Hull York Medical School, University of Hull, Hull UK

[2] Department of Academic Cardiology, University of Hull, UK

[3] European Food Safety Authority, Parma, Italy

[4]Weill Cornell Medical College Qatar, Education City PO Box 24144, Doha, Qatar

[5] Department of Clinical Chemistry, Sidra Medical and Research Center, PO Box 26999, Doha, Qatar

Corresponding author

Dr SL Atkin, Weill Cornell Medical College Qatar, Education City, PO Box 24144, Doha, Qatar.

Tel: +97455639807
Fax +97444928422
Email: sla2002@qatar-med.cornell.edu

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38

39 Abbreviations. CVR = cardiovascular risk; SPI = soy with isoflavones; SP soy protein
40 alone; CVD = cardiovascular disease; hsCRP= high sensitive C-reactive protein; CV=
41 coefficient of variation; HDL= high density lipoprotein cholesterol; LDL= low density
42 lipoprotein cholesterol;

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Abstract

Background: Hormone replacement therapy may be beneficial for cardiovascular disease risk (CVR) in post-menopausal women. Soy isoflavones may act as selective estrogen receptor modulators. The aim of this study was to evaluate whether soy isoflavones had an effect on CVR markers.

Methods: The expected 10-year risk of cardiovascular disease and mortality were calculated as a secondary endpoint from a double blind randomised parallel study involving 200 women (mean age 55 years, Caucasian, Hull, UK, 2012) in the early menopause who were randomised to 15g soy protein with 66mg isoflavone (SPI) or 15g soy protein alone (depleted of all isoflavones; SP) given as a snack bar between meals daily for 6 months. Age, diabetes, smoking, blood pressure and lipid profiles were used to calculate CVR using the Framingham CVR engine.

Results: SPI treatment resulted in a significant reduction in the metabolic parameters and systolic blood pressure compared to SP ($p<0.01$). There were no changes in fasting lipid profile and diastolic blood pressure with either treatment. At 6 months, changes in these parameters with SPI treatment were reflected in a calculated 27% ($p<0.01$) reduction in 10 year coronary heart disease risk, a 37% ($p<0.01$) reduction in myocardial infarction risk, a 24% ($p<0.04$) reduction in cardiovascular disease and 42% ($p<0.02$) reduction in cardiovascular disease death risk.

Conclusions: Supplementation with soy protein with isoflavones for 6 months significantly improved CVR markers and calculated CVR at 6 months during early menopause compared to soy protein without isoflavones.

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Introduction

Cardiovascular disease (CVD) is uncommon in premenopausal women, but at the menopause there is an increased and recognised cardiovascular disease risk (CVR) for coronary heart disease (CHD) (1). Analysis of the Women's Health Initiative study suggested that women treated with hormone replacement therapy (HRT) did not have an increased risk of CHD and indeed it may result in reduced CVR if estrogen was given within 10 years of their menopause compared to those who were not on HRT (2). Soy isoflavones can act as selective estrogen receptor modulators that may have beneficial effects on CVR indices (3, 4). Although there are studies comparing the effect of whole soy, soy protein and isoflavones showing variable effect on cardiovascular disease risk markers (5-8), there are no studies looking into the effect of combined soy protein and isoflavones with isoflavone free comparator in post-menopausal women.

The isoflavones are heterocyclic phenols that mainly comprise genistein, daidzein and glycitein that have both in vitro and in vivo estrogenic effects due to their structure that is similar in structure to 17 beta estradiol (3). Equol is produced by the metabolism of the isoflavone daidzein by intestinal bacteria. In Western countries, 30% to 50% of individuals metabolize daidzein into equol and are known as equol producers. It has been suggested that equol production may be the source of benefit from isoflavones(9). Isoflavones can potentially improve cardiovascular health by maintaining endothelial integrity and increase nitric oxide, prostacyclin release leading to endothelium-dependent vasodilation (10). Isoflavones can also inhibit vascular smooth muscle proliferation and contraction by activating cAMP- and cGMP-dependent pathways and decreasing Ca^{2+} influx and release (10). Isoflavones

have also been shown to reduce oxidative stress, inhibit angiogenesis and attenuate vascular inflammation (10).

