Soy isoflavone supplementation tends to improve specific immune responses in postmenopausal women

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ABSTRACT

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Immune dysfunction in postmenopausal women tends to decrease health-related quality of life (HRQoL). The present study's objective was to evaluate the effect of daily supplementation of 100 mg soy isoflavones on specific immune responses among healthy postmenopausal women. The study design was a community-based double blind randomized controlled trial involving 60 healthy postmenopausal women, aged between 48–60 years, in the Mampang Prapatan district of South Jakarta. Participants were randomized to receive either 100 mg soy-isoflavone + 500 mg calcium (intervention group) or 500 mg calcium only (control group). Both supplements were taken daily for 12 weeks, from January to April 2010. Specific immune responses (measured by serum Ig G and CD4$^+$) were assessed at baseline and after supplementation. Statistical analysis using independent t-test was performed to evaluate the effect of soy isoflavone supplementation on specific immune responses. Fifty-six (93.3%) participants completed the study without any significant side-effects or adverse events. Daily supplementation of 100 mg soy isoflavones for 12 weeks did not significantly increase the humoral specific immune response (p=0.242), but tended to improve the cellular specific immune response (p= 0.850). Other findings of this study were that soy isoflavone supplementation tends to improve specific immune responses in postmenopausal women with normal body mass index and adequate daily dietary isoflavone intakes. Short-term soy isoflavone supplementation is unable to improve the humoral and cellular specific immune responses in postmenopausal women aged 48 to 60 years.

Keywords : Soy-isoflavone, specific immune response, postmenopausal women

INTRODUCTION

Menopause, according to the definition of the World Health Organization and American Association of Clinical Endocrinologists, is the last natural menstruation, which is the time of permanent cessation of menstruation due to loss of activity or nonfunctioning of ovarian follicles caused by structural and functional changes, thereby causing decreased secretion of ovarian steroidal sex hormones, particularly estrogens and progesterones.[1,2] The decrease

in steroidal sex hormone concentrations in the body, particularly estrogens, does not change the menstrual pattern, but impacts on general health, particularly as somatic/urogenital and vasomotor abnormalities, known as menopausal symptoms.[3]

In addition to risk of coronary heart disease, osteoporosis and several malignancies, as a result of the aging process, postmenopausal women also undergo reduction of the immunity of the body, such that menopausal women become more susceptible to infectious diseases, and commonly also to various degenerative disorders.[4] Several studies have shown that postmenopausal women have decreased total numbers of lymphocytes, in comparison to women of reproductive age, as a result of decrease in antibody-producing B lymphocytes (which produce antibodies) and cytokine-producing T helper (Th) cells, although the existence of other mechanisms involved in the control of lymphocyte numbers in women cannot be eliminated.[5]

The results of the study conducted by the Women's Health Initiative, showing an increased risk of breast cancer, coronary heart disease, stroke, and venous thromboembolism (VTE) in users of hormonal therapy, has caused some menopausal women to refuse hormone therapy (HT) and to seek alternative treatments capable of filling their need for managing the menopausal abnormalities.[6,7] Phytoestrogens also known as "herbal estrogens" are natural substances from plants with chemical structures and effects that are similar to those of estrogens. These compounds bind to estrogen receptors (ER-á and ER-â),

and have both estrogenic and weakly anti-estrogenic activities, depending on endogenous estrogen and estrogen receptor concentrations, such that phytoestrogens are also known as selective estrogen receptor modulators (SERMs) with both agonist and antagonist effects.[8,9]

Isoflavones are among the best phytoestrogens, being found in soy beans, which are are much consumed by Indonesian communities and other Asian populations. Since the human immune system is partially influenced by steroidal hormones, it may be assumed that soy isoflavones are capable of affecting the immune system through estrogen-receptor-mediated mechanisms. Epidemiological data indicate that the prevalence of chronic and degenerative disorders is significantly lower in Asia populations consuming soy-rich foods, in comparison to American or European populations.[10]

The present study had as objective to determine the increase in humoral and cellular specific immune responses in postmenopausal women after soy isoflavone supplementation.

