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Effect of isoflavone on plasma nitrite/nitrate, homocysteine, and lipid levels in Turkish women in the early postmenopausal period: a randomized controlled trial

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Aim: The aim of the study was to evaluate the effect of isoflavones on cardiovascular risk markers including plasma nitrite/nitrate, homocysteine, and lipid levels in Turkish women in the early postmenopausal period.

Materials and Methods: Ninety participants between 42 and 59 years of age were randomly assigned to receive twice a day either isoflavone tablet (n:45) or placebo tablets (n = 45). Plasma nitrite/nitrate, homocysteine, and lipid levels were measured at baseline and after the 6 months of treatment.

Results: After 6 months, isoflavone resulted in a statistically significant decrease in total cholesterol, low-density lipoproteins, triglyceride levels, serum homocysteine and an increase in high-density lipoproteins and serum nitrites/nitrates. Lipoprotein-a level did not change in both groups.

Conclusions: Six months of treatment with isoflavones had a favorable effect on serum nitrites/nitrates, homocysteine and lipid levels in Turkish women in the early postmenopausal period. Although there is not enough evidence yet from large randomized clinical trials to make a recommendation about the use of phytoestrogens for prevention of cardiovascular disease in postmenopausal years, our results show that phytoestrogens improve the biomarkers of some cardiovascular risk markers in Turkish women in the early postmenopausal period.

Key Words: Menopause, isoflavone, nitrite/nitrate, homocysteine, lipid

Erken dönem postmenopozal Türk kadınlarında isoflavon'un plazma nitrit/nitrat, homosistein ve lipid düzeylerine etkisi: randomize, kontrollü çalışma

Amaç: Bu çalışmanın amacı erken postmenapozal dönemdeki Türk kadınlarında isoflavonların plazma nitrit/nitrat, homosistein ve lipit düzeyleri gibi kardiovasküler risk faktörleri üzerine olan etkilerini değerlendirmektir.

Yöntem ve Gereç: Yaşları 42-59 arasında değişen 90 katılımcı günde 2 kez isoflavon veya plasebo tablet alacak şekilde rastgele olarak belirlendi. Plazma nitrit/nitrat, homosistein ve lipit düzeyleri başlangıçta ve tedavinin 6. ayında ölçüldü.

Bulgular: Altı ay sonra isoflavon, total kolesterol, LDL, serum homosistein ve trigliserit düzeylerinde istatistiksel olarak belirgin bir düşme, HDL ve serum nitrit/nitratlarında ise yükselme sağladı. Lp(a) düzeylerinde her iki grupta da bir değişiklik görülmedi.

Sonuç: Altı aylık bir isoflavon tedavisinin erken dönem postmenapozal Türk kadınlarında serum nitrit/nitrat, homosistein ve lipit düzeyleri üzerine olumlu bir etkisi oldu. Postmenapozal dönemde kardiovasküler hastalıklardan korunmak amacıyla fitoöstrojenlerin kullanılmasının tavsiye edilebilmesi için henüz geniş ve randomize çalışmalarca desteklenen yeterli kanıtlar olmamasına rağmen bizim sonuçlarımız erken dönem postmenapozal Türk kadınlarında fitoöstrojenlerin bazı kardiovasküler risk parametrelerinde gelişmelerde bulunduğunu göstermiştir.

Anahtar Sözcükler: Menopoz, isoflavon, nitrit/nitrat, homosistein, lipit

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Introduction

Cardiovascular disease (CVD) is known as the leading cause of death among women in developed nations and the lifetime risk of death is 31% in postmenopausal women versus a 3% risk of dying of breast cancer (1). It is well recognized that CVD incidence increases substantially with aging and accelerates after menopause, purportedly due to the loss of estrogen protection. Several observational studies have shown a lower risk of CVD among postmenopausal users of hormone therapy (HT) compared with non-users (2,3). However, recent reports about the ineffectiveness of the HT on CVD (4,5) and the adverse effects including increased incidence of endometrial disease, breast cancer, and thromboembolic events have prompted a search for alternative treatment options, such as phytoestrogenic molecules, for the prevention of CVD, osteoporosis, and hormone-related cancers. Interest in the use of phytoestrogens for postmenopausal women's health has been encouraged by observations of a lower prevalence of menopausal symptoms and CVD among women in Asian populations where soy is an important component of the traditional diet compared to Western societies. Phytoestrogens are a class of plant-derived compounds that appear to have an estrogen-like activity on the cardiovascular system without affecting other systems.

