Effects of Dietary Soybean Estrogens on the Reproductive Tract in Female Rats 44242

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Abstract. **The estrogenic actions of dietary phytoestrogens have raised concerns regarding the potential DES-like developmental effects on the female genital tract, but the growing evidence of cardioprotective benefits of dietary soybean estrogens provides the impetus to assess the effects of these compounds in adult female models of the menopause. We conducted an experiment in ovariectomized rats to determine the independent effects of dietary soybean estrogens (SBE) and the interactions of these agents with the commonly used pharmaceutical estrogen preparation (conjugated equine estrogens, CEE) in the vagina and uterus. We looked at the effects of SBE and CEE, alone and in combination, on uterine weight, body weight, vaginal cytology, uterine luminal epithelial height, and immunohistochemical staining for proliferating cell nuclear antigen (PCNA), lactoferrin (Ltf), and apoptosis. Ovariectomized rats were fed diets containing casein or soybean protein (SBE, low dose = 11.6 mg isoflavones/1800 cal; high dose = 117.8 mg/1800 cal), with no CEE, low dose CEE (0.313 mg/1800 cal), or high dose CEE (0.625 mg/1800 cal) added. In this study, SBE did not demonstrate estrogenic activity for uterine weight or vaginal cytology. We also found no estrogenic effects of these doses of SBE for PCNA, apoptosis, Ltf staining, or for LEH measurements. In addition, our results regarding the interactions of SBE and CEE do not show any evidence that the combination is additive in effect. On the contrary, the LEH response induced by low levels of CEE, was reduced by high levels of SBE. Furthermore, the Ltf response induced by CEE also was reduced by high levels of SBE. This suggests that high doses of SBE may antagonize the estrogen-agonist actions of low doses of CEE in the rat uterus. Our results in the ovariectomized rat model of menopause suggest that dietary soybean estrogens will not elicit a pattern of effects that simply recapitulates those of steroidal estrogens.**

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The reproductive failure in sheep grazed on estrogenic pastures has been largely attributed to the effects of phytoestrogens on the uterus and cervix (1). These female reproductive tract effects include an increase in thickness and keratinization of vaginal epithelium, enlarged cervix, increased uterine weight, and endometrial hyperplasia, all of which are similar to the changes induced by steroidal estrogens (2, 3).

These estrogenic actions of dietary phytoestrogens have raised concerns regarding the potential DES-like developmental effects on the female genital tract, but the growing evidence of cardioprotective benefits of dietary soybean estrogens in postreproductive age women provides the impetus to assess the effects of these compounds in adult female models of the menopause. Whether women choose to use hormone replacement therapy, or seek a dietary alternative to such therapy, the population of postreproductive age women who will be exposed to exogenous estrogens will increase.

We conducted an experiment in ovariectomized rats to determine the independent effects of dietary soybean estrogens (SBE) and the interactions of these agents with the commonly used pharmaceutical estrogen preparation (conjugated equine estrogens, CEE) in the vagina and uterus. Using markers of estrogenic activity, we assessed the effects

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SBE alone on the reproductive tract of ovariectomized rats, and contrasted this with the effects of CEE. In addition, we measured the interactions of SBE and CEE on the same tissues. The methods we used included immunohistochemical staining for proliferating cell nuclear antigen (PCNA), lactoferrin (Ltf), and apoptosis; uterine and body weights; vaginal cytology; and uterine luminal epithelial height (LEH).

Materials and Methods

Female Harlan Sprague-Dawley rats (n = 127), 40 days old were received from Charles River Laboratories (Raleigh, NC) and placed on casein diets (see Table I). Following an adjustment period, the rats were ovariectomized and placed on diets containing either casein or soy protein (SBE, low dose = 11.6 mg isoflavones/1800 cal. diet; high dose = 117.8 mg isoflavones/1800 cal), with no CEE (Premarin, Wyeth-Ayerst, Radnor, PA), low dose CEE (0.313 mg CEE/1800 cal), or high dose CEE (0.625 mg CEE/1800 cal) added (see Table II). The isoflavone dosages were based on previous work using soybean diets in rats (4). Another group of rats was sham ovariectomized and placed on casein or SBE diets, with no CEE added. SBE isolate was provided by Protein Technologies International (St. Louis, MO) as SUPRO 670 and SUPRO 670-IF. The latter is alcohol-extracted to reduce the isoflavone content of the soybean isolate to its lowest attainable level (0.173%). All diets were formulated in the Comparative Medicine Clinical Research Center diet laboratory.

