Stimulatory Influence of Soy Protein Isolate on Breast Secretion in Pre- and Postmenopausal Women[1]

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Abstract

Soy foods have been reported to have protective effects against premenopausal breast cancer in Asian women. No studies have been reported on potential physiological effects of dietary soy consumption on breast gland function. We evaluated the influence of the long-term ingestion of a commercial soy protein isolate on breast secretory activity. We hypothesized that the features of nipple aspirate fluid (NAF) of non-Asian women would be altered so as to resemble those previously found in Asian women.

At monthly intervals for 1 year, 24 normal pre- and postmenopausal white women, ages 30 to 58, underwent nipple aspiration of breast fluid and gave blood and 24-h urine samples for biochemical studies. No soy was administered in months 1–3 and 10–12. Between months 4–9 the women ingested daily 38 g of soy protein isolate containing 38 mg of genistein. NAF volume, gross cystic disease fluid protein (GCDFP-15) concentration, and NAF cytology were used as biomarkers of possible effects of soy protein isolate on the breast. In addition, plasma concentrations of estradiol, progesterone, sex hormone binding globulin, prolactin, cholesterol, high density lipoprotein-cholesterol, and triglycerides were measured. Compliance was assessed by measurements of genistein and daidzein and their metabolites in 24-h urine samples. Excellent compliance with the study protocol was obtained.

Compared with NAF volumes obtained in months 1–3, a 2–6-fold increase in NAF volume ensued during months 4–9 in all premenopausal women. A minimal increase or no response was found in postmenopausal women. No changes were found in plasma prolactin, sex hormone binding globulin, cholesterol, high density lipoprotein cholesterol, and triglyceride concentrations. Compared with concentrations found in months 1–3 (no soy), plasma estradiol concentrations were elevated erratically throughout a "composite" menstrual cycle during the months of soy consumption. No significant changes were seen in plasma progesterone concentrations. No significant changes were found in plasma estrogen levels in postmenopausal women. A moderate decrease occurred in the mean concentration of GCDFP-15 in NAF in premenopausal women during the months of soy ingestion. Of potential concern was the cytological detection of epithelial hyperplasia in 7 of 24 women (29.2%) during the months they were consuming soy protein isolate. The findings did not support our *a priori* hypothesis. Instead, this pilot study indicates that prolonged consumption of soy protein isolate has a stimulatory effect on the premenopausal female breast, characterized by increased secretion of breast fluid, the appearance of hyperplastic epithelial cells, and elevated levels of plasma estradiol. These findings are suggestive of an estrogenic stimulus from the isoflavones genistein and daidzein contained in soy protein isolate.

Introduction

Incidence and mortality rates of breast cancer in Chinese and Japanese women in Asia are reported to be significantly lower than those of Chinese and Japanese women living in the United States (1). In addition, a lower proportion of breast biopsies from Asian women contain hyperplasia, atypical hyperplasia, and apocrine metaplasia than breast biopsies from American women (2, 3). The increased rates among Chinese and Japanese women in the United States have been attributed to various factors associated with the adoption of the American lifestyle, involving changes in reproductive practices, and to putative exposures to cancer-initiating and -promoting chemicals in the environment and the American diet (reviewed in Ref. 4). Alternatively, it has been hypothesized that the adoption of American diets by Asian women may be accompanied by a decreased consumption of inhibitory or protective factors that may be present in the traditional Asian diet, thus increasing the risk of benign breast disease and breast cancer (5, 6).

Foods derived from soybeans are one class of such "protective" foods that are consumed in significantly higher amounts in China and Japan than in the United States. Even now, the American diet contains little or no soy (7). Soy beans

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contain significant concentrations of the isoflavones, genistein and daidzein, reported to have both estrogenic and antiestrogenic activity (8–10), and are considered by some investigators to be protective against breast cancer (11–13). Epidemiological studies on Japanese and Chinese women in Japan, Hawaii, and Singapore indicate a reduced risk of premenopausal breast cancer associated with the consumption of soy (14–16). A more recent case-control study in Shanghai and Tianjin, China, found no association with soy (17).

Although a number of studies of the effects of soy on mammary cancer in rats have been reported, there are no reports of the effects of soy protein ingestion on the human female breast. In prior studies we found that, compared with white and African-American women, NAF[3] was less frequently obtained from Chinese and Japanese women, was of lower volume, lighter in color, and contained lower mean levels of GCDFP (GCDFP-15), a glycoprotein secreted by breast apocrine epithelial cells and found in high concentration in the NAFs of American women (18–20). In addition, hyperplastic and atypical epithelial cells were less frequently found in NAF from Chinese and Japanese women than in NAF from white and African-American women (21). The present study made in white and African-American women examined the effect on these characteristics of breast fluid secretions associated with the daily consumption of a commercial soy protein isolate for a 6-month period. We hypothesized that the characteristics of the NAF of these women might be modified by soy protein so as to resemble those of Asian women. We also investigated the effect of soy on plasma levels of estradiol, progesterone, prolactin, SHBG, cholesterol, HDL cholesterol, and triglycerides.

Materials and Methods

The subjects in this pilot study were 14 premenopausal and 10 postmenopausal women, ages 29 to 58, who had previously volunteered for our studies of NAF (Table 1). One premenopausal woman was using oral contraceptives and four postmenopausal women were using replacement estrogens. Based on our working hypothesis that the soy protein supplement might produce a decrease in the volume of NAF, the subjects selected for this study were nonpregnant women who had been found previously to yield breast fluid by nipple aspiration.

All women filled out a brief epidemiological and medical questionnaire at the onset of the study and at monthly intervals throughout the study. Information was collected on weight, menstrual history, time since onset of last menstrual period, duration of menstrual period, use of medications, general health, and any side effects experienced that might be related to the soy protein use. The women agreed to supplement their daily diet for a 6-month period with two packets of a commercial soy protein isolate (kindly provided by Protein Technology International, St. Louis, MO). Each packet contained 18.7 g of protein and 18.7 mg of genistein (in the form of mixed glucosidic conjugates) or a total of 37.4 mg of genistein per day (Table 2). The women were instructed to dissolve the soy protein in water, fruit juice, yogurt, etc., or, if preferred, to mix it into cereal or other food. Most women used fruit juice or milk.