Nitric oxide (NO) is an endothelium-derived vasoactive factor that is important in cardiovascular diseases. NO causes vasodilatation, inhibits both platelet aggregation, and endothelin-1 production, suppresses smooth-muscle proliferation, and acts as an anti-atherogenic factor. Continuous release of nitric oxide from the endothelium helps to maintain basal vascular tone, and alterations in nitric oxide release allow auto-regulation of blood flow (6).

The principal isoflavones found in soy proteins and soy foods are daidzein, genistein, and glycitein. Genistein demonstrates, either in vitro or in experimental studies, NO- and endothelium-dependent effects, thus raising the possibility that this compound may have the potential to be a cardio-protective agent (7,8). However, a limited number of studies exist in humans about the positive endothelium-dependent effects of phytoestrogens, and results are inconsistent (9, 10).

Total homocysteine (tHcy) is also a strong and independent risk factor for CVD. In menopausal years, which are associated with a decrease in estrogen levels, women are exposed to have higher plasma tHcy concentrations. It is already well documented that HT has positive effects on plasma tHcy concentrations in menopausal years (11). Contrary to the HT results, some studies report no effect of isoflavone supplementation on plasma tHcy levels in menopausal women (12, 13) while one study reports a modest reduction (9) and another one reports a significant reduction of plasma tHcy in postmenopausal women (14).

The relationship between isoflavones and lipid metabolism is controversial, too. The results of previous controlled clinical trials in human populations have been inconsistent and raised serious questions regarding the hypothesis that soy (9,15,16) and/or isoflavones lower lipids in a clinically relevant way.

The purpose of this prospective randomized study with a 6-month follow-up period is to evaluate the effect of isoflavones on plasma nitrite/nitrate, tHcy, and lipid levels in Turkish women in the early postmenopausal period.

Methods

In this study, 90 healthy postmenopausal women were randomly selected from the Menopause Clinic of the Department of Obstetrics and Gynecology, Faculty of Medicine, Fatih University, Ankara, Turkey. This was a single center, double-blind, placebo-controlled randomized study. Approval for this study was obtained from the Institutional Review Board of the Faculty of Medicine, Fatih University. The volunteers received written and verbal information on the purpose and the procedures of the study and an informed written consent was obtained from all of them.

The women who underwent natural menopause, had not a menstrual period in the preceding year, and with a follicle stimulating hormone (FSH) level greater than 40 IU/l and a serum estradiol (E_2) level of 30 pg/ml or less were included in the study. Exclusion criteria were clinical or laboratory abnormalities suggesting any cardiovascular,

hepatic, cerebrovascular disease, thromboembolism/ thrombosis or renal disorders and history or presence of any malignant disorder, uncontrolled hypertension (systolic blood pressure >170 mmHg and/or diastolic blood pressure >105 mmHg), glucose intolerance (fasting glucose >110 mg/dl) or alterations in lipid metabolism (Low Density Lipoproteins [LDL] >200 mg/dl), use of oral or transdermal estrogen, progestin, or other steroids in the preceding year, and smoking habit of more than 10 cigarettes per day. Women were not administered statins, natural products with presumed estrogenic activity or drugs possibly affecting climacteric symptoms or metabolism, and absorption of phytoestrogens (e.g. antibiotics during the previous 3 months). All patients received dietary instructions in an isocaloric, fat-restricted diet offering 30% energy from fat, <10% energy from saturated fatty acid, and a cholesterol intake of <300 mg/day. During the study, the women were encouraged to lead normal lives, with no changes in dietary, alcohol consumption, or physical activity habits that might influence the outcome, which were obtained by questionnaires before the initiation of the study, after 3 months, and at the end of the treatment period. Dietary isoflavone and lignan intake was assessed with a food frequency questionnaire covering the habitual diet during the year preceding enrollment. This intake was shown to be typical of the Western population (1-2 mg/day) (17). Patient compliance was reinforced by a physician by means of a questionnaire that was completed by the subjects before the initiation of the study, after 3 months, and at the end of the treatment period.

