

A RANDOMISED, DOUBLE BLIND, PLACEBO CONTROLLED STUDY TO EVALUATE THE EFFECT OF ISOFLAVONES ON ADP AND EPINEPHRINE INDUCED PLATELET AGGREGATION IN OOPHORECTOMISED WOMEN

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Summary

The present study was conducted with an objective to probe into the cardioprotective mechanism of isoflavones by evaluating their platelet aggregation inhibitory potential in oophorectomised women in a randomised, double blind, parallel, placebo controlled manner. 43 women were randomised to receive 75mg/day isoflavones tablet or placebo for 12 weeks. ADP and epinephrine induced platelet aggregation was determined at baseline and at the end of study. Platelet aggregation was not significantly altered with ADP (student's paired t test, P value for isoflavones: 0.19; for placebo: 0.70) or epinephrine (student's paired t test, P value for isoflavones: 0.65; for placebo: 0.64) after 12 weeks in either of the drug or placebo groups. Also, the median change in platelet aggregation at the end of 12 weeks with study drug was not significantly different as compared to the placebo with either ADP (unpaired t test, P value: 0.27) or epinephrine (unpaired t test, P value: 0.52). There was no statistically significant difference in the proportion of patients experiencing adverse events in two groups (P value: 1.00).

Key Words: Isoflavones, ADP, Epinephrine, Platelet Aggregation, Oophorectomised Women

Introduction

Isoflavones are the most common form of phytoestrogens, a group of naturally occurring plant compounds with demonstrated estrogenic and/or antiestrogenic activity. There are three main classes of phytoestrogens: isoflavones, coumestans and lignans. The major dietary sources of these include: legumes and soyabean products (isoflavones); whole grain cereals, fruits, seeds and alcoholic sources (lignans); bean sprouts and fodder crops (coumestans). Among isoflavones, the most investigated compounds with regard to oestrogenicity are genistein, daidzein, biochanin A and formononetin. Genistein (4',5,7-trihydroxyisoflavone) is the most active principle with the highest binding affinity for the estrogen receptor. ^[1]

Phytoestrogens hold great promise as botanical dietary supplements for postmenopausal women. There has been a recent interest in the use of phytoestrogens due to their positive effect on cardiovascular health. The Food and Drug Administration (FDA) in the USA has approved a health claim for soya since laboratory investigations, clinical trials and epidemiological data indicate that high soya consumption is associated with a lower risk of coronary artery disease. ^[2] Although there is substantial evidence to indicate that it is the isoflavone fraction that provides the anti-thrombogenic and anti-atherogenic effects of soya, however the mechanisms by which isoflavones exert these beneficial effects still remains partly understood. ^[3]

Previous studies have demonstrated the beneficial potential of isoflavones to improve endothelial dysfunction ^[4,5] reduce arterial stiffness ^[6] and exert antithrombotic activity by virtue of their stimulation of prostacyclin production by binding to estrogen receptor beta on endothelial cells ^[7] and causing down-regulation of Protein S expression, an anticoagulant, by estrogen receptor independent mechanisms. ^[8] The purpose of the present study was to expand the current knowledge concerning the molecular mechanisms by which genistein and daidzein might exert anti-thrombogenic and anti-atherogenic effects.

Extensive literature review revealed few conflicting reports of the potential of isoflavones to inhibit platelet aggregation. ^[11-16] Moreover, there is lack of such data in postmenopausal women, the group which is most commonly prescribed with isoflavones for the relief of menopausal symptoms. Hence, the present study was planned to evaluate the effect of isoflavones on platelet aggregation inhibitory potential in oophorectomised menopausal women.

Subjects and methods

Subjects: All women who reported to the Obstetrics and Gynaecology out patient department of Post Graduate Institute of Medical Education and Research, Chandigarh, India were screened for inclusion in the study. They were included in the study if they had undergone bilateral oophorectomy, were less than 55 years of age and were willing to comply with the protocol. They were excluded if any of the following criteria were present: (i) already on isoflavones (ii) taken hormone replacement therapy (HRT) / estrogen replacement therapy (ERT) within previous 8 weeks (iii) presence of renal and/or hepatic disease (iv) active major psychiatric disorders (v) history of thrombophlebitis or thromboembolism or cerebrovascular disorders (vi) uncontrolled hypertension with blood pressure > 180/100mmHg (vii) uncontrolled diabetes (viii) presence of active infection or malignancy (ix) present or past history of soya or nut related food allergies.

