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Randomized placebo-controlled trial of an isoflavone supplement and menopausal symptoms in women

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Key words: ISOFLAVONES, RANDOMIZED TRIAL, HOT FLUSHES, MENOPAUSE

ABSTRACT

Objective To test the hypothesis that increasing the intake of isoflavones by dietary supplementation may produce a therapeutic effect in reducing the incidence and severity of hot flushes in menopausal women.

Methods Fifty-one postmenopausal women were randomized to placebo and active (one tablet per day of a 40-mg isoflavone supplement) groups in a cross-over design trial. After a 1-week run-in period, subjects were commenced on a 12-week period of treatment (active or placebo), followed by a 1-month placebo wash-out period, then crossed over to the alternative treatment regimen for a further 14 weeks. Symptom diaries were maintained throughout the trial and at the start and end of treatment. Plasma sex hormone binding globulin (SHBG) assay, full blood count, biochemical profiles, vaginal swabs and vaginal ultrasound scans were performed and isoflavones determined in 24-h urine collections by high-pressure liquid chromatography (HPLC) analysis.

Results There was no significant difference between active and placebo groups in the reduction in hot flushes between start and finish time-points. Analysis performed on interim data time-points revealed a substantially greater reduction in flushing in the active group than placebo at 4 and 8 weeks after commencement of treatment, but this was not statistically significant. There were no significant differences between groups for Greene scores or in SHBG levels, hematological or biochemical parameters and vaginal swab or ultrasound findings. The combined values for all subjects, regardless of treatment group, revealed a strong negative correlation between the level of urinary isoflavone excretion and the incidence of hot flushes.

Conclusions These data do not indicate a therapeutic benefit from dietary supplementation with isoflavones in women experiencing menopausal symptoms, but do indicate that the apparent placebo effect in many studies of menopausal symptoms may be attributable to dietary sources of isoflavones. The study also demonstrates that 3 months of isoflavone supplementation did not cause adverse events or endometrial changes.

INTRODUCTION

The reported incidence of acute menopausal symptoms, notably hot flushes, the most common symptom of the climacteric, varies from 70 to 80% in menopausal women in Europe[1], to 57% in Malaysia[2], and 18%, 15% and 14% in China, Japan and Singapore, respectively[3]. While racial

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and cultural differences may account for some of this variation, the lack of physical evidence of vasomotor symptoms, such as hot flushes in Asian and Central American women[4], suggests that the lower incidence in these communities reflects a real biological difference in the expression of menopausal symptoms.

A possible explanation for these observed variations may be related to diet and, in particular, differences in the content of estrogenic compounds in the diet. Naturally occurring substances with estrogenic activity (phytoestrogens) occur widely throughout the plant kingdom. The overwhelming estrogenic activity is found in phenolic compounds such as flavones, flavonols, flavanones, lignans, chalcones and isoflavones. These compounds have a steric structure similar to that of steroidal estrogens, allowing them to bind to the human estrogen receptor. The subtle differences in structure mean that their affinity for and activation of the receptor is weak, with a binding affinity between 10^{-2} and 10^{-4} of that of 17β-estradiol[5,6]. However, these substances can be present in the blood at levels up to 10 000 times that of steroidal estrogens[7], suggesting significant potential for them to exert estrogenic activity in the body.

Much scientific attention has focused on isoflavones. These appear to be of particular significance to humans because, compared to the other classes of phytoestrogens, they have a relatively high estrogenic potency and occur at higher levels in the human diet[8]. Isoflavones are found almost solely in legumes, and the greater reliance on legumes for dietary protein in communities such as Japan, China, South-East Asia and Central America means that those communities typically have substantially higher dietary isoflavone levels than Western countries such as North America, Western Europe and Australasia[9,10].

