Original Article

Effect of soy isoflavone supplementation on vascular endothelial function and oxidative stress in postmenopausal women: a community randomized controlled trial

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A 12-month randomized double blind controlled trial was conducted among 182 Indonesian postmenopausal women aged 47 to 60 years to determine the effect of 100 mg/day soy isoflavone supplementation on vascular endothelial function such as vascular cell adhesion molecule-1 (VCAM-1), nitric oxide (NO) and malondialdehyde (MDA) as oxidative stress marker. The subjects were randomized to the intervention group receiving tablets consisting of 100 mg soy isoflavones and calcium carbonate 500 mg, and to the control group receiving 500 mg calcium carbonate. The concentrations of VCAM-1, NO and MDA were measured at baseline, and post-supplementation at 6 months and 12 months. After supplementation, the MDA concentrations were significantly lower in the soy isoflavone group compared with the control group (p=0.001). The concentrations of VCAM-1 and NO were not affected (p=0.992 and p=0.759, respectively). In all group the MDA concentration increased compared with baseline concentrations but the relative change of MDA concentrations was significantly lower in the soy isoflavone group compared with the control group. This study demonstrates that supplemental intake of soy isoflavones for 6 months and 12 months had an effect on oxidative stress by decreasing MDA concentration, but did not improve vascular endothelial function.

Key Words: soy isoflavone, supplementation, vascular endothelial function, oxidative stress, postmenopausal

INTRODUCTION

The prevalence of cardiovascular disease (CVD) is considerably higher in postmenopausal women than in premenopausal women.[1] Epidemiologic data indicate that Japanese and Asian women have lower CVD rates as compared to women in Western countries. This suggest that CVD may be associated with the higher dietary soy isoflavone intakes in Asian women.[2-4]

Hormonal therapy (HT) for reducing the impact of postmenopause is still controversial.[5] Several studies demonstrated that HT reduced the CVD risk by 50%, whereas others showed increased CVD cardiovascular prevalence rates.[6] Hormonal therapy is also expensive and thus affordable to only a small percentage of postmenopausal women.

Another form of therapy currently proposed is the use of isoflavones. These phytoestrogens are found in a variety of commonly consumed foods, such as soy bean products, and are considered to be the best among the phytoestrogens. Clinical studies on the in-vivo effects of isoflavones on endothelial function have yielded equivocal results.[2] There are numerous markers for endothelial function such as Von Willebrand factor (VWF), endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and nitric oxide (NO).[1,2] Soy isoflavones are also known to affect oxidative stress,[7-10] and it is this antioxidant effect of isoflavones that may protect the body from lipid oxidation and increase plasma antioxidant concentration.[7] The results of various clinical studies on the effects of soy isoflavones on oxidative stress are still subject to debate. One of the markers of lipid oxidation is malondialdehyde (MDA).[8-10]

The objective of the present study was to determine whether supplementation with soy isoflavones 100

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mg/day for 6 and 12 months would have an effect on vascular endothelial function and oxidative stress as indicated by decreased VCAM-1, increased NO and decreased MDA concentrations, respectively.