The Framingham Risk Score is an algorithm commonly used to estimate the 10-year cardiovascular risk of an individual without diabetes inputting various variables including age, sex, smoking status, total cholesterol, LDL-cholesterol, systolic blood pressure and use of anti-hypertensive medications (11). This has been used in prospective studies to assess the cardiovascular risk (12). We have previously shown a reduction in cardiovascular disease risk markers using this soy/isoflavone preparation in men (4). Therefore, a post hoc analysis of cardiovascular risk using the Framingham Risk Score was undertaken in this randomised, double blind, parallel study in which the primary end point was a change in bone turnover markers (13).

Materials and methods

Two hundred Caucasian women from the Hull and East Riding of Yorkshire, UK within two years of the onset of their menopause (FSH greater than 20 mU/L and amenorrhoea for one year) were recruited after screening 334 women who responded to newspaper advertisements (13). None of the patients were taking any prescription or over the counter medications. Women with a previous history of medication that could interfere with bone metabolism including steroids, bisphosphonates, thyroxine or hormone replacement therapy were excluded. All women were non-smokers and no subject had type 2 diabetes. Women with significant hepatic or renal impairment, who were allergic to soy products and those who had antibiotic exposure in the three months prior to the study, were also excluded. The study was undertaken at the Diabetes, Endocrinology and Metabolism centre, Hull Royal Infirmary, UK.

Two hundred women were randomised into either the SPI group (15 g soy protein with 66 mg of isoflavones) or SP group (15 g soy protein alone, isoflavone free) daily for a period of six months, administered as below.

The primary outcome of this study was to assess the plasma bone turnover markers (13). The secondary outcomes for this study were the assessment of cardiovascular disease risk markers including insulin resistance, lipids, and hsCRP, but their assessment within the Framingham risk engine was a new analysis within this dataset.

During study visits (baseline, three months and six months), participants were instructed to maintain their normal level of physical activity throughout the study. In addition, participants were required to avoid food products containing soy, alcohol, vitamin or mineral supplementation, and over-the-counter medications. No other changes in the diet were recommended. Dietary reinforcement was undertaken at each visit by a registered dietician, together with measurement of serum isoflavone concentrations to ensure compliance. There was telephone contact by study personnel, six and 18 weeks after study visits to ensure compliance. Analysis of compliance with the study preparation was undertaken by counting the returned sachets. All participants gave their written informed consent for this study that had been approved by the Research Ethics Committee (East Yorkshire & North Lincolnshire Research Ethics Committee, ref: 09/1304/45).

Study product

The intervention comprised a snack bar containing 7.5 g isolated soy protein powder (Solcon F, Solbar Industries, Israel) with 33 mg of isoflavones (SPI) (Solgen 40, Solbar Industries, Ashdod, Israel) given twice daily between meals (15 g soy protein and 66 mg of isoflavones per day), or 7.5 g of the isolated soy protein alone given twice daily (15 g soy protein per day without isoflavones per day) as control (SP). The latter had

an isoflavone concentration of less than 300 parts per billion following serial alcohol extraction by Dishman Ltd, India(13); and product isoflavones assayed by FERA, Sand Hutton, UK(13). Analysis showed the composition of the dose materials to be 54% genistein, 35% daidzein, and 12% glycitein as aglycones and further confirmed that 90% of phytoestrogens were in the primary glucoside form, with the remaining 10% as aglycones or acetyl and malonyl glucosides. The soy with and without isoflavones was analysed using AOCS official method Ba 4d-90 "Nitrogen-ammonia-protein modified Kjeldahl method titanium dioxide + copper sulphate catalyst" that determines total nitrogen content and protein. The snack bars were eaten twice daily between meals for 6 months. The soy protein and the isoflavones were from a single batch that was designated for the study. The study bars were specifically commissioned, prepared (soy with and without isoflavones, mixed with water and cold compressed into a snack bar) and packaged by Halo foods, Swindon, UK. Soy bars of similar macronutrient content were identical in size, shape, texture and both arms were in identical packaging; a taste panel prior to the study could not distinguish a difference in taste between the 2 preparations. There was no difference in side effects or drop outs that would distinguish between the 2 products.

Randomisation

The randomisation was performed by Essential Nutrition Ltd, UK as detailed(13), using a computer generated randomisation sequence was used to provide balanced blocks of patient numbers for each of the two treatment groups. Compliance was documented by return of the empty wrappers and uneaten bars.