Studies have been reported in which isoflavone-rich foodstuffs, such as soy, produce a moderate decrease in the incidence and severity of hot flushes when added to the diet of menopausal women[11]. However, the difficulty with such dietary challenge studies is the confounding factor of dietary change. To test the hypothesis that dietary intake of isoflavones may have a therapeutic benefit in this regard, a double-blind, placebo-controlled, cross-over trial was conducted in menopausal women, in which the test group received supplementation of dietary isoflavone intake provided as a semi-purified isoflavone extract in tablet form.

METHODS

Study location

The study was conducted at the Menopause Clinic at the Royal North Shore Hospital in Sydney, Australia and was approved by the Human Ethical Review Committee of that hospital.

Subjects

All subjects were unpaid volunteers from whom written informed consent was obtained prior to recruitment into the trial. Women were recruited according to the criteria given in Table 1, and 51 women were finally accepted into the study.

Isoflavone preparation

The product used to supplement dietary isoflavone levels in test subjects was a proprietary preparation, Promensil® (Novogen Ltd, Sydney). Promensil is a standardized isoflavone supplement prepared from red clover extract in 500-mg tablet form containing 40 mg per tablet of total isoflavones and containing the four primary isoflavones: genistein, daidzein and their methylated precursors biochanin and formononetin. A placebo tablet was formulated with a similar appearance and taste.

Study design

Subjects were assessed for 1 week to ensure a minimum of three hot flush episodes per day on average. They were then allocated randomly and blindly to either the active or the placebo group and treatment commenced (one active or one placebo tablet taken daily in the morning) for 3 months, followed by a 1-month 'wash-out' on placebo tablets, followed by 3 months on the

Table 1 Inclusion and exclusion criteria

Inclusion criteria	*Exclusion criteria*
Aged 45–65 years	intercurrent medical problems
More than three flushes per day	HRT or antibiotics in previous 3 months
	FSH < 30 mIU/ml
	menstruation in previous 6 months
	hysterectomy
	vegetarian (> 10 g legumes per day)

HRT, hormone replacement therapy; FSH, follicle stimulating hormone

reverse treatment arm. The final arm was extended by 2 weeks to allow for any change in reporting habits associated with apprehension at the completion of the trial.

At the start and completion of the first treatment arm and at the completion of the second treatment arm, the following procedures were performed:

(1) A routine medical examination, performed by the same clinician throughout;

(2) Blood collection for various hematological and serological assays;

(3) A 24-h urine sample collection for isoflavone analysis;

(4) Determination of endometrial thickness by transvaginal ultrasound;

(5) A vaginal smear to assess vaginal maturation index.

A clinical nurse examined subjects on a monthly basis and recorded body weight and blood pressure. Subjects maintained a diary card in which they recorded symptoms daily throughout the trial, including the number of hot flushes experienced each day, and other symptoms were scored as non-existent, mild, moderate or severe on the Greene menopause symptom scale[12].

Analyses

All laboratory analyses were conducted at Pacific Laboratory Medicine Services, Royal North Shore Hospital, Sydney. Fasting blood samples were collected for a full blood count, liver function tests, and serum levels of follicle stimulating hormone (FSH), estradiol and sex hormone binding globulin (SHBG). Full blood count and liver function were measured using automated techniques. Serum FSH was measured using an immunoassay technique (CHIRON, ACS-180, MA, USA), and SHBG was measured by a non-competitive 'liquid-phase' immunoradiometric assay (IRMA, Orion Diagnostica, Espoo, Finland).

Urinary isoflavone levels of genistein, daidzein, formononetin and biochanin A were measured by high-pressure liquid chromatography (HPLC) using a modified method described by Setchell and colleagues[13] and Franke and co-workers[14]. From each urine sample, a 30-ml aliquot was mixed with 100 µl of glucuronidase and the mixture was incubated for 24 h at 37 °C, after which it was extracted on a C-18 solid phase extraction column (Waters Pty Ltd, Sydney). Isoflavones were eluted with 3 ml of methanol, and 10 µl of the extract was injected into the HPLC system.

The HPLC system consisted of a 25-cm, 5-µm, C-18 stationary phase column (Symmetry, Waters, Sydney) with a gradient acetonitrile/water mobile phase. The assay has a limit of detection for each of the isoflavones of 5 ng/ml.

