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THE EFFECT OF ISOFLAVONES SUPPLEMENTATION ON SEX STEROIDS, LIPIDS AND INFLAMMATION MARKERS IN ELDERLY MEN

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Abstract: *Background/Objective:* To analyze the biological impact of soy isoflavones intake over specific cardiovascular risk factors, we investigated whether an oral supplement influenced serum levels of lipoprotein, steroids sex hormones and some inflammatory markers in elderly men. *Subject/Methods:* Thirty healthy men, aged 65-80 years, were invited to take part in this randomized control study, to receive either 100 mg/day of isoflavones supplement (n=15) or identical placebo capsules (n=15). Blood samples obtained at baseline and after 24 weeks were analyzed for total isoflavones, lipoprotein, triglycerides, fibrinogen, adiponectin, leptin, insulin, estradiol, testosterone, DHEA, DHEAS, sex hormone-binding globulin, plasminogen activator inhibitor-1 (PAI-1), P-selectin, high sensitive C-reactive protein (hsCRP) and platelet thromboxane A2 receptor density. Body weight and blood pressure were also registered at baseline and at the end of the treatment. Changes in variables within and between groups were analyzed through ANOVA for repeated measures. *Results:* The levels of PAI, hsCRP and p-selectin were decreased significantly ($p<0.05$) after the treatment period only in the intervention group. All the other studied parameters did not change significantly in either group. *Conclusion:* During this 24–weeks intervention, soy isoflavone intake modified serum levels of PAI-1, hsCRP and P-selectin in elderly men. Further studies with diverse markers of inflammation are necessary to clarify the specific effect of isoflavones on immune response.

Key words: Isoflavone, inflammatory marker, elderly men.

Introduction

Epidemiological studies have shown that the incidences of cardiovascular diseases are commonly high in Western Europe, United States of America, and Latin America, but low in Asian populations who consume large amounts of soy bean foods (1, 2). Soy products are rich in isoflavones, such as genistein and daidzein. Isoflavones have been suggested as the principal chemical constituents responsible for the potential preventive effect of soy bean against cardiovascular risk factors (3). Different mechanisms have been proposed to explain the protective effects of soy isoflavones on cardiovascular disease (CVD), including estrogen-like effects, prevention of LDL oxidation and anti-platelets aggregation (4, 5); however, the main interest has been to study the effects of isoflavones on lipoprotein status. The reason for this focus is that serum lipoproteins have been the most studied risk factor for CVD. In epidemiological and observational studies, the consumption of soy isoflavones has been negatively related to circulating total or LDL cholesterol (6) and triacylglycerol (7) and positively related to HDL (8). In clinical trials in both, women and men, the effects of isoflavones on serum lipoprotein are controversial; some studies suggest that soy-derived isoflavones decrease serum total and LDL-cholesterol (9), whereas many others have observed no effect (10, 11).

The impact of isoflavones in the circulating levels of sex hormones has been extensively studied. In pre and post-menopausal women, consumption of soy isoflavones had no effect on circulating total estradiol (E2), estrone or SHBG (12, 13). On the other hand, some studies in adult men treated with soy isoflavones have found increases in SHBG together with a fall in free testosterone and DHT (14), while in many other studies no changes have been observed (15, 16, 17).

Platelets may become activated at sites of vascular injury and inflammation. Activated platelets rapidly adhere to one another (platelet aggregation) or to exposed subendothelial matrix. These events are pre-requisite not only to physiological haemostasis but also to pathological thrombosis (18, 19).

Growing body of evidences suggest that systemic

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inflammation plays a key role in the pathogenesis of CVD (20), and based on genistein's ability to down-regulate cytokine-induced signal transduction in immune cells and to suppress cell-mediated inflammatory response and atherosclerosis in experimental models, isoflavones have been proposed as possible anti-inflammatory agents and thus antithrombotic (20).

Little information is available with regard to the effect of soy supplementation on circulating inflammatory markers in men (21). In this intervention study, with the purpose to analyze the biological impact of soy isoflavones against some risk factors of CVD, we investigated whether daily consumption of an isoflavone supplement influenced serum of lipoprotein, steroids sex hormones and some inflammatory markers in elderly men.

Subjects and methods

The study protocol was reviewed and approved by the Institute of Nutrition and Food Technology, University of Chile Review Board Human Subjects Committee.

