Soy Isoflavone Intake and Estrogen Excretion Patterns in Young Women: Effect of Probiotic Administration

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Abstract. *Background: Soy isoflavones may lower breast cancer risk through altered hepatic estrogen metabolism, leading to increased urinary excretion ratios of 2-hydroxyestrone ($2OHE_1$) to 16α-hydroxyestrone ($16αOHE_1$). Materials and Methods: Urinary excretion of $2OHE_1/16αOHE_1$ was measured in 36 healthy, pre-menstrual women before and after ingestion of a soy-protein formula containing 120 mg of isoflavone daily for one month. Since isoflavone absorption and metabolism depends on intestinal bacteria, effects of co-administration of Lactobacillus GG ($2x10^{12}$) on estrogen ratios and isoflavone excretion were studied. Urinary isoflavone excretion measurements assessed compliance. Results: Soy isoflavone ingestion induced quantitative differences in urinary excretion of estrogen metabolites and isoflavones but failed to alter $2OHE_1/16αOHE_1$ ratios. Co-administration of Lactobacillus GG with soy reduced excretion of total and individual isoflavones by 40% (p=0.08), without altering $2OHE_1/16αOHE_1$ ratios. Conclusion: Isoflavone-rich soy protein administration alone, or with probiotic supplement, did not alter urinary excretion of estrogen metabolites in pre-menopausal women. However, adding concentrated probiotics may alter isoflavone bioavailability.*

The low incidence of breast cancer in Asian countries has led to the suggestion that soy consumption may reduce the risk of breast cancer (1, 2). Recent epidemiological studies, however, indicate that this association is quite variable (3, 4). Soy

isoflavones, known as phytoestrogens, have estrogenic and anti-estrogenic properties (5), and these compounds are currently used extensively as an alternative to traditional hormone replacement therapy (6). It has been reported that over 30% of women in the US currently use soy supplements (7). A mechanism for the potential beneficial action of the major soy isoflavones, genestein and daidzein, may be through binding and competition with estrogens at estrogen receptors in target organs, such as the uterus, breast and brain (5). Alternatively, soy isoflavones may act by altering estrogen catabolism to less active compounds, thereby lowering breast cancer risk and decreasing other endocrine-related symptoms (8-11). The primary aim of the present study was to determine whether consumption of an isoflavone-rich soy supplement in healthy free-living pre-menopausal women alters estrogen catabolism in a manner that might reduce the subsequent risk of breast cancer through changing estrogen metabolites to less active compounds. Estrone is metabolized by two major mutually exclusive pathways, one leading to formation of 2-hydroxyestrone ($2OHE_1$) and the other to 16α-hydroxyestrone ($16αOHE_1$) (10, 11). A third minor pathway to $4OHE_1$, a putative genotoxic estrogen has been described (12, 13), but was not analyzed in the present study. Since $16αOHE_1$ acts as an estrogen receptors agonist and the $2OHE_1$ form acts as an estrogen antagonist (5), it was proposed that facilitating the $2OHE_1$ pathway would lead to decreased breast cancer risk and that measurement of urinary $2OHE_1/16αOHE_1$ ratios would serve as a surrogate marker of breast cancer risk (10).

Recent interest has also been focused on two intestinal bacterial daidzein metabolites, equol and O-desmethylan-golensin (ODMA) (14). Following ingestion of soy isoflavones 30-50% of the human population metabolize daidzein to equol and 80-90% to ODMA. Several studies suggest that equol or ODMA production from daidzein may confer on subpopulations of women more protection from breast cancer compared to the general population (15). The mechanism responsible is unclear, but *in vitro* studies suggest that equol, in contrast to estradiol acts by binding to estrogen receptor β (ERβ) (14). We therefore also assessed the relationship

between urinary equol/ODMA excretion and changes in urinary $2OHE_1/16\alpha OHE_1$ ratios.

