Effects of Genistein or Soy Milk During Late Gestation and Lactation on Adult Uterine Organization in the Rat

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In utero **and lactational exposure to estrogenic agents has been shown to influence morphological and functional development of reproductive tissues. Thus, consumption of dietary phytoestrogens, such as isoflavones, during pregnancy and lactation could influence important periods of development, when the fetus and neonate are more sensitive to estrogen exposure. In this study, reproductive outcomes after developmental exposure to isoflavones were examined in Long-Evans rats maternally exposed to isoflavones via a commercial soy beverage or as the isolated isoflavone, genistein. Most reproductive endpoints examined at birth, weaning, and 2 months of age were not significantly modified in pups of either sex after lactational exposure to soy milk (provided to the dams in place of drinking water) from birth until weaning. However, soy milk exposure induced a significant increase in progesterone receptor (PR) in the uterine glandular epithelium of the 2-month-old pups. In pregnant dams treated with genistein (GEN; 15 mg/kg body weight) by gavage, from Gestational Day 14 through weaning, PR expression in the uterine glandular epithelium from 2-month-old GEN-treated females (postexposure) was also significantly increased. Diethylstilbesterol (DES) also stimulated uterine PR expression only in the glandular but not luminal epithelial cells. However, unlike DES,** ***in utero*****/lactational exposure to GEN did not increase expression of the proliferation marker, proliferating cell nuclear antigen (PCNA), in the luminal epithelial cells of the 2-month-old rat uteri. These experiments demonstrate that developmental exposure to dietary isoflavones, at levels comparable to the ranges of human exposure, modify expression of the estrogen-regulated PR in the uterus of sexually mature rats weeks after exposure ended. Since the PR is essential for regulating key female reproductive processes, such as uterine proliferation, implantation, and maintenance of pregnancy, its increased expression suggests that soy phytoestrogen exposure during reproductive development may have long-term reproductive health consequences.** Exp Biol Med 229:108–117, 2004

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While considerable attention has been focused on the developmental effects of environmental contaminants, comparatively less attention has been directed toward potential actions of hormonally active dietary factors, such as the phytoestrogens. As the public has become increasingly aware of the health benefits associated with consuming an Asian-style diet, consumption of foods and health products containing dietary phytoestrogens has risen (1). While these chemicals may have health-related benefits in some age-groups, they may also present a risk to immature estrogen-sensitive target tissues, such as the developing reproductive tract.

Previous research has demonstrated that infants are exposed to phytoestrogens via breast milk and soy-based infant formulas, which contain between 17.2 and 21.9 mg/L isoflavones (2, 3). Consumption of soy products by women can increase the concentration of phytoestrogens in breast milk by 10-fold (4). In addition, a moderate challenge with 20 g roasted soybeans (equivalent to 37 mg isoflavones) results in 2.0 μM/L total isoflavones (daidzein, genistein, and glycitein) detectable in breast milk (5). The increase in phytoestrogen levels in breast milk is of concern because genistein can be

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transferred from the lactating dam to the neonate via the milk (3). Since it has been shown that infants can digest and absorb dietary phytoestrogens in active forms (6) and since neonates are generally more susceptible than adults to perturbations of the sex steroid milieu (7), exposure to these naturally occurring estrogenic compounds may pose a developmental hazard to the developing offspring and affect target tissue functions later in life. These findings and the concerns over the possible effects of other environmental estrogens have led to questions about the safety of phytoestrogens in breast milk and soy-based infant formula (8, 9).

In addition to the concerns about phytoestrogen exposure to neonates, consumption of isoflavones during pregnancy can also result in exposure of the fetus to these dietary phytoestrogens. Dietary phytoestrogens are detectable in amniotic fluid samples obtained during the second trimester from >90% of women undergoing routine amniocentesis (10). Therefore, the mother's consumption of foods containing isoflavones during pregnancy can result in exposure of the human fetus during this critical window of development.

Exposure to exogenous sex steroid agonists, antagonists, or modulators of endogenous sex steroid hormone metabolism is known to alter patterns of normal sexual dimorphic development in multiple target tissues (11, 12). Numerous studies have demonstrated that both naturally occurring and xenobiotic estrogens can affect markers of sexually dimorphic development (13–19). *In utero* and neonatal exposure to genistein and diethylstilbesterol (DES) has been shown to result in altered weaning anogenital distance (AGD) and pubertal onset, increased size of the sexually dimorphic nucleus of the preoptic area of the hypothalamus, and altered pituitary responsiveness to GnRH (13, 14, 20). In addition, phytoestrogens have been shown to cause both direct and indirect adverse effects on the reproductive tract of animals. These effects include infertility, persistent vaginal cornification, hemorrhagic ovarian follicles, and premature vaginal opening (21–24).

Although it has been shown that phytoestrogens can adversely effect reproductive function in animals, current animal studies provide little insight into the effects of *in utero* exposure to phytoestrogens and DES on uterine physiology, particularly patterns of gonadal steroid receptor and proliferation of uterine epithelium. This study tested the hypothesis that *in utero* and lactational exposure to dietary phytoestrogens would increase the expression of estrogen and progesterone receptors. Therefore, the objectives of this study were to determine how developmental exposure to isoflavones, either those contained in soy milk or the isolated isoflavone genistein, influences expression of estrogen receptor (ER), progesterone receptor (PR), and proliferating cell nuclear antigen (PCNA) in the sexually mature rat uterus.

Materials and Methods

Animals. Adult Long-Evans hooded rats were used in this study. These studies were performed in accordance with the Guidelines for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee of Duke University. Rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained under a 12:12-hr light:dark schedule with controlled temperature and humidity. Food and water were available *ad libitum*. For at least 3 weeks prior to mating, dams and sires were fed a modified version of the AIN-93G Purified Diet formulated for the growth, pregnancy, and lactational phases of rodents (Harlan Teklad, Madison, WI). In the custom diet, the soybean oil present in AIN-93G was replaced with corn oil. Dams, sires, and pups were maintained on this diet throughout the study.