Study Participants

Sixty men, aged 65-80 years, were recruited from Santiago Metropolitan Area, and gave written informed consent to participate in this study. Of the sixty men who expressed interest in the study, thirty eligible men were invited to participate. We excluded men with any type of hormonal treatment during the last 24 weeks, currently using lipid-lowering drugs, soybean-derived products or dietary herbal supplements. Other exclusion criteria were: cigarette smoking within last 5 years, diabetes, heavy alcohol consumption (>30 g/day), hypertension and coexisting major illnesses. A fasting blood sample was obtained to perform the following test: routine clinical laboratory (serum lipoprotein, glucose, insulin, hepatic and renal screening test), 17β-estradiol (E2), testosterone (T), free testosterone, DHEAS, DHEA, SHBG, and thromboxane A2 (TxA2) receptor density. Volunteers were then randomly assigned to taken twice daily capsules of a soy-germ isoflavones extract (50 mg isoflavones per capsule: SoyLife ® Netherlands B.V.) or identical placebo capsules during 24 consecutives weeks; one capsule was taken after breakfast and other capsule after dinner. The study coordinator and investigation team performing the blood collection, and assays were blinded to the group assignment.

Laboratory measurements

Routine laboratory and hormone serum levels

Commercially available RIA kit (Diagnostic Product, Los Angeles, CA, USA) were employed to measure concentration of E2, testosterone, DHEA, DHEAS and insulin. Free testosterone was calculated from the reported measured serum concentration of testosterone and SHBG by use of the low of mass action (22). Total plasma cholesterol (TC), HDL-cholesterol, and total triacylglycerols (TG) were quantified by commercially available kits (Sigma Diagnostics, St Louis, MO, USA). LDL cholesterol (LDL-C) was calculated by using the Friedewald equation (23).

Anthropometric data were collected by a designated research nurse and physician, ascertaining height (to the nearest 0.5 cm), weight (to the nearest 0.1 kg), in addition to systolic and diastolic blood pressure measurements. Morning fasting blood samples were collected from all subjects on an assigned date. Serum glucose, renal and hepatic profiles were measured using automated laboratory methods. Serum total leptin and adiponectin were evaluated in one run using a commercially available RIA (Linco Research, Inc., St. Louis, MO, USA). PAI-1, P-selectin, and hsCRP were quantified using ELISA kit (R&D System, Inc, Minneapolis, MN, USA). The intra-assay coefficients are 7.8% for leptin and 8.6% for adiponectin, 7.1% for P-selectin, 7.3% for PAI-1 and 6.3% for hsCPR and the inter-assay coefficients are 6.2% for leptin and 7.9% for adiponectin, 5.3% for P-selectin, 7.9% for PAI-1 and 9.3% for hsCPR.

Total isoflavones in plasma and total isoflavones in soy-germ extract capsules were analyzed by HPLC, as described previously (24).

Radioligand binding assay for thromboxane A2 receptor determination

Experiments of binding [^{3}H]-SQ-29548 to platelet were carried out essentially as previously described (24). Briefly, aliquots of platelet suspensions (0.4 ml) were diluted to a final volume of 0.8 ml in assay buffer (140 mmol/L NaCl, 5 mmol/L KCl, 5.6 mmol/L dextrose, 25 mmol/L Tris-HCl. pH 7.4) contained 1,0 mmol/L aspirin and 0.1% BSA in the presence of 8 nmol/L [^{3}H]-SQ-29548 (38 Ci/mmol; PerkinElmer NEN), and then incubated at 37 °C for 30 min. The reaction was terminated by the addition of 2 ml of ice- cold assay buffer followed by filtration under reduced pressure through Whatman GFC glass filter. The tubes and filter were washed with assay buffer (four times with 2 ml) and the radioactivity was counted. Specific binding was defined as the total amount of radioactivity bound minus radioactivity observed in the presence of a thousand times of SQ-29548. Saturation binding curves were constructed using increasing concentration of [^{3}H]-SQ-29548 (0.1- 9 nmol/L). The dissociation concentration (kd) and maximum receptor density (Bmax) were analyzed by Graph PadPRISM (version 3, Software, San Diego, CA, USA).

Statistical analysis

Results are expressed as means ± S.D. Significance was considered at $p<0.05$. Differences between the groups at baseline were evaluated by using Student's t test. ANOVA for repeated measurements was employed to study changes in parameters between and within treatment group. Data analysis was performed using Statistica for Window® version 17.0.2.