An additional goal was to determine whether the consumption of probiotics together with the soy supplement might enhance the intestinal absorption and enterohepatic recirculation of soy isoflavones estrogen metabolites by increasing bacterial deconjugating enzymes in the intestinal lumen and/or altering enzymes in the intestinal mucosa. It is generally recognized that isoflavones are deconjugated and degraded in large part by gut bacteria such as Clostridium, Butyrivibrio, and Eubacteria (16, 17). Whether probiotics might alter bacterial populations involved in deconjugation reactions presently is uncertain at present (18). A preparation of *Lactobacillus* GG was used since this organism has been shown to survive passage through the gastrointestinal tract and because it has low β glucuronidase and can increase β-glucosidase activity in humans (19-21).

Materials and Methods

The study was conducted in two parts. Phase I was a longitudinal study in which 36 healthy pre-menopausal women received soy supplementation for four weeks followed by a washout period of four weeks. Outcome measures were recorded at baseline just prior to the start of soy supplementation, at the end of the four-week soy supplementation period, and at the end of the washout period. All investigators were blinded to the timing of the urine and blood samples that were analyzed. Informed consent was obtained from all eligible subjects who were recruited through advertisements placed in a local newspaper, in the hospital bulletin, and *via* flyers posted in the immediate area. All volunteers were screened to determine whether they met the study criteria before being entered into the study.

Exclusion criteria included women taking birth control pills or describing very irregular or prolonged menstrual periods, women who were taking soy products daily or who were allergic to soy products, who had taken antibiotics in the previous 10 weeks, had a history of hormone-related cancers including breast cancer, small bowel malabsorption or chronic pancreatic disease, women with severe heart disease, diabetes, or other major chronic conditions, who had undergone hysterectomy or oophorectomy, or women with a BMI greater than 36.

Subjects had a brief medical history taken and a physical examination was performed including height, weight, and waist circumference measurements, and a baseline breast examination to exclude patients with undiagnosed masses. All subjects filled out the Block Modified, Semi Quantitative Food Frequency Questionnaire, a 132 item, semi-quantitative food frequency questionnaire, which included detailed quantitation of the genistein and daidzein content of their diets under the supervision of a research nutritionist.

The soy product used consisted predominantly of conjugated isoflavones with over 90% in the conjugated form (genistin 45%, daidzin 40%, glycetin 6%). Small amounts of other isoflavones accounted for 7.5% of the total (Narula Research, Chapel Hill, North Carolina, USA). Volunteers took a one ounce serving of the soy product twice daily, containing approximately 28.4 grams of soy protein and approximately 60 mg of all isoflavone isoforms. Total

genistein and daidzein aglycone equivalents consumed were 40 mg and 20 mg per day, respectively. The soy supplement contained 100 calories per serving totaling an additional 200 calories daily, and participants were helped by the nutritionist to reduce an equivalent number of calories from their regular food intake to prevent weight gain. Compliance was evaluated in part by measuring the amount of soy protein returned in cans at the end of the study period, and in addition, measurements of urinary isoflavone levels which were obtained at baseline and after each subsequent monthly visit. Hematological and overnight urine collection studies were performed on the 7-8th day of the menstrual cycle.

Overnight urine was collected in plastic containers with 1g ascorbic acid per liter as a preservative. Urinary concentrations of genistein and daidzein were measured using high performance liquid chromatography using the HPLC method of Franke and Cantor (22). Conjugates were enzymatically digested in a 2 stage enzymatic procedure described in detail (23), using β-glucuronidase/arylsulfatase and genistein and daidzein were separated using a NovaPak C18 reversed-phase column, combined with a C18 guard column. Analyses were performed with a UV spectrophotometer and concentrations were quantified by comparing the peak area with authentic standards obtained from Sigma Chemical (St. Louis, MO, USA). Urinary daidzein metabolites, equol, and ODMA were assessed by GC-MS techniques (24) at the Fred Hutchinson Cancer Institute (Seattle, WA, USA). The analyses of urinary $2OHE_1$ and $16\alpha OHE_1$ metabolites were performed by competitive solid-phase immunoassay at the Institute for Biomedical Research (Hackensack, NJ, USA) (25, 26). The phenotypes were defined as positive if measurable metabolites were detected.