Ovarian cyclicity was monitored by daily vaginal smears. On a day of proestrus, the rats were allowed to mate overnight. Copulation was confirmed by detection of sperm in the vaginal smear on the ensuing morning of estrus. In the genistein study, four dams were randomly assigned to one of the five treatment groups: corn oil (control), low (1×) and high (10×) dose DES (low DES and high DES, respectively), Genistein (GEN), and GEN + low DES. Dams were treated from Gestational Day (GD) 14 to PND 21 when the pups were weaned. The day of delivery was defined as Postnatal Day (PND) 1. The mean litter size (means of 10.7 for control, 12.3 for low DES, 10.8 for GEN, 11.0 for GEN + DES, and 10.7 for high DES) and sex ratio in each of the treatment groups did not vary significantly. The number of pups exposed to each treatment was as follows: 18 male and 25 female pups, control; 28 male and 19 female pups, low DES; 20 males and 23 females, GEN; 26 male and 17 female pups, GEN + DES; and 12 male and 17 female pups, high DES.

In the soy milk study, to evaluate the effect of total soy isoflavones exposure on pup development during the time of lactation, on the day of delivery five dams were randomly assigned to the rice milk control (two dams) or the soy milk (three dams) treatment group. The mean litter size was 12.4 and did not significantly vary. On the day of delivery, water was replaced by plain 2% fat soy milk ("Westsoy" brand, WESTBRAE Natural Foods, Carson, CA) or plain 2% fat rice milk ("Rice Dream Organic Original" brand, Imagine Foods, Palo Alto CA) fortified with rice protein powder ("Nutribiotic of Lakeport" brand; Lakeport, CA) to match the protein composition of the soy milk product. The milk was replaced daily in order to maintain freshness. The dams were given either rice milk or soy milk from delivery to the time of weaning, corresponding to PND 1 through PND 21 of pup development. The pups had some direct but limited exposure once they were old enough to begin drinking from the water bottle; however, this is loosely comparable to human infant exposure indirectly via mother's milk and directly via infant formulas. At PND 21, the rats were given water *ad libitum* until the end of the study. A total of 14 male and 22 female pups exposed to soy milk and 8 male and 18 female rice milk control pups were evaluated for the following endpoints: birth and weaning AGD, birth and

weaning body weight (BW), age of onset of puberty, and average cycle length. At PND 60, organs were collected, weighed, and then fixed for histopathological analysis.

Treatments

Genistein Study. The hormonal compounds used in this study were diethylstilbestrol (DES; Steraloids, Wilton, NH) and genistein (GEN; ICN Biomedicals, Costa Mesa, CA). Doses used in this study were based on prior scientific publications and calculated estimates of exposures in human populations. Based on prior studies by Thigpen and colleagues (25, 26), the calculated daily dose of DES was 0.06 to 0.09 μg/day, and thus for the low dose of DES or the GEN + DES group, we administered 0.1 μg/day of DES per dam, which is approximately 0.5 μg/kg BW. The high DES dose group was administered at 5.0 μg/kg BW.

For the estrogenic isoflavone genistein, if all dietary protein were to be isoflavone-rich soy, then the upper limit of daily dietary intake of genistein by a rat is on the order of 10–20 mg/kg BW (27–30). We chose to dose the dams in our present study with 15 mg GEN/kg BW, which is, on a body-weight basis, 10–15 times the daily dose ingested by humans consuming a traditional Asian diet; however, on a caloric intake basis, this dose is virtually identical to the amount of isoflavone ingested by simply using soy as the exclusive protein source in the diet (as it is produced on the commercial scale as food components for livestock, experimental animals, or humans). Note that on a body-weight basis, the dose of 5 mg genistein/day previously administered subcutaneously to pregnant dams (18) is approximately 15–20 mg/kg/day. While oral absorption is certainly less than the 100% delivery by subcutaneous dosing, the substantial enterohepatic recirculation of oral isoflavones (especially genistein) greatly delays ultimate excretion and, thus, prolongs bioavailability of these agents. The treatment groups were as follows: corn oil (vehicle control), low DES (0.5 μg/kg BW), high DES (5.0 μg/kg BW), GEN (15 mg/kg BW), and GEN + DES (GEN, 15 mg/kg BW; low DES, 0.5 μg/kg BW). Dams were gavaged from GD 14 through weaning (PND 21) with either corn oil alone (unexposed controls) or the estrogen agent in corn oil, with the exception that DES was not administered from GD 20 through the day of birth (to minimize the risk of dystocia/delayed parturition). By gavage treating the dams, the nursing pups did not have any direct exposure to GEN or the other treatments. The day of delivery was designated PND 1. No litters were culled.

Soy Milk Study. Daily fluid volume consumption by pregnant and lactating rats ranges from 30 to 65 ml (31). Product specifications for the commercial rice and soy milk products that we used indicate that the isoflavone content of the two, rice milk and rice powder, is negligible, whereas there are approximately 31 mg of isoflavones (genistein and daidzein in an approximately 3:2 ratio) per 8-ounce (240 ml) serving in the soy milk. Thus, daily intake of isoflavones by each dam assigned to the soy milk group is estimated to have been greater than 3.5 mg/day but less than 8.5 mg/day. Therefore, on a body-weight basis, the daily intake by dams can be estimated to have been between 10 and 30 mg/kg BW of soy isoflavones.