The study protocol was approved by the Institutional Ethics Committee and all the women participating in the study signed a written informed consent prior to enrolment. The study was conducted in accordance with ICMR ethical guidelines for biomedical research on human subjects. Eligible women were randomized so that they had an equal probability of assignment to either of the two groups. The randomization code was developed using random number table to select random permuted blocks of length four. The identity of the drugs was hidden by packing in numbered opaque envelopes to ensure concealment of the sequence until assigned. Randomization, allocation sequence generation and packing of envelopes was done by investigators not directly involved in dispensing and evaluating the treatments so that it was double blind - both the patient and evaluating clinician not aware of the assigned treatment. The participants remained on the same allocation throughout the study period if they continued. The randomization code was revealed to the investigators once recruitment, data collection, and laboratory analyses were complete. The participants attended the clinic at the time of randomization (baseline) and at interval of every three week for 12 weeks.

Study interventions: The participants were randomized to either placebo or study drug to be administered once a day orally for 12 weeks. The study drug tablet was obtained as free gift sample from Dr Reddy's Laboratory, Hyderabad (there was no conflict of interest) which contained 75 mg of soy isoflavones extract standardized to provide isoflavones 40% Genistein and Genistin 25%; Daidzein and Daidzin 15%. Placebo tablet matching in colour, size, shape and taste was used as control treatment. Patients were advised to take one tablet daily at bedtime with 150 ml of plain water for a total 12 week period. All the patients were instructed to stop the dietary isoflavones consumption by avoiding soy, seeds and sprouts, beans and legumes during the study period. However, concomitant treatments for other illnesses were allowed. The patients were asked to bring the drug envelopes during subsequent visits and compliance was assessed by pill counting. More than 80 percent compliance was considered as adequate.

Objective: The primary objective of the study was to evaluate the potential of isoflavones to alter ADP and epinephrine induced platelet aggregation. Women were also assessed for health status as appearance of any adverse events was also taken in account during each visit.

Measurement of platelet aggregation: For the estimation of platelet aggregation, fasting blood sample was taken at the start of study medications and at the end of three month study period. 4.5 ml blood was taken in 0.5 ml of 3.2% trisodium citrate vacutainer for platelet aggregation and the test was performed within three hours of sampling keeping the sample at room temperature.

Principle: The absorbance of platelet rich plasma falls as platelets aggregate. The amount and the rate of fall are dependent on platelet reactivity to the added agonist if other variables, such as temperature, platelet count and mixing speed are controlled. The absorbance changes are monitored on a chart recorder. The results can be expressed as a) either a percentage fall in absorbance measured at 3 minutes after the addition of an agonist or after achieving plateau phase (whichever is earlier) , b) by the initial slope of the aggregation tracing. This indicates the rate of aggregation, but does not show whether or not secondary aggregation occurred , c) by the minimum amount of the agonist required to induce a secondary response.

Method: The procedure as described by British Society for Haematology for guidelines on platelet function testing was followed^[9]. Briefly, whole blood was taken and one part of tri-sodium citrate was added to nine parts of blood. The sample was kept at room temperature as cold causes activation of platelets. Gentle centrifugation of the sample at 800 rpm for fifteen minutes yielded platelet rich plasma (PRP) and a part of this platelet rich plasma was used for the test. The remaining part was again centrifuged at 3000 rpm for 15 minutes to get platelet poor plasma (PPP). Platelet count of PRP was done and the count between $200-400 \times 10^9 /L$ was taken for adequate platelet aggregation response. In case the platelet count was high, adjustment was done by diluting with patients's platelet poor plasma.

The readings were recorded in a platelet aggregometer (Chrono- Log Co, USA). The aggregometer was switched on about 30 min before doing the tests to allow the heating block to warm upto 37°C. Stirring speed was set to 900 rpm and 450 microliters of PRP was pipetted. The tube was placed in heating block. Equal volume of PPP was placed in the other block. After 1 min, magnetic stirrer was inserted into the plasma. The transmission on the chart recorder was set to between 10-90%. Appropriate amount of the agonist was added and the change in absorbance was recorded until the response reached a plateau or for 3 min (whichever earlier). Different agonists used for the assay were ADP (10µL of 10µM) and epinephrine (10µL of 10µM). Dilutions were done as per manufacturer's instructions (ADP was diluted in normal saline and epinephrine was diluted in distilled water). A control sample was put up each time with the test. Analysis was done by measuring the percentage fall in absorbance by directly reading from the chart paper.