METHODS

Research design

This was an experimental study of community-based double blind randomized controlled trial design to investigate the effect of supplementation of soy isoflavones 100 mg for 12 weeks on humoral and cellular specific immune responses in postmenopausal women. This study was conducted from January to mid-April 2010.
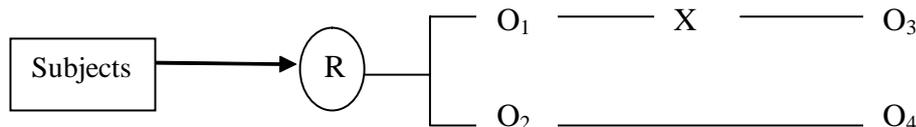

Figure 1. Diagram of research design

Notes : R = randomization; O = observation; X = intervention; O1 and O2 = pre-supplementation; O3 and O4 = post-supplementation

Research subjects

The study population comprised postmenopausal women (minimally one year without menstruation). The inclusion criteria were: postmenopausal women aged 48-60 years; healthy (capable of independently performing activities of daily living and capable of communication, not consuming menopause-related hormonal medications within the last three months, and agreeing not to consume other vitamins/minerals during the 12 weeks of supplementation, never having had hysterectomy and/or oophorectomy. The exclusion criteria for the subjects were: past history of diabetes mellitus, renal or hepatic disorders, heart disease, and hypertension.

Sample size

The study sample was divided into two independent groups, namely the intervention group and the control group. The number of subjects in each group was calculated according to the following formula for hypothesis testing of means difference of two independent groups[11]

$$n = 2\left[\frac{(z_\alpha + z_\beta)\,sd}{(\overline{X}_1 - \overline{X}_2)}\right]^2 \text{ x design effect}$$

SD = standard deviation of the two groups
$\overline{X}_1 - \overline{X}_2$ = expected clinical difference (ä)
á $= 0.05 \rightarrow z_á = 1.96$
â $= 20\% \rightarrow z_á = 0.84$

Based on the results of a previous study on the effect of soy isoflavone administration on immune function in healthy postmenopausal women conducted by Ryan-Borchers et al.,[10] who measured $CD4^+$ concentrations in blood plasma, a standard deviation (SD) of 6.9 was obtained, and the expected effect size (ä) after administration of soy isoflavones for 3 months being 15%, the calculated sample size n for each group was 12 subjects. Assuming a drop-out rate of 20% during the study, the required sample size for each group was 15 subjects. Based on the above calculation and a design effect of 2, a total of 30 subjects was required for each group, therefore the total sample size required was 60 postmenopausal women.

Sample selection

This experimental study was conducted in the catchment area of the Mampang Prapatan Subdistrict Health Center, South Jakarta. The Mampang Prapatan subdistrict was chosen as the study area by purposive sampling. Selection of *kelurahan* (villages), *RW* (hamlets) and *RT* (neighborhoods) to be involved in this study was performed by cluster random sampling, whereas selection of subjects in *RT*s was by proportional simple random sampling.

Intervention

The study subjects were divided into two groups, as the isoflavone and the control group, respectively. Random allocation was performed with a block size of 4 on 60 subjects by random number generation. The intervention was conducted by administering the supplement at a dose of one tablet per day for 12 consecutive weeks. The supplement tablets were prepared by PT Ikapharmindo Putramas with a special license from *Badan Pengawasan Obat dan Makanan Republik Indonesia (BPOM-RI)*. The tablets were labelled A and B by PT Ikapharmindo Putramas, where the tablets labelled A contained 250 mg of 40% soybean isoflavone extract (equivalent to 100 mg soy isoflavones) + 500 mg calcium, while the other type of tablet only contained 500 mg calcium. To minimize the effect of bias on the study results, the tablets were administered in a double-blind manner.

Soy isoflavone intake

Daily dietary intakes (carbohydrates, lipids, proteins, and isoflavones) were assessed and calculated by trained nutritionists, based on the quality and quantities of the foods consumed. Assessment was done by using the food record method, in which the subjects

themselves recorded their daily food consumpton three times weekly, on 2 working days and 1 holiday (Saturday or Sunday). Assessment of the quantity and quality of dietary isoflavone intakes was based on the completeness of foods containing soy isoflavones and their weekly frequency of consumption, by means of the Semi-Quantitative Food Frequency Questionnaire (SQ-FFQ), which contains a list of isoflavone-containing foods. The subjects were grouped into quintiles (Q_1 to Q_5), according to their dietary isoflavone intakes, as follows: Q_1 were the subjects considered to have poor dietary isoflavone intakes, while subjects in Q_2–Q_5 were considered to have adequate dietary isoflavone intakes.