Endpoints Evaluated. On PND 1, litter size, sex, anogenital distance (AGD), and BW were recorded, and individual pups were tattooed for identification. Pups were inspected daily and marks refreshed whenever needed. Each pup was weighed on day of birth, on day of weaning, weekly until puberty, and then monthly until sacrificed. AGD was measured in duplicate with Vernier calipers to the nearest 0.3 mm (PND 1) and to the nearest 1.0 mm on PND 21, and the average measurement for each time point was recorded (16). Body weights were obtained weekly for general documentation of development. Results of these measurements have been reported previously (20). The age of puberty was determined by inspecting the offspring for the day of vaginal opening or preputial separation. On vaginal opening, vaginal cytology was assessed daily for 3 weeks by vaginal lavage to monitor cyclicity as a measure of ovarian function and to determine reproductive senescence of dams and pups (on reaching puberty). The age of puberty was determined by inspecting the female offspring daily after PND 30 to assess the day of vaginal opening. An eye-dropper was used to collect a saline suspension of vaginal cells. A drop of the aspirate was air-dried on a microscope slide, and smears were stained with 1% toluidine blue and read according to established procedures (32). The puberty data and initial cyclicity data for the genistein study were previously published (20).

Necropsies and Tissue Collection. At necropsy, each rat was euthanized by an intraperitoneal injection of pentobarbital. The reproductive systems were removed before thoracotomy and cardiac perfusion for brain fixation. All pups were necropsied at 2 months of age (PND 50–60), with the female pups necropsied on a day of estrus confirmed by vaginal smear cytology and inspection of the fallopian tubes for ova. The brain, uterus, ovaries, and mammary glands were collected and fixed for future histopathological analyses. The males were necropsied, and weights of the testes, prostate, epididymis, and seminal vesicles were determined. One testicle and half the prostate and seminal vesicles were snap-frozen in liquid nitrogen. The other testicle, epididymis, and remaining portions of the prostate and seminal vesicles were placed in Bouin's fixative for 1 day, washed in running tap water, and transferred to 70% ethanol.

Uterine Histomorphometry. At necropsy, uteri were removed and fixed in 4% paraformaldehyde, washed, and transferred to 70% ethanol 24 hrs later. After fixation, three representative pieces from the proximal, middle, and distal portions of one uterine horns from each animal were dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin for sectioning; 5-μm–thick sections were cut and mounted on glass slides. Sections

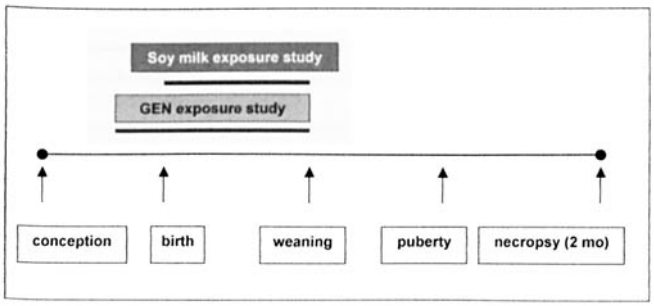

Figure 1. Treatment scheme for the soy milk and genistein studies. The breeders were switched from standard rat chow to an isoflavone-free diet prior to mating to prevent unintended exposure to the phytoestrogens. All study rats were maintained on the isoflavone-free diet until necropsy. Treatments were given to the dams from PND 1 through PND 21 for the soy milk study and from GD 14 though PND 21 for the genistein study. The female pups were necropsied in estrus at 2 months of age. In the soy milk study, the male pups were also necropsied at 2 months of age.

were deparaffinized in xylene and rehydrated in graded ethanol solutions. After Van Gieson's staining (33), luminal epithelium height (LEH) was measured using an image analysis system (Media Cybernetics Image-Pro Plus, version 4.0, Silver Spring, MD).

Immunohistochemistry for PCNA, ERα, and PR. Slides were deparaffinized in xylene, rehydrated in a graded ethanol series, and transferred to Antigen Retrieval Citra (pH 6.0) (Biogenex, San Remon, CA) and then heated for 2.5 mins in a microwave oven, allowed to cool, and then washed in PBS. Nonspecific binding was blocked with 10% normal goat serum in PBS for 30 mins, followed by incubation with PCNA monoclonal antibody (19A2 clone from Biogenex) at an optimal dilution of 1:20 in 10% goat serum in PBS or the ERα (1D5 clone) and PR (10A9 clone) (Immunotech, Westbrook, ME) for 24 hrs at 4°C. Slides were incubated with normal mouse IgG1 as a negative control. Sections were incubated with the biotinylated secondary antibody for 30 mins and for 30 mins with alkaline phosphatase conjugated streptavidin for 30 mins. Sections were washed four times for 1 min each in Autobuffer (Biomedia Corp., Foster City, CA) and incubated with alkaline phosphatase substrate kit I (Vector Laboratories, Inc., Burlingame, CA) for 10 mins. Sections were then counterstained in Mayer's hematoxylin, dehydrated through graded ethanol solutions, cleared in xylene, and mounted with Permount for bright-field microcopy. Images were acquired digitally using a microscope coupled to an Image Analysis System (Image Pro Plus 4.0, Silver Spring, MD) used for histomorphometry.

Cell Counting. All ERα and PR immunopositive cells in the luminal and glandular epithelium were counted in ×40 field. For proliferating cell nuclear antigen (PCNA), immunopositive cells in the luminal epithelium only were counted. The average number of immunopositive cells was obtained by counting three separate randomly selected fields per section. The positively stained cells were expressed as a percentage of the total luminal epithelium (immunopositive and immunonegative) cells counted.

Statistics. Data were analyzed by one-way analysis of variance in conjunction with a multiple range test using SigmaStat software (Jandel Scientific, San Jose, CA) and included testing for normality of distribution and homogeneity of variance. On these bases, although less robust than parametric procedures, we chose to use a nonparametric ranking test (Kruskal-Wallis) for making comparisons between groups. In all cases, $P < 0.05$ was required to determine statistical significance.