Platelet aggregation was performed in a blinded manner. The status of the drug (whether predrug or postdrug) and drug group was not known. All the analysis were done at the end of the study period. During analysis the status of the sample was kept blinded till final calculations were made.

Statistical analysis

Data was expressed as Mean \pm SD unless specified, numbers (percentages) and median (interquartile range). Proportions were compared using chi-square tests with continuity correction or Fisher's exact test when appropriate. The within groups and between group comparisons were carried out using paired Student's t test and unpaired Student's t test, respectively. Two sided significance tests were used throughout. P value less than 0.05 was considered significant.

Results

A total 43 underwent randomization, with 21 and 22 assigned to the isoflavone and placebo groups respectively (Fig 1). In the isoflavone group, one patient withdrew consent later on due to non specific reasons and one discontinued treatment because of adverse effects. In the placebo group, two patients discontinued treatment due to adverse effects. Two patients in isoflavone group and three patients in placebo group were lost to follow up and could not be contacted.

Baseline characteristics: At baseline, the two study groups were similar in terms of age, body mass index, time since surgery (bilateral oophorectomy), past use of HRT, coexisting and past medical history (Table 1). The most common cause of surgery in both the groups was fibroid uterus. Three patients in the isoflavones group and one patient in the placebo group had taken HRT in the past on account of post hysterectomy or complaints of menorrhagia. The duration of HRT ranged from two months to five years but all the patients had stopped the intake of HRT between 6 to 48 months prior to enrolment to the present study.

Platelet aggregation: At baseline, the mean \pm SD values of platelet aggregation with ADP (drug: 64.71 \pm 18.52; placebo: 54.29 \pm 13.63) and epinephrine (drug: 52.43 \pm 12.54; placebo: 50.29 \pm 17.68) were not significantly different in the two groups (p value: ADP- 0.253; Epinephrine- 0.798, respectively). Platelet aggregation was not significantly altered with ADP or epinephrine after 12 weeks in the two groups (Table 2). The alteration in platelet aggregation at the end of 3 months with isoflavones was not significantly different as compared to the placebo with either ADP or epinephrine (Table 3).

Figure 1: CONSORT flowchart:

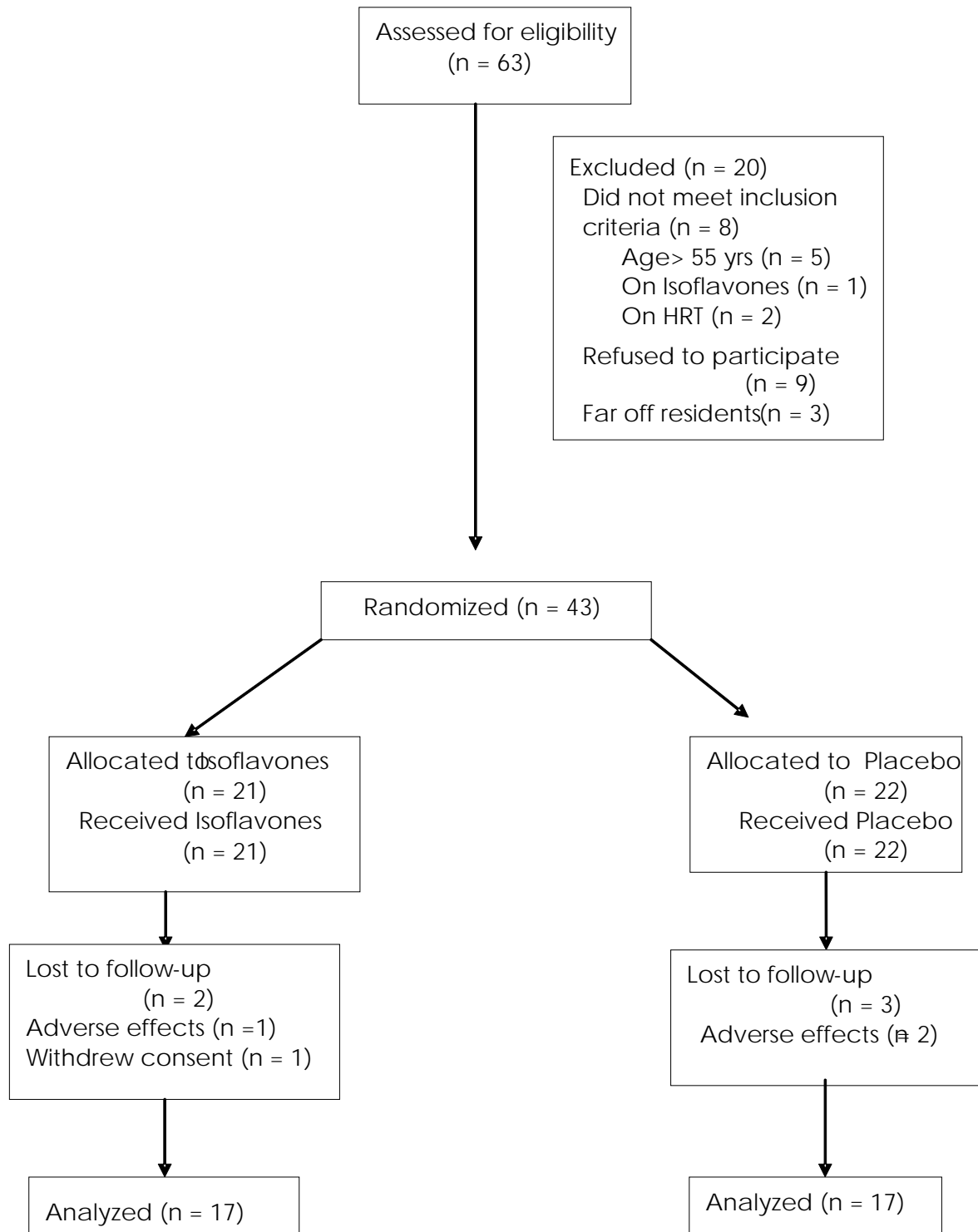


TABLE 1. DEMOGRAPHIC PROFILE AND CLINICAL CHARACTERISTICS OF PATIENTS IN ISOFLAVONE AND PLACEBO GROUPS AT BASELINE:

Characteristics	Isoflavones (n=21)	Placebo (n=22)	P value
Age in years (Mean \pm SD)	49.14 \pm 6.83	46.28 \pm 6.14	0.15
BMI in kg/m ² (Mean \pm SD)	24.99 \pm 4.58	24.94 \pm 4.03	0.97
Time since oophorectomy, in months; Median (IQR)	6 (1.5-33)	4.5 (1-21.75)	0.44
Past HRT use, n (%)	3 (14.3)	1 (4.5)	0.34
Coexisting illness, n (%)			
Hypertension	9 (42.8)	9 (40.9)	0.90
Type 2 diabetes	3 (14.3)	2 (9.1)	1
Paraumbilical h ernia	0	1 (4.5)	1
Joint diseases	0	1 (4.5)	1
Other	0	2 (9.1)	0.49
Past history (n %)			
Gall stones	1 (4.7)	1 (4.5)	1
Renal stones	1 (4.7)	0	0.49

TABLE 2. EFFECT OF ISOFLAVONES AND PLACEBO ON PLATELET AGGREGATION AT 12 WEEKS COMPARED TO BASELINE.

Parameter	Treatment Group	Time of estimation		P value
		0 week	12 week	
ADP induced platelet aggregation ^s	Isoflavones	64.71 \pm 18.52	60.29 \pm 12.88	0.19
	Placebo	54.29 \pm 13.63	56.14 \pm 14.53	0.70
Epinephrine induced platelet aggregation ^s	Isoflavones	52.43 \pm 12.54	50.29 \pm 6.75	0.65
	Placebo	50.29 \pm 17.68	54.43 \pm 24.09	0.64

^sPlatelet aggregation measured as percent light transmission as in optical aggregometry.

TABLE 3. MEDIAN (IQR) CHANGE IN PLATELET AGGREGATION WITH ISOFLAVONES AND PLACEBO FROM BASELINE AT 12 WEEKS.

Parameter	Isoflavones	Placebo	P value
ADP induced platelet aggregation	-4 (-8 – 0.5)	-4 (-5.5 – 4.5)	0.27
Epinephrine induced platelet aggregation	2 (-9.5 – 5.5)	-3 (-4.5 – 2.5)	0.52

One patient out of 21 enrolled (2%) in the isoflavone group discontinued treatment after 4 weeks because of disturbed sleep, anxiety and restlessness. In the placebo group, two out of 22 patients (5%) experienced adverse effects in the form of generalized body aches and increased fatiguability and they discontinued treatment at 2 weeks and 2 days respectively. The proportion of patients experiencing adverse events was similar between two groups (P=1.00).