The study design is shown in Fig. 1. In this study, each woman served as her own control. At approximately monthly intervals for 12 visits, the women underwent nipple aspiration for breast fluid, gave a blood sample, and on alternate months provided a 24-h urine sample. Soy protein consumption began the day following the third visit and continued to the day of the ninth visit. Compliance was assessed by measurements of urinary excretion of genistein, daidzein, and their metabolites in 24-h urine samples.

[3] The abbreviations used are: NAF, nipple aspirate fluid; GCDFP, gross cystic disease fluid protein; SHBG, sex hormone binding globulin; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography.

Table 1 Subjects by age, menopausal status, and hormone use

Status and age	Use of oral contraceptives	Menopausal estrogen use
Premenopausal		
29	No	No
37	No	No
41	No	No
42	No	No
43	No	No
44	No	No
45	No	No
45	No	No
47	No	No
49	No	No
49	No	No
50	No	No
50	Yes	No
50	No	No
Postmenopausal		
45	No	No
47	No	Yes[a]
47	No	No
50	No	No
50	No	Yes
50	No	No
53	No	No
53	No	Yes
54	No	Yes
57	No	No

[a] Patient has pituitary adenoma with amenorrhea; she also uses bromocryptine.

Table 2 Composition of soy beverage powder packet[a]

Protein (g)	18.7
Fat (g)	1.1
Ash (g)	2.8
Carbohydrate (g)	4.3
Genistein (mg)	18.0
KCal	102

[a] Kindly provided by Protein Technology Research and Development International (St. Louis, MO).

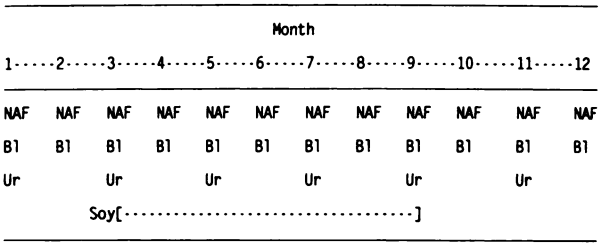

Fig. 1. Study design. See text for details. *Bl*, blood; *Ur*, 24-h urine.

Study Measures

The following characteristics of NAF were used as evidence of an effect of genistein on the breast: (*a*) a statistically significant

change in the volume of breast fluid obtained from one or both breasts. The highest volume of NAF obtained from the right or left breast at each visit was also used as a single measure of NAF volume for that month; (*b*) change in color of the NAF. The color of the NAF was recorded as one of the following: colorless, white, pale yellow, dark yellow, brown, or green. Findings in a previous study indicated that the color might be used as an estimate of the concentration of lipid-soluble steroids in NAFs (22). Compared with lighter fluids, darker fluids contain higher concentrations of lipid-soluble steroids such as cholesterol, cholesterol epoxides, estrogens, and fluorescent products of lipid peroxidation; (*c*) a statistically significant change in concentration of GCDFP-15 in NAF. GCDFP-15 is a glycoprotein secreted by apocrine metaplastic epithelial cells present in the breast ducts and, typically, in gross cystic breasts (23, 24). High concentrations of GCDFP-15 are found in NAF from white and African-American women compared with Asian women (20). The concentration of GCDFP-15 in NAF samples was measured by RIA with the solid-phase technique of Haagensen *et al.* (23) using ^{131}I-labeled antibody to GCDFP-15; the results were expressed as ng/ml of breast fluid; and (*d*) alterations in the morphology of exfoliated epithelial cells found in the NAF. NAF cytology was evaluated using the techniques and criteria described by King *et al.* (25), in which epithelial cells were classified as normal, hyperplastic, or atypical.

Initially, 37 women volunteered for the study, but 13 dropped out within the first 2–5 months because of loss of interest in the study, because they did not like the taste of or the consistency of the unflavored soy protein, or because of unrelated medical illnesses. These women were excluded from the present analyses, leaving 24 women who completed 9–12 months in the study. All 24 women remained in the study for the first 9 months, 21 of 24 (88%) for 10 months, 16 of 24 (67%) for 11 months, and 15 of 24 (63%) for 12 months. No untoward clinical symptoms were reported that could be attributed to daily ingestion of the commercial soy protein supplement. Of interest, a 50-year-old woman underwent menopause during the study. Her menstrual periods stopped at the fifth month of the study and transiently resumed at the tenth month, before ceasing altogether shortly after completion of the study. A 47-year-old postmenopausal woman disclosed that 10 years earlier she had been diagnosed as having a pituitary adenoma and was currently using bromocriptine to control the condition. She was also using menopausal estrogen. Both women were retained in the study.

NAF was obtained with a Sartorius-type breast pump consisting of a 15-cc syringe attached to a small cup by a short piece of plastic tubing (26). The cup was placed over the nipple and the plunger of the syringe withdrawn to the 7–8 ml mark and held for up to 15 s or until fluid appeared at the surface of the nipple. The fluid at the nipple surface was collected in capillary tubes, the ends were sealed with Cytocrit, and the length of the column of fluid was measured in millimeters. A millimeter on the capillary tube is approximately equivalent to 1 μl. At each visit, an attempt was made to extract all of the fluid available, using at least three repeated aspirations on each breast. A portion of the NAF sample from each woman was used for cytological evaluation, and the remaining fluid was stored at −20°C for later biochemical measurements. Compliance of the women with regard to consuming the soy beverage was assessed by the isoflavone content of the 24-h urine samples. These were frozen at −80°C after collection and shipped to the laboratory of Dr. Barnes.