Groups were subgrouped by gender in the analysis. Since the outcomes in this study are sexually dimorphic and since we know that in litter-bearing mammals the pup's intrauterine position (IUP) profoundly affects AGD (up to 50% within a sex), central nervous system nuclear volumes (up to 50% within a sex), aggressive behavior, and so on, these data were analyzed on the basis of individuals rather than litters. Failure to either explicitly account for the IUP effect or to preserve the IUP-related increase in variance in outcomes would actually be a much greater threat to interpretation than possible metabolic or other differences between dams. Since IUP was not determined in these experiments, the influence of IUP is a variable factor that contributes to the error term.

Results

To determine if exposure to isoflavones during critical periods of development has long-term effects on the reproductive tract, even after exposure has ended, two animal studies were conducted. In the first, the effects of soy milk containing isoflavones (31 mg/8 oz) on reproductive tract development of male and female pups exposed during lactation were examined (soy milk study). This study is designed to mimic exposure of the pups to soy, containing isoflavones, ingested by the mother during lactation and direct exposure from soy-based infant formulas. In the second, the effects of the predominant isoflavone in soy, genistein, on the uterus was analyzed after *in utero* and lactational exposure (genistein study). The genistein study examines whether this phytoestrogen component in soy is able to modify adult uterine responsiveness when the mother consumes this isoflavone during pregnancy and lactation. Both treatment schemes are shown in Figure 1.

Estrogen (DES) as well as other reproductive endocrine disrupters have been shown by numerous studies to cause reproductive malformations or initiate cancer in adulthood. Skakkebeck hypothesizes that this is due to an alteration in the differentiating stem cells and that this alteration is not reversible (34). Therefore, evaluating the reproductive organs after exposure has ended and when the rat pups have reached sexual maturity (both males and females) is of importance in understanding the long-term effects due to permanent alteration of reproductive tissue via endocrine disruption during development. For the males, fertility

Table 1. Effects of Lactational Exposure to Soy Milk on Birth, Weaning, and Pubertal Indices in Male Pups[a]

Group	N	bAGD (mm)	bBW (g)	bAGDI	wAGD (mm)	wBW (g)	wAGDI	Age at preputial separation (days)
Rice milk controls	8	2.27 ± 0.10	6.15 ± 0.22	0.36 ± 0.02	13.24 ± 0.32	42.06 ± 0.90	0.32 ± 0.01	31.00 ± 0.38
Soy milk	14	2.46 ± 0.18	6.09 ± 0.28	0.41 ± 0.04	13.81 ± 0.39	44.58 ± 2.09	0.31 ± 0.01	31.31 ± 0.40

[a] *N* indicates sample size; AGD, anogenital distance at birth; bBW, body weight at birth; bAGDI, anogenital distance index (AGD/BW) at birth; wAGD, weaning anogenital distance; wBW, weaning body weight; wAGDI, weaning anogenital distance index. Results are shown as mean ± SE.

begins around 2 months of age. For the females, the chosen age for necropsy allows 3 weeks for testing cyclicity after onset of puberty (from vaginal opening on PND ~34-38 until PND 50-60).

Soy Milk Study. In this study, either soy milk or rice milk was provided to the mother instead of drinking water from birth through weaning, and any resulting effects on the pups were examined. At birth, effects of soy milk exposure (vs. the rice milk control group) on BW, AGD, or AGD index (AGDI; the ratio of AGD/BW) in male (Table 1) and female pups (Table 2) did not differ significantly. At weaning, the two groups did not significantly differ regarding AGD in either the male (Table 1) or the female pups (Table 2); however, compared to the rice milk controls, the soy milk exposed female pups had significantly increased BW and, thus, had a significantly decreased AGDI (Table 2).

To determine if exposure to soy milk modified reproductive endpoints after weaning, after exposure had ended, onset of puberty, cyclicity in females, male reproductive tract organ weights at 2 months of age (sexually mature pups), and hormone responsive markers in the uteri were assessed. There were no significant differences between soy milk–exposed and rice milk control pups regarding onset of puberty in either male (Table 1) or female pups (Table 2). Additionally, in the female pups, no significant differences between soy milk–exposed and rice milk control pups regarding initial cycle length could be demonstrated (Table 2). In addition, there were no differences in the two groups of male pups regarding reproductive organ weights (testicle, seminal vesicles, or prostate), except for the epididymis when indexed to necropsy BW at 2 months of age (Table 3).

To assess the potential of the uterus to respond to hormonal stimuli, histomorphometric and immunohistochemical analyses of randomly selected subgroups of six uteri from both the soy milk–exposed and the rice milk control groups were performed. Again, little effect of soy milk was evident since there were no detectable differences in gland number, LEH, or PCNA staining between the two groups (Table 4). However, though ERα expression in both luminal epithelial cells (LEC) and glandular epithelial cells (GEC) and PR expression in LEC did not differ between the two treatment groups, PR expression in GEC was increased in the females that had been exposed to soy milk approximately 6 weeks earlier during lactation (Table 5).

Genistein Study. To determine if the isoflavones present in the soy milk were responsible for the altered PR expression, exposure to the isolated isoflavone, genistein (GEN), the predominant isoflavone in soy, was tested for influences on the uterine hormonally responsive markers examined in the soy milk study. However, since developmental exposure to isoflavones also occurs when the mothers ingest these phytoestrogens during pregnancy, both *in utero* and lactational exposure were examined. Since GEN has been reported to have both estrogenic and antiestrogenic activities, groups treated with a known estrogen alone and in combination with GEN were included to assess these respective activities. DES was chosen as the estrogenic control, given that it is known to influence reproductive outcomes in exposed pups. Because of the weak estrogenic activity of GEN, a low dose of DES is included for comparison.

