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Soy isoflavones have an antiestrogenic effect and alter mammary promoter hypermethylation in healthy premenopausal women 1

Abstract

We hypothesized that soy isoflavones would have dose related estrogenic and methylation effects. 34 healthy premenopausal women were prospectively enrolled and randomized in double-blind fashion to receive either 40 mg or 140 mg isoflavones daily through one menstrual cycle. Breast specific (NAF) and systemic (serum) estrogenic effects were assessed measuring the estrogenic marker complement (C)3 and changes in cytology, while methylation effects were evaluated in mammary ductoscopy (MD) specimens using methylation specific PCR assessment of five genes (p16, RASSF1A, RAR β 2, ER, and CCND2) associated with breast carcinogenesis. Serum genistein significantly increased post treatment in women consuming both isoflavone doses. Neither NAF nor MD cytology significantly changed after either low or high dose isoflavones. Serum C3 levels post treatment were inversely related to change in serum genistein ($r = -0.76$, $p = 0.0045$) in women consuming low dose isoflavones. RAR β 2 hypermethylation increased post treatment correlated with the post treatment level of genistein among all subjects ($r = 0.67$, $p = 0.0017$) and in women receiving high dose isoflavones ($r = 0.68$, $p = 0.021$). At the low dose, CCND2 hypermethylation increase correlated with post treatment genistein levels ($r = 0.79$, $p = 0.011$). The inverse correlation between C3 and genistein suggests an antiestrogenic effect. Isoflavones induced dose specific changes in RAR β 2 and CCND2 gene methylation which correlated with genistein levels. This work provides novel insights into estrogenic and methylation effects of dietary isoflavones. 2

Keywords

Nipple aspirate fluid, mammary ductoscopy, isoflavones, estrogenic, methylation

Disciplines

Cancer Biology | Food Science | Human and Clinical Nutrition | Other Genetics and Genomics | Women's Health

Comments

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SOY ISOFLAVONES HAVE AN ANTIESTROGENIC EFFECT AND ALTER MAMMARY PROMOTER HYPERMETHYLATION IN HEALTHY PREMENOPAUSAL WOMEN¹

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Running Head: estrogenic and methylation effects of isoflavones

Key words: nipple aspirate fluid, mammary ductoscopy, isoflavones, estrogenic, methylation

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ABSTRACT

We hypothesized that soy isoflavones would have dose related estrogenic and methylation effects. 34 healthy premenopausal women were prospectively enrolled and randomized in double-blind fashion to receive either 40 mg or 140 mg isoflavones daily through one menstrual cycle. Breast specific (NAF) and systemic (serum) estrogenic effects were assessed measuring the estrogenic marker complement (C)3 and changes in cytology, while methylation effects were evaluated in mammary ductoscopy (MD) specimens using methylation specific PCR assessment of five genes (*p16*, *RASSF1A*, *RAR β 2*, *ER*, and *CCND2*) associated with breast carcinogenesis. Serum genistein significantly increased post treatment in women consuming both isoflavone doses. Neither NAF nor MD cytology significantly changed after either low or high dose isoflavones. Serum C3 levels post treatment were inversely related to change in serum genistein ($r = -0.76$, $p = 0.0045$) in women consuming low dose isoflavones. *RAR β 2* hypermethylation increased post treatment correlated with the post treatment level of genistein among all subjects ($r = 0.67$, $p = 0.0017$) and in women receiving high dose isoflavones ($r = 0.68$, $p = 0.021$). At the low dose, *CCND2* hypermethylation increase correlated with post treatment genistein levels ($r = 0.79$, $p = 0.011$). The inverse correlation between C3 and genistein suggests an antiestrogenic effect. Isoflavones induced dose specific changes in *RAR β 2* and *CCND2* gene methylation which correlated with genistein levels. This work provides novel insights into estrogenic and methylation effects of dietary isoflavones.