Urine Measurements. Thawed urines (3 ml) were diluted to 5 ml to give final concentrations of 150 mM ammonium acetate buffer (pH 7) containing 500 mM triethylammonium sulfate. 4-Methylumbelliferone sulfate (100 nmol), 4-methylumbelliferone glucuronide (100 nmol), and biochanin A (100 nmol; Sigma Chemical Co., St. Louis, MO) were added as internal standards. All samples were then passed over 0.3-g Sep-pak C_{18} cartridges (Waters, Milford, MA), which were subsequently washed with 5 ml of 10 mM ammonium acetate. The absorbed isoflavones were eluted with 3 ml of methanol. The recovery of isoflavone metabolites by this procedure was 82% for sulfate esters and 98% for β-glucuronides.[4]

After evaporation of the methanol, the pH of the extract was adjusted to 5.0 by the addition of 250 μl of 1 M ammonium acetate buffer, and β-glucuronidase/sulfatase (363 units of β-glucuronidase and 15–40 units of sulfatase, type H-1; Sigma) was added; the sample was incubated at 37°C overnight. The aglucones were recovered by solid phase extraction using the C_{18} Sep-pak cartridges as described above. After evaporation to dryness, the extracts were redissolved in 400 μl 80% aqueous ethanol prior to HPLC-mass spectrometry analysis. They were diluted as necessary to bring them into the linear response range in the mass spectrometry assay.

HPLC-Mass Spectrometry Analysis. HPLC analysis was performed on the extracts on a 15 cm × 4.6-mm internal diameter 300 Å pore size, C_8 reversed-phase HPLC column. The solvent gradient (0–50% acetonitrile in 10 mM ammonium acetate over 15 min) was created by a Hewlett Packard model 1050 quaternary pumping system at a flow rate of 1.0 ml/min. Urine extracts were introduced using a model 7125 Rheodyne injector fitted with a 20-μl sample loop. After chromatographic separation, the eluate stream was diluted with 13 μl/min of ammonium hydroxide provided by a Harvard infusion pump and passed into the HN-APCI interface. The orifice potential of the PE-Sciex (Concord, Ontario, Canada) API III triple quadrupole mass spectrometer was set at −60 V.

Multiple reaction ion monitoring was carried out by selection of individual parent molecular ions in the first quadrupole and measurement of specific daughter fragment ions in the third quadrupole following collision-induced dissociation in the second quadrupole. Parent/daughter ion pairs for the isoflavones, daidzein (253/132), dihydrodaidzein (255/135), *O*-desmethyl-angolensin (257/108), and genistein (269/133), and the internal standard, 4-MU (175/112), were chosen for this analysis. Integration of peak areas was carried out using the program MacQuan, provided by PE-Sciex. Areas were corrected by the peak area of the added internal standard and compared to the areas of a set of known isoflavonoid standards to estimate the urine isoflavonoid concentrations. Genistein was recovered and purified from soy molasses as described previously (27–29). The isoflavonoids, *O*-desmethylangolensin and dihydrodaidzein, were kindly provided by Dr. Kristiina Wähälä (Department of Chemistry, University of Helsinki, Finland). Daidzein was purchased from LC Labs (Woburn, MA).

Plasma Measurements. Plasma levels of estradiol, progesterone, and prolactin were determined by the Reproductive Endocrinology/RIA Laboratory in the University of California-San Francisco Department of Obstetrics and Gynecology. The plasma concentrations of SHBG, triglycerides, cholesterol, and HDL were determined by the Clinical Laboratories of the University of California-San Francisco Medical Center. Be-

[4] L. Coward, M. Kirk, J. Sfakianos, and S. Barnes, unpublished observations.

Table 3 Influence of soy protein isolate on NAF volume and GCDFP-15 concentration

Influence of soy protein isolate on mean NAF volumes from right and left breasts, highest volume from right or left breast (volume), and GCDFP-15 concentration in pre- and postmenopausal women.

	Month 1–3, no soy		Month 4–6, on soy		Month 7–9, on soy		Month 10–12, no soy		*P*
	(*n*)	mean ± SD	(*n*)	mean ± SD	(*n*)	mean ± SD	(*n*)	mean ± SD	
Premenopausal women									
Rt vol (μl)	(42)	12.7 ± 10.9	(28)	17.8 ± 20.2	(27)	30.0 ± 29.3	(31)	34.1 ± 34.6	0.029
Lt vol (μl)	(42)	10.7 ± 16.8	(28)	18.6 ± 24.9	(27)	26.8 ± 37.0	(31)	31.0 ± 43.6	0.036
Vol (μl)	(42)	16.1 ± 16.1	(28)	22.3 ± 23.7	(27)	36.2 ± 37.3	(31)	42.3 ± 40.9	0.001
GCDFP-15 (ng/ml)	(25)	30.7 ± 21.3	(19)	19.4 ± 17.9	(19)	15.4 ± 11.4	(21)	20.4 ± 17.9	0.037
Postmenopausal women									
Rt vol (μl)	(30)	12.4 ± 12.8	(30)	14.1 ± 15.8	(30)	16.9 ± 22.3	(22)	14.4 ± 21.1	NS[a]
Lt vol (μl)	(30)	11.3 ± 14.7	(30)	9.9 ± 10.7	(30)	17.3 ± 19.8	(22)	15.3 ± 13.5	NS
Vol (μl)	(30)	16.1 ± 16.5	(30)	16.3 ± 15.7	(30)	23.4 ± 23.5	(22)	23.3 ± 20.4	NS
GCDFP-15 (ng/ml)	(20)	41.9 ± 41.9	(24)	28.2 ± 39.9	(27)	44.1 ± 87.4	(17)	23.3 ± 20.4	NS

[a] NS, not significant.

cause of unequal allocation of specimens for these various tests, not all women had each test performed at each visit.

Data Analysis. The data are presented by: (*a*) visit months 1–12; and (*b*) grouped by months 1–3 (no soy), months 4–6 (on soy), months 6–9 (on soy), and months 10–12 (off soy). Standard statistical measures were used for analysis of the data, including means, SD, ANOVA, and *t* tests.