As expected, the high DES dose induced estrogen responses, including increased LEH. However, like the low dose DES alone and combined with GEN, GEN did not

Table 2. Effects of Lactational Exposure to Soy Milk on Birth, Weaning, and Pubertal Indices in Female Pups[a]

Group	N	bAGD (mm)	bBW (g)	bAGDI	wAGD (mm)	wBW (g)	wAGDI	Age at vaginal opening (days)	Initial cycle length (days)
Rice milk controls	18	0.93 ± 0.03	6.22 ± 0.10	0.151 ± 0.005	8.22 ± 0.17	40.75 ± 0.99	0.208 ± 0.006	35.12 ± 0.43	4.29 ± 0.27
Soy milk	22	1.06 ± 0.05	6.19 ± 0.18	0.169 ± 0.004	8.74 ± 0.25	45.71 ± 1.32*	0.191 ± 0.005*	34.15 ± 0.44	4.21 ± 0.19

[a] *N* indicates sample size; bAGD, anogenital distance at birth; bBW, body weight at birth; bAGDI, anogenital distance index (AGD/BW) at birth; wAGD, weaning anogenital distance; wBW, weaning body weight; wAGDI, weaning anogenital distance index. Results are shown as mean ± SE.

* $P < 0.05$, indicating statistical significance compared with control group (rice milk).

Table 3. Effects of Lactational Exposure to Soy Milk or Rice Milk on Reproductive Organ Weights in Male Pups at 2 Months of Age[a]

Group	N	Testis/nBW ×1000	Epididymis/ nBW ×10,000	Seminal vesicles/ nBW ×1000	Prostate/ nBW ×1000
Rice milk controls	8	4.32 ± 0.16	9.25 ± 0.46	2.64 ± 0.42	1.15 ± 0.08
Soy milk	14	4.56 ± 0.17	7.95 ± 0.35*	2.14 ± 0.28	1.14 ± 0.09

[a] *N* indicates sample size; organ weights are in mg; nBW, body weight at necropsy. Results are shown as mean ± SE.
* $P < 0.05$, indicating statistical significance compared to control (rice milk).

increase LEH compared to the control group. LEH was significantly increased in the high DES dose group in comparison to all treatment groups, including control, GEN, and GEN + DES groups, but did not differ from that in the low DES group (Fig. 2). The marker of uterine proliferation, PCNA, showed that both low and high dose DES treatments increased PCNA staining in comparison to controls (Fig. 3). However, as with soy milk exposure, GEN alone did not significantly increase PCNA staining in LEC. Indicative of its potent estrogenic activity, the high dose of DES was the only treatment that increased ERα expression in LEC and GEC, while all other exposures had no effect on ERα expression (Fig. 4).

In utero/lactational exposure to GEN resulted in increased expression of PR in the GEC similar to that observed with lactational exposure to soy milk. In fact, all treatment groups increased PR expression in GEC compared to the control group (Figs. 5 and 6). However, there were no treatment-related effects on PR expression in LEC. In addition, GEN did not augment or inhibit the effects of the low dose DES (GEN + DES) on PR in GEC. However, since PR expression of the low and high dose DES were similar, augmentation or inhibition of the low dose DES by GEN may not be discernable.

Discussion

In both of the studies examined herein, expression of the estrogen-responsive PR was modified in the GEC of the adult uterus after developmental exposure to dietary isoflavones at levels comparable to those consumed in the human diet. The increased expression of PR in GEC occurred with lactational exposure to the commercial beverage containing dietary isoflavones as well as with *in utero*/lactational exposure to the isolated isoflavone, GEN. PR expression in the GEC was the only endpoint modified in both studies after pre- and/or postnatal exposure. The altered expression of this hormonally responsive marker indicates that long-term effects of early isoflavone exposure may alter the ability of the uterine tissue to respond to hormonal stimuli, such as during times of reduced estrogen or progestin availability. Altered hormonal responsiveness may, accordingly, have the potential to impact important functions, such as pregnancy, implantation, and uterine susceptibility to cancer. Therefore, further studies are needed to determine how the observed changes in PR expression potentially impact female reproductive health.

The high dose of DES increased all the estrogen-responsive markers examined in the uterus, including LEH, PCNA, ERα, and PR expression in GEC, as might be predicted on the basis of its potent estrogenic activity. GEN more closely mimicked the low dose DES, as would be expected, because of its weaker estrogenic activity. This was evident with both treatments having no discernable effect on LEH as well as PCNA and ERα expression. However, both GEN and low dose DES increased PR levels in GEC similar to the high dose DES.

Results in the genistein study on PCNA expression suggest that GEN may exert some estrogen antagonistic activity when administered in combination with the low dose DES. Relative to the control group (dams given corn oil only), GEN alone did not significantly increase PCNA staining in LEC. However, the mean values for the GEN alone and GEN + DES groups were intermediate to those of the control and to both DES groups (high and low doses). These data suggest but do not establish that for this endpoint, GEN may exert low potency agonist activity (GEN alone); however, when given in combination with a more potent estrogen (GEN + DES), GEN may be a partial antagonist.

Not all estrogens exert the same effect on any given tissue or endpoint, so differences in the low dose DES and GEN groups (PCNA expression; Fig. 3) may be due to availability and/or relative dose or to selective activities

Table 4. Effects of Lactational Exposure to Soy Milk on Uterine Development (Gland Number, Luminal Epithelium Height, and PCNA) in 2-Month-Old Female Pups in Estrus[a]

Group	N	Gland number	LEH (μm)	% PCNA positive in LEC
Rice milk controls	6	20.50 ± 2.73	32.45 ± 1.09	6.96 ± 0.80
Soy milk	6	16.06 ± 2.06	32.64 ± 1.08	10.05 ± 1.29

[a] *N* indicates sample size; LEH, luminal epithelial height; PCNA, proliferating cell nuclear antigen; LEC, luminal epithelial cells. Results are shown as mean ± SE.