INTRODUCTION

In a case-control study, a significant reduction in breast cancer risk was found in women who regularly consumed isoflavones (1). The isoflavones genistein and daidzein have a chemical structure similar to estrogen and bind to the active site (2, 3) of the estrogen receptor (ER). Ovarian hormone levels decreased in premenopausal women after taking 113-207 mg/d isoflavones for one menstrual cycle (4). Six month ingestion of 37 mg/day genistein increased plasma estradiol, nipple aspirate fluid (NAF) volume and epithelial hyperplasia in NAF in a subset of premenopausal women (5).

Genistein and daidzein belong to a larger class of compounds called polyphenols, which affect DNA methylation (6). Genistein, and to a lesser extent daidzein, reversed DNA hypermethylation and reactivated *RAR β* , *p16*, and *MGMT* in mammary cancer cells *in vitro* (7, 8), whereas two animal studies observed increased methylation effects with treatment (9, 10). Thus, the demethylation effects of genistein and daidzein on tumor suppressor genes *in vitro* appears to be at odds with the methylating effects of genistein in the two animal studies. In theory, prevention or reversal of hypermethylation-induced inactivation of key tumor suppressor genes by genistein and daidzein could be an effective approach for cancer prevention.

We examined the response of genes frequently methylated in breast cancer to orally administered genistein and daidzein in the breast tissue of healthy premenopausal women using mammary ductoscopy (MD). Our attempts to use methylation arrays were unsuccessful, so we report treatment related methylation effects using quantitative methylation specific PCR (qMS-PCR). Estrogenic effects of isoflavones were determined by measuring circulating levels of the estrogenic marker complement (C)3 (11) and evaluating the cytologic profile of breast ductal epithelial cells. We correlated the estrogenic and methylation changes with isoflavone dose and with circulating levels of genistein.

MATERIALS AND METHODS

Subjects

Premenopausal subjects (19-54 y) with no history of atypia, *in situ* or invasive breast cancer were recruited after Internal Review Board approval. Oral contraceptive use was permitted, although no changes in use were allowed during the study. Women who were pregnant, lactating, or had nursed within 20 months of study enrollment were excluded. Subjects consuming supplements in the past month containing alfalfa, black cohosh, flax meal, flax seed, ginseng, hops, licorice, red clover, thyme, tumeric, verbana, vitex agnus castus, or using Chinese, Ayurvedic or Tibetan medicines were excluded. Subjects were administered a food frequency questionnaire to determine their daily isoflavone consumption. Subjects ingesting > 3 mg isoflavones/day from foods were required to eliminate these foods for at least one month prior to starting the trial, and were counseled regarding soy containing foods to avoid.

A prospective, double-blind, randomized trial was conducted in 34 subjects with two doses of isoflavones, with 15 randomly assigned to consume one low dose capsule (20 mg isoflavones: 13.3 mg genistein and 6.7 mg daidzein) twice daily for a total daily phytoestrogen dose of 40 mg or and 19 assigned to consume one high dose capsule (70 mg isoflavones: 46.7 mg genistein and 23.3 mg daidzein) twice daily for a total daily phytoestrogen dose of 140 mg through one menstrual cycle. Subjects began and ended supplement treatment during the first 10 days of their menstrual cycle. Isoflavone preparations were provided as a single lot by the Solae Company, St. Louis, MO. Each subject served as their own control by collecting samples before and after treatment. Compliance was assessed by a capsule calendar and collection of unused capsules.

Specimen collection

NAF, blood and MD samples were collected before and one menstrual cycle after isoflavone intervention and prepared for cytologic and biologic analysis as previously described (12, 13). Whereas NAF samples from the left and right breast were kept separate, MD samples from each breast were combined to increase the total sample available for methylation and cytologic analyses.

C3 studies

A matched (pre- and post treatment) set of serum and NAF from the same breast was analyzed for C3 using an Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI).