All unfavorable effects were accepted as adverse effects such as breast tenderness, vaginal bleeding, abdominal distention, and constipation.

Participants were randomly assigned to receive daily either an isoflavone tablet (n = 45), which provides 29.8 mg genistein, 7.8 mg daidzein, and 2.4 mg glycitein per tablet (Isoflavin®, Mikro-Gen, Istanbul, Turkey) or placebo tablets (n = 45, 250 mg starch per tablet) of identical appearance twice a day. Placebo pills were prepared with the same appearance by the manufacturer company of the isoflavone tablets. The randomization codes of the placebo and isoflavone tablets were obtained from the company at the end of the study. A block randomization was used in order to ensure an equal number of patients in each group. An independent research assistant carried out the concealed randomization procedure. The random numbers were computer generated, and slips bearing the allocated groups were placed in opaque, sealed envelopes. Block size for the randomization was 5. Envelopes were placed into a bag and then a staff member who was blinded to the research protocol selected the patients into the treatment groups. Researchers dealing with the baseline and outcome data were blinded to the intervention assignments.

Before the initiation of therapy, detailed personal and family histories were taken and all patients underwent gynecologic and physical examination, which included: pap smear, transvaginal ultrasonography (TVS), breast examination and mammography, thyroid, renal and liver function tests, and blood coagulation tests. Laboratory tests, including serum lipid profile, fasting glucose, plasma levels of nitrite/nitrate, tHcy, lipoprotein (a) [Lp (a)], and serum FSH and serum E_2 levels, were conducted before the initiation and after 6 months of therapy.

Blood pressure and body mass index (kg/m^2) were recorded at baseline and 6 months later in all patients. Blood pressure was measured twice, at the right upper arm in sitting position and with a cuff of appropriate size after a 15-min rest for each subject. Two blood pressure readings were taken at 5-min intervals at each visit. The mean of the 2 measurements was taken as the reading of the subjects.

Blood samples were collected after an overnight fast between 08:00 AM and 10:00 AM. immediately before the start of the study and on the last day of 6-month therapies. Blood glucose was assessed in serum immediately. For other assessments, serum was separated by centrifugation and serum aliquots were kept frozen at -80 °C for subsequent analyses.

Nitric oxide production was assessed by monitoring plasma levels of nitrites/nitrates, 2 stable oxidation products of nitric oxide metabolism, by commercially available Nitric Oxide Assay Kits (Assay Designs Inc, USA). Nitrite/nitrate levels were

measured colorimetrically and concentrations were determined using the nitrite/nitrate standard curve. The interaction of NO was measured by determination of both nitrite and nitrate concentrations in the sample. A specific control serum was use to check the calibration of the assay. The sensitivity of the assay was 0.625 uM/l. The inter-assay and intra-assay coefficients of variation for the lowest mean concentrations of nitrate determined in this assay (18.67 and 20.86 μM/l) were 6.9 % and 5.3 %, respectively.

tHcy assay was performed using the commercially available Microtiter Plate Assay. Homocysteine Microtiter Plate Assay is an EIA (Elisa)-like assay for the determination of tHcy in blood. The assay employs a genetically engineered Homocysteine Binding Protein (HBP) as the capturing reagent. Plasma samples are pretreated in vials with a reducing agent, TCEP, to reduce the protein bound Hcy to free Hcy, which is subsequently converted to S-adenosyl-L-homocysteine (SAH) by SAH hydrolase and quantitated by the HBP in a competition assay between free SAH from samples and tracer SAH-HRP conjugate. The sensitivity of the assay was 0.625 uM/l. The intra-assay coefficient of variation for the lowest mean concentrations of homocysteine determined in this assay (7.0 μmol/l) was %4.1. The dynamic range of the assay was between 1.5-60 μmol/l.

Levels of total cholesterol (TC), high-density lipoproteins (HDL), LDL, and triglycerides were assayed enzymatically with a Hitachi 912 automated analyzer with kits from Roche Diagnostics. Lp (a) was measured by an immunonephelometric assay on a nephelometric analyser (Behring, Marburg, Germany). FSH and E_2 were measured by chemiluminescent assays (Immulite One bioDPC, bioDPC, USA).