Analysis of data and statistical analyses were performed by a consultant group, Quintiles Pty Ltd, Sydney. All data were included in the analysis on an intention-to-treat basis. Analyses were performed on data provided as Excel spreadsheets and converted to SAS7 datasets for analysis. Non-parametric data were analyzed using the Wilcoxon repeated measures analysis of variance (ANOVA) test.

RESULTS

Of the 51 patients randomized to the study, 26 were allocated initially to the placebo group and 25 to the active group. Withdrawal by eight women (one before enrollment, four after 3 months of treatment in the active group and three after 3 months of placebo treatment) resulted in data generated for 44 women on 3 months of placebo tablets and 43 women on 3 months of Promensil tablets. Seven women withdrew for personal reasons; the eighth woman withdrew for medical reasons not related to the study. The demographic data are given in Table 2.

Weight was monitored monthly throughout the study. There was no difference in weight for participants, whether on the placebo or active treatment arms. These results are given in Table 3.

The frequency of flushes and Greene score data for the two individual phases and the phases combined are given in Table 4. Greene score and flush frequency decreased in both placebo and active groups during the 3 months of treatment, based either on data from the two groups combined or on data from the two phases individually. The baseline values for flush count and Greene score were significantly different at screening baseline and at cross-over baseline ($p = 0.003$). Although there was no significant difference between active and placebo groups in the reduction in hot flushes between start and finish (12 weeks) time-points,

Table 2 Demographic data (mean ± standard error): observations from 51 subjects recruited to study

Age at trial start (years)	54 ± 4.1
Age at last menstrual period (years)	50 ± 3.6
Height (cm)	165 ± 5.9

Table 3 Weight data (kg) at baseline and at 12 weeks (mean ± standard error): observations from number of subjects shown in parentheses in each phase

	Baseline	*Week 12*	*p Value*
Combined phases			
Placebo group (n = 26)	67 ± 11.0	67 ± 11.5	0.91
Promensil group (n = 25)	71 ± 11.6	71 ± 12.4	0.96
Phase I			
Placebo group (n = 26)	67 ± 11.0	67 ± 11.9	0.87
Promensil group (n = 25)	70 ± 11.6	70 ± 12.3	0.88
Phase II			
Placebo group (n = 26)	71 ± 12.5	70 ± 12.8	0.83
Promensil group (n = 25)	67 ± 12.2	67 ± 11.6	1.00

Table 4 Flush and Greene score data at baseline and at 12 weeks (mean ± standard error): observations from number of subjects shown in parentheses

		Hot flush count		*Greene score*	
Study phase	*Treatment group*	*Baseline*	*Week 12*	*Baseline*	*Week 12*
Phase I	placebo (n = 26)	6.42 ± 2.59	3.95 ± 2.63	12.35 ± 9.04	8.31 ± 8.45
	Promensil (n = 25)	6.2 ± 2.65	4.83 ± 3.36	10.96 ± 6.5	6.63 ± 4.91
Cross-over	placebo (n = 22)	4.39 ± 2.78	3.45 ± 2.98	6.13 ± 4.38	5.25 ± 3.37
	Promensil (n = 19)	4.44 ± 2.65	3.57 ± 3.00	9.38 ± 10.57	7.86 ± 8.17
Combined	placebo (n = 46)	5.49 ± 2.84	3.72 ± 2.77	9.5 ± 7.86	6.93 ± 6.76
	Promensil (n = 42)	5.4 ± 2.77	4.22 ± 3.22	10.24 ± 8.53	7.23 ± 6.65

analysis performed on interim data time-points revealed a substantially greater reduction in flushing in the Promensil group, compared to the placebo group, at 4 and 8 weeks after commencement of treatment (Figure 1), although this did not reach statistical significance on a repeated measures ANOVA. However, by 12 weeks, the reduction in flushing in the placebo group was equivalent to that observed in the active treatment group. There were no significant differences between groups in Greene scores at any time-point (p = 0.158).