Determination of body mass index (BMI)

The subjects were measured as to height and body weight. Height was measured using a portable microtoise to the nearest 0.1 cm and weight was measured using Sage portable scales to the nearest 0.1 kg. The BMI criteria used were determined by the World Health Organization. According to these criteria, BMI is classified as normal (18.5 - 25.0 kg/m²), mildly overweight (25.1 – 27.0 kg/m²), and severely overweight (> 27.0 kg/m²).[12]

Laboratory measurements

At baseline and after supplementation, from each study subject in both groups (control and intervention), a 10 ml venous blood sample was collected by a trained laboratory technician from PRODIA Clinical Laboratories. The plasma isoflavone, $CD4^+$ and immunoglobulin G (IgG) concentrations were determined at baseline (before supplementation) and after 12 consecutive weeks of supplementation.

Isoflavone concentrations

Plasma isoflavone concentrations were measured by high performance liquid chromatography (HPLC) at the DKI Jakarta Provincial Health Laboratory *(Laboratorium*

Kesehatan Daerah, Labkesda, Provinsi DKI Jakarta). Helix pomatia glucuronidase-sulfatase H-2 was added to serum (1 mL), and the mixture was incubated at 37 °C for 15 to 18 h to hydrolyse genistein and daidzein. To the sample was added trichloroacetic acid and the isoflavones extracted with 7.5 ml ethyl acetate. Extracted isoflavones were dissolved in 80% methanol in water for HPLC.

Imunoglobulin G and $CD4^+$ concentrations

Laboratory determination of IgG concentrations was performed at PRODIA Clinical Laboratories, by means of turbidimetry (with Advia 1800 reagent from ADVIA Chemistry Systems – Bayer Health Care), with a coefficient of variation (CV) of 2.7%. Determination of $CD4^+$ concentrations was performed at Dharmais Cancer Hospital Laboratory, using flow cytometry (with BD TriTest™ reagent from Becton Dickinson – USA), with a coefficient of variation (CV) of 2.58%. The abovementioned laboratories had passed ISO 9001:2000 and ISO 9001:2008 certification.

Ethical clearance

This study received ethical clearance from the Commission for Research Ethics, Faculty of Public Health, University of Indonesia.

Statistical analysis

The study data were tested for normality of distribution using the Kolmogorov-Smirnov test. Effect differences between the isoflavone group and the control group were assessed by independent t-test**.** All analyses used the Statistical Package for Social Sciences (SPSS) for Windows version 17. P-values <0.05 were considered statistically significant.

RESULTS

Characteristics of subjects

Screening of postmenopausal women selected for participation as study subjects

yielded 80 postmenopausal women, among whom 18 women did not meet the inclusion criteria and 2 women declined to participate. The remaining 60 subjects were assigned to two groups. After 12 weeks of supplementation, 3 subjects from the isoflavone group and 1 subject from control group dropped out from the study, because they had to move outside the study area. Therefore 56 subjects were available for analysis per protocol and at the completion of the study, namely 27 subjects in the isoflavone group and 29 subjects in the control group (Figure 2).

Among subjects participating in this study, the youngest subject was 48 years old and the oldest 60 years. As shown in Table 1 (below)