Results

Nipple Aspirate Fluid

Volume

Premenopausal Women. The amount of NAF obtained from premenopausal women at each monthly visit began to increase incrementally after the third month when soy protein consumption began. Mean NAF volumes increased from 16.1 ± 16.1 μl in months 1–3 to 42.3 ± 40.9 μl by month 11 (ANOVA $P = 0.001$; Table 3). A high concordance was found in the volumes of NAF obtained from the right and left breasts. The highest mean volume obtained from the right or the left breast at each monthly visit demonstrated a highly significant increase in mean NAF volume associated with soy protein consumption. Following discontinuance of soy after month 9, the volume of NAF obtained decreased in the majority of women. In a few women, however, the volumes continued to increase into months 10–12 after the soy was discontinued. Typical examples of these changes are shown for three premenopausal women in Fig. 2 (*left*).

Postmenopausal Women. A modest, but statistically insignificant, increase in mean NAF volume was found between months 8 to 11 in the postmenopausal women. No significant increases in mean NAF volumes were obtained from right or left breasts, or as the highest volume from either breast when the data were grouped by 3-month visits (Table 3). However, in the four postmenopausal women who were using menopausal estrogen replacement therapy, a distinct increase in NAF volumes was observed during the period of soy consumption. One example is shown in Fig. 2, *right lower graph*. No increase in NAF volume during soy use occurred in the six women not using menopausal estrogen. Two examples are shown in Fig. 2, *right upper* and *middle graphs*.

GCDFP-15

Mean concentrations of GCDFP-15 in NAF were somewhat higher in postmenopausal than in premenopausal women (Table 3). Mean GCDFP-15 concentrations in NAF decreased in premenopausal women during the months on soy, reaching their lowest concentration in months 7–9. In two premenopausal women, distinct increases in GCDFP-15 occurred during the period of soy consumption, reaching a peak level at 8 and at 5 months, respectively (Fig. 3). No significant change in mean GCDFP-15 concentration was observed in the NAF of most postmenopausal women; however, in three women, increases in GCDFP-15, similar to that found in premenopausal women, were observed during the 6-month period of soy consumption.

Color of NAF

No significant shifts in the color of the nipple aspirates were observed during the course of the study.

NAF Cytology

During the months 1–3, prior to soy use, hyperplastic epithelial cells were found in only one of the 24 women (4.2%). In this premenopausal subject, the hyperplastic cells were observed only at her first visit. In contrast, hyperplastic cells were found in NAF of 7 of 24 women (29.2%) when the women were consuming or had ceased the consumption of soy protein isolate (Fisher exact test, $P = 0.021$). Hyperplastic cells were present in the NAF in 4 of 14 (28.6%) premenopausal and 3 of 10 (30%) postmenopausal women on one to five occasions during the months of soy consumption and in months 10–12 after soy was discontinued.

Plasma Estradiol, Progesterone, SHBG, and Prolactin

Estradiol and Progesterone

Premenopausal Women. Because of variability in dates of clinic visits and in the day of menstrual cycles at each visit among the 14 premenopausal women, the mean serum estradiol and progesterone concentrations varied widely over the 12-month period of the study. As expected, mean plasma estradiol and progesterone concentrations in premenopausal women were significantly greater than those in postmenopausal women Table 4. No statistically significant changes were found in SHBG concentrations during the study. Prolactin plasma levels remained relatively constant throughout the 12-month period of study in all women, and no relationship was found between prolactin levels and NAF volumes during the time of soy protein consumption. In the woman with the prolactinoma, NAF volumes increased during soy consumption, and although her prolactin levels were consistently elevated between 40–50

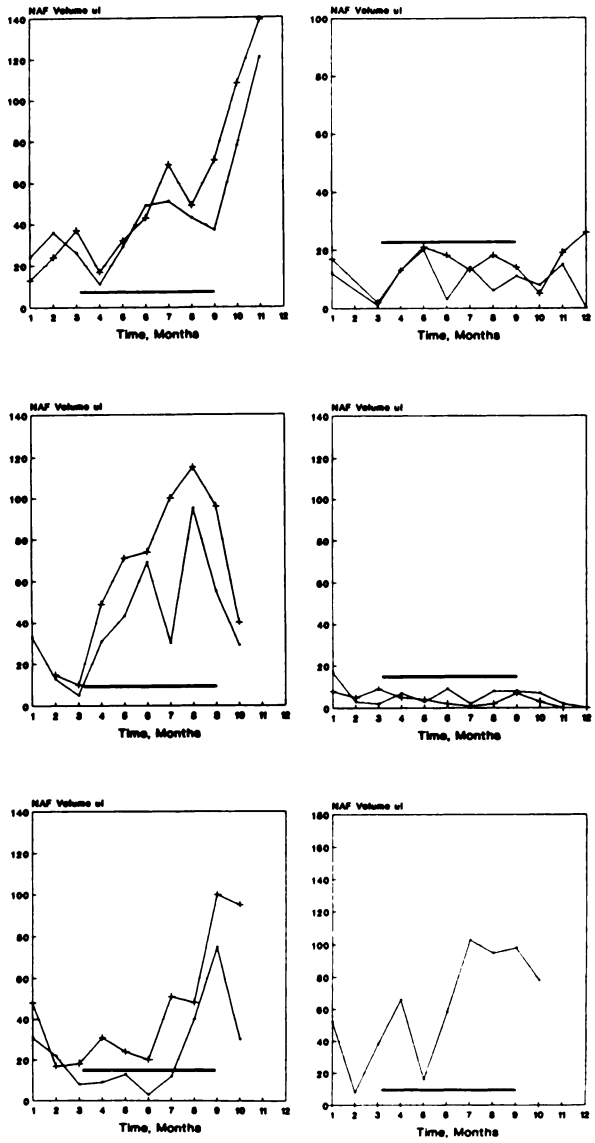

Fig. 2. Effect of soy protein on NAF volume. *Left column,* premenopausal women: *top panel,* age 29; *middle panel,* age 43; *lower panel,* age 46. *Right column,* postmenopausal women: *top* and *middle panels,* lack of effect in women aged 47 and 51, respectively; *lower panel,* increase in NAF volume from right breast of woman aged 52 using menopausal estrogen. *Horizontal lines,* periods of soy consumption.

ng/ml, the volumes of NAF obtained were similar to those found in premenopausal women.