No differences were observed between treatment and placebo groups in terms of full blood count, biochemical parameters or SHBG levels. Analysis of the maturation index data also revealed no correlation between those who improved and those who deteriorated, suggesting a constant therapeutic effect. The evaluable number in each group was too small to allow analysis of the significance of differences between groups. Transvaginal ultrasound showed no difference in endometrial thickness between treatment groups at trial entry and exit (Table 5).

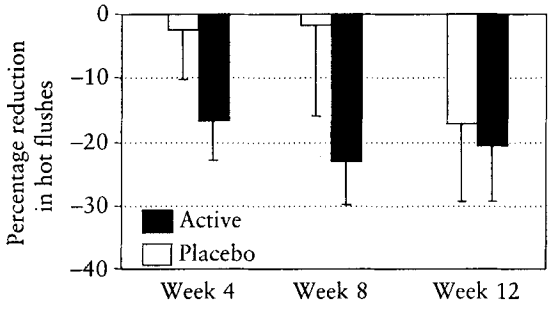

Figure 1 Percentage reduction in hot flushes at 4, 8 and 12 weeks. Histograms represent means and vertical bars are standard errors of observations from 42 and 46 subjects for Promensil and placebo groups, respectively

After urine isoflavone analysis was performed, it became apparent that many subjects in the placebo group displayed high urinary isoflavone excretion at the end of each study phase. The baseline and week-12 urinary excretion levels for placebo and active treatment arms are given in Table 6. There was no change in mean total isoflavone levels between baseline and 3 months of placebo treatment (phase I and phase II data

combined), but the difference in total isoflavone level between baseline and the end of 3 months of 40 mg per day supplementation was significantly different ($p < 0.001$).

The combined values for all subjects, regardless of treatment group, revealed a strong correlation between the level of total urinary isoflavone excretion (the sum of daidzein, genistein, formononetin and biochanin) and the incidence of hot flushes (Figure 2). Although there was a strong correlation between total isoflavone excretion and reduction in flushes, analysis of correlations between excretion of each of the individual isoflavones and flushing reduction demonstrated that daidzein excretion was the most strongly correlated with change in flushing incidence (Figure 3).

DISCUSSION

This study examined the effect on menopausal symptoms and on safety parameters of dietary isoflavone supplementation provided by a daily dose of a tablet containing 40 mg of phytoestrogens obtained from red clover, using a double-blind, placebo-controlled, cross-over model.

No effect of phytoestrogens was found, compared to placebo treatment, on red cell, white cell or platelet parameters normally measured in a full blood count. Similarly, no changes were detected in serum electrolytes, urea, creatinine or liver function tests, and no change in body weight was observed.

In previous studies of the estrogenic effects of phytoestrogens in sheep, Nwannenna and colleagues[15], using a dose of 6.1 g of phytoestrogen per day in ewes weighing a mean 64 kg, reported significant and reversible estrogenic changes in the appearance and size of the vulva and an increase in the size of the uterus. Although these effects were still less than those observed following the use of 17β-estradiol, these findings raise the possibility that phytoestrogens may exert similar effects on the human genital tract in a dose-related manner. Indeed, it has been suggested that, in any studies of potentially estrogenic substances, endometrial safety should be assessed[16]. For these reasons, vaginal smears and transvaginal ultrasound scans were performed on all patients. However, at the dose tested here, no increased estrogenic effect was detected, compared to placebo, on the vaginal epithelium (using either Kupperman index or maturation index), nor on endometrial thickness by transvaginal ultrasound. Indeed, in the few biopsies performed because of

Table 5 Transvaginal ultrasound data of endometrial thickness (mm) at baseline and at 12 weeks (mean ± standard error): observations from number of subjects shown in parentheses

	Baseline	*Week 12*	*p Value*
Placebo ($n = 26$)	3.17 ± 1.5	3.19 ± 1.8	0.95
Promensil ($n = 25$)	3.17 ± 1.5	3.25 ± 1.8	0.8