Phase II was a three-period crossover study in 32 pre-menopausal women age 25-45, who received the soy protein mixture (1 month), soy protein plus probiotics (1 month), and probiotics alone (1 month) (12 women in this study also participated in the Phase I study.). The study was designed to follow a balanced 3x3 Latin Square design to ensure that treatment means would be adjusted to reduce potential carryover effects between treatments. Hence, there were 6 different treatment sequences applied randomly to the volunteers. Urine and blood samples were collected at the end of each 4-week treatment period on day 7-8 of the participant's menstrual cycles. Intake procedures and nutrient assessment by Food Frequency Questionnaire (FFQ) were similar to those described above.

The probiotic bacteria used were *Lactobacilli* GG in the form of capsules stated to contain approximately 2×10^{10} organisms (courtesy of Con Agra Inc., Omaha, NE, USA). Two probiotic capsules were taken with food twice daily. Quantification of organisms from two different batches indicated that there were approximately 1×10^{12} organisms per probiotic capsule at the onset of the study. All capsules were kept refrigerated. Compliance was assessed by examining the amount soy protein returned in the original cans, the amount of soy isoflavones appearing in the urine, and pill counts.

Statistical methods. Baseline measures, including urinary isoflavone and hormone levels, age, body mass index (BMI), waist/hip ratio, caloric intake, smoking and alcohol use, diet and exercise were analyzed descriptively by treatment using summary statistics and frequency distributions.

Testing of the primary hypotheses was based on all randomized subjects who completed the study, regardless of their level of compliance. The data from subjects who dropped out and were replaced by another subject were excluded from the analyses.

Table I. *Demographics.*

	Phase I (n=36)	Phase II (n=31)
	Mean percent	
African American	28	29
Hispanic	22	19
Asian	11	10
Caucasian	40	42
Smokers	19	10
F.H. Breast Cancer	25	23
Age - Mean (range)	34.5 (23-44)	34.4 (26-45)
BMI - Mean (range)	23.3 (16.5-33)	25.0 (19-36)

Demographics and BMI (body mass index) in the number (n) of volunteers enrolled for whom data was available. F.H.: family history; BMI: body mass index.

Table II. *Mean dietary intake.*

	Phase I (n=35)	Phase II (n=31)
Energy (Kcal)	1429±15.6	1207±13.5
Soy isoflavone/2000iKcal	5.51±0.38	8.3±0.4
Fiber (g)	17.0±0.3	17.3±0.28
Fat % Energy	33.2±0.2	33.0±0.23
CHO% Energy	48.6±0.2	47.6±0.2
Alcohol % Energy	3.67±0.13	4.89±0.2

Dietary intake calculated from data obtained at the baseline, screening visit in the number (n) of subjects for whom data was available ±SEM. CHO: carbohydrate.

Table III. *Urinary isoflavone data – phase I study.*

	Total	Genistein µg/mg creatinine	Daidzein µg/mg creatinine	$2OHE_1/16\pm OHE_1$ ratio
Baseline	2.61±4	0.75±1.5	1.86±2.9	1.63±0.6
Soy	15.4±17*	7.41±8.2*	10.3±10.4*	1.62±0.6
Washout	1.58±3	0.36±0.8	1.22±2.3	1.66±0.8

Data presented as mean±SD in overnight urine collections in all of the subjects for whom data was available *$p < 0.01$ *versus* baseline by paired *t*-test.

Table IV. *Urinary isoflavone data – phase II study.*

	Total	Genistein µg/mg creatinine	Daidzein µg/mg creatinine	$2OHE_1/16\pm OHE_1$ ratio
Baseline	2.11±7	0.69±1.9	1.42±5.0	1.71±1
Soy	29.6±37.0*	11.2±15*	18.4±23.0*	1.63±1
Soy + Probiotics	18.0±22*†	7.0±9.5*†	11.0±12.7*†	1.59±1
Probiotics	4.8±9	1.81±3.7	3.0±5.3	1.53±0.9

Data presented as mean±SD in overnight urine collections in all of the subjects for whom data was available *$p < 0.01$ *versus* baseline and probiotic period by paired *t*-test.
†< 0.05 versus probiotic period by paired *t*-test.
< 0.08 versus probiotic period by Tukey's multiple comparison test.

In Phase I of the study, paired *t*-tests were used comparing the levels of biomarkers at baseline and at the end of the soy supplementation. Secondary analyses included paired *t*-tests to compare the measures obtained at the end of treatment and the end of the washout period to evaluate potential carry-over effects.