Statistical analysis

Data analysis was performed using SPSS for Windows, version 11.5. Student's t-test, paired-t test, chi square test, Pearson correlation, and multiple regression analyses were used when and as appropriate. A P value of <0.05 was accepted as significant. According to Shapiro-Wilk W tests, all repeated measures are normally distributed about the mean.

Based on the literature we expected isoflavones to reduce the serum total cholesterol and LDL-cholesterol (8). We considered a 20% decrease in 6 months acceptable and power analysis identified 90 patients (45 for each group) as the total sample size required to detect the difference in therapeutic success with a power of 80% at a 5% significance level. We could not consider the effects of isoflavones on plasma nitrite/nitrate and tHcy levels for calculation of power analysis as there are very few studies conducted in humans and with small numbers of subjects.

Results

Ninety subjects were included in the study and received study medication. Clinical data of the 2 randomized groups are shown in Table 1. Participants were between 42 and 59 years of age (1 to 6 years of menopause). All groups had a similar age, BMI, age at menopause, and years postmenopausal. All women had incapacitating hot flushes and other climacteric symptoms. Education levels, food, vegetable, coffee, tea, and soy-related food consumption, exercise, and smoking habits of both groups were similar. The levels of FSH and E_2, and systolic and diastolic blood pressures were not significantly different in both groups.

When Pearson correlation and multiple regression analyses were performed to determine the relation between NO and tHcy with other cardiovascular risk factors, including age, body mass index, baseline lipid profiles, baseline LPA, CRP, there were no statistically significant associations.

Compliance was controlled by counting the left-over pills and habitual diets were assessed by a dietician. In the placebo group 2 patients were excluded from the study because of taking the drugs irregularly. During the 6 month-period, there were 8 dropouts; 2 (4.4%) dropouts in the isoflavone group and 6 (17.7%) dropouts in the placebo group, which left 43 and 37 participants, respectively, for the per protocol analysis (χ^2 = 1.55, P = 0.213). In the isoflavone group, 1 patient dropped out because she moved to another city and did not come to follow up and 1 patient decided to stop medication for personal reasons. In the placebo group, dropout

Table 1. Baseline subject characteristics in isoflavone and placebo-treated groups.

	Placebo n = 45	Isoflavone n = 45	P value
Age (years)	51.0 ± 4.8	52 ± 5.4	NS*
BMI (kg/m^2)	27.4 ± 3.2	26.9 ± 3	NS
Menopause age	47.7 ± 2.7	47.9 ± 3.7	NS
Years postmenopausal	4.0 ± 1.6	3.3 ± 1.8	NS
Systolic blood pressure (mmHg)	115 ± 11	113 ± 12	NS
Diastolic blood pressure (mmHg)	78 ±7	80 ± 9	NS
FSH mIU/ml	58.6 ± 16	74.7 ± 30	NS
E2 pg/ml	20 ± 2.6	20.4 ± 6.6	NS

Values are mean ± SD.
*NS = No Significance (P > 0.05)

reason was the lack of effect as these patients were complaining hot flushes at the beginning and during the study period.

Adverse effects were similar in both groups (2 isoflavone, 1 placebo) and the primary one was breast tenderness. Intolerance of the supplements or gastrointestinal effects, including constipation, diarrhea, and weight gain, were not observed in both groups.

After 6 months, isoflavone resulted in a significant decrease in TC, LDL, triglyceride levels, and serum tHcy (P = 0.003, P = 0.001, P = 0.008, and P = 0.003, respectively) and a significant increase in HDL and serum nitrite/nitrate (P = 0.002 and P = 0.031, respectively) (Table 2).

Fasting glucose, TC, and LDL levels of isoflavone and placebo groups were similar at baseline and after 6 months of therapy (Table 2). Triglyceride and tHcy levels were not different at baseline between the groups but in isoflavone group both decreased significantly compared with placebo after 6 months (P = 0.037 and P = 0.031, respectively). HDL and serum nitrite/nitrate levels were similar in both groups at baseline but in the isoflavone group both increased significantly compared with placebo after treatment (P = 0.021 and P = 0.000, respectively) (Table 2). Lp(a) levels in isoflavone and placebo groups were similar at baseline and showed no changes in either group.