Cytologic review

The Pap-stained slides were examined in blinded fashion as previously described (14, 15).

qMS-PCR

One μg salmon sperm carrier DNA was added to DNA extracted from each MD sample, which was then sodium bisulfite treated. A two-step PCR strategy was employed for *p16*, *RASSF1A*, *RAR β* , *ER*, and *CCND2*, all frequently hypermethylated in breast cancer (16-18) and some known to be demethylated by genistein (6, 8). First round PCR of MD DNA, 100% methylated DNA (positive) and water (negative) controls, was carried out for the five genes using an AmpliTaq Gold PCR kit (Applied Biosystems, Foster city, CA). For second round SYBR green-based qMSP, diluted PCR products were amplified with specific primers of the five genes for both methylated and unmethylated DNA. The primer sets for qMSP have previously been reported (19, 20). The percent of methylated DNA in a each sample was calculated (21). DNA that was 100% methylated or 100% unmethylated was used to generate a standard curve to quantify the percent methylated DNA in each sample.

qMS-PCR validation: To verify the specificity of second round qMS-PCR products, selected amplicons for *p16*, *RASSF1A*, *RAR β* , *ER*, and *CCND2* were subcloned using the TOPO-TA cloning system (Invitrogen, Carlsbad, CA). Plasmid DNA of 5-6 insert positive clones was isolated and sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Quantification of genistein in serum

After preparing stock and working solutions of genistein, serum samples were analyzed as previously described (22). After sample centrifugation, the supernatant was added to a β -glucuronidase solution, incubated, mixed with 2 N HCl and then loaded on a Waters C₁₈ sep-vac-pak column (Waters Corp., Milford, MA), vacuum washed, the columns eluted with methanol and

the eluants taken to dryness. High performance liquid chromatography was carried out on reconstituted samples using an ESA Model 582 pump with ESA Model 5600-A coularray detector (ESA, Inc., Chelmsford, MA) and a Thermal Separation Product autosampler (20 μ L injection).

Statistical analysis

Both within group and between group analyses were conducted. Within group analyses to detect significant pre to post changes in cytology were done using the sign test. The Wilcoxon signed ranks test was employed to test for significant within group changes in quantitative variables such as C3 and the fraction of methylated *p16*, *RASSF1A*, *RAR β 2*, *ER*, and *CCND2*. Spearman's correlation coefficients were computed to investigate relationships between quantitative variables such as change in genistein level and changes in C3 and the fraction of methylated *p16*, *RASSF1A*, *RAR β 2*, *ER*, and *CCND2*. The Wilcoxon rank sum test was employed to test for between group differences for quantitative variables such as the change in genistein level from pre to post.

RESULTS

Subjects

Of the 198 women interested in the study, 34 enrolled and completed it. Median age (37 vs 36 y) and family history of breast cancer (6 vs. 5) in the low vs. high dosage groups were similar. A median of 94% of the recommended isoflavone capsules were consumed. qMS-PCR was performed on 26 matched MD samples, the remaining samples having inadequate DNA.

Isoflavone levels in capsules and in serum

Two independent laboratories analyzed capsules at baseline. Capsules presumed to contain 20 mg isoflavones contained 18.5 mg and 19.1 mg isoflavones, whereas 70 mg capsules contained 70.2 and 64.4 mg isoflavones. Isoflavone content was evaluated at six monthly intervals thereafter, with a CV over time of < 10%. Baseline serum genistein levels (Table 1) increased after treatment ($p < 0.01$ for both doses, median change 144 ng/mL in the low dose and 507 ng/mL in the high dose group). Serum genistein change was greater after high than low dose treatment ($p < 0.001$).

No Estrogenic Effect Observed With Isoflavones

A nonsignificant trend toward lower C3 levels was observed after isoflavone treatment (Table 1). Among women in the low but not the high dose group, post treatment serum C3 levels were inversely related to the change in serum genistein ($r = -0.76$, $p = 0.0045$). NAF C3 levels were not significantly influenced by genistein levels. There was no significant effect of isoflavone treatment, regardless of dose or genistein level, on either NAF or MD cytology.

qMS-PCR results

Matched samples from 26 subjects (13 receiving low and 13 receiving high dose of isoflavones) were analyzed by qMS-PCR analysis for five genes (Table 2). There were no significant treatment related changes for any of the genes, considering all subjects or by dose. However, considering all subjects, the change in *RAR β 2* was correlated (Figure 1A) with both the post treatment level of genistein ($r = 0.67$, $p = 0.0017$). In general, *RAR β 2* methylation decreased post treatment at circulating genistein levels below 600 ng/mL, with the opposite effect for circulating levels of genistein greater than 600 ng/mL.