MATERIALS AND METHODS

Study design and subjects

This study was a randomized double-blind controlled interventional trial of 12 months' duration, conducted at Mampang District Health Center, South Jakarta, between January 2010 and February 2011. Sample size of postmenopausal women per group was estimated to be adequate to detect a 20% difference in the mean values of VCAM-1, MDA and NO between the treatment groups, using a two tailed test, and assuming an alpha of 0.05 and power of 80%.[8,11,12] The study was conducted in two phases. In the first phase, the subjects were screened by questionnaire, with the following inclusion criteria: healthy postmenopausal women aged 45 to 60 years, with natural cessation of menstruation of minimally one year and less than 10 years, BMI of $\leq$35 kg/m^2, not receiving medications and supplements in the previous 6 months (HT, glucocorticoids, anticoagulants, antihyperlipidemic agents, antihypertensive drugs, supplements containing isoflavones and oral antidiabetic agent), bilirubin concentration of $\leq$2.0 mg/dL, serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) concentrations within reference range, creatinine $\leq$1.5 mg/dL, ambulatory, and capable of verbal communication. The exclusion criteria were: menopause as results of total hysterectomy, bilateral oophorectomy, radiation, or chemotherapy; chronic or terminal diseases, diabetes mellitus, stroke and myocardial infarction/heart attack, severe psychosis and mastectomy. All subjects participating in this study signed informed consent after receiving information on the purpose of the study at baseline. Subjects meeting the inclusion and exclusion criteria underwent physical examination, performed by two trained nurses, comprising of determination of blood pressure, pulse rate, body weight, height, and waist and hip circumference. After the screening, 200 women were randomized within blocks of 4, with one half of the participants assigned to soy isoflavone tablets and the remaining subjects assigned to control tablets. Investigators, research staff, and subjects were blinded to the treatment codes. After a 12-hour fast, the subjects underwent laboratory screening tests, comprising fasting blood glucose, complete lipid profile, creatinine, SGOT, SGPT, bilirubin, and measurement of estradiol, soy isoflavones, MDA, NO and VCAM-1 concentrations. Ethical clearance was issued by the Human Ethics Committee of the Faculty of Medicine University of Indonesia.

Soy isoflavone and control tablets

The soy isoflavone tablets consisted of 100 mg soy extract (40% soy isoflavone content, consisting of genistein 56%, daidzein 41% and glycitein 3%) and 500 mg calcium carbonate. The control tablets contained 500 mg calcium carbonate only. The soy extract was imported from Hui Song Pharmaceuticals China with lot number SYB-080301 and were prepared into tablets by Ikapharmindo Pharmaceuticals in Indonesia with batch number 700307. An investigator (AH) at a site remote from the field area prepared the allocation sequence. Soy isoflavones in the soy extract were identified by high performance liquid chromatography (HPLC) at Bogor Indonesia. The isoflavone and control tablets, produced by Ikapharmindo Pharmaceuticals, were indistinguishable in physical and sensorial characteristics.

Compliance measures

Subject compliance was verified by direct observation and recording of tablet intake and monthly counting of remaining tablets. For evaluation of subject compliance and adverse events, the principal investigator monitored or rechecked the subjects by visiting the study area twice weekly and randomly interviewing 5 study subjects. For the 6-month trial, subjects were categorized as drop-outs if they failed to take supplement tablets for 7 consecutive days or took less than 90% of the required number of 151 tablets. Similarly, for the 12-month trial, the drop-out criterion was failure to take tablets for 14 consecutive days, or taking of less than 90% of 328 tablets.

Soy and nutrient intakes

During the study, the subjects stayed at home and were visited for collection of recall diet and semi-quantitative food frequency data at baseline and at 6 and 12 months post-supplementation. Calculation of semi-quantitative soy food frequency was performed based on analysis of soy isoflavone-containing foods, commonly consumed by the subjects and available at traditional markets. The nutritionists were not involved in the study and were blinded to the treatment codes.

Clinical blood chemistry

Serum samples were drawn three times at baseline, 6 months, and 12 months post-supplementation. Sera for assessment of soy isoflavones, MDA, NO, and VCAM-1 concentrations were stored at -70℃, pending assessment. Serum soy isoflavones (genistein, daidzein) were assessed by means of liquid chromatograph mass spectrophotometry (LCMS). Serum MDA was treated with thiobarbituric acid (TBA) to form MDA-TBA compounds, the amounts of which were measured spectrophotometrically at 532 nm wavelength. Before determination of lipid concentrations, the lipoprotein fraction was precipitated with trichloroacetic acid (TCA). NO was measured on the basis of the results of nitrite and nitrate reduction in the blood sample, using Griess' method. VCAM-1 measurement was by ELISA. Intra-assay and inter-assay coefficients of variation (CV) for soy isoflavone, MDA, NO and VCAM-1 assessment were 1.54%, 1.55%, 3.1%, 3.2%, 3.1%, 3.5%, 2.11%, and 2.7%, respectively.