the results of comparability testing between the isoflavone and control groups showed that several important characteristics (such as age, marital status, educational level, employment, physical exercise, smoking, coffee consumption) as well as a number of clinical characteristics (such as body mass index, duration of menopause, estradiol concentration, dietary intake) at the start of the study (baseline data) were not significantly different in both groups. Mean IgG and $CD4^+$ concentrations at baseline were also not significantly different in both intervention groups. This indicates that the random allocation with a block size of 4 succeeded in uniformly distributing all non-intervention variables.
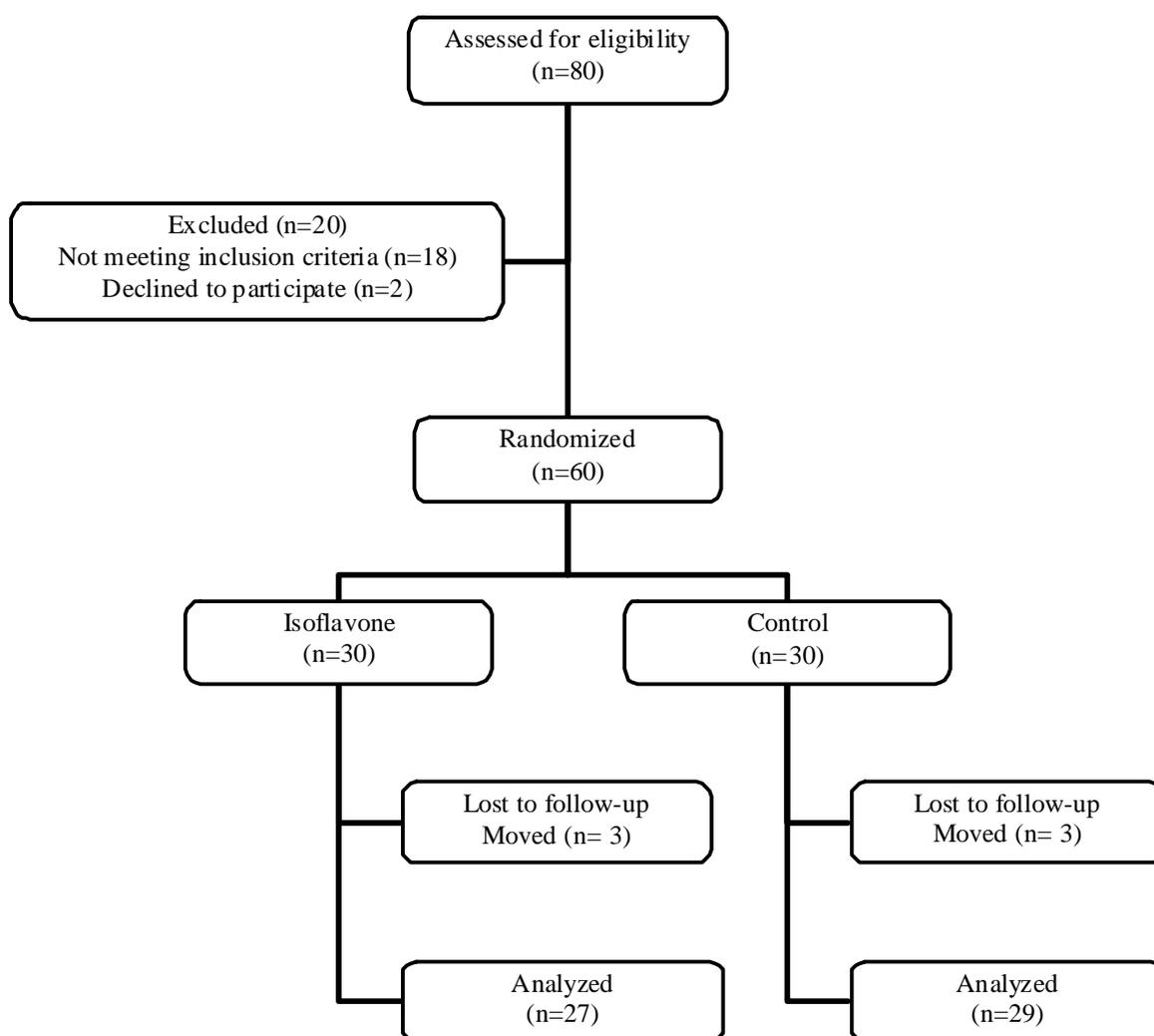

Figure 2. Flowchart for screening & recruitment of study subjects, allocation of intervention, follow-up, and data analysis

Table 1. Distribution of demographic,habitual, and clinical characteristics of
subjects at baseline, by intervention group