At the higher (but not the lower) dose, *RARβ2* methylation changes were correlated (Figure 1B) with genistein post treatment ($r=0.68$, $p=0.021$) and genistein change ($r=0.68$, $p=0.022$). At the lower dose (Figure 1C), change in *CCND2* methylation correlated with genistein level post treatment ($r=0.79$, $p=0.011$). *CCND2* methylation generally decreased in subjects with post treatment genistein levels less than 200 ng/mL, and increased in subjects with higher post treatment genistein levels.

In each of the three graphs of Figure 1, there is a single observation which was markedly changed by isoflavone intervention. Notably, Spearman's correlation, which was used to calculate associations between genistein levels and methylation, is based on ranks and therefore not severely impacted by a single extreme observation. Nonetheless, we determined the association between genistein and methylation change after excluding the single extreme observation from each analysis. After doing this, each association (*RARβ2* methylation change-all subjects with genistein: $r=0.51$, $p=0.03$; *RARβ2* methylation change-high dose isoflavone group with genistein: $r=0.50$, $p=0.14$; and *CCND2* methylation change-low dose isoflavone group with genistein: $r=0.43$, $p=0.29$) remained strong, with significance limited by sample size.

DISCUSSION

Whether genistein and daidzein act on the ER to increase or decrease tumor growth is likely related to the dose ingested and their subsequent circulating levels. Serum isoflavone levels increased in a dose dependent manner when consuming the supplement. Within group levels varied, likely related to known individual differences in isoflavone metabolism and excretion (23). Because of this variation, we evaluated the influence of both isoflavone dose and circulating genistein levels on estrogenic and methylation markers.

Both *RARβ2* and *CCND2* methylation decreased with low and increased with high circulating levels of genistein (Figure 1). Our findings at higher circulating genistein levels are consistent with preclinical studies demonstrating increased methylation after treatment (9, 10), and suggest that circulating genistein level is a better predictor of methylation effect than isoflavone dose.

Of the two estrogenic parameters evaluated, C3 was influenced by the intervention but cytology was not. This is perhaps not surprising, as changes in protein expression generally precede changes in cell morphology (24). Previously an estrogenic effect, based on higher NAF pS2 levels, was observed after two weeks of low dose isoflavones (24), however we observed a trend toward lower C3 levels after one menstrual cycle treatment. Several factors, including different populations studied, menstrual cycle start and end times, and study design may account for the different results.

A recent study detected hypermethylation of *RARβ* and *CCND2* in 21% and 5% respectively, of healthy BRCA gene mutation carriers (25). We observed baseline methylation levels in the breasts of healthy premenopausal women which were generally low but not zero, thereby providing useful benchmark information for future methylation studies. Our finding that the direction of changes in *RARβ2* and *CCND2* methylation depended on circulating levels of genistein suggests different mechanisms of action for high versus low levels of isoflavones.

In summary, among healthy premenopausal women consuming isoflavones for one menstrual cycle we found no estrogenic effects, and preliminary evidence of an antiestrogenic

effect. Isoflavones caused significant changes in the methylation levels of *RAR β 2* and *CCND2* in the breast, the direction of which was dependent on the circulating levels of genistein. This study provides a novel, non-invasive approach to define the impact of dietary isoflavones on mammary cells and therefore the risk of breast cancer. The degree to which soy isoflavones influence breast hypermethylation deserves further study.