In Phase II, the effects of the three dietary interventions on urinary isoflavone and estrogen metabolite levels and their ratios were evaluated through mixed-model factorial ANOVA, with treatment included as a fixed effect, and treatment sequence and period included as random effects. Paired *t*-tests also were used to compare biomarker levels obtained at baseline *versus* following each treatment, with the *p*-values adjusted for multiple comparisons using the Bonferroni criterion. All analyses were performed using SAS Version 9 (SAS Institute, Cary, NC, USA).

Results

The volunteers consisted of young women with a mean age of 34.5 years, about 40% Caucasian, 28% African American, 20% Hispanic and 10% Asian (Table I). There were relatively few smokers and approximately one quarter of the subjects had a family history of breast cancer. The subjects overall were of normal weight, with a mean BMI of about 24 (Table I). Their dietary intake overall, was similar to that of the United States population, except that calculated energy intake was somewhat lower than the US mean (Table II). Volunteer subjects did not change weight significantly during the study. There were no significant adverse events recorded. A few volunteers reported anecdotal reduction in pain during menstruation, but there was no pattern of elongation of the menstrual cycle in subjects due to soy consumption.

In Phase I of the study, approximately one third of the test isoflavones consumed over a 24 hour period appeared in the urine. Three of the 36 subjects were found to excrete no detectable urinary isoflavones when reportedly taking soy products, indicating that they were non-compliant. Urinary isoflavone data from Phase I shown in Table III includes data on all the subjects in the study; however, omitting the subjects who were non-compliant did not significantly change mean urinary isoflavone content. As shown by the large standard deviations, there were wide interindividual differences in soy isoflavone excretion at baseline and during soy ingestion. Isoflavone-rich soy protein isolate consumption did not alter urinary $2OHE_1$ to $16\alpha OHE_1$ ratios from those measured at baseline or during the washout period (Table III), although
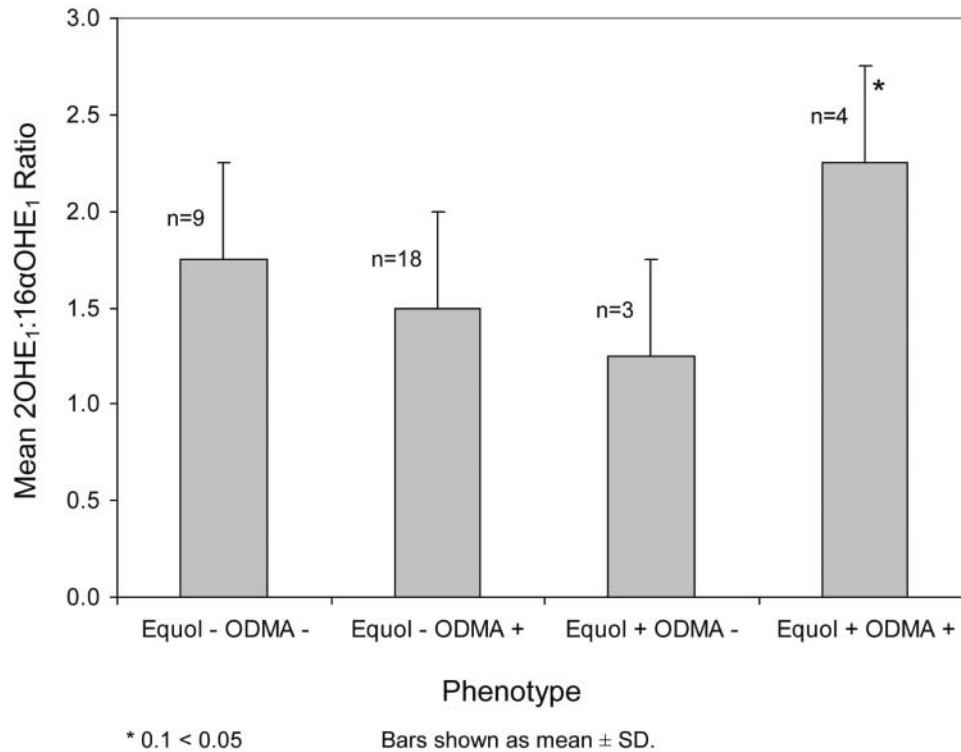
Figure 1. *2OHE₁: 16αOHE₁ ratio as function of phenotype.*