Table 5. Effects of Lactational Exposure to Soy Milk on the Percentage of ERα- and PR-positive Cells in Uterine Luminal Epithelium (LEC) and Glandular Epithelium (GEC) Cells in 2-Month-Old Female Pups in Estrus[a]

Treatment	Sample size	ERα (%) in LEC	ERα (%) in GEC	PR (%) in LEC	PR (%) in GEC
Rice milk controls	6	66.18 ± 3.82	90.16 ± 1.67	59.11 ± 4.34	83.39 ± 3.32
Soy milk	6	64.34 ± 4.10	90.13 ± 1.80	63.70 ± 7.37	93.38 ± 1.06*

[a] ERα, estrogen receptor alpha; PR, progesterone receptor; LEC, luminal epithelial cells; GEC, gland epithelial cells. Results are shown as mean ± SE.
* $P < 0.05$, indicating statistical significance compared to control (rice milk).

of each. Although both DES and genistein are complete agonists when bound to rat and human ERα and ERβ *in vitro*, genistein exerts stronger interactions via ERβ (35). In contrast, estradiol and DES interact more strongly with rat ERα than with ERβ (35). The molecular mechanisms of developmental effects of DES and genistein are incompletely understood, yet both may act on the developing uterus through ER pathways. However, only the high dose DES was able to modify ERα expression in uterine epithelial cells and, thus, potential estrogen responsiveness.

When comparing male and female reproductive endpoints in the soy milk–exposed versus rice milk control study to those results previously reported for the genistein study (20), including birth and weaning AGDI, onset of puberty, male organ weights, and initial cycle length, most endpoints were unaffected after developmental isoflavone exposure in both studies. However, for the females, GEN (like DES) increased weaning AGDI, unlike the decrease in observed with soy milk exposure. Also, compared to the controls (dams given corn oil only), the onset of puberty occurred earlier in the GEN-treated males, in contrast to the lack of effect detected in the soy milk study. The epididymal weight was also decreased in males exposed to soy milk but not to GEN; however, high DES also resulted in a decreased weight in the epididymis. Although similar doses were used for both studies, the isoflavones in the soy milk were not exclusively genistein since daidzein and glycitein are also present in soy. Also, the soy milk study examined only lactational exposure compared to the *in utero*/lactational exposure tested in the genistein study. Therefore, the few differences observed in the reproductive endpoints between the two studies may be related to interactions or relative estrogenic activities between these phytoestrogens and/or due to the timing of isoflavone exposure.

One report examining *in utero*/lactational exposure to genistein found that a high dose of genistein (300 mg/kg) resulted in increased epididymal weights and decreased prostate weights at PND 70 (36). However, these changes were not evident in the soy milk study (see Table 3). In the soy milk study, the dose of the combined isoflavones was at least 10-fold lower than the dose inducing the effects on

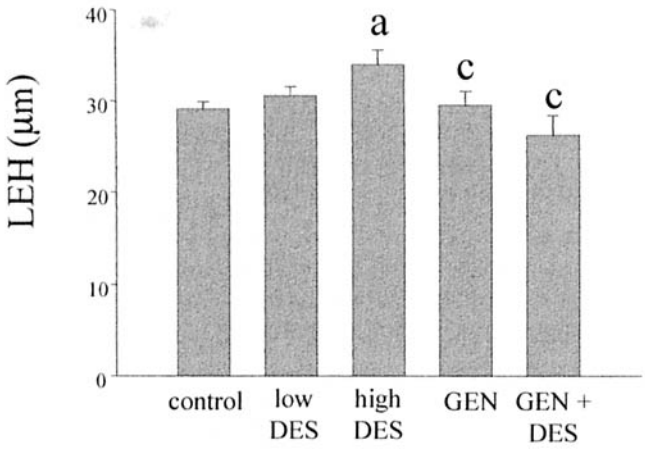

Figure 2. Uterine luminal epithelial height at 2 months of age, on day of estrus, after *in utero*/lactational exposure to the estrogen treatments. Five-micrometer sections were deparaffinized in xylene and rehydrated in graded ethanol solutions. After Van Gieson's staining, luminal epithelial height (LEH) was measured using the image analysis system. Eight rats from each group were analyzed in this experiment. Error bars indicate the standard error of the mean; a, versus control, $P < 0.05$; c, versus high DES, $P < 0.05$.

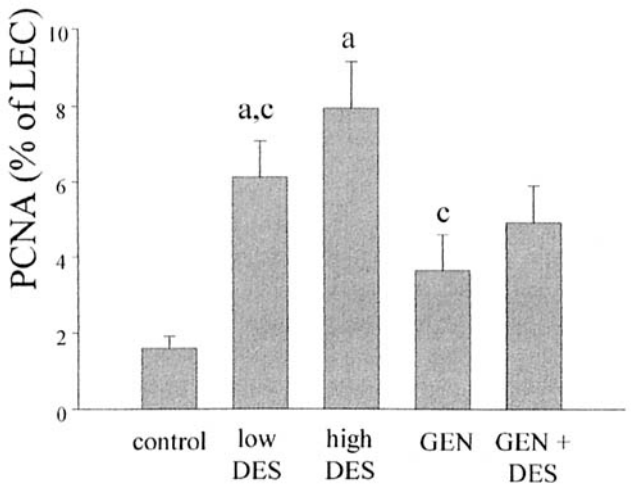

Figure 3. PCNA expression in the uterine luminal epithelium cells at 2 months of age, on day of estrus, after *in utero*/lactational exposure to the estrogen treatments. After immunohistochemical staining, PCNA immunopositive cells in the luminal epithelium only were counted. The average number of immunopositive cells was obtained by counting three separate randomly selected fields per section. The positively stained cells were expressed as a percentage of the total luminal epithelial cells (LEC) counted (immunopositive and immunonegative). Eight rats from each group were analyzed in this experiment. Error bars indicate the standard error of the mean; a, versus control, $P < 0.05$; c, versus high DES, $P < 0.05$.