Table 6 Total urinary isoflavone levels (mg) at baseline and after 3 months of placebo or active treatment: phase I and phase II data have been combined to generate 3-month time-point data (mean ± standard error): observations from number of subjects shown in parentheses

	Baseline	*Week 12*
Placebo ($n = 26$)	1.3 ± 1.5	1.2 ± 1.8
Promensil ($n = 25$)	0.9 ± 1.4	5.5 ± 4.1

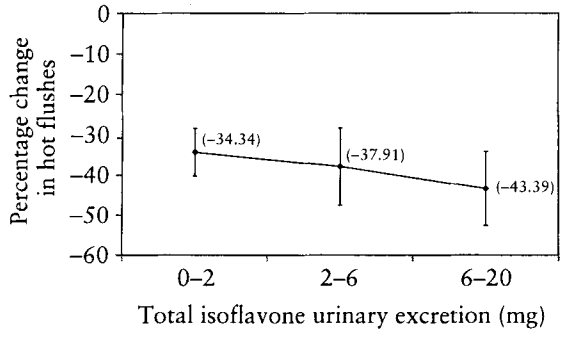

Figure 2 Correlation between percentage change in hot flushes and total urinary isoflavone excretion in all subjects, regardless of treatment group. Plotted points represent mean reduction in flushing for subjects in 0–2 mg, 2–6 mg and 6–20 mg excretion ranges, respectively. Vertical bars are standard errors

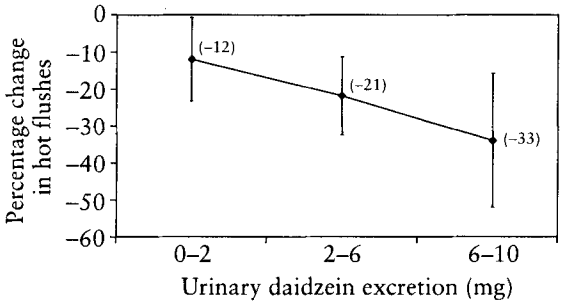

Figure 3 Correlation between percentage change in hot flushes and urinary daidzein excretion in all subjects, regardless of treatment group. Plotted points represent mean reduction in flushing for subjects in 0–2 mg, 2–6 mg and 6–20 mg excretion ranges, respectively. Vertical bars are standard errors

perceived endometrial thickening on ultrasound scan, atrophy was the finding in all but one case (in which the patient was at the end of the placebo phase of the study).

These findings demonstrate that, despite the estrogenic potential of isoflavones, at the doses used in this study and over a 3-month observation period, they had no estrogenic effect on the endometrium, they did not induce weight gain and they appeared to be safe with respect to other hematological and biochemical parameters. However, further studies are required to verify the long-term safety of isoflavone supplementation.

This study failed to show a statistically significant difference between 40 mg of isoflavones (one Promensil tablet per day) and placebo treatment with respect to alleviating menopausal symptoms. There was no significant improvement in symptoms in subjects in the active treatment group, compared to the placebo group, after 12 weeks of treatment. It was noted that there was a very high placebo response at this time-point, but analysis of interim time-points revealed a lower response in placebo groups at 4 and 8 weeks, resulting in a substantial although non-significant difference in flushing incidence between active and placebo groups at these times. The data were characterized by a high variability in responses, with some subjects experiencing a substantial decrease in symptoms, others only a modest decrease and others no change at all. This was shown to be mirrored by high variability in urinary excretion of phytoestrogen metabolites. However, when urinary excretion for individual subjects, regardless of treatment group, was compared with change in incidence of hot flushes, a strong negative correlation was observed, especially with respect to daidzein (Figure 3). This indicates that availability of isoflavones or their metabolites, whether from dietary intake or from deliberate supplementation, may contribute to alleviation of these symptoms.