"Composite" Menstrual Cycle

To determine if a relationship existed between NAF volume and day in the menstrual cycle and the possible influence of soy consumption, a "composite" menstrual cycle was developed using data obtained at each monthly visit from the premenopausal women. The volumes of NAF and the concentrations of estradiol, progesterone, and prolactin were recorded by the day from the onset of the previous menstrual period.

As shown in Fig. 4, the individual data on NAF volume and plasma estradiol by day in the menstrual cycle were plotted, and computer generated best-fit curves were produced for months 1–3 (no soy), months 4–6 (on soy), months 7–9 (on soy), and months 10–12 (off soy). The data points were omitted for clarity.

Months 1–3 (No Soy). Concentrations of estradiol were low in the first 7 days of the follicular phase, then progressively increased, reaching a peak of 138 pg/ml at day 15, and then declined during the luteal phase. By day 24, estradiol concentrations had fallen to levels below 60 pg/ml. Individual NAF volumes remained under 20 μl throughout the cycle (Fig. 4, *upper left*).

Months 4–6 and 7–9 (On Soy). Individual estradiol concentrations were irregularly elevated above 80 pg/ml throughout much of the follicular and luteal phases of the cycle. NAF volumes were mostly below 20 μl in the follicular phase of cycle but rose progressively during the luteal phase, reaching volumes of 50 and 65 μl between days 21–27 in months 7–9 (Fig. 4, *upper right* and *lower left*).

Months 10–12 (Off Soy). During the 10–12 months (off soy), the plasma estradiol concentrations began to resemble those seen in months 1–3, with a peak at mid cycle (Fig. 4, *lower right*). Of interest, NAF volumes progressively rose in the follicular phase, and then at day 15 abruptly fell to levels under 20 μl during the luteal phase of the cycle.

Progesterone concentrations were very low during the follicular phase of the cycle, rose to peak levels between days 16–21, and then progressively declined to low levels by day 25–28. During months 4–9 and 10–12, progesterone levels did not appear to differ significantly from those found in months 1–3.

Plasma Cholesterol, HDL, and Triglycerides

No statistically significant changes were observed in the plasma levels of cholesterol, HDL cholesterol and triglyceride levels during the 12-month study period.

Urinary Excretion of Genistein and Daidzein

Genistein and daidzein and their metabolites were low or undetectable in 24-h urines obtained during months 1–3 (no soy), but markedly high concentrations were found during the period of soy consumption, indicating that the women were in compliance with the study plan (Table 5). During months 3–9 (on soy), the mean 24-h urinary output of genistein was 3.12 mg, 7.4% of the genistein ingested in the soy beverage each day. In contrast, urinary excretion of daidzein (6.01 mg) and its metabolites dihydrodaidzein (3.14 mg) and *O*-desmethylangolensin (2.27 mg) accounted for 42.1% of the daidzein ingested in the soy beverage. Urinary excretion of isoflavones tended to increase with the length of time using the soy beverage.

Aside from initial dissatisfaction with the taste of the soy protein isolate, the women reported no unusual systemic symptoms, significant weight change, breast symptoms, or abnormalities of menstruation that could be attributed to soy ingestion. As indicated by the 24-h urine studies, compliance with the study appeared to be excellent. Daidzein and its metabolites were excreted in the urine to a greater extent than genistein, thereby confirming observations hitherto limited to short-term feeding studies (30, 31).

Discussion

The hypothesis that soy may have a protective action against breast and other cancers has been reviewed recently by Messina

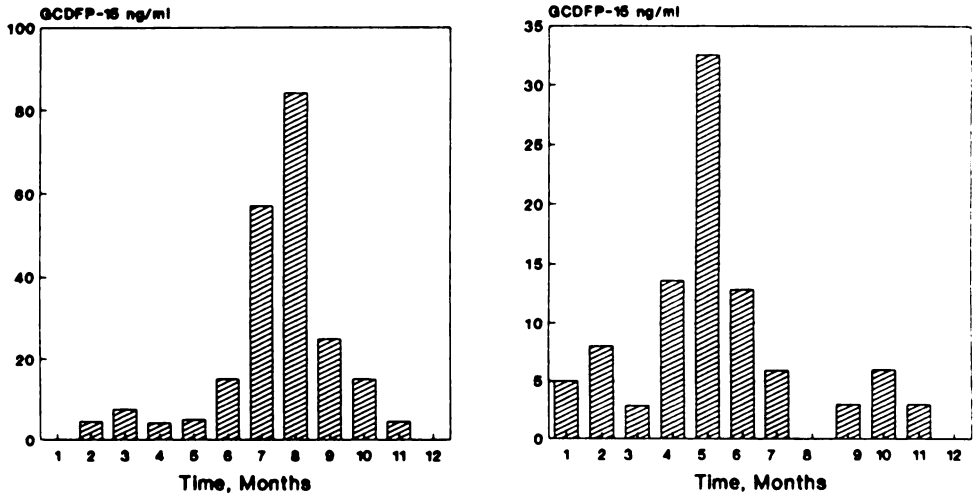

Fig. 3. Examples of an apparent stimulatory effect of soy consumption on GCDFP-15 concentrations in NAF in a 29-year-old premenopausal woman (*left*) and a 53-year-old postmenopausal woman not using estrogen (*right*).

Table 4 Influence of soy protein isolate on concentration of plasma hormones

Lack of effect of soy protein consumption on plasma cholesterol, HDL and triglycerides.