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TABLE 1

Genistein and Complement 3 Concentrations in Serum and Nipple Aspirate Fluid (NAF) Before and After Isoflavone Intervention

	<u>20 mg Isoflavones¹</u>			<u>70 mg Isoflavones¹</u>		
	Before	After	Δ	Before	After	Δ
<u>Serum genistein (ng/mL)</u>						
Mean	17.3(12) ²	172.8(12)	155.5(12)	26.3(15)	620.5(15)	594.3(15)
SD ³	16.7	85.0	92.5	48.2	360.6	367.3
Median	15.8	171.1	143.6	14.8	528.5	507.3
<u>Serum C3 (ng/mL)</u>						
Mean	24138(14)	23252(14)	-886(14)	18432(17)	16157(17)	-2275(17)
SD	13395	12478	6843	12512	12837	15594
Median	27673	17828	446	14191	12040	-1925
<u>NAF C3 (ng/mg)⁴</u>						
Mean	0.37(9)	0.15(9)	-0.23(9)	0.35(14)	0.51(14)	0.16(14)
SD	0.37	0.15	0.43	0.44	0.95	0.81
Median	0.23	0.070	-0.03	0.14	0.12	-0.00
<u>NAF C3 (ng/mL)</u>						
Mean	49.6(9)	20.5(9)	-29.1(9)	48.4(14)	62.2(14)	13.8(14)
SD	65.1	30.4	77.5	70.7	152.9	113.5
Median	17.6	15.5	-4.3	23.4	13.1	-0.4

1: The difference in serum genistein was significant for both the low and high dose isoflavones groups (p<0.01 for both). Differences in serum C3, NAF C3 (ng/mL) and NAF C3 (ng/mg) were not significant for either dose group.

2: Numbers in parenthesis represent subjects with matched before and after treatment samples.

3+: SD: standard deviation

4: ng/mg: ng C3 per mg total NAF protein

TABLE 2

Percent DNA Methylated in Five Genes in Healthy Premenopausal Women Before and After Treatment with Soy Isoflavones

	<u>20 mg Isoflavones¹</u>			<u>70 mg Isoflavones¹</u>		
	Before	After	Δ	Before	After	Δ
<u><i>p16</i></u>						
Mean	0.37(12)	0.30(12)	-0.06(12)	0.72(11)	0.84(11)	0.11(11)
SD ²	0.55	0.41	0.67	1.49	1.60	2.22
Median	0.12	0.16	-0.01	0.17	0.14	-0.02
<u><i>RASSF-1A</i></u>						
Mean	1.48(13)	1.24(13)	-0.24(13)	1.22(9)	1.21(9)	-0.01(9)
SD	2.32	1.61	1.17	0.92	1.73	1.59
Median	0.82	0.99	-0.00	0.98	0.70	-0.26
<u><i>RARβ2</i></u>						
Mean	0.18(10)	0.12(10)	-0.06(10)	0.18(10)	0.57(10)	0.39(10)
SD	0.23	0.16	0.10	0.27	1.09	1.15
Median	0.05	0.05	-0.03	0.05	0.18	0.00
<u><i>ER</i></u>						
Mean	0.45(9)	0.53(9)	0.08(9)	2.11(9)	0.89(9)	-1.22(9)
SD	0.40	0.79	0.74	4.76	2.35	5.30
Median	0.19	0.66	0.05	0.22	0.10	-0.14
<u><i>CCND2</i></u>						
Mean	0.05(11)	0.06(11)	0.00(11)	0.03(11)	0.02(11)	-0.00(11)
SD	0.06	0.09	0.09	0.03	0.03	0.03
Median	0.03	0.00	0.00	0.02	0.01	0.00

1: Differences in *p16*, *RASSF-1A*, *RARβ2*, *ER* and *CCND2* were not significant for either dose group. Results were excluded if unreliable. Only matched results are shown.

2: SD: standard deviation

Figure 1. Change in methylation of *RARβ2* and *CCND2* correlated with serum genistein level. Points above zero represent an increase in DNA methylation, points below zero represent a decrease. Methylation of *RARβ2* for all subjects is shown in panel **(A)** and only subjects consuming high dose isoflavones in panel **(B)**. When serum genistein levels were less than 600 ng/mL a decrease in *RARβ2* methylation was observed, whereas with serum genistein levels over 600 ng/mL increased methylation was seen. Similar effects on *CCND2* methylation are shown in panel **(C)**. For *CCND2*, a genistein level of 200 ng/mL provided the conversion point for methylation.

Figure 1