Rats were housed in single cages to ensure adequate dosing of each individual. They were maintained in barrier rooms under a 14:10 hr light:dark cycle. Food was provided *ad libitum,* as was water *via* an automatic watering system. Guidelines established by our institutional Animal Care and Use Committee and state and federal regulations were followed for all procedures involving animals.

Table I. Diet Composition

Ingredients (g/100 g)	Casein diet	Low SBE	High SBE
Casein	8.00	—	—
Lactalbumin	8.00	—	—
Dextrin	6.00	6.00	6.00
Sucrose	5.00	5.00	5.00
Wheat flour	36.00	36.00	36.00
Wheat bran	4.50	4.50	4.50
Applesauce	4.50	4.50	4.50
Alphacel	1.89	2.12	2.07
Lard	5.00	5.00	5.00
Beef tallow	7.00	7.25	7.25
Butter	3.00	3.00	3.00
Safflower oil	3.50	3.50	2.95
Crystalline cholesterol	0.0145	0.0145	0.0145
Complete vitamin mix	2.50	2.50	2.50
Ausman-Hayes mineral mix	5.00	5.00	5.00
Calcium carbonate	0.10	—	—
SUPRO 670-IF®	—	15.40	—
SUPRO 670®	—	—	16.00
DL-methionine	—	0.22	0.22

Note. CEE doses: low = 0.313 mg CEE/1800 cal; high = 0.625 mg/1800 cal. Low SBE = SUPRO 670-IF (phytoestrogen-reduced soy isolate) equivalent to 11.6 mg isoflavones/1800 cal. High SBE = SUPRO 670 (phytoestrogen-containing soy isolate) equivalent to 117.8 mg/1800 cal.

Table II. Number of Rats Receiving Casein or Soy Diets and Estrogen Replacement Treatments

Group #	n	Diet	Treatment	Condition
1	11	Casein	No CEE	OVX
2	8	Casein	No CEE	Sham OVX
3	11	Low SBE	No CEE	OVX
4	10	Low SBE	No CEE	Sham OVX
5	11	High SBE	No CEE	OVX
6	10	High SBE	No CEE	Sham OVX
7	11	Casein	Low CEE	OVX
8	11	Low SBE	Low CEE	OVX
9	11	High SBE	Low CEE	OVX
10	12	Casein	High CEE	OVX
11	11	Low SBE	High CEE	OVX
12	11	High SBE	High CEE	OVX

Note. OVX = ovariectomized; CEE = conjugated equine estrogens; SBE = soybean estrogens.

The animals were fed the diets for 2 months. Vaginal cytology samples were taken daily for 1 week at the beginning of diet treatment and 1 week at the end of the study. Vaginal cytology samples were obtained by aspiration.

At the end of the study, animals were euthanized by carbon dioxide inhalation. At necropsy, the uteri were collected and fixed overnight in 10% neutral buffered formalin (at 4°C) and then transferred to 70% ethanol for storage before being processed for paraffin sectioning.

Tissue sections were mounted on poly-L-lysine coated slides for PCNA immunohistochemical staining; other sections were mounted on Probe-On Plus charged slides (Fisher Scientific, Pittsburgh, PA) for the apoptosis and lactoferrin immunohistochemical staining.