Discussion

In the present study, after 6 months of treatment, serum nitrite/nitrate, homocysteine, and lipid levels were decreased by the favorable effects of isoflavones in Turkish women in the early postmenopausal period.

In 2004 Sobey et al. investigated the effects of short-term phytoestrogen treatment in male rats and demonstrated that short term administration of daidzein or 17-β-estradiol modulates cerebral artery reactivity in males by enhancing synthesis and release of endothelium-derived NO (7). Genistein has also been proven to stimulate the release of NO in animal experiments (8). Our results clearly suggest that isoflavone therapy improves plasma nitrite/nitrate levels in Turkish women in the early postmenopausal period. This result is in accordance with those of previous studies in terms of positive effects of isoflavones on plasma nitrite/nitrate levels (10, 18). As NO causes vasodilatation, inhibits both platelet aggregation and endothelin-1 production, and achieves an increase in serum nitrite-nitrate concentrations after 6 month of treatment with isoflavone, NO may also have the potential to be a cardio protective agent. Several mechanisms may be responsible for this effect. Isoflavone therapy may regulate NO by increased activity of the endothelial NO synthase. It has been recently shown that the phytoestrogen genistein therapy produces acute

Table 2. Effect of isoflavone and placebo on biochemical parameters.

Variable	Before	After	Baseline	P value for changes compared with	
				Isoflavone-Placebo Before	Isoflavone-Placebo After
Fasting glucose					
Isoflavone	90.1 ± 9.9	89.5 ± 8.2	*NS	NS	NS
Placebo	94.1 ± 12.8	92.0 ± 15	NS		
Total cholesterol					
Isoflavone	263.8 ± 37	246.8 ± 29.6	0.003	NS	NS
Placebo	243.6 ± 29,5	249.5 ± 29.3	NS		
LDL cholesterol					
Isoflavone	164.5 ± 28.2	147.3 ± 23.4	0.001	NS	NS
Placebo	157.3 ± 25.2	158.3 ± 19.8	NS		
Triglyceride					
Isoflavone	150.6 ± 50.7	120.4 ± 43	0.008		
Placebo	156.7 ± 64.8	156.1 ± 50.1	NS	NS	0.037
HDL cholesterol					
Isoflavone	59.5 ± 13.4	64.9 ± 12.7	0.002	NS	0.021
Placebo	53.2 ± 10.8	55.4 ± 10.8	NS		
Nitrit*e*/nitrate (micromol/l)					
Isoflavone	27.8 ± 9.3	33 ± 8.2	0.031		
Placebo	25 ± 7.6	24 ± 7.4	NS	NS	0.000
Homocystein*e* (mmol/l)					
Isoflavone	7.5 ± 1	6.7 ± 0.9	0.003	NS	0.031
Placebo	8.7 ± 1.8	8.6 ± 1.5	NS		
LPa (mg/dl)					
Isoflavone	29.5 ± 29.5	34 ± 43.3	NS	NS	NS
Placebo	27.7 ± 21.1	32.1 ± 20.2	NS		

* No Significance

nitric oxide-dependent dilatation of human forearm vasculature with similar potency to 17ß-estradiol (19). Squadrito et al. demonstrated that the mean baseline ratio of nitric oxide to endothelin was significantly increased after six months of genistein treatment (20). In another study of Squadrito, 1 year of genistein therapy improved the endothelium function in postmenopausal women to a similar extent as did an estrogen/progestin regimen (18). These results, taken together, strongly suggest that genistein therapy, either through a genomic and receptor mediated effect or alternatively via a non-genomic effect, improves endothelium dependent vasodilation.