Variable	Intervention group		p
	Isoflavone (n=27)	Control (n=29)	
Age (years) *	53.0 (3.2)	54.0 (3.4)	0.261[a]
Marital status **			
Married (w/ living husband)	59.2	75.9	0.254[b]
Unmarried/widowed	40.8	24.1	
Educational level **			
Low ($\leq$ SD)	62.9	68.9	0.635[b]
Intermediate (SMP/SMA)	33.3	31.1	
High ($\geq$ D1)	3.8	0	
Employment **			
Employed	37.0	37.9	0.945[b]
Unemployed	63.0	62.1	
Physical exercise **			
Yes, regular	29.6	20.7	0.151[b]
Yes, irregular	40.8	24.1	
Never	29.6	55.2	
Smoking **			
No	92.6	93.1	1.000[c]
Yes	7.4	6.9	
Coffee drinking **			
No	85.2	79.3	0.731[c]
Yes	14.2	20.7	
Body mass index (kg/m2)*	27.4 (4.8)	26.5 (3.7)	0.438
Normal **	40.7	31.0	0.143
Overweight **	59.3	69.0	
Duration of menopause (years) *	4.3 (2.5)	5.0 (2.4)	0.238
Early (1-4 years) **	59.2	44.8	0.280
Late ($\geq$ 5 years)**	40.8	55.2	
Hormones/"hormone like substance"			
Estradiol conc. (pg/mL) *	7.7 (4.1)	7.0 (3.6)	0.519
Isoflavone conc. (ng/L) *	4.8 (3.7)	4.1 (2.7)	0.363
Daily dietary intake			
Proteins (g) *	41.9 (13.7)	49.5 (18.7)	0.093

* Mean (SD) ; ** Number of subjects (n) in %; [a] Independent t-test; [b] Chi-square test; [c] Fisher's exact test

Effect of isoflavone supplementation on specific immune responses

In this study, the specific immune responses assessed were the humoral immune response with IgG concentration as parameter and the cellular immune response with CD4$^+$ concentration as parameter.

In Table 2 it is apparent that after supplementation the isoflavone group had higher mean IgG and CD4$^+$ concentrations, in comparison with the control group, although the difference was not statistically significant.

In Table 3, the mean IgG concentration after supplementation decreased in both the isoflavone and the control subgroups based on duration of menopause, with a larger decrease in the isoflavone group. In subgroups based on body mass index, mean IgG concentration in the isoflavone group with normal BMI was slightly increased after supplementation (1%), while mean IgG concentration in the control group decreased by 3.6%. In subgroups based on dietary isoflavone intake, mean IgG concentration after supplementation in the

Table 2. Mean IgG and CD4$^+$ concentration after supplementation of soy isoflavones for 12 weeks, by intervention group

| Specific immune response | Intervention group | | p $^{b)}$ |
	Isoflavone (n = 27)	Control (n = 29)	
IgG (mg/dL) $^{a)}$	1516.1 (228.0)	1441.5 (242.6)	0.242
CD4$^+$ (cells/μL)$^{a)}$	773.5 (264.3)	759.3 (292.4)	0.850

Legend: $^{a)}$ Mean (SD); $^{b)}$ p value from independent t-test

isoflavone group with poor isoflavone intake increased by 4.6%, as compared with baseline concentrations, while in the control group the concentration decreased by 4.4%. In the subgroup of subjects with "overweight BMI" and "adequate isoflavone intake", mean IgG after supplementation of 100mg soy isoflavones for 12 weeks decreased by 10.7% and 8.3%, respectively, while the decrease in mean IgG concentration in the isoflavone was larger than that in the control group. In general, these results of subgroup analyses indicate that isoflavone supplementation for 12 weeks had no positive effect, in other words, it was incapable of increasing the humoral response, with the exception of the subgroup with normal body mass index and poor isoflavone intake.