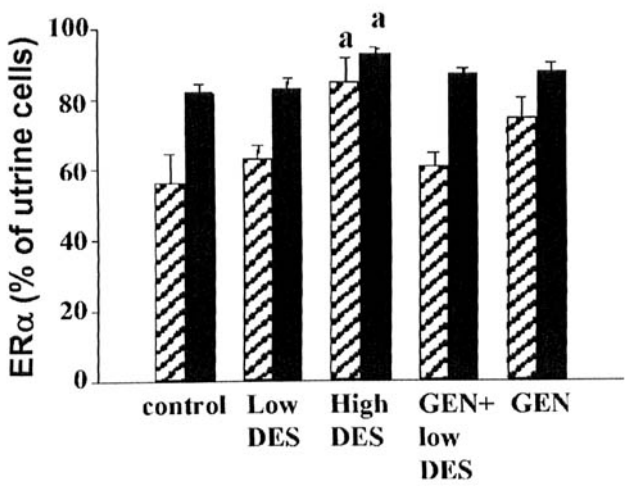

Figure 4. Percentage of uterine cells staining positive for ERα in luminal and glandular epithelial cells at 2 months of age, on day of estrus, after *in utero*/lactational exposure to the estrogen treatments. After immunohistochemical staining, all ERα immunopositive cells in the luminal and glandular epithelium were counted in ×40 field. The average number of immunopositive cells was obtained by counting three separate randomly selected fields per section. The positively stained cells were expressed as a percentage of the total luminal (LEC) or glandular (GEC) epithelial cells counted (immunopositive and immunonegative). Black bar indicates GEC; striped bar, LEC. Eight rats from each group were analyzed in this experiment. Error bars indicate the standard error of the mean; a, $P < 0.05$ compared to control.

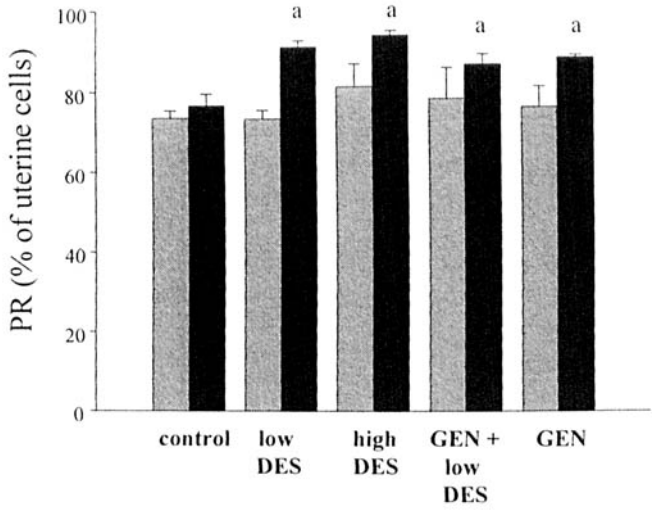

Figure 5. Percentage of uterine cells staining positive for progesterone receptor in the luminal and glandular epithelial cells at 2 months of age, on day of estrus, after *in utero*/lactational exposure to the estrogen treatments. After immunohistochemical staining, all PR immunopositive cells in the luminal and glandular epithelium were counted in ×40 field. The average number of immunopositive cells was obtained by counting three separate randomly selected fields per section. The positively stained cells were expressed as a percentage of the total luminal (LEC) or glandular (GEC) epithelial cells counted (immunopositive and immunonegative). Black bar indicates GEC; striped bar, LEC. Eight rats from each group were analyzed in this experiment. Error bars indicate the standard error of the mean; a, $P < 0.05$ compared to control.

the male sex organs, and exposure occurred only during lactation. However, our results in the soy milk study are consistent with the report by Nagao *et al.* (37) demonstrating that genistein exposure on PND 1–5 at higher doses, ranging from 12.5 to 100 mg/kg, than in the soy milk study did alter sex organ weights in postpubertal male rats. In addition, this short-term lactational exposure altered fertility in females and uterine histopathology only at the 100-mg/kg dose of genistein (37).

Reports with lower doses of genistein have shown little effect on the developing male and female pups. Kang *et al.* (38) reported that maternal exposures to genistein at 0.4- and 4.0-mg/kg doses during gestation and lactation did not modify birth AGD, vaginal opening, sperm counts, or male or female reproductive organ weights at PND 49, 70, or 100, except for prostate weight at PND 70 in the 4.0-mg/kg genistein group. Another report from Lewis *et al.* (39) demonstrated that there were no effects on males or females after direct exposure to genistein during the period from birth to PND 21 from a dosage of 4.0 mg/kg genistein, including reproductive organ weights in 12-week-old offspring, onset of puberty, and estrous cycling (39). In the study by Awoniyi *et al.* (30), *in utero* and lactational exposure (GD 17–PND 21) to a low dose of genistein (5 mg/kg of diet) did not modify uterine weight in females at PND 70, vaginal opening, or histologic features of the reproductive tract, although some irregularity was noted in the length of the estrous cycle. The data from these studies correlate with our findings that developmental exposure to genistein or soy milk has little influence on most reproductive endpoints investigated. However, in our study, isoflavones did influence uterine PR expression, which was not examined in these other studies.

One study by Cotroneo *et al.* (40) did investigate the effects of genistein on PR as well as ERα expression in the rat uterus after prepubertal or *in utero*/lactational exposure. At PND 21, uterine ERα was reduced and PR increased in female rats injected with genistein (500 mg/kg) on PND 16, 18, and 20, but only the decrease in ERα expression persisted at PND 50. In our studies, using considerably lower doses, ERα was not altered, but PR expression was increased in the 2-month-old females after developmental exposure to isoflavones. After *in utero*/lactational exposure to a lower dose of genistein (250 mg/kg of diet) that is closer to the dose used in our study, Cotroneo *et al.* reported that neither ERα nor PR was modified in the 21-day-old female uteri (40). The increase in PR expression detected in our soy milk and genistein studies may be due to several differences in the study designs, including the strain of rats (Long-Evans vs. Sprague-Dawley), examining sexually mature versus immature uteri, and the method for assessing receptor expression (immunohistochemistry vs. Western blot). In our studies, the increase in PR immunostaining was detected only in the GEC, whereas Western blots used for their analysis will detect PR in both the luminal and glandular epithelial as well as stromal cells. Accordingly, differences in the GEC may be obscured when all uterine compartments are examined together. Therefore, although Controneo *et al.*