there were marked differences in ratios between different subjects. Although the genistein content of the soy protein supplement was 12% greater than that of daidzein, 30% more daidzein was excreted in urine (Table III), as others have noted (8, 9) suggesting that this metabolite is more bio-available than genistein. There was no association between any of the demographic or dietary factors with the ratios of urinary estrogen metabolites during soy supplementation.

Analysis of the two major daidzein metabolites in the 34 subjects for whom data was available showed that 26% had the equol-ODMA-phenotype, 58% Equol-ODMA+, 9% equol+ODMA−, and 12% equol+ODMA+, indicating that the ODMA pathway was favored in this population, as reported previously (14). A trend toward an increase in the $2OHE_1/16\alpha OHE_1$ ratio was seen in subjects expressing the equol+ODMA+ phenotype when compared to equol+ODMA− or the two equol negative phenotypes (Figure 1). However the number of individuals in this subgroup was too small to achieve statistical significance.

Thirty-one volunteers completed Phase II of the study; there were 12 dropouts and all but five were replaced. The baseline demographics of subjects in this study were similar to those in the Phase I study (Table I). Baseline dietary intakes also were similar except that reported soy isoflavone intake was 50% higher and mean caloric intake somewhat lower (Table II).

Regardless of the sequence of treatments, urinary isoflavone excretion increased markedly from a baseline of approximately 2 μg/mg to over 30 μg/mg creatinine with two non-compliant subjects showing no increase in urinary isoflavone content. When probiotics were provided with the soy supplement, there was a 40% reduction in total urinary isoflavone, genistein and daidzein excretion when compared to the soy-supplemented study period without probiotics. This reduction in isoflavone excretion was significant when analyzed by paired *t*-test ($p<0.05$), but showed only a trend by Tukey's Multiple Comparison test ($p=0.08$) (Table IV).

There were no differences in the urinary $2OHE_1/16\alpha OHE_1$ ratios, nor in the urinary concentrations of individual metabolites, during any of the experimental periods.

Discussion

The present study was designed to determine whether the consumption of an isoflavone rich soy supplement would alter estrogen metabolism in healthy free-living, pre-menopausal women in a manner that might be associated with a lower risk of breast cancer by enhancing conversion of estrone toward the $2OHE_1$ rather than the $16\alpha OHE_1$ metabolic pathway. The relative importance of the two

pathways was determined by measuring urinary estrogen metabolites, since approximately one-third of isoflavones are recovered in the urine (8, 9).

The results of Phase I of the study showed that even when the urinary isoflavone metabolites increased by over 8-fold, the $2OHE_1/16\alpha OHE_1$ ratio did not differ significantly. Of 34 compliant subjects taking the soy supplement, 13 (about 40%) showed a lower ratio, and 20 (about 60%) a higher ratio than at the baseline. This data contrasts with that of Lu *et al*. (8) who described increased urinary excretion of $2OHE_1$ while $16\alpha OHE_1$ remained unchanged during ingestion of an isoflavone- rich product. The reasons for these differing results are unclear but could be related to differences in the soy products and/or the treatment regimens. While the results of this study do not nullify the estrogen metabolite theory (10), they do indicate that in healthy pre-menopausal women, a soy isoflavone supplement of up to 120 mg per day, has no systematic effects on estrogen metabolism. The data also implies that if high soy intakes reduce menopausal symptoms and/or breast cancer risk, it may do so *via* a yet-undefined mechanism.