Results

The two groups were well matched for all baseline variables, including age, blood pressure, lipoprotein, body weigh, (Table 1), sex hormones (Table 2), inflammation markers, adipokines, etc, except for P-selectin levels which were significantly higher among isoflavone treated subjects. All volunteers were healthy, except for mild hypertension detected in three men. Hypercholesterolemia (total cholesterol over 200 mg/dL) was present in two cases and hypertriglyceridemia (serum fasting triglycerides over 150 mg/dL) in one subject. Adverse reactions associated with to the soy supplement were not reported, and one individual abandoned the study for personal reasons.

Total isoflavones, measured in the soy-germ extract capsules, contained 24.4 ± 3.4 mg daidzein, 17.6 ± 2.8 mg glycitein and 6.2 + 0.9 mg genistein; thus, the subjects in the experimental group ingested approximately 100 mg aglycone isoflavone equivalent weights daily. All men assigned to isoflavones group showed a clear rise in plasma isoflavone levels (Table 2); thus, from negligible levels at baseline, isoflavone serum level rose 18-fold in the treatment group (25.3 ± 3.7 to 473 ± 75 nmol/L), remaining unchanged in the placebo group.

No significant changes were observed in blood pressure, body weight and lipoprotein serum levels (Table 1) either in the placebo or isoflavone group. Also, no significant changes in total testosterone, free testosterone, DHEAS, DAEA and SHBG serum levels were obtained (Table 2). However, in subjects treated with isoflavones serum levels of P-selectin, hsCRP and PAI-1 significantly decreased after 24 weeks (Table 3). The serum concentration of fibrinogen, leptin and adiponectin did not change significantly in either treatment group.

We evaluated binding of [^{3}H]-SQ-29548 to platelets, where the Bmax and Kd were determined by Scatchard analysis (data not shown). [^{3}H]-SQ-29548 bound to TxA2 receptor in a saturable manner, and the analysis (Table 4) revealed that the Kd and the Bmax before and after isoflavone treatment were similar;1.7 ± 0.27 nmol/L versus 1.9 ± 0.3 nmol/L($p = 0.41$) and 146 ± 23 fmol/10^8 platelets versus 151 ± 25 fmol /10^8 ($p = 0.56$), respectively. In the control group the Kd before and after placebo

Table 1
Lipoprotein profiles and blood pressure in elderly men treated with isoflavone or placebo for 24 weeks

	Placebo group (15)		Isoflavones group (15)		p-Value
	Baseline	Treatment	Baseline	Treatment	
BMI (kg/m^2)	26.7 ± 3.2	27.2 ± 2.8	27.4 ± 2.5	27.1 ± 2.2	0.69
Total-cholesterol (mmol/L)	5.1 ± 1.0	4.8 ± 0.9	5.2 ± 1.0	4.9 ± 1.0	0.30
HDL-cholesterol (mmol/L)	1.3 ± 0.4	1.2 ± 0.3	1.4 ± 0.3	1.3 ± 0.3	0.51
LDL-cholesterol (mmol/L)	3.1 ± 0.5	2.9 ± 0.6	2.8 ± 0.8	3.0 ± 0.8	0.70
Triglycerides (mmol/L)	1.4 ± 0.5	1.7 ± 0.7	1.3 ± 0.6	1.5 ± 0.6	0.41
Apo A-1 (g/L)	1.5 ± 0.2	1.4 ± 0.3	1.5 ± 0.3	1.4 ± 0.4	0.37
Apo B (g/L)	1.1 ± 0.3	1.2 ± 0.4	1.2 ± 0.4	1.1 ± 0.3	0.48
SBP (mmHg)	139.4 ± 21.3	140.4 ± 22.4	138.3 ± 20.5	139.7 ± 21.7	0.45
DBP (mmHg)	81.3 ± 9.7	80.6 ± 10.1	82.6 ± 10.5	81.8 ± 10.8	0.54

Values represent mean ± S.D. BMI, body mass index. SBP, systolic blood pressure. DBP. diastolic blood pressure.

