Phytoestrogen Influences on the Development of Behavior and Gonadotropin Function (43836)

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Abstract. **The effect of phytoestrogens on the sexual differentiation of gonadotropin function was examined by neonatal exposure of pups through milk of rat dams fed a coumestrol (100 μg/g), control, or chow diet during the "critical period" of the first 10 postnatal days or throughout the 21 days of lactation. In females, exposure to coumestrol throughout the period of lactation produced growth suppression and an acyclic condition in early adulthood resembling the premature anovulatory syndrome. When the period of treatment was restricted to the first 10 postnatal days, however, no effects on vaginal cyclicity were seen. The 10-day exposure period produced more marked effects in males, resulting in transitory reductions in body weight in weanling males and reductions in mount and ejaculation frequency and a prolongation of the latencies to mount and ejaculate. Testicular weights and plasma testosterone levels did not differ among treatment groups, suggesting that the deficits in male sexual behavior were not due to deficits in adult gonadal function. Few effects of chow treatment were observed. However, significant differences from controls were apparent for weight at vaginal opening in females, and mount rate for chow-treated males was intermediate between that of controls and that of the coumestrol-treated group. These data provide evidence that lactational exposure to phytoestrogen diets can alter neuroendocrine development in both female and male rats.**

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The isoflavonoids are a group of plant chemicals found in human and animal foods that resemble steroidal estrogens and mimic many of their actions (1). They are of interest to human health because they occur in substantial quantities in human urine (2). Their high concentrations in the urine of Asian women, known to be resistant to breast cancer, and their low concentrations in breast cancer patients have suggested that they might play a role in the prevention of estrogen-dependent carcinoma (3, 4, 5). However, the well-documented role of estrogens in sexual development (6) suggests that isoflavonoids might exert some adverse effects as well (7, 8). Estrogens and androgens regulate sexual differentiation in mammals, altering the inherently female pattern of development to induce the male phenotype. Consequently, exogenous substances that mimic or influence estrogen or androgen action can alter the genotypic phenotype, resulting in defeminization and masculinization of females and feminization and demasculinization of males. In females, these alterations result in the development of male patterns of sexual behavior and orientation and in the loss of cyclical gonadotropin secretion. In female rats, these changes are expressed as a premature anovulatory syndrome characterized by persistent cornification of the vaginal epithelia and an inability to exhibit a luteinizing hormone (LH) surge (9). In males, these alterations result in the retention of LH surge capacity and in the loss of male-typical patterns of sexual behavior and orientation (10).

There are reasons to suspect that isoflavonoids may influence sexual development. Sheep that have grazed on isoflavonoid-rich pasture for prolonged pe-

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riods of time develop an infertility syndrome known as "clover disease" that resembles the premature anovulatory syndrome observed in rats following perinatal estrogen exposure (11, 12). Perinatal administration of isoflavonoids to rodents by subcutaneous injection induces premature anovulation (13), reproductive tract abnormalities (14), and altered pituitary responsiveness to gonadotropin releasing hormone (GnRH) (15).

Clearly, the balance among these actions has important implications for the medicinal and nutritional use of these phytochemicals. Consequently, our goals in this research have been to identify the specific actions and sites of action of isoflavonoids and to evaluate their effectiveness as dietary constituents. We have addressed these goals through experimental studies of phytoestrogen action using a rat model of estrogen action and a paradigm of dietary treatments with concentrations found naturally in plant foods. We focused on the action of coumestrol in these experiments because it is the isoflavonoid with the highest affinity for the estrogen receptor and therefore should provide a good index of the most extreme actions of these plant chemicals. These investigations have demonstrated that coumestrol is active in the reproductive tract (16) and the central nervous system (CNS) (12, 17) at concentrations found naturally in plants and influence estrogen-dependent reproductive processes. Recently, we have shown that lactational exposure to coumestrol induces the premature anovulatory syndrome (17). Here, we extend these findings by comparing the effects of duration of treatment and examining the effects on male as well as female reproductive function.