An increased plasma tHcy level was found to be an independent risk factor for CVD, similar to that of hyperlipidemia and smoking. In our study, at the end of the treatment period, in isoflavone group, plasma tHcy levels were significantly reduced compared to baseline values and placebo. Because most of the health benefits of isoflavones are thought to be mediated in part by an estrogenic mechanism, a decrease of plasma tHcy might be expected. Recent studies report that soy protein with isoflavones reduces plasma tHcy levels in hyperlipidemic and diabetic men and women (9, 21). In contrast, D'anna et al. (13) reported that the phytoestrogen genistein (54 mg/dl), after 6 months of treatment, does not modify circulating tHcy and CRP plasma levels from baseline. Studies investigating the effect of isoflavones on plasma tHcy used isolated soy protein rather than isolated isoflavones. A multicenter, double-blind, crossover intervention trial in 89 postmenopausal women

showed that a soy isoflavone extract incorporated in a food matrix, after 8 weeks of treatment, does not reduce circulating homocysteine levels (12). In our study we preferred the use of standardized isoflavones instead of isolated soy protein. Differing results between studies may result from the source of isoflavones and the treatment period.

It is plausible that the lower incidence of CVDs in populations ingesting diets high in phytoestrogen is due to an improved lipid profile. Soy isoflavones, like estrogens used for HT, stimulate HDL and decrease LDL synthesis in the liver, which initially seems to be suggestive of an anti-atherosclerotic effect, similar to that of estradiol. Results from double-blind, placebo-controlled trials of mildly hypercholesterolemic individuals have shown that 20 to 50 g of soy protein daily significantly reduces LDL levels (22). In 2004, a meta-analysis of 8 randomized controlled trials in humans showed that consumption of soy protein with a high isoflavone content significantly decreased serum LDL concentration compared with the same soy protein intake with low isoflavone content in hypercholesterolemic and normocholesterolemic individuals (23). Although in our study, patients' baseline cholesterol levels were moderately elevated, after 6 months of treatment, total cholesterol, LDL, and triglyceride levels decreased whereas HDL levels increased. Contrary to our results, Kreijkamp-Kaspers et al. (24) demonstrated that 25.6 g soy protein containing 99 mg of isoflavones administered daily for 12 months had no effect in improving plasma lipids as well as cognitive functions and bone mineral density, but the median plasma genistein levels in this study were found to be as low as 0.6 μM (24). Similarly, Lichtenstein et al. (25) performed a comprehensive study to examine the effects of soy proteins and soy-derived isoflavones on blood lipid parameters and demonstrated only a minimal reduction of blood cholesterol levels. Soy foods used in these studies were the source of isoflavones with limited bioavailability due to the food matrix in which they were incorporated and the insufficient effect of isoflavones on the lipid levels may be due to this.

In 2 meta analyses published by Hughes and Howes, clinical efficacy of soy isoflavones was clearly attributable to the composition and dosage of soy isoflavones (genistein and daidzein ratio) (26, 27). It is reported that estrogen receptor β gene polymorphism may alter the beneficial cardio protective role of isoflavones in postmenopausal women (28). In a study conducted by Yildiz et al., similar results with our trial on lipid profile obtained in menopausal women in early years were considered to be a strong predictive marker that Turkish population genotype may favor the isoflavones, especially genistein activity (29). As in our trial, it is advisable to inform that women in early ages following menopause may gain more benefit from isoflavones.

Lp(a) is another independent risk factor for coronary heart diseases. A recent publication from the HERS emphasizes the importance of Lp(a) as a predictor of CVD (30). In our study, the positive effects of isoflavones on plasma lipids were clarified but Lp(a) levels were not affected.

Commercially available isoflavones are mostly nutritional supplements and label claims do not always reflect the isoflavone content properly (31). The trials held with nutritional supplements with no or little regulation achieve limited efficacy with controversial results. Our trial, conducted with Isoflavin® tablet, which is under regulation of health authority and complies with all requirements of United States Pharmacopoeia, will surely help to understand the efficacy of isoflavones on our populations.

Conclusions

In this study we have shown that phytoestrogen isoflavones, after 6 months of treatment, increased serum nitrites/nitrates and high-density lipoproteins and decreased triglyceride and tHcy levels in Turkish women in the early postmenopausal period. Although there is not enough evidence yet from large randomized clinical trials to make a recommendation for the use of phytoestrogens for prevention of cardiovascular diseases in postmenopausal years, our results show that phytoestrogens in early postmenopausal years improve the biomarkers of cardiovascular diseases in Turkish population.

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