It is well established that the consumption of soy products containing high concentrations of daidzein results in intestinal bacterial conversion to equol in about 30 to 50% of the population, and that 90% convert daidzein to ODMA (14). Although individuals may vary in the concentrations of these metabolites, their proportions remain stable over time (14). Women converting daidzein to equol may be at reduced risk of breast cancer, in part due to reduced levels of plasma E_1 (15), since equol binds preferentially to ERβ (14). Our subjects showed that the ODMA pathway was favored in accordance with the data of others (14). A trend to increased $2OHE_1/16\alpha OHE_1$ ratios was seen in the equol+/ODMA+ phenotype compared to the equol+/ODMA- phenotype, suggesting that a subpopulation of pre-menopausal women consuming soy isoflavones have a metabolic phenotype that may lower the risk for breast cancer as also suggested recently by Atkinson *et al*. (14)

In Phase II of the study, we sought to determine whether the administration of a large concentration of probiotic bacteria with the isoflavone-rich soy formula, might modify isoflavone and estrogen metabolism in such a way as to increase $2OHE_1/16\alpha OHE_1$ ratios. The soy product alone increased total urinary isoflavones about 14-fold over baseline levels. When probiotics were administered with the soy supplement, total urinary isoflavone output and that of genestein and daidzein fell by about 40% ($p<0.08$) but without changing the estrogen metabolite ratios significantly. Similar results were recently reported by Nettleton *et al*. in a cohort of postmenopausal women consuming a soy product with a probiotic (27).

It is well established that isoflavones, at least in part, are deconjugated and degraded by gut bacteria such as Clostridia, Butyrivibrio and Bacteroides species (18). The precise reason

why an individual subjects differ in their isoflavone metabolism is unclear (14). However such individual differences would be expected to influence isoflavone blood levels and thus their biologic effects. Gut microflora are a key determinant of isoflavone bioavailability since food isoflavones are conjugated and require hydrolytic cleavage by intestinal β-glycosidases before intestinal absorption (28). The bioavailability of genistein and daidzein therefore is determined by gut bacteria through a balance between hydrolytic (deconjugating) and degradative (ring fission) reactions (28), although recent studies suggest some deconjugating activity by intestinal epithelial cells (29). Indigenous microflora also are able to modulate the expression of intestinal epithelial genes (30). Isoflavone blood levels would be expected to increase if degradation following hydrolysis is limited, and might decrease if hydrolytic cleavage was blocked and degradation increased.

Urinary equol and ODMA concentration were not measured in Phase II of the study. However, Nettleton *et al*. (31) recently have reported that Lactobacillus 10^9CFU did not alter plasma isoflavone levels or change the number of equol producers in postmenopausal women. In a parallel-arm study in premenopausal women, Bonorden *et al*. (32) reported that a 2 month intervention with a probiotic mixture containing 10^9CFU lactobacillus acidophilus and Bifido bacterium langum did not alter equol excretion or plasma concentrations of estradiol sulfate.. Hence, it appears unlikely that under the conditions of our study probiotics would have changed the proportion of equol or ODMA excretors.

In the present study, the administration of 10^{12}CFU *Lactobacillus* GG appeared to decrease urinary isoflavone excretion, suggesting that this organism might enhance isoflavone deconjugation and/or block isoflavone degradation leading to increased circulating levels of isoflavones. Since blood levels of isoflavones were not measured in the present study, this remains to be directly demonstrated. Nettleton *et al*. (31) did not report changes in blood levels of phytoestrogens but this group used a much lower content of probiotic bacteria.

Overall, we found no effects of ethnicity, smoking, BMI, or dietary intake variables on isoflavone excretion, equol/ODMA production, or urinary $2OHE_1/16\alpha OHE_1$ ratios. There are conflicting epidemiological reports on the effects of the food matrix or habitual diet on soy isoflavone metabolism, uptake, and disposition. Adlercreutz *et al*. (33) reported that the ratio of fat to fiber was positively associated with equol production in a Japanese population. In contrast, others reported that equol excretion was more frequent in Western populations consuming high levels of fiber and carbohydrates and low fat to fiber ratios (21, 34). The roles of ethnicity and the diet in isoflavone disposition require further study.

In summary, the present study failed to demonstrate that consumption of an isoflavone-rich soy protein isolate

induces significant changes in the hepatic metabolism of estrogens as judged by the urinary excretion of $2OHE_1$ and $16\alpha OHE_1$. Co-administration of the soy isolate with high concentration of a probiotic may alter the metabolism of isoflavones, but does not appear to change urinary $2OHE_1/16\alpha OHE_1$ ratios.

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