From Table 3 it may been seen that in general the subgroup analyses also demonstrated increased CD4$^+$ concentrations

Table 3. Relative difference percentage (ä) of mean IgG concentration before and after supplementation, by intervention group, for all subjects and selected subgroups

| Subgroups | Mean IgG | | | Mean CD4$^+$ | | |
	Isoflavone (n=27)	Control (n=29)	p*	Isoflavone (n=27)	Control (n=29)	p*
Duration of menopause						
Early menopasue	- 6.9	- 2.8	0.319	2.8	-1.5	0.886
Late menopause	- 5.1	- 2.9	0.381	6.0	1.3	0.626
Body mass index						
Normal	1.0	- 3.6	0.073	14.2	-1.2	0.559
Overweight	-10.7	- 4.4	0.925	- 1.4	0.5	0.945
Dietary isoflavone intake**		- 4.4				
Poor	4.6	- 4.0	0.951	-0.1	-5.7	0.938
Adequate	- 8.3		0.158	5.1	1.6	0.955

*Results of independent t-test on mean IgG and CD4$^+$ concentration postsupplementation between isoflavone group and control group; **Dietary isoflavone intake: poor<0.13 g/d; adequate: 0.13 - 0.44 g/d

ä: Relative difference percentage of mean IgG concentration before and after supplementation, calculated according to the formula :

$$\frac{\text{IgG concentration after supplementation} - \text{IgG concentration before supplementation}}{\text{IgG concentration before supplementation}} \times 100\%$$

ä: Relative difference percentage of mean CD4$^+$ concentration before and after supplementation, calculated according to the formula :

$$\frac{\text{CD4}^+ \text{ concentration after supplementation} - \text{CD4}^+ \text{ concentration before supplementation}}{\text{CD4}^+ \text{ concentration before supplementation}} \times 100\%$$

postsupplementation in the isoflavone subgroups, both in those in early and late menopause. Although the increase in CD4$^+$ concentrations after 12 weeks of isoflavone supplementation was statistically not significant, in the late menopausal subgroup this increase was 4 times that in the control group, revealing a tendency for a raised CD4$^+$ concentration in the late menopausal subgroup postsupplementation.

Among the subgroups with normal BMI, the mean CD4$^+$ concentration increased by 14.2% in the isoflavone group, but decreased by 1.2% in the control group. In addition, there was also a reduction in mean CD4$^+$of 1.4% in the isoflavone subgroup with above normal BMI. Similarly, in the subgroups with adequate dietary isoflavone intake, the CD4$^+$ concentrations in both the intervention and the control group showed an increase, with a higher increase in the former (5.1% vs. 1.6%). Thus it may be concluded that supplementation of soy isoflavones 100mg/day for 12 weeks tends to raise CD4$^+$ concentrations in postmenopausal women, particularly in the subgroups with normal BMI and those with adequate dietary isoflavone intakes.

Side effects and adverse events

None of the subjects in the control group experienced adverse effects after consumption of their supplementation tablets for 12 weeks, whereas in the isoflavone group there were 3 subjects (11.1%) who reported having mild symptoms of nausea in the first week of supplementation, with the symptoms decreasing in the second week and disappearing thereafter.

DISCUSSION

In this study, the results indicate that there were no significant differences in IgG and CD4$^+$ concentrations after supplementation of soy isoflavones 100 mg per day for 12 weeks in postmenopausal women. Supplementation of soy isoflavones 100 mg per day for 12 weeks tended to increase CD4$^+$ concentrations, but

had an immunosuppressant effect on IgG. The increase in CD4$^+$ concentrations after supplementation of soy isoflavones tended to be more pronounced in the subgroup of women in late menopause, with normal BMI and adequate dietary isoflavone intake.

Studies on the effects of soy isoflavone supplementation on specific immune responses in postmenopausal women are still very limited in number. The concentrations of estradiol (which is also an immunomodulator) in post-menopausal women are extremely low (<20 mg/dL). In this connection it should be noted that the soy isoflavones (genistein, and daidzein), whose structures are similar to those of estrogens (17 â-estradiols), will bind to estrogen receptors (ER-á and ER-â), and therefore are administered as a supplement and expected to be capable of affecting immune functions in humans, as they exert agonist and weakly antagonist effects on estrogens.[13] Estradiol has equal affinities to both type of receptors, whereas isoflavones bind more strongly to ER-â.[14,15] In postmenopausal women both types of estrogen receptor also have reduced functionalities and affinities, especially for isoflavones with their considerably lower potency in comparison to estradiol (E2).[16-18] To date it is still unclear what immune mechanisms are involved, whether through binding of estradiol to ER-á or to ER-â receptors. Several investigators have concluded that lymphocyte potentiation probably does not involve estrogen receptor (ER)-dependent mechanisms, because daidzein, but not genistein, is capable of potentiating lymphocytes, while both types of isoflavone have equal affinities for estrogen receptors in ER-sensitive tissues.[19,20]