Statistical analysis

Demographic and physical characteristics, and blood chemistry of the study subjects at baseline were compared between treatment groups to assess the effectiveness of randomization and to identify potentially confounding variables. In these analyses, differences for categorical variables were examined by chi-square test, and for continuous variables the means were compared by independent t-test. The effect of supplementation on dependent

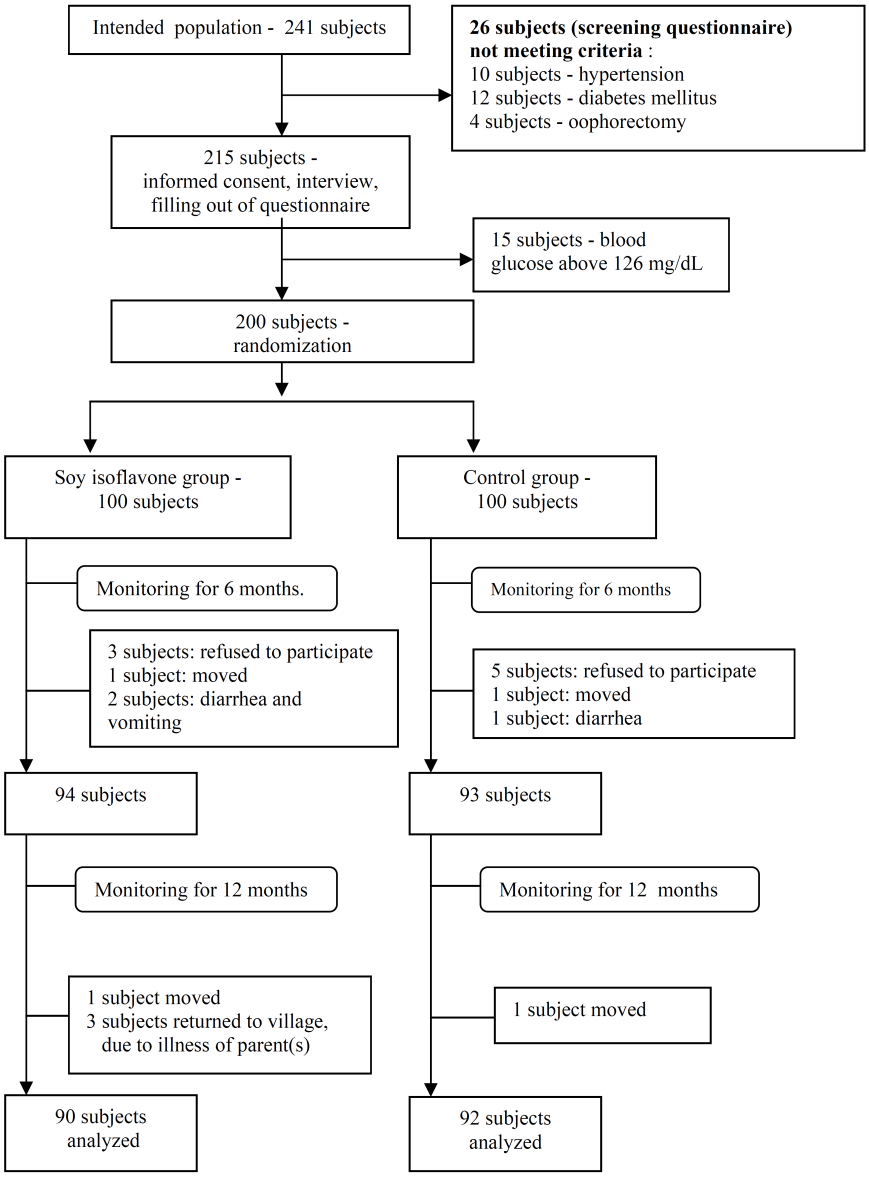

Figure 1. Flow of subject participation during study

variables was analyzed using general linear mixed model for repeated measurements (GLM) of ANOVA. Significance was set at $p<0.05$. Manufacturers were not involved in the study design and data analysis.