PCNA staining was performed according to a modification of the protocol of Foley *et al.* (5). Briefly, the slides were dried overnight, deparaffinized through three changes of xylene, hydrated to distilled water, and incubated with PC-10 antibody (Novocastra Laboratory, Newcastle-on-Tyne, UK) in capillary gap slides at 35°C for 30 min. A link antibody (biotinylated anti-mouse antibody, Biogenex Super-Sensitive StrAviGen, Biogenex, San Ramon, CA) was applied, and the slides incubated at room temperature for 30 min; then the label antibody was applied (SuperSensitive Streptavidin Alkaline Phosphatase, Biogenex) and incubated at room temperature for 30 min. The chromogen (Alkaline Phosphatase Substrate Kit I, Vector Laboratories, Burlingame, CA) was applied for 15 min at room temperature, under dark conditions. Slides were counterstained with Mayer's hematoxylin for 5–10 min, and dehydrated to xylene for coverslipping.

Apoptosis staining was performed using a digoxigenin-nucleotide antibody kit (ApopTag, Oncor Co., Gaithers-

burg, MD) that labels DNA fragments with terminal deoxynucleotidyl-transferase (TdT). The TdT-digoxigenin optimally stains 3'-OH ends of DNA fragments that are localized in apoptotic cells (6). Briefly, tissue sections mounted on charged slides (Probe-On Plus) were deparaffinized, digested with proteinase K, incubated with equilibration buffer (ApopTag, Oncor) and incubated with TdT (ApopTag, Oncor) for 1 hour. The reaction was stopped with stop solution (ApopTag, Oncor), and label antibody was applied for 2 hr at room temperature (AP-conjugate, Boehringer Mannheim Corp., Indianapolis, IN); the chromogen was then applied for 10 min under dark conditions. Slides were counterstained with Mayer's hematoxylin as above.

Sections were counted using a Macintosh-based image analysis system, using video acquisition of 40X-magnification color images (VK-C370 camera Hitachi Corp., Lyndhurst, NJ; and a Scion LG-3 capture board, Scion Corp., Frederick, MD) which were then processed using the public domain NIH Image program (written by Wayne Rasband, National Institutes of Health, available from the Internet by anonymous FTP from zippy.nimh.nih.gov). Using a grid to randomize counting, 100 cells were evaluated based on a modification of the grid-filtering system of Lindholm (7), from both the superficial and deep endometrial glands. For the purposes of analysis, cells with any amount of staining were considered positive. The positively stained cells were expressed as a percentage of the total for both superficial and deep glands in each section. Luminal epithelial height was measured according to the method of Branham *et al.* (8) without modification. Data analysis was performed using the JMP statistical program (SAS Institute, Cary, NC); means were compared using ANOVA and Student's *t*-test.

Lactoferrin staining was performed according to the protocol of Walmer *et al.* (9). Staining intensity scores were assessed for the endometrial epithelium based on a scale of 0 (no visible staining) to 2 (intense staining of the entire cell).

Results

The addition of CEE to casein or either phytoestrogen diet resulted in vaginal smears that were similarly estrogenized (see Fig. 1). Comparison of total days of cornified vaginal smears during dietary exposure showed that neither low nor high SBE diets had any effect upon vaginal cytology, relative to the casein controls.

While CEE increased body weight and uterine weight, SBE alone at either low or high doses had no statistically significant effect (ANOVA; see Fig. 2). Furthermore, even when uterine weight was normalized for body weight, there were no significant differences between the casein group and either the low SBE or high SBE groups (data not shown). The interaction of SBE and CEE resulted in body weights similar to those of animals fed CEE alone.

In both the casein and the high SBE diet groups, proliferating cell nuclear antigen (PCNA) staining showed low