	Month 1–3, no soy		Month 4–6, on soy		Month 7–9, on soy		Month 10–12, no soy		*P*
	(*n*)	mean ± SD	(*n*)	mean ± SD	(*n*)	mean ± SD	(*n*)	mean ± SD	
Premenopausal women									
Estradiol (pg/ml)	(21)	81.20 ± 59.20	(23)	88.50 ± 64.3	(22)	103.70 ± 70.30	(27)	89.80 ± 61.10	NS[a]
Progesterone (mg/ml)	(21)	4.75 ± 5.16	(23)	2.43 ± 3.6	(22)	3.20 ± 4.82	(27)	3.05 ± 4.03	NS
Prolactin (ng/ml)	(21)	13.20 ± 5.80	(23)	14.30 ± 6.4	(22)	13.60 ± 5.00	(25)	13.90 ± 5.04	NS
SHBG (nmol/liter)	(4)	67.50 ± 31.00	(9)	43.60 ± 18.5	(9)	42.00 ± 17.70	(10)	50.00 ± 18.50	NS
Postmenopausal women									
Estradiol (pg/ml)	(15)	53.30 ± 56.60	(28)	45.60 ± 52.80	(25)	52.80 ± 78.30	(21)	39.09 ± 34.60	NS
Progesterone (mg/ml)	(15)	0.44 ± 0.11	(28)	0.41 ± 0.05	(25)	0.40 ± 0.40	(21)	0.51 ± 0.40	NS
Prolactin (ng/ml)	(15)	15.50 ± 14.14	(27)	12.50 ± 12.05	(25)	11.84 ± 9.02	(21)	12.71 ± 12.57	NS
SHBG (nmol/liter)	(1)	36.00	(14)	54.28 ± 30.10	(8)	52.00 ± 34.29	(7)	74.14 ± 41.15	NS

[a] NS, not significant.

et al. (7). A number of animal studies have shown protective effects of soy protein against spontaneous and carcinogen-induced mammary cancers. Barnes *et al.* (32) found that feeding a soy protein isolate to rats markedly reduced the incidence of mammary tumors induced by 7,12-dimethylbenz(*a*)anthracene and *N*-methyl-*N'*-nitrosourea. Further studies indicated that the effect was due to the genistein in the soy (33).

Soy beans contain two isoflavones, genistein and daidzein, having an estrogenic activity 1000 times less than that of estradiol (10–12). Genistein, the major isoflavone in soy, has been shown *in vitro* to compete for estrogen receptors; to inhibit receptor tyrosine kinase and intracellular signaling pathways, topoisomerases, and mitosis; and to affect other aspects of intracellular metabolism (7). When competing for estrogen receptor binding sites, isoflavone phytoestrogens can also have both estrogen agonist and antagonist activity (34). There are, however, no reported toxic effects in humans from eating soy protein. Asian women, who for centuries have consumed soy as a staple of the diet, have not experienced any evident adverse effects on their hormonal and reproductive systems. Epidemiological support for an inhibitory influence of soy on breast cancer was provided by studies in Hawaii by Nomura *et al.* (15) and by Hirayama (14) in Japan. Both studies found that the fermented soybean paste, miso, appeared to have a protective effect against breast cancer in premenopausal women. More recently, using a case-control study design, Lee *et al.* (16) reported a significantly lower risk of breast cancer in premenopausal Chinese women in Singapore who consumed soy. No protection by soy was found in postmenopausal women in any of these three studies. The nature, amount, and duration of time that the soy products had been consumed were not given in these reports. These findings of a protective effect of soy on breast cancer was not confirmed in a case-control study of premenopausal and postmenopausal women in Shanghai and Tianjin, China, by Yuan *et al.* (17).

The findings in the present study did not support our *a priori* hypothesis that the long-term consumption of a diet high in soy protein isolate would lead to a decrease in NAF volumes or lighter color of NAF. Instead, as indicated by a progressive increase in the yield of NAF and the appearance of hyperplastic epithelial cells in the NAF specimens of 30% of the women, the prolonged daily consumption of soy protein isolate appeared to have a stimulatory effect on the premenopausal female breast. These findings are suggestive of a persistent estrogenic stimulus, likely from the ingestion of the soy protein isolate.

Several mechanisms were considered to explain the increased volumes of NAF found in association with soy consumption. These included: (*a*) an effect of prolactin release stimulated by the monthly nipple aspiration procedure; (*b*) a response to the increased daily intake of protein; and (*c*) an estrogenic influence on the breast in response to the genistein and