RESULTS

Study subjects

From 441 postmenopausal women aged 47 to 60 years, 241 women agreed to become study subjects, among whom 215 subjects met the inclusion and exclusion criteria. After physical examination and laboratory investigations, 15 women with fasting blood glucose concentrations above 126 mg/dL were excluded from the study. The remaining 200 subjects were randomized equally into the isoflavone and control groups; of these 18 withdrew during the follow-up period, 4 by moving out of the study area, 6 through illness, and 8 by withdrawing their consent. At 12 months post-supplementation the number of subjects in the soy isoflavone and control groups was 90 and 92, respectively (Figure 1). The compliance rate at 6 months post-supplementation was 99.94% in the soy isoflavone group and 99.97% in the control group. After 12 months supplementation the compliance rate was 99.97% in the soy isoflavone group and 99.98% in the control group. In addition to counting the remaining tablets, evaluation of compliance rate was also done by measuring the soy isoflavone concentrations in the blood, at 6 and 12 months post-supplementation. There were significant differences in blood soy isoflavone concentrations between the soy isoflavone and control groups with $p=0.001$ (data not shown).

Baseline subject characteristics (Tables 1 and 2) showed no significant differences in demographic data, physical characteristics, and laboratory measurements, indicating successful randomization of the subjects. The results from 24-hour food recall and semi-quantitative food frequency data at baseline, at 6 months and 12 months post-supplementation also showed no significant differences (data not shown).

GLM results showed that at 6 and 12 months post-supplementation of soy isoflavones 100 mg/day, the MDA concentrations in the soy isoflavone group was significantly lower than those in the control group at $p=0.001$ (Tables 3). In all group the MDA concentration

Table 1. Distribution of demographic and physical characteristics of the subjects at baseline by treatment group

Characteristic	Soy isoflavone group (n=92)	Control group (n=90)	*p*-value
Age, years	52.8 (6.6)	53.6 (3.5)	0.747†
Age at menarche, years (range)	15 (13-15)	14 (13-15)	0.218†
Age at menopause, years	48.9 (3.2)	49.2 (3.3)	0.697†
Duration of menopause, years† (range)	4 (3-6)	4 (2.1-6)	0.543†
Educational level, n (%)			
Academy	3 (3)	1 (1)	
Senior high school	11 (12)	7 (8)	
Junior high school	16 (18)	20 (22)	0.726‡
Primary school	42 (47)	48 (51)	
Did not finish primary school	9 (10)	8 (9)	
No education	9 (10)	8 (9)	
Employment status, n (%)			
Employed	49 (54)	35 (38)	0.224‡
Unemployed	41 (46)	57 (62)	
Weight, kg	59.2 (10.9)	59.5 (11.7)	0.823†
Height, cm	149 (5.1)	149 (5.3)	0.391†
Body mass index, kg/m^2	26.8 (4.8)	26.7 (4.7)	0.863†
Waist circumference, cm	83.6 (10.4)	84.2 (10.5)	0.686†
Hip circumference, cm	97.0 (8.0)	96.7 (8.6)	0.803†
Systolic blood pressure, mmHg	125 (22.9)	124 (19.6)	0.665†
Diastolic blood pressure, mmHg	78.6 (13.4)	78.6 (11.9)	0.964†
Pulse rate, per minute	78 (7)	77 (7)	0.323†

Values are Mean (SD); †calculated by independent t test, ‡chi-square test, significant at $p<0.05$.