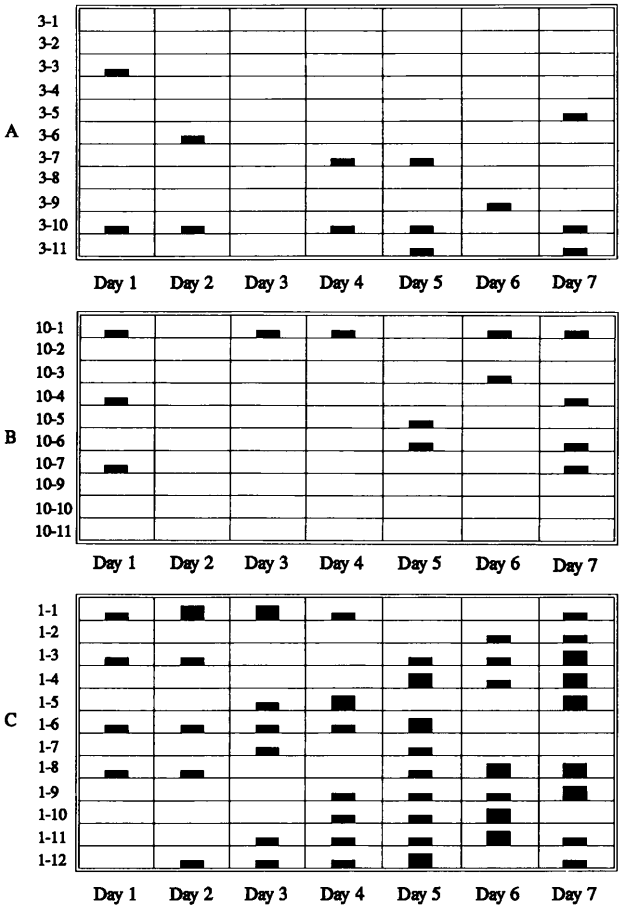

Figure 1. Daily vaginal cytology smears from ovariectomized rats fed (A) casein diet; (B) high soy (SBE) diet; or (C) casein with high CEE. Casein-fed and soy-fed animals demonstrate hypoestrogenized vaginal smears, whereas CEE-fed animals demonstrate more estrogenized vaginal cytology. One week's data are shown. Numbers at left represent individual animals. Full-height bars indicate fully cornified smears, whereas half-height bars indicate partial cornification.

levels of proliferation (see Fig. 3). The addition of CEE to all three diets resulted in a significant increase in proliferation ($P < 0.05$). It is noteworthy that PCNA staining in the high SBE/high CEE group did not differ from the casein/high CEE group, and the low SBE/low CEE group did not differ significantly from the high SBE/low CEE group.

Apoptosis staining seemed to parallel the results of the PCNA staining: higher levels of apoptosis were seen in all groups given CEE (Fig. 4). Regarding the surface endometrial cells, relative to either casein alone or high SBE alone groups, apoptosis was significantly increased in the high SBE/high CEE group ($P < 0.05$). Only the high SBE/high CEE group differed significantly from the low SBE-alone group (Fig. 4). In the endometrial glands, the high SBE/high CEE, casein/high CEE, and high SBE/low CEE groups were not different from each other. These groups did differ significantly from the high SBE-alone, low SBE-alone, and casein groups ($P < 0.05$) (Fig. 4). The low SBE/low CEE group appeared to be intermediate regarding apoptosis, in that it did not differ significantly from any other groups.