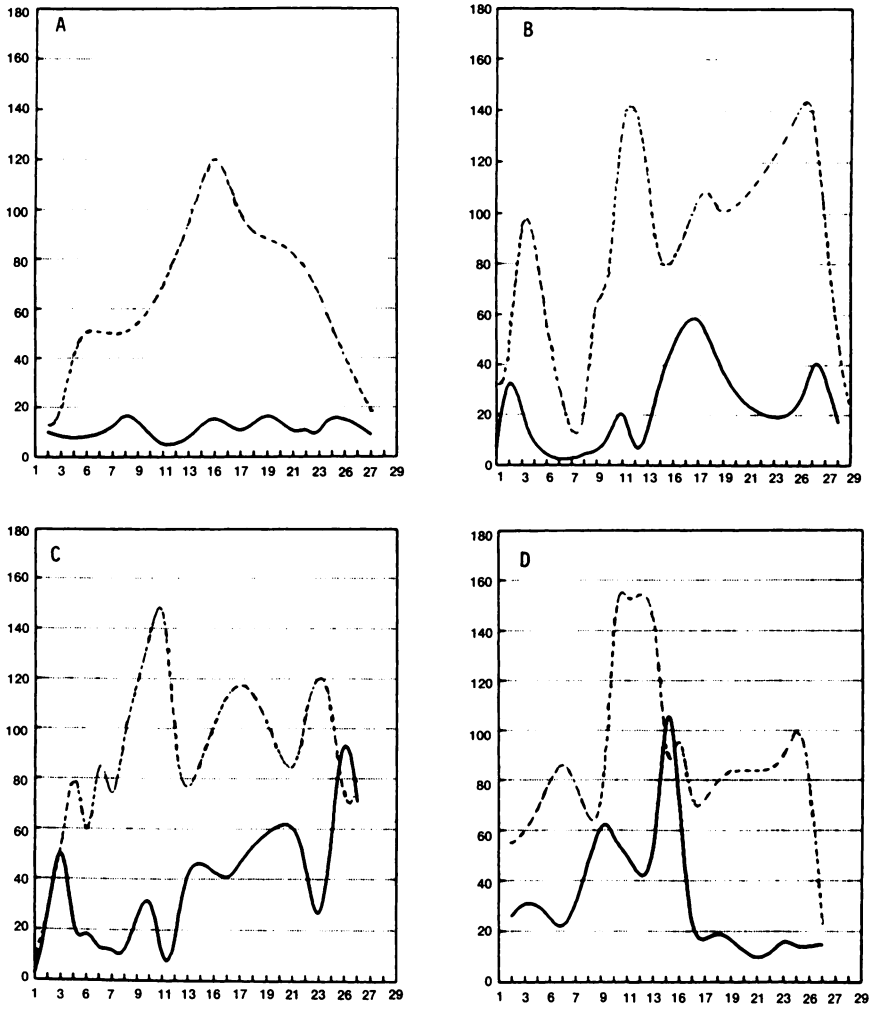

Fig. 4. Plasma estradiol concentrations and NAF volumes in "composite" menstrual cycle shown as computer generated best-fit curves. Data points are omitted. *Upper dashed line*, plasma estradiol concentration. Data obtained from women in months 1–3 (no soy; *A*), months 4–6 (*B*), months 7–9 (on soy; *C*), and months 10–12 (off soy; *D*). *Upper curve*, estradiol; *lower curve*, NAF volume.

daidzein in the soy supplement. Because of the absence of any significant change in plasma prolactin concentrations throughout the course of the study, we rejected the possibility that repeated monthly nipple aspirations may have stimulated the secretion of breast fluid. Also, the very brief time required for the nipple aspiration procedure made this mechanism unlikely. That periodically repeated nipple aspiration is not associated with an increasing yield of fluid is further supported by an unreported study we conducted in 20 premenopausal women in whom NAF was obtained at bimonthly intervals over a period of 2–1/2 years. No significant increase in NAF volume was observed. We also consider it unlikely that the daily addition to the diet of 35 g of soy protein, *per se*, is responsible for stimulation of breast fluid secretion. Most of the women tended to adjust their total diet with respect to the soy supplement, and no significant change in weights of the women was observed. However, in the absence of detailed dietary studies, this possibility cannot be entirely excluded.

Most plausible, the elevated NAF volumes and the cytological hyperplasia associated with the ingestion of soy protein are estrogenic responses to the genistein and daidzein in the soy supplement. As noted above, genistein is reported to inhibit protein tyrosine kinase, a component of the estrogen receptor binding sites, and to compete with estradiol for estrogen receptors in target organs. This suggests that our findings may be a result of competitive binding of estradiol and genistein to the estrogen receptors in breast epithelium. Competitive binding of genistein to estrogen receptor-positive MCF7 and T-47D cell lines is reported to be dose dependent, with agonistic effects at low doses and antagonistic effects at high doses (35).

Evidence suggestive of an effect of soy protein isolate on the menstrual cycle has been reported in two studies. Cassidy *et al.* (36) studied the effect of 60 g of soy protein containing 45 mg of isoflavones given daily to six women who were confined to a metabolic suite for one complete menstrual cycle. Compared with control menstrual cycles studied in the month prior to entry into the metabolic suite, a slight prolongation of the follicular phase length and/or delayed menstruation was found with the soy. Plasma estradiol concentrations were elevated, but no change was found in SHBG levels. A decrease in plasma cholesterol concentration occurred in the follicular phase of the cycle. In a preliminary report presented at a workshop on soy, Goldin[5] presented data on pre-

[5] B. R. Goldin. The effect of feeding soy and whole rye on urinary isoflavonoid and lignin excretion and on the concentration of plasma and excretion of urinary sex hormones. Presented at the National Cancer Institute Workshop, "Dietary Phytoestrogens: Cancer Cause or Prevention?" Herndon, VA, September 21–23, 1994.

Table 5 Influence of soy protein on 24-h urinary isoflavone excretion (μg/ml)

Isoflavone	Month[a]	Number	Mean[b]	SD	Median	*P*
Daidzein	1–3	21	113.6	144.4	49.0	0.000
	4–9	40	6006.5	5119.5	5340.5	
	10–12	22	562.8	1505.9	45.0	
DHD	1–3	21	149.1	393.1	38.0	0.000
	4–9	40	3136.5	3165.2	2441	
	10–12	23	351.5	849.5	27.0	
ODMA	1–3	21	36.8	91.1	0.0	0.000
	4–9	40	2271.5	2554.5	1744	
	10–12	24	276.4	758.7	16.5	
Genistein	1–3	21	196.6	319.5	72.0	0.000
	4–9	40	3121.1	2514.0	2429.0	
	10–12	23	477.9	1127.6	56.0	
Daidzein/genistein ratio	1–3	18	1.34	0.94	1.33	0.455
	4–9	40	1.88	0.80	1.89	NS
	10–12	22	1.53	2.77	0.76	
DDO/G radio	1–3	16	2.19	1.49	2.24	0.075
	4–9	40	3.82	1.71	3.67	
	10–12	19	3.75	4.01	2.35	

[a] Months 1–3: No Soy; Months 4–9: On Soy; Months 10–12: Off Soy
[b] Mean isoflavone concentration in 24-h urine samples before, during, and after soy consumption.
[c] DHD, dihydro-daidzein; ODMA, *O*-des-methyl angolensin.

and postmenopausal women who consumed food containing 15 mg of genistein and 5 mg of daidzein daily for three months. Cyclic variation in urinary excretion of isoflavonoid was observed, with the highest excretion in the luteal phase and lowest in the follicular phase. A concomitant decrease was seen in plasma LH levels. Soy was reported to depress urinary estrogen excretion among postmenopausal women.