Table 2
Hormones profile and isoflavones level in elderly men treated with isoflavones or placebo for 24 weeks

	Placebo group (15)		Isoflavones group (15)		p-Value
	Baseline	Treatment	Baseline	Treatment	
Total testosterone (nmol/L)	12.3 ± 4.2	10.8 ± 3.4	12.9 ± 3.6	13.2 ± 4.8	0.41
Free testosterone (nmol/L)	5.4 ± 1.3	4.8 ± 1.2	5.7 ± 1.4	5.9 ±1.3	0.36
SHBG (nmol/L)	37.2 ± 7.8	35.2 ± 9.8	43.2 ± 12.1	41.0 ± 12.2	0.30
DHEA (nmol/L)	12.8 ± 2.6	12.1 ± 3.4	13.2 ± 3.6	13.7 ± 2.7	0.53
DHEAS (μmol/L)	2.9 ± 0.7	2.6 ± 0.8	3.1 ± 0.8	3.0 ± 0.9	0.47
Total isoflavones (nmol/L)	27.2 ± 3.5	28.3 ± 3.9	25.3 ± 3.7	473 ± 75	0.002

Value represent mean ± S.D; $P< 0.05$, statistically significant.

Table 3
Plasma concentrations of inflammation markers in elderly men treated with Isoflavones or placebo for 24 weeks

	Placebo group (n=15)		Isoflavone group (15)		P-value
	Baseline	Treatment	Baseline	Treatment	
hsCRP (mg/L)	3.9 ± 1.1	4,2 ± 2,1	3.8 ± 1,2	2,7 ± 0.82	0.02
sP-selectin (ng/ml)	29.5 ± 8.1	29,0 ± 8,6	41,7 ± 9,2	29,8 ± 10.7	0.002
Adiponectin (μg/mL)	11.4 ± 5.1	10.5 ± 4.3	13.4 ± 5.5	12.9 ± 5.4	0.34
Leptin (ng/mL)	8,6 ± 4.5	8.8 ± 4.5	7.9 ± 2.6	7.7 ± 2.7	0.42
PAI-1 (ng/mL)	15.1 ± 2.3	14,1 ± 3,4	15.2 ± 2,2	12.1 ± 2.3	0.03
Fibrinogen (mg/mL)	2.4 ± 0.4	2.3 ± 0.47	2.4 ± 0.28	2.3 ± 0.25	0.35

Values represent mean ± SD; hsCRP, high sensitive C-reactive protein. PAI-1, plasminogen activator inhibitor-1; P< 0.05, statistically significant

Table 4
Effect of isoflavones on Kd and Bmax for specific binding of [3H]- SQ-29548 in platelets of elderly men

	Placebo group (n=15)		Isoflavone group (15)		P-value
	Baseline	Treatment	Baseline	Treatment	
Kd (pmol/L)	1.8 ± 0.29	1.7 ± 0.3	1.7 ± 0.27	1.9 ± 0.3	0.41
Bmax (fmol/108 platelets)	159 ± 26	153 ± 34	145 ± 23	151 ± 25	0.56

Kd = dissociation constant; Bmax = maximal number of binding sites; [3H]- SQ-29548, thromboxane A2 antagonist; Statistical significance $p < 0.05$; Values represent mean ± SD.

treatment was 1.8 ± 0.29 nmol/L versus 1.7 ± 0.32 nmol/L ($p = 0.59$) and Bmax before and after treatment was 159 ± 26 fmol/ 10^8 platelet versus 153 ± 34 fmol/10^8 platelet ($p = 0.47$), respectively.

Discussion

The results of the present double-blind, randomized placebo-controlled study indicate that a supplementation with soy-germ derive isoflavones had significant effects in reducing serum levels of PAI-1, hsCRP and P-selectin, but was ineffective in modifying cholesterol, triglycerides, fibrinogen, adiponectin and leptin concentrations in health elderly men. In addition, there were no significant changes in lipoprotein fractions and apoproteins associated with the treatment, and soy isoflavones had no significant effect in reducing the platelet TxA2 receptor density. The testis and adrenal steroidogenic function and production of SHBG were also unaffected by isoflavone treatment.

Isoflavones have been promoted as good candidates for the beneficial lipoprotein changes attributed to soy intake (25). However, our results along with previous investigations on the effect of isoflavones supplements in women and men, do no support this general hypothesis (10, 11). More over, the meta-analysis of Taku et al, of studies published between 1966 and 2007, found that ingestion of an average of 70 mg soy isoflavones per day for 1-3 months did not significantly change total cholesterol and LDL-C compared with placebo, in normocholesterolemic menopausal women (26).