Table 2. Descriptive statistics for blood chemistry measurement at baseline by treatment group

Parameter	Soy isoflavone group (n=90)	Control group (n=92)	*p*-value
Estradiol, pg/mL, (range)	5.63 (5-9.7)	5 (5-8.3)	0.111
Fasting glucose, mg/dL	89.1 (13.8)	86.6 (11.5)	0.180
Cholesterol, total, mg/dL	209.9 (38.4)	204.2 (34.8)	0.290
Cholesterol, HDL, mg/dL	55.4 (11.4)	58.9 (12.6)	0.060
Cholesterol, LDL, mg/dL	134.1 (34.8)	125.5 (32.1)	0.086
Triglycerides, mg/dL	121.8 (42.5)	108.7 (42.2)	0.178
SGOT, U/L	20.8 (5.2)	20.5 (5.2)	0.740
SGPT, U/L	14.3 (7.9)	13.4 (6.7)	0.897
Bilirubin, total, mg/dL	0.46 (0.19)	0.49 (0.21)	0.287
Protein, g/dL	7.6 (0.5)	7.5 (0.6)	0.271
Albumin, g/dL	4.4 (0.3)	4.5 (0.3)	0.260
Creatinine, mg/dL	0.67 (0.18)	0.63 (0.12)	0.083
Soy isoflavone			
Genistein, ng/mL	77.8 (63.7)	79.4 (59.5)	0.592
Daidzein, ng/mL	16.1 (3.2)	11.6 (9.2)	0.471
VCAM-1, ng/mL	855 (214)	840 (325)	0.723
NO, μmol/L	9.9 (6.8)	9.1 (5.5)	0.380
MDA, nmol/mL	0.77 (0.24)	0.81 (0.23)	0.338

Values are mean (SD)
HDL cholesterol; high density lipoprotein cholesterol, LDL cholesterol: low density lipoprotein cholesterol, SGOT: serum glutamic oxaloacetic transaminase, SGPT: serum glutamic pyruvic transaminase, VCAM: vascular cell adhesion molecule, NO: nitric oxide, MDA: malondialdehyde, *p* values determined by independent t test; significant at $p<0.05$.

increased compared with baseline concentration but the relative change of MDA concentrations was significantly lower in the soy isoflavone group compared with the control group. This indicates that supplementation with soy isoflavones 100 mg/day for 6 months already showed antioxidant effects in the soy isoflavone group compared with the control group, as did also supplementation for 12 months. From the results of the GLM it was found that at 6 and 12 months post-supplementation, the VCAM-1 concentrations did not show significant differences between the soy isoflavone and the control group ($p=0.992$), which was also the case with the NO concentrations between the two groups ($p=0.759$).

Adverse events

Most of subjects did not have any subjective complaints, both in the isoflavone group and the control group (55.9% vs 58.4%). No subjects dropped out from this study for clinical reasons. The subjective complaints in both treatment groups were pain (in the knees, legs, waist), leg stiffness, paresthesia, headache, and increased appetite (data not shown). Statistical analysis with the chi square test did not find any significant differences ($p=0.531$). Adverse events from laboratory examination, such as liver functions (protein, albumin, SGOT, SGPT) were not significantly different except for creatinine ($p=0.007$), (data not shown) which however were clinically not sign-

Table 3. Relative change of mean VCAM-1, NO, MDA concentrations by supplementation group from baseline, 6 months and 12 months supplementation, according to a general linear model

Study group	Baseline/0 months	6 months	Relative change (%) 0-6 months	12 months	Relative change (%) 0-12 months
VCAM-1, ng/mL					
Soy isoflavone (n=90)	855 (214)	841 (223)	-1.7	854 (219)	-0,1
Control (n=92)	840 (325)	862 (338)	2.6	854 (219)	2.3
NO, μmol/L					
Soy isoflavone (n=90)	9.9 (6.8)	10.1 (6.7)	2.0	10.1 (8.5)	2.0
Control n=(92)	9.1 (5.5)	9.9 (7.1)	8.8	8.9 (6.9)	-2.2
MDA, nmol/mL					
Soy isoflavone (n=90)	0.77 (0.24)	0.89 (0.26)[a]	15.6*	0.93 (0.26)[c]	16.0*
Control (n=92)	0.81 (0.23)	0.96 (0.26)[b]	18.5*	0.96 (0.25)[d]	18.5*