Recently, the effect of a daily consumption for 1 month of soy protein by six women, ages 22–29 years, was reported by Lu *et al.* (37). These investigators found a decrease in plasma estrogen concentrations persisting for two to three menstrual cycles following discontinuing the soy. The amount of genistein ingested daily in this study was about one-half that consumed by the women in the study of Cassidy *et al.* (36). These disparate findings on plasma estrogen concentrations during the menstrual cycle may be due to variations in the quantity of soy and genistein consumed and resulting plasma levels of genistein, affecting the normal feedback regulation of the hypothalamo-ovarian axis by gonadotrophic hormones. Tamoxifen, which has estrogen antagonist and agonist properties, is reported to affect plasma estrogen levels as a result of such a feedback mechanism (38). In the present study of the "composite" menstrual cycle, the sporadically elevated plasma estradiol levels found during the 6 months of soy consumption may represent evidence of such competitive binding to the estrogen receptors. Of added interest, the increase in NAF volume in premenopausal women seen during soy protein consumption was found to occur in the luteal phase of the cycle. However, we did not study the effect of soy on the menstrual cycles of individual women, and we consider our findings as only suggestive. In the present study, no significant change in length of menstrual cycles of individual women was found.

The failure of soy to produce an increase in NAF volume in the six postmenopausal women not using menopausal estrogen replacement may be indicative of the loss of estrogen receptors in the involuting breast epithelium. In the three postmenopausal women in whom soy consumption was associated with increased NAF volumes, functional estrogen receptors may persist in their breast epithelia because of their use of menopausal estrogen replacement therapy. Baird *et al.* (39) found no evidence of an estrogenic effect of genistein in postmenopausal women; however, these investigators did not study breast fluid secretion.

Of potential concern was the finding of hyperplastic epithelial cells in nipple aspirates in 30% of women one or more times during the time they were consuming soy protein or in the months after they had completed the soy regimen. Although statistically significant, the number of women is very small, and the results should be considered as suggestive. Because estradiol is known to stimulate breast epithelial proliferation and hyperplasia (40), it is reasonable to attribute the hyperplastic response seen in our study to the result of a combined estrogenic stimulus from high levels of endogenous estrogen and of genistein and daidzein. In a prospective study of NAF cytology and breast cancer risk in 2700 women, NAF hyperplasia was found by Wrensch *et al.* (41) to indicate a modest increased risk of breast cancer, but the risk was much lower than that found with NAF epithelial atypia. Although no atypical epithelial cells were found in NAF in the present study, further research on the effects of long-term soy consumption on breast epithelium is warranted.

The glycoprotein, GCDFP-15, is produced by apocrine metaplastic epithelial cells of the breast and is found in high concentration in the ducts and breast cysts of benign breast disease and in breast cancer, presumably as a response to hormonal stimulation (24). Significant increases in GCDFP-15 plasma levels have been reported in patients with metastatic breast cancer during androgen therapy (23), and *in vitro* studies of breast cell lines have shown stimulatory effects of androgen on GCDFP-15 synthesis (42, 43). Unfortunately, we did not measure plasma testosterone concentrations. In the present study, a 30% decrease in the mean concentration of GCDFP-15 in NAF was found in premenopausal women during the months of soy consumption. The underlying mechanism for this is unclear. This may represent a dilutional effect associated with the increase in NAF volume or a decrease in androgenic stimulation of breast apocrine epithelial cells. The increases in GCDFP-15 seen in two women during soy ingestion may

represent transient, idiosyncratic increases in androgenic stimulation resulting from an antiestrogenic action of genistein. Because of the widespread prevalence of benign cystic disease in premenopausal American women compared with Asian women, further studies of soy would be of interest. Soy has been reported to lower cholesterol levels in subjects with hypercholesterolemia (42, 43) but not in those with normal cholesterol and lipid levels. In our study, plasma cholesterol levels were within relatively normal limits in all women, and no significant effects of soy protein consumption were found on plasma cholesterol, HDL cholesterol, and triglycerides.

Here are some final caveats regarding this study. This was a small pilot study to determine if we could detect an effect on breast fluid secretion by soy protein consumption. Based on our preliminary findings, further studies are warranted on a larger sample size. Because the data used to construct the "composite" menstrual cycle data were obtained from many women at differing days in their cycles, these observations will need to be confirmed by additional long-term studies of soy on the menstrual cycles of individual women, including measurements of testosterone levels. As noted earlier, we did not attempt to control the total diet of the women in the study. The diets of the subjects were *ad lib*, supplemented by the soy protein. It is possible that the absorption and metabolism of the isoflavones can be altered by various components of the diet. It is also likely that the amount of genistein consumed in our study, *i.e.*, 38 mg/day, is considerably higher than the amount normally eaten by the majority of Asian populations and may exert pharmacological rather than physiological effects on the breast. Data are lacking on the actual amount of genistein and daidzein consumed by Chinese and Japanese in Asia and in Western countries. It is likely that consumption varies widely within and between countries, by socioeconomic status, and by age at which soy consumption is begun. M. Lee[6] has conducted dietary studies on Chinese in San Francisco that indicate that American-born Chinese women consume significantly less soy foods than recently immigrant Chinese women living in Chinatown.

This pilot study of NAF from women consuming soy protein isolate daily for 6 months revealed apparent estrogenic effects on the breast fluid secretions. In view of the increasing use of soy protein food products in Western populations, more detailed investigations of the effects of soy on the physiology of the female breast appear highly desirable.

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