Study measurements

During the baseline, three months and six month study visits, and following an over-night fast, anthropometric parameters were measured and blood samples collected, stored at -80oC and insulin batch analysed at the end of the study. Blood pressure was measured after the participants had been seated quietly for at least five minutes with the right arm supported at heart level. Blood pressure measurements were performed using an automated device (NPB-3900; Nellcor Puritan Bennett, Pleasanton, CA) during each study visit. Two readings were obtained at the beginning of each visit at least one minute apart and the average of the readings was taken. Fasting venous blood samples were collected and prepared as previously described (13). Briefly, blood was separated by centrifugation at 2000 g for 15 min at 4^{o}C, and the aliquots stored at -80^{o}C within one hour of collection. Plasma glucose was measured using a Synchron DxC analyzer (Beckman-Coulter, UK), and serum insulin was assayed using an ultra-sensitive chemiluminescent one-step immunoenzymetic 'sandwich' assay performed on a Unicel DXi Immunoassay system (Beckman-Coulter, UK). The coefficient of variation (CV) of this method was 8%, calculated using duplicate study samples. The analytical sensitivity was 2 µU/mL. Insulin resistance was calculated using HOMA-IR (Insulin x glucose)/22.5) (14).

Total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL) levels were measured enzymatically using a Synchron DxC analyzer (Beckman-Coulter, UK). Low-density lipoprotein cholesterol (LDL) was calculated using the Friedewald equation. At a mean total cholesterol of 4.9mmol/l combined within and between (intralab) CV was 0.7%; at a mean HDL of 0.9mmol/l combined within and between CV was 1.0%; at a mean triglyceride level of 1.61mmol/l combined within and between CV was 0.94%; at a mean hsCRP of 8.4mmol/l combined within and between CV was 1.1%).

The isoflavones in serum were extracted and analysed by LGC, Fordham, Cambridgeshire, UK using isotope-dilution LC-MS/MS (15). LC-MS/MS was conducted using a Sciex 4000 Qtrap with separation achieved using a C18 column and mobile phases of water and acetonitrile, both containing acetic acid(16).

The calculated risk scores between groups using the Framingham equation (11) (based on age, total cholesterol, HDL and systolic blood pressure: smoking and diabetes were exclusion criteria in this study and therefore set to zero in the calculation) were performed at 6 months as this was the pre-determined end point of the study

Statistical analysis

Sample size was powered for changes in bone markers and not specifically for cardiovascular risk (13): a post hoc power analysis for CVR would have been poor statistical practice and as such was not conducted. An intention to treat analysis was undertaken; however, the data from withdrawals were included as part of intention-to-treat analysis. Baseline values were not compared statistically given that this was a randomised controlled trial. For each group (SPI and SP) separately a paired difference (six-months minus baseline) of means was calculated, the two paired means were then compared using an independent t-test; the p-value is the probability of the difference of the difference being a false positive. This is referred to in Table 2 as the 'difference of the difference' and 95% confidence interval gives the precision of the difference of the difference in the tables. This difference of the difference at 6 months is reflected in **Figure 1** for the calculated cardiovascular risk. A paired t test for baseline to 3 months and 3 months to six-months within groups was performed for the metabolic factors and cardiovascular risk to assess trend. Data was analysed using the Stata statistical computer package (StataCorp. *Stata Statistical Software. Release 13.* College Station, Texas, 2013).

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Results

120 women completed six months of the study, 60 in the SPI group and 60 in the SP group with an overall dropout rate of 40%: the main reasons for dropping out of the study have been detailed previously (13).

The baseline anthropometric, metabolic, plasma isoflavone levels were comparable between the two groups and may be seen in Table 1.

Serum Diadzein, genistein and equol were increased in the SPI group confirming compliance ($p<0.001$) whilst those in the SP group did not differ between baseline, 3 months and 6 months; bone marker concentrations changed significantly during the study as described elsewhere (13). Empty wrappers and uneaten bars were returned and counted by the study team. If compliance was less than 75% then the subject was to be withdrawn from the study: those that completed the study had a compliance of more than 90%.

Changes in the metabolic parameters after 6 months are shown in Table 2 with decreased fasting glucose, fasting insulin and HOMA-IR. Lipid parameters (total cholesterol, LDL, HDL and triglycerides) and hsCRP were unchanged between treatment groups. There was a significant reduction in systolic blood pressure at six months between SP and SPI supplementation though diastolic blood pressure was unchanged. (Table 2).

There was no difference in the baseline characteristics of those that dropped out of the study versus those that completed the study.