Discussion

Phytoestrogens are a diverse group of naturally occurring non steroidal plant compounds that have the ability to cause estrogenic or/and antiestrogenic effects because of their structural similarity with estradiol (17 β -estradiol). Isoflavones are the most common form of phytoestrogens. ^[1] The two main isoflavonoids (genistein and diadzein) are present in all soy bean foods as glycosides either as aglycone (unconjugated form) or as beta-glycoside (conjugated form), which are not active estrogenically. These glycosides are readily hydrolysed to estrogenically active aglycones either as a result of processing and preparation of soy food or as result of the metabolism by intestinal microflora. Further metabolism of isoflavonoids takes place by conjugating the aglycone with glucouronic acid and to a lesser extent with sulfuric acid. ^[10]

Isoflavones have been implicated to be cardioprotective by virtue of several direct and indirect vascular effects. One of the postulated mechanisms is inhibition of platelet aggregation where numerous *in vitro* and *in vivo* studies have shown variable results in this regard. *In-vitro* studies with isoflavones have demonstrated their potential to inhibit thrombin ^[11] collagen ^[3, 12] and arachidonic acid ^[13] induced platelet aggregation. In a study by Choo et al the inhibitory potential of daidzin and daidzein against ADP and collagen induced platelet aggregation *in vitro* and *ex vivo* (rat platelets) has been demonstrated. ^[14] Both compounds exhibited anti-platelet activity against the two agonists with daidzein being more potent. The results of these studies is in contrast to the findings of the present study in which isoflavones have shown no significant inhibitory activity on ADP and epinephrine induced platelet aggregation. One possible explanation for such an observed difference might be the fact that crude preparations of isoflavones were used in these

studies while we used standardised soy extract and also the degree of metabolism was not studied by biochemical estimations. This is important since extensive metabolism of some isoflavones may be responsible for a decrease in their biological activity as demonstrated by Rimbach et al that genistein-4'-sulfate and genistein-4'-7-disulfate were less potent than genistein, at inhibiting collagen-induced platelet aggregation.^[15] The types of agonists used might also suffice to explain the observed variations, since different agonists act through varied mechanisms to cause platelet aggregation which might not be possibly counteracted by isoflavones under diverse settings.

The results of the present study corroborate with the findings of a study by Gooderham et al in which the effect of consuming a soy protein isolate beverage powder (60 gm/day) v/s casein supplement given for 28 days, on platelet aggregation was assessed in 20 healthy male subjects. Although the soy protein supplemented group exhibited a dramatic rise in plasma isoflavone concentrations, but no significant differences in collagen or 9,11-dideoxy-11 α , 9 α -epoxymethanoprostaglandin F2 α induced platelet aggregation was observed in platelet rich plasma from the two groups.^[16]

The exact molecular mechanisms by which isoflavones affect platelet aggregation is not exactly clear and is currently under research. Few possible hypothesis reported include protein tyrosine kinase inhibition^[11,17] inhibition of thromboxane A2 linking to its platelet receptor^[13,18], modification of platelet cyclic AMP via the inhibition of phosphodiesterase activity^[19] and stimulation of adenylate cyclase, leading to increased cyclic AMP levels.^[20]

Few limitations in the present study can be the lack of pharmacokinetic data for isoflavones, which would have provided an opportunity to ascertain the compliance and bioavailability and understand the kinetic – dynamic correlation for the agents. However, the concentration of their metabolites differ widely among individuals even after administration of controlled quantity of food supplements due to gut microflora, antibiotic use, bowel disease, gender difference and concomitant dietary intake.^[21, 22]

Secondly, it was a fixed dose study for a limited duration, whereas, the postmenopausal women consume soy products over prolonged periods of time and the use of active comparator in the form of aspirin, a reference drug used widely as an antiplatelet aggregation agent in clinical practice, would have been more justifiable.

Conclusion

In this study conducted on surgically induced menopausal women, the effect of 75 mg/day isoflavones on ADP and epinephrine induced platelet aggregation was assessed and no significant antiplatelet activity was found with either of the agonists at the end of 3 month study period as compared to the placebo group. However, future studies for longer duration can be planned with different doses of isoflavones, using active comparator group and including objective pharmacokinetic measurements to provide a conclusive proof regarding the use of isoflavones in clinical practice.

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