As many subjects in the placebo group displayed higher urinary isoflavone excretion at the end of the trial than at entry, the apparently high placebo response, especially at 12 weeks, needs to be interpreted with caution. As these substances only appear in urine after isoflavone ingestion, it is clear that, even in the absence of deliberate supplementation in the placebo group, many subjects must have been obtaining isoflavones from their diet, despite urging all participants to maintain their normal diet. Thus, the fact that the placebo response appeared to increase over the course of the trial (see Figure 1) may have reflected a change in dietary habits over this time. It is possible that subjects in the placebo group, confronted with unabating symptoms and having been informed of the potential benefits of dietary sources containing isoflavones, may have adjusted their diets over the course of the trial. Thus, by 12 weeks, the response in the placebo group may have masked an effect of isoflavone supplementation in groups receiving active tablets.

This apparently high placebo effect is consistent with data published from other trials studying menopausal symptoms[17,18], and suggests that, in the absence of data on urinary isoflavone excretion, it would be difficult to draw conclusions regarding effects of supplementation with either hormone replacement therapy (HRT) or plant-derived isoflavones (phytoestrogens) on menopausal symptoms. It also highlights the need for accurate dietary records to be maintained, a feature which should be incorporated into future studies, especially in view of the increasing availability of isoflavone-enriched processed foods.

The current knowledge of isoflavone metabolism stems predominantly from sheep studies. In this species, after ingestion of phytoestrogens, free-aglycone forms of genistein, daidzein, biochanin A and formononetin are produced by acid or enzymatic hydrolysis of the conjugated isoflavones which are then absorbed, appearing in the blood and urine as glucuronide conjugates[19]. Biochanin A and formononetin may also be metabolized by gut flora to form genistein and daidzein, respectively, and daidzein may be reduced further by gut flora to equol and various other metabolites. Genistein is reported to be metabolized, largely within the gut, by ring cleavage to the non-estrogenic compound paraethyl phenol.

In humans, formononetin, daidzein, genistein, equol, methylequol, dihydrodaidzein and *o*-desmethylangiolensin have all been detected in urine; human fecal flora have been shown to be able to produce equol from soy broth; and enzymes capable of carrying out the reduction and deoxygenation of daidzein to equol have been identified in human gut flora. It is reasonable, therefore, to assume that metabolism of dietary phytoestrogens in humans is similar to that in sheep. To an extent, the variable urinary isoflavone levels observed for participants in this study may have been a reflection of individual variation in humans in levels of metabolism of the dietary isoflavones once they are absorbed. The reasons for this are uncertain. Clearly, as all participants were female, gender is not involved, but the variability may be related to the composition

of the intestinal flora, intestinal transit time, the variability in the redox potential of the large intestine or even diet[20]. However, Kelly and colleagues[21] have demonstrated consistent levels in individuals repeatedly tested, suggesting that factors unrelated to dietary variation may be involved.

While the study reported here demonstrates that isoflavone supplementation provided by one tablet per day of Promensil caused no adverse effects, at least over a 3-month period, in terms of female genital tract morphology, body weight, full blood count or biochemical profile, it has not been possible to demonstrate a therapeutic benefit above that obtained from dietary intake of isoflavones. However, an isoflavone supplement may have a beneficial effect in management of menopausal symptoms in patients where dietary intake is low. This is consistent with epidemiological studies[22] that have shown that communities with a dietary phytoestrogen intake similar to that used in the active arm of this study experience fewer menopausal symptoms than those seen in communities whose diet is based on the 'Western' model.

Apart from demonstrating the potential for dietary isoflavone supplementation to assist in alleviation of acute symptoms of the menopause, this study emphasizes problems in interpretation of placebo data associated with intervention studies using naturally occurring dietary compounds. There is a need for further larger studies investigating the clinical effectiveness of isoflavone supplementation on menopausal symptoms, and their pharmacokinetic properties, in which more careful documentation of dietary patterns and a revised definition of a placebo response are observed.

Conflict of interest G.E.K. is Managing Director and L.W. is a Clinical Research Associate employed by Novogen Ltd. R.J.B., C.T., T.M. nil.

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