The within group calculation risk at 3 months, and 3 months to 6 months was performed to determine trend across the time period and is shown in Figure 1. The calculated 10 year risk for coronary heart disease showed a 27% reduction at 6 months comparing

SPI with SP ($p<0.01$), though only the within group change for SPI, but not SP, showed a significant reduction at 3 months and a subsequent further reduction at 6 months. The calculated 10 year myocardial infarction risk showed a 37% reduction at 6 months between SPI and SP ($p<0.01$); the within group change for SPI, but not SP, showed a significant reduction at 3 months and a subsequent further reduction at 6 months. The calculated 10 year cardiovascular disease risk showed a 24% reduction at 6 months between SPI and SP ($p<0.04$); the within group change for SPI, but not SP, showed a significant reduction at 3 months and a subsequent further reduction at 6 months. The calculated 10 year cardiovascular death risk showed a 42% reduction at 6 months between SPI and SP ($p<0.02$); the within group change for SPI, but not SP, showed a significant reduction at 3 months and a subsequent further reduction at 6 months (Figure 1). Stroke and death from coronary heart disease did not differ at 6 months between SP and SPI treatment (Figure 1); however, it is of interest that risk of stroke decreased within groups for both the SPI and SP groups.

No one isoflavone measured (genistein, diadzein, equol) in the SPI group showed a difference in Framingham score compared to each other ($p>0.05$), and there was no difference between equol producers (n=38) and equol non-producers (n=22) for cardiovascular risk (data not shown). The prevalence of equol producers was 19% in this study which is comparable to that seen in the Caucasian population (9).

Discussion

The calculation of the CVR parameters showed a significant reduction in calculated 10-year coronary heart disease (27%), myocardial infarction (37%), cardiovascular risk (24%) and death due to cardiovascular disease (42%) with SPI supplementation using the Framingham equation (11, 17). This is in accord with an observational study using dietary recall where high isoflavone intake was associated with reduced risk of cerebral and myocardial infarction that was more pronounced for postmenopausal women (5, 18). A Japanese study of the traditional soy food natto showed a decrease in CVD mortality(6). Others have shown that soy protein along with isoflavone supplementation may reduce subclinical atherosclerosis in women at low-risk for cardiovascular disease who were <5 years postmenopausal (7). The effect of the soy/isoflavones SPI preparation on CVR parameters and indices reflects those seen in a study using the same preparation in hypogondal men with type 2 diabetes (4). Stroke risk did not differ at 6 months between SP and SPI treatment; however, it is of interest that risk of stroke decreased within groups for both the SPI and SP groups. The risk of cerebral infarction has been noted to decrease with soy intake, particularly in postmenopausal women (18) and in the natto study, a decrease of stroke was only seen at the highest quartiles of soy intake, above that of this study(6). A meta-analysis of eleven trials demonstrated that soy isoflavone intake resulted in a mean decrease of 2.5 mmHg for systolic blood pressure compared to placebo (19); however, there was significant heterogeneity between the studies. A 4–5 mmHg reduction in systolic blood pressure can reduce CVD risk by 8–20% (20). In the current study, there was a 3.2mmHg reduction in systolic blood pressure with soy protein and isoflavone supplementation for 6 months. An improvement in systolic pressure alone was seen in a study using the same isoflavone preparation with soy protein as here(21), but in a

study in type 2 diabetes patients treated with 132mg tablets of isoflavone alone without soy protein there was no effect on systolic blood pressure (5). This suggests that a synergistic matrix effect between the soy protein with the isoflavones may be responsible for any cardiovascular disease changes since both supplements contains the same amount of protein.

Given that this was a healthy volunteer population without other cardiovascular comorbidities and therefore were not likely to have had any additional cardiovascular risk; thus repeating this study in a population of greater risk may likely see increased benefits. There were no significant changes for body mass index, diastolic blood pressure, hsCRP and lipid profile, and the reduction in predicted 10-year cardiovascular disease risk from the Framingham risk score that was derived from the decreased systolic blood pressure.

There was a significant reduction in systolic blood pressure with three months of SPI that did not improve further at 6 months, but no changes were seen with SP, and diastolic blood pressure remained unchanged with treatment. Participants' age and systolic blood pressure are the two most potent risk factors included in the Framingham risk equation, so although lipids were no different between the groups, presumably the overall cardiovascular risk calculation was being driven by the observed SBP difference.