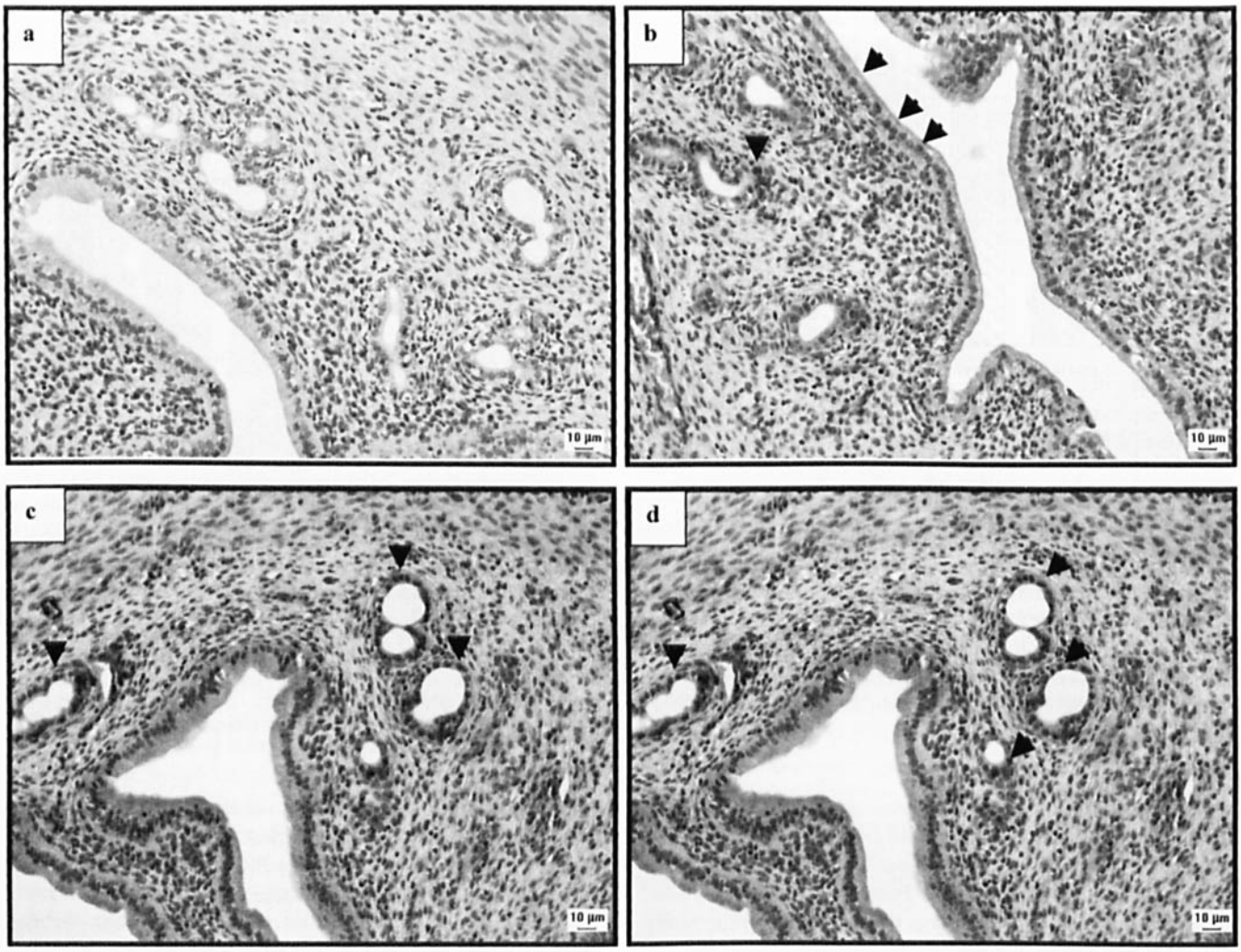

Figure 6. Progesterone receptor immunostaining in the rat uterus after *in utero*/lactational exposure. Progesterone receptors (PR) were demonstrated immunohistochemically in the epithelium and stroma of the rat uterus using a monoclonal antibody (PR10A) and alkaline phosphatase as described in *Materials and Methods.* No staining was detected in a control section (a) in which the primary antibody was omitted, whereas immunopositive cells (arrowheads) were found in the luminal and glandular epithelium of animals of the control group (b). Strong immunopositive staining was detected in the glandular epithelium of DES (c) and genistein (d) treated rats.

concluded that pharmacologic but not physiologic concentrations of genistein may modulate sex steroid receptor expression in the rat uterus, our data from both the soy milk and genistein studies indicate that dietary levels of exposure (and dosing regimens that approximate the upper dietary range) can modulate expression of at least one sex steroid receptor in the rat uterus.

In the genistein study, the reference estrogen, DES, at low doses and the common dietary phytoestrogen, GEN, at levels comparable to the upper range of human exposure affected only some of the tested markers of uterine development in Long-Evans hooded rats. Whether these effects are caused exclusively by lactational, exclusively by *in utero*, or by the combination of the two periods of exposure cannot be distinguished. Since much of the development that occurs in the neonatal rat (during early lactation) is comparable to the latter two trimesters in human gestation (41), the effects of the lactational exposure interval in this study imply that possible effects of both *in utero* and lactational exposure of humans should be investigated.

In conclusion, developmental exposure of rats to isoflavones, at levels of exposure that mimic those found in humans in North America, induces effects on an estrogen-responsive marker in the uterus long after exposure has ended. Since the PR influences several processes important to women's reproductive health, these sets of observations sustain concerns that there is potential for long-term health effects from developmental exposures to dietary estrogens for the human fetus/neonate.

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