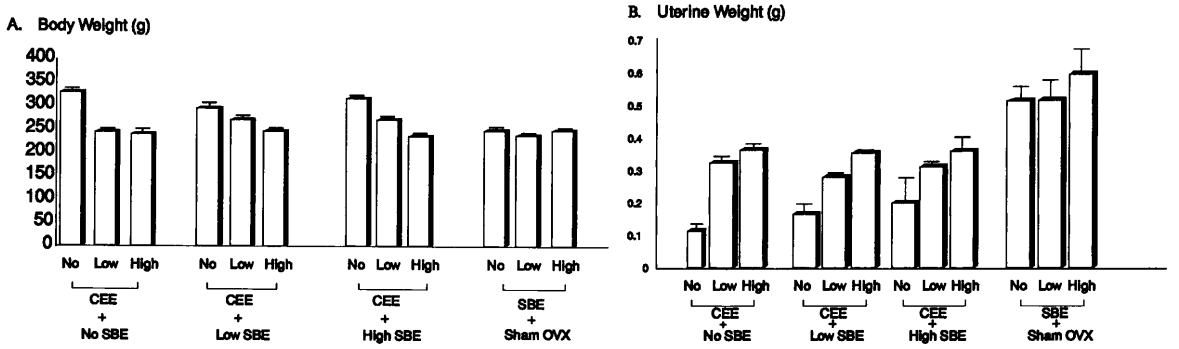

Figure 2. (A) Body weight; and (B) uterine weight data from ovariectomized and sham ovariectomized rats fed casein diets or soy diets, with differing doses of CEE. Error bars indicate standard error of the mean. Animals fed casein or SBE alone have significantly higher body weights ($P < 0.05$), whereas CEE-fed animals are similar in weight to the intact (sham OVX) rats. Sham OVX animals have significantly higher uterine weights ($P < 0.05$); the soy-alone animals are not significantly different from casein-fed animals.

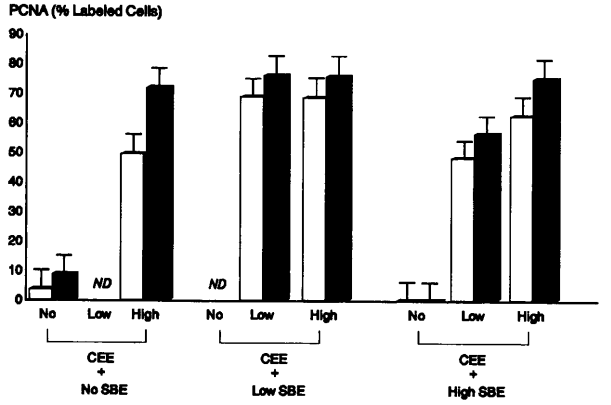

Figure 3. Effects of CEE and SBE on rat uteri. Proliferating cell nuclear antigen (PCNA) staining of endometrial surface cells and glands from selected groups. For each pair of bars, the open bar refers to surface and the dark bar refers to gland cells. ND = group was not done. Error bars indicate the standard error of the mean. Stained cells are expressed as a percentage of the total cells counted in each section.

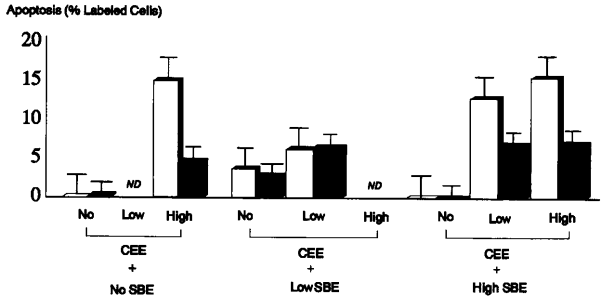

Figure 4. Effects of CEE and SBE on rat uteri. ApopTag staining of endometrium from selected dietary groups. For each pair of bars, the open bar refers to surface and the dark bar refers to gland cells. ND = not done. Error bars indicate the standard error of the mean. Stained cells are expressed as a percent of the total cells counted in each section.

Lactoferrin staining demonstrated no estrogenic effect of SBE and was strongly positive for the CEE-alone treatment group (Fig. 5). The addition of CEE to SBE resulted in positive staining; however, in the high SBE/high CEE group, the intensity of the staining was decreased compared to the CEE-alone group. This suggests a blunting of the

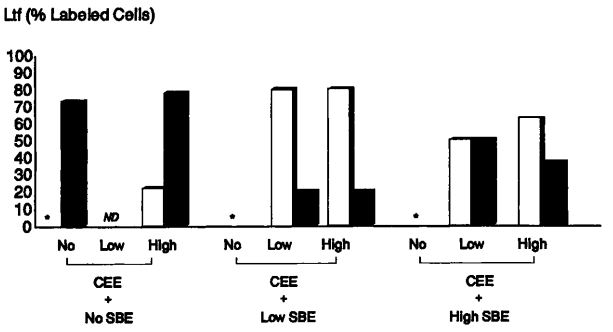

Figure 5. Effects of CEE and SBE on lactoferrin (Ltf) expression in the rat uterine epithelial cells. Each section was assessed for intensity of staining, which was scored as 0 (no stain), 1 (weak stain), or 2 (intense stain). Weakly and strongly stained cells were expressed as a percentage of the total labeled cells. For each pair of bars, the open bar indicates low-intensity Ltf staining, and the dark bar indicates high-intensity Ltf staining. ND = not done; * = no positive Ltf staining. All groups differed significantly by nonparametric (Kruskal-Wallis test) statistical analysis ($P < 0.05$).