There were no changes in the total cholesterol, LDL, HDL or triglyceride levels by the soy preparations between groups at 6 months, results that are in accord with others where the placebo used was cellulose (5) and lipid parameters were unchanged. This is the converse reported for a soy with a cassein comparator study that reported a 4% reduction in LDL (22). Reductions in both total cholesterol and LDL, but not HDL were detailed in a meta-analysis (23), though differences in study design and small study

311 numbers, soy preparation, isoflavone composition (glucoside or aglycone forms) would
312 all contribute to the discrepant findings here and in other studies. However, 15g/day of
313 soy were used in this study that may have been too little to reduce cholesterol, thought
314 to be due to the soy protein affect, and a Food and Drug Administration claim called
315 for 25g/ day to be effective. There were no differences in the cardiovascular risk
316 parameters between producers and non-producers of equol in accord with the 28
317 negative studies reported in a recent meta-analysis (24). It is not known whether these
318 cardiovascular beneficial effects would continue in the future with the cessation of soy
319 treatment, akin to the metabolic memory seen in diabetes (25), or would be short term
320 with only an effect whilst taking the soy preparation.
321 Dietary intake of isoflavones in Asian soy diets has been estimated to be in the range
322 of 30-50 mg per day of combined isoflavone aglycone equivalents(26, 27). In Western
323 countries an average daily intake of approximately 2 mg isoflavones is seen though
324 estimated to be 16mg in vegetarians(28); therefore, the dose of 66mg of isoflavones
325 used in this study may be considered to be in the pharmacological range.
326 The strength of this study is that this study is unique in using a soy preparation well
327 defined from a single batch that was truly isoflavone free that could determine the
328 contribution to any cardiovascular disease risk effect by the soy protein alone. No
329 treatment effects on the individual parameters were seen for soy protein alone,
330 suggesting that the soy protein by itself is inactive. Whilst there was no difference in
331 the protein composition between soy with and without isoflavones following serial
332 alcohol washing, the serial alcohol washing could have altered the tertiary structure of
333 the protein and removed other components besides isoflavones. The limitations of this
334 study include that the cardiovascular disease risk markers were not the primary aim of
335 the study. However, the study was over powered for the primary outcome and

analysed as an intention to treat thus minimizing the anticipated dropout rate. The dropout was around 40% as anticipated so that the power of the study was not compromised. This approach circumvented the concerns of a potential type 2 error for the primary variable. Furthermore, the changes in the CVR markers were in accord with another large study using the same preparation (4). The features of those that dropped of the study did not differ between groups nor differed to those that completed. Plasma isoflavone concentrations increased in the SPI alone confirming compliance, whilst the SP group did not change from baseline excluding exogenous isoflavone ingestion. Whilst dietary advice was given at each visit, formal dietary assessment to determine macronutrient intake was not undertaken so it is possible that the ingestion of the extra 15g of soy protein may have subtly altered dietary habits that may have contributed to the results.

In conclusion, there was a beneficial effect on systolic blood pressure with soy and isoflavone intake over 6 months in this population of women in their early menopause, and the reduction in systolic blood pressure was reflected in cardiovascular disease risk calculated by the Framingham equation.

Author's contributions

All authors have read and approved the final manuscript.

T. Sathyapalan was involved in study design, conducted research, wrote paper

M Aye conducted research and data collection

A Rigby performed statistical analysis

N Thatcher was involved in research design

S Dargham was involved in statistical analysis and wrote paper

ES Kilpatrick was involved in research design, sample analysis, wrote paper

SL Atkin was involved in study design development, data analysis, wrote paper and primary responsibility for final content

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Legend to Figure 1

Trend in cardiovascular disease risk reduction with soy protein and isoflavone (SPI) and soy protein alone (SP) showing the within group changes from baseline to 3 months and from 3 months to 6 months using Framingham criteria. Data show the progressive fall in the risk parameter over the 6 month period of the study for the SPI treated group for A), CHD; B), CHD death; D, MI; E), CVD; F), CVD death, but not for C), stroke.

CHD – 10 year coronary heart disease risk. MI – 10 year myocardial infarction risk. Stroke – 10 year stroke risk. CVD – 10 year cardiovascular risk. CHD death – 10 year risk for death due to coronary heart disease. CVD death – 10 year risk for death due to cardiovascular disease. Error bars are SEM.