Values are mean (SD). VCAM: vascular cell adhesion molecule, NO: nitric oxide, MDA: malondialdehyde. Each parameter was analyzed using GLM of ANOVA, [a,b,c,d] post hoc test (difference between groups) significant at $p<0.05$, *ANOVA significant at $p<0.001$

ificant (0.66 mg/dL vs 0.60 mg/dL).

DISCUSSION

After 6 months and 12 months of soy isoflavone supplementation, only MDA concentrations significantly increased in both groups, compared with baseline values. In contrast, VCAM-1 and NO concentrations showed no significant differences between groups. Increased MDA concentrations were also obtained by Vega-Lopez *et al*[8] and Wiseman *et al*.[10] In our study, increased postsupplementation MDA concentrations were possibly caused by high subject compliance in taking the supplementation (up to 90%), increased lipid peroxidation by free radicals, and maximal isoflavone concentrations.[8,9] In adults, highest peak plasma concentration from both isoflavone (genistein and daidzein) were at 6 hours after administration, but in our study we only used a-single tablet/day and urinary excretion rates ranged from 16 to 66% after the ingested dose.[15] Lipid peroxidation may be increased either through age-related decreases in estrogen concentrations or by high LDL cholesterol concentrations,[13,14] as in the study by Vega-Lopez *et al*.[8] on subjects with hypercholesterolemia. In our study one of the sources of increasing MDA in both groups were intake of PUFA, but the results of 24 hours recall of PUFA in the study subjects showed no significant differences ($p<0.473$; data not shown) between the two groups at baseline, 6 months and 12 months after supplementation. Therefore intakes of PUFA were presumably not the reason for the MDA increases in both groups. Maximal soy isoflavone concentrations are found during prolonged or continuous supplementation, or when a plateau concentration is reached.[15] In the present study, at 6 months and 12 months post-supplementation, genistein and daidzein concentrations did not increase significantly, thus possibly resulting in smaller (but still significant) mean MDA differences between both groups at 12 months post-supplementation. Other studies similarly found no increase in blood isoflavone concentrations after continuous soy isoflavone supplementation, resulting in constant MDA concentrations.[15,16]

Supporting our findings on VCAM-1 concentrations, several studies also failed to detect significant differences in VCAM-1 post-supplementation concentrations between isoflavone and control groups.[17-20] However, other studies reported decreased VCAM-1 post-supplementation concentrations.[11,21,22] These variable results on the relationship between isoflavones and adhesion molecules may probably be explained by duration of supplementation, dosage, and bioavailability of the soy isoflavone supplement. The studies showing no effect of soy isoflavones (given at dosages equivalent to ours) used a shorter supplementation period of 6 weeks,[18-20] while our study was of longer duration, which was sufficient to show any isoflavone effects. Higher dosages and differing daidzein to genistein ratios might possibly demonstrate an effect of isoflavones on adhesion molecules. The study of Teede *et al*,[22] comparing formononetin (daidzein precursor) and biochanin (genistein precursor), found an effect on adhesion molecules in the formononetin group but not in the biochanin group, indicating a greater effect of daidzein on adhesion molecules. This may be the reason for our differring VCAM-1 results, as our isoflavone tablets contained more genistein than daidzein, but this remains conjectural. Dietary intake of soy isoflavones might also be a factor. Our study subjects had relatively high daily soy isoflavone intakes, whereas most studies showing significant results were conducted in Western countries with low soy isoflavone intakes.