The subgroups of subjects with normal BMI in this study showed an increase in CD4$^+$ concentrations of 14.2%, whereas the subgroup with above normal BMI tended to have reduced CD4$^+$ concentrations after supplementation. These data indicate that circulating estradiol concentrations in

postmenopausal women did not influence the effects of isoflavone supplementation. Data from several in vitro in animal studies demonstrate that isoflavones may either enhance or impair immunocompetence, depending on dietary isoflavone intake, isoflavone concentration, and most importantly, sensitivity of the target tissue and route of administration of the isoflavones.[21] The results of the present study indicate that the subjects in the subgroup with adequate dietary isoflavone intakes showed a better response to supplementation of 100 mg soy isoflavones for 12 weeks, in the form of increased CD4+ concentrations after supplementation. Several investigators have cited the estimated daily dietary isoflavone intakes in Asians to be approximately 50–200 mg.[22] A study among postmenopausal women showed that the dietary isoflavone intake was 69.5 mg/d.[23]

In-vitro studies using high doses of genistein (as a type of flavonoid) have shown that genistein is capable of inducing apoptosis and inhibiting angiogenesis, both of which are involved in cell proliferation and consequently also in immune responses. In the studies by Wang and colleagues on cultures of mitogen-activated murine lymphocytes, addition of daidzein in physiological concentrations to the culture medium was capable of potentiating the lymphocytes and increasing the production of interleukins (IL-2 and IL-3).[24] The study conducted by Paes on administration of soy isoflavones 100 mg/day for 4 weeks, found that the supplementation did not induce significant changes in lymphocyte proliferation and production of the cytokines IL-2 and interferon (IFN), and was incapable of increasing immune responses in postmenopausal women.[25] Ryan-Borchers et al. administered a daily supplementation of 70 mg soy isoflavones to healthy postmenopausal women for 16 weeks, and succeeded in increasing the population of B lymphocytes by 12.2% as compared to the control group, but there were no significant differences in the numbers of T lymphocytes and natural killer (NK) cells.[10]

These conflicting study results may be due to several differences regarding the study subjects, types of isoflavone used, route of administration, and duration of supplementation. Yellayi administered genistein injections directly to oophorectomized mice, resulting in a reduction in the proportions of CD4+ and CD8+ cells.[26]

The results of this study indicate that supplementation of 100 mg soy isoflavones was capable of increasing cellular immune responses in postmenopausal women, but only in those with certain characteristics. This study also demonstrates that supplementation of 100 mg soy isoflavones was incapable of increasing humoral immune responses, and even tended to have immunosuppressant effects on postmenopausal women with certain characteristics. Therefore it may be concluded that the effects of soy isoflavone supplementation are associated with characteristics of postmenopausal women, thus preventing generalization of the results to all. In other words, the management of postmenopausal women still requires an individual approach and cannot be generalized.

There are many questions emerging around the optimal dose and duration of supplementation required for effecting the expected clinical response. Some authors question the length of administration of supplements in several studies, considereing them to be too short for obtaining a clinical response.

This study has several limitations, one of the limitations being that the outcome variables used only one immune response indicator, namely IgG concentration for assessing humoral specific immune responses, and CD4+ concentration for assessing cellular specific immune responses. Other immune response indicators were not assessed in this study, as a consequence of limited funding. Another limitation was that the subjects remained in their homes, making it difficult to ensure that they did not consume other supplements in addition to the supplements administered in this study.

CONCLUSIONS

Supplementation with 100 mg soy isoflavones for 12 weeks in healthy postmenopausal women did not result in increased humoral and cellular immune responses. Postmenopausal women with normal BMI tended to have increased IgG concentrations, while those with adequate daily dietary intakes of isoflavones tended to have increased CD4$^+$ concentrations. Further studies are required to explore the effects of soy isoflavones in immunologically challenged subjects.

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