effects of pharmaceutical estrogens on the induction of Ltf expression in the uterine epithelium.

Luminal epithelial height (LEH) measurements in the casein, low SBE, and high SBE groups did not differ, but the addition of CEE to the diets resulted in increased LEH (Fig. 6). The high SBE/high CEE group demonstrated a reduction in LEH relative to the high CEE-alone and the low SBE/high CEE groups ($P < 0.05$).

Discussion

We assessed several markers of estrogen action in the ovariectomized rat. Uterine weight and vaginal cytology did not demonstrate estrogenic activity of SBE at the doses studied. This is in contrast to earlier studies using uterine weight in mice and rats as biological assays for estrogenic activity in plant materials (10–12). In those studies, it was shown as early as 1953 that exposure of mice or rats to estrogenic forages resulted in increased uterine weight; however, these increases occurred at dosages greater than those used in the current study. We also found no estrogenic effects of these doses of phytoestrogens in the PCNA and apoptosis staining, or for LEH and Ltf measurements.

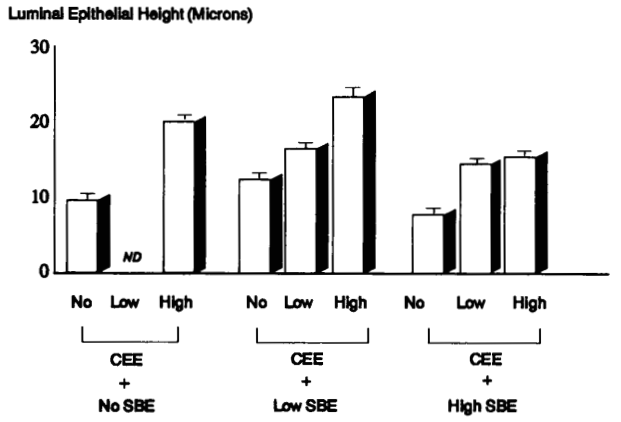

Figure 6. Effects of CEE and SBE on luminal epithelial height (in microns) in rat uteri for selected groups. Error bars indicate the standard error of the mean. ND = not done.

In addition, our results regarding the interactions of SBE and CEE do not show any evidence that the combination is synergistic in effect. Furthermore, the Ltf response induced by CEE also was reduced by high levels of SBE. This suggests that high doses of SBE may antagonize the estrogen-agonist actions of low doses of CEE in the rat uterus, or that there may be cell-type specificity of response within the uterus.

The failure of SBE to affect the vaginal cytology suggests that these compounds would have limited utility in managing the symptoms of atrophic vaginitis in postmenopausal women. On the other hand, the lack of estrogen-agonist effects of SBE alone on the endometrium, and the partial antagonism of a histomorphometric marker of CEE-stimulatory action on the endometrium by high levels of SBE, suggest that the addition of SBE to the estrogen replacement therapy of postmenopausal women may be beneficial. Dietary soybean phytoestrogens may complement the cardioprotective effects of estrogen replacement therapy, allowing reductions in the required doses of steroidal estrogens. Such dose reduction combined with the direct antagonism of some aspects of steroidal estrogen stimulation of the endometrium by the phytoestrogens may concurrently reduce the risk of endometrial hyperplasia or endometrial cancer in menopausal women. In terms of reproductive tract actions in the menopausal woman, our results in this ovariectomized rat model suggest that dietary soybean estrogens will not elicit a pattern of effects that simply recapitulates those of steroidal estrogens.

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