Theoretically, exposure to high levels of dietary estrogens could alter the hypothalamic-pituitary-gonadal axis in men in a similar manner to that of an estradiol agonist. In the present study there was no significant change in E2, testosterone, free testosterone, DHEAS, DHEA and SHBG serum levels associated with isoflavones intake. Studies have indicated that isoflavone have estrogenic properties, but its metabolic and hormonal effects in both men and women seem to be controversial (12-17). However, a recently published meta-analysis by Hamilton-Reeves et al. (16) concluded that neither soy protein nor isoflavone intake affect circulating levels of total T, SHBG, free T, or the free androgen index, in agreement with the data of the present study.

Platelets contribute critically to the development and progression of CVD, and in the last years several studies have provided evidences that the isoflavones can modulate platelet´s activity. Evidence of platelet involvement in this syndrome includes increased TxA2 synthesis and increased platelet TxA2 receptor density (24, 27). In the present study, isoflavone supplementation had no effect on the latter. This result is in contrast with our previous studies, in menopause women, where the intake of a similar isoflavone supplement declined platelet TxA2 receptor density (~30% compared with control) (24). This observed discrepancy is not easy to explain, but is it likely that the higher plasma T concentration of men, compared with menopause women, could alter the effect of isoflavones. The administration of testosterone to young men, at a clinical replacement dose, increased TxA2 platelet receptor density (28). Moreover, in castrated men with prostate cancer the TxA2 platelets receptor density is decreased (29).

Platelets' sole role was once thought to be for thrombosis, but an expanding body of evidence has shown them to be important in a number of inflammatory responses, including the interaction between activated vascular endothelial cells and circulating monocytes, via adhesion molecules (19): e.g. one of the mechanisms by which platelets and leukocytes interact is via binding of P-selectin on platelets to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes. Soluble P-selectin is a platelet marker rather than an endothelial cell marker. There is a notion that isoflavones exert lipid-independent vascular benefits. Accordingly, in this investigation, a marked reduction in P-selectin concentration was observed after isoflavone supplementation. Prior studies reported null effect of isoflavones on serum levels of inflammatory markers (21). It is likely, however, that the impact of isoflavones on this specific marker depends on the physiological status of the subjects. In the current study, baseline of serum P-selectin was significantly ($p< 0.02$) higher in the isoflavone treated compared to the placebo group. We think that effect of isoflavone on P-selectin should be interpreted with caution that either be simply reflect regression to the mean or a significant effect that would need to be confirmed.

Combining clinical findings with experimental observations, CRP is considered a direct mediator of CVD and atherosclerosis (30). The finding of the present study show a significant ($p<0.03$) decrease in plasma hsCRP concentration after supplementation with isoflavone. Interestingly, in a recent study, it has been observed that soy isoflavones for 12 weeks resulted in a significant decrease in serum hs-CRP level in men with prior ischaemic stroke (31). These results confirm anti-inflammatory mechanism to explain the beneficial effects of dietary isoflavones

Increased levels of PAI-1 in the vasculature and other tissues have been known to associate with thrombosis and fibrosis, respectively. During the initiation of thrombus formation, release of PAI-1 by platelets represents the most likely primary source of PAI-1 (32). However, different types of cells have the capacity to produce PAI-1 in response to various inflammatory cytokines (33). The multiplicities of potential sources of PAI-1 as a response factor have implications for PAI-1 function in both physiological and pathophysiological conditions. Like CRP and fibrinogen, PAI-1 levels in plasma have been shown to increase in response both to acute trauma, such as local tissue injury (34) and to chronic inflammatory states, predisposing to CVD (35). In the present research serum PAI-1 levels decreased by isoflavone treatment, but serum fibrinogen concentration remain unchanged. In postmenopausal women several studies have shown no effect of isoflavone on serum PAI-1 level (36, 37).

The mechanism by which soy isoflavone decreases the plasma levels of PAI-1, P-selectin and CRP is not known, but by means of the platelet activation it might be. Thus, isoflavones may block of calcium channel (27), binding to thromboxane A2 receptors (38, 39), or interfere with different platelet signaling pathways triggered by thrombin (40).

The present findings, although requiring confirmation in a larger trial, show that in elderly men soy isoflavones treatment result in reduction of some inflammation and procoagulation markers, suggesting that soy isoflavone have biologically significant effects on the hemostatic system. However, further mechanistic research will be necessary to definitively assess the safety and efficacy of soy isoflavones.

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