Our mean baseline NO concentrations were substantially lower than those in the studies by Hall *et al*,[12] Nikander *et al*,[23] and Chan *et al*,[24] suggesting ethnic influences. Among the studies reporting increased NO concentrations after soy isoflavone supplementation are those of Hall *et al*,[12] Squadrito *et al*,[25,26] and Hallund *et al*[27] To explain these variable results on NO concentrations, the possible factors to be considered are duration of supplementation, dosage and bioavailability of soy isoflavones, type of soy isoflavones, subjects characteristics, and time of blood sample collection, as well as NO assessment. The cross-over study by Nikander *et al*,[23] demonstrating no effect of soy isoflavones, was of 3 months' duration, presumably too short to observe any supplementation effects. In contrast, our longer supplementation periods tended to increase NO concentrations in the isoflavone group.

Hall *et al*,[12] used a dosage of 80 mg isoflavones per day (containing genistein 57%, daidzein 39.6 % and glycitein 3.4%), whereas our dosage was 100 mg/day, with a similar composition. However, their study showed increased NO concentrations at peak blood isoflavone concentrations (7 hours after oral administration). In our study and

in others demonstrating no increased NO concentrations, blood samples were collected in the morning, when the isoflavone concentrations were not at their peak. This may explain why soy isoflavone supplementation studies conducted continuously within a given period, did not show significant differences in NO concentrations. The probable mechanisms underlying reduced NO concentrations in postmenopausal women are decreased NO production or inactivation by peroxynitrite ($ONOO^-$) formation.[28]

The implication of this study is that supplementation with soy isoflavone 100 mg/day may be used as alternative supplement for postmenopausal women because it is capable of reducing oxidative stress by lowering MDA concentration in isoflavone group compared with control group. The limitations in our study are that menopausal status was not confirmed by determination of follicle stimulating hormone concentration and that isoflavones were administered in a single dose, not in divided doses. In conclusion, supplementation with soy isoflavones 100 mg/day for 6 months and 12 months is capable of reducing oxidative stress through lowering the MDA concentration, but does not improve vascular endothelial function.

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AUTHOR DISCLOSURES

Pusparini, Rahayuningsih Dharma, Fransiscus D Suyatna, Muchtaruddin Mansyur and Adi Hidajat disclose no conflict of interest.

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Original Article

Effect of soy isoflavone supplementation on vascular endothelial function and oxidative stress in postmenopausal women : a community randomized controlled trial

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大豆異黃酮補充劑對於停經後婦女血管內皮細胞功能及氧化壓力的影響：社區隨機對照試驗

共 182 位，年齡介於 47 至 60 歲，已停經的印尼婦女，參與為期一年的隨機雙盲對照試驗。研究目的為評估停經後婦女，每日攝取 100 毫克大豆異黃酮補充劑，對於血管內皮功能，如血管細胞黏附分子-1(VCAM-1)、一氧化氮(NO)，以及氧化壓力指標-丙二醛(MDA)的影響。受試者被隨機分派至介入組及控制組，介入組攝取含有 100 毫克大豆異黃酮及 500 毫克碳酸鈣的錠片，控制組僅攝取 500 毫克碳酸鈣片。於介入前、介入後 6 個月、及介入一年，三個時間點檢測受試者血管細胞黏附分子-1、一氧化氮以及丙二醛濃度。研究結果顯示，介入組在攝取大豆異黃酮補充劑後，比起控制組，有較低的丙二醛濃度(p=0.001)，但血管細胞黏附分子-1 及一氧化氮濃度則無顯著差異(p=0.992 與 p=0.759)。兩組的丙二醛濃度皆增加，但介入組的相對改變量顯著低於控制組。本研究證實，攝取大豆異黃酮補充劑 6 個月與 12 個月，有助於減少丙二醛的濃度，藉以降低氧化壓力產生，但無法改善血管內皮功能。

關鍵字：大豆異黃酮、補充劑、血管內皮功能、氧化壓力、更年期