Materials and Methods

Animals. Timed-pregnant Sprague-Dawley rats were obtained on the 15th day of gestation. The animals were maintained on a 12:12-hr light:dark cycle (lights on 0630) and provided food and water *ad libitum*. Because commercial rodent chow contains some isoflavonoids (4), all animals were fed a semipurified formulation, the AIN semipurified rat-mouse diet (U.S. Biochemical Corp., Cincinnati, OH) containing the following ingredients: casein-high nitrogen 20%; DL-methionine 0.3%; cornstarch 15%; sucrose 50%; fiber-celufil 5%; corn oil 5%; mineral mixture 3.5%; vitamin mixture 1%; choline bitartrate 0.2%. Dams were treated with the semipurified control diet from the 15th day of gestation until birth.

Experimental Treatment. In the first study, female pups from five dams were pooled and randomly assigned to litters (Camm, Wayne, NJ) on the day of delivery (postnatal Day [PND] 1). Dams were treated with either the control diet (n = 2) or a 0.01% coumestrol diet (n = 3) from birth until Day 21. The phytoestrogen-treated animals received a custom formulated AIN diet (U.S. Biochemical) containing the isoflavonoid coumestrol at a concentration of 0.01%, a concentration that doubles uterine weight over a 90-hr period in immature female rats. Control animals continued to receive the AIN diet. Pups were exposed to coumestrol (n = 12) or to control diets (n = 11) during lactation. At weaning, they were placed on control diets until vaginal opening, when they were placed on a standard chow diet.

In the second study, both male and female pups from eight dams (Harlan, Indianapolis, IN) were pooled and randomly assigned to litters on the day of delivery. Dams were treated with either the control diet (n = 2), a chow diet (n = 3) or a 0.01% coumestrol diet (n = 1) during the first 10 PNDs, the critical period when rat CNS function appears to be most sensitive to the organizing effects of sex steroids (6). All dams were then placed on a control diet until weaning on PND 21. At weaning, pups were placed on chow diets for the remainder of the study.

Vaginal Cyclicity. Vaginal smears were taken daily by lavage on PND 99–101, 105–109, 132–136, 139–143, and 270. Animals were classified by vaginal smear pattern as follows: (i) cornified (cornified cells predominant in more than 70% of smears); (ii) leukocytic (leukocytes predominant in more than 70% of smears); or (iii) cyclic (no more than 70% of smears cornified or leukocytic).

Sexual Behavior. Sexual behavior was tested in intact adult males. Four to five males per treatment group were tested. Tests for male sexual behavior were performed out in a 12-inch diameter plexiglass arena during the dark phase of the cycle under dim red light. Testing began 2 hr after the onset of darkness. Stimulus animals were ovariectomized females unfamiliar to the male who were rendered sexually receptive by subcutaneous injection of estradiol benzoate 48 hr before testing and progesterone in 0.1 ml sesame oil, 4–6 hr before testing. Males were allowed to adapt the arena for 10 min prior to addition of the stimulus female and the start of the test. All mounts, intromissions, and ejaculations were recorded during a 30-min test period. The arena was thoroughly cleaned after each test.

Statistical Analyses. Analysis of variance was used to test the significance of differences between control, coumestrol-, and chow-treated animals in body weight, age at vaginal opening, and male sexual behavior. When significant treatment effects were found, differences between treatment groups were further tested using the multivariate t distribution calculated by Dunnett for the simultaneous comparison of several treatments with a control or standard (18). When treatment groups differed significantly in variance, the effects of treatments were analyzed using the

nonparametric Kruskal-Wallis analysis of variance, and Dunn's test was used to test differences between treatment groups. The Chi-square test was used to test the significance of differences in vaginal cyclicity between treatment groups.

Results

Vaginal Opening. Table 1 shows that the phytoestrogen diets did not alter age at vaginal opening, but weight at vaginal opening was significantly lighter in both the coumestrol- and chow-treated females. These differences in body weight were apparent in both studies, however, the differences were much less marked following the 10-day treatment than following the 21-day treatment.

We have previously shown that differences in weight at vaginal opening were due in part to suppression of postnatal growth in coumestrol-treated females (18). Table II shows that the 10-day treatment produced no significant differences among treatment groups in female body weight at 4 or 6 weeks of age. However, significant body weight differences were evident among males, with coumestrol-treated males lighter than control males, at 4 weeks of age.

Vaginal Cycles. Table III shows changes over time in the percentage of females exhibiting cornified vaginal smears. Following 21 days of perinatal treatment, 83% of the coumestrol-treated females displayed cornified vaginal smears by 132 days, suggesting a persistent estrous state. In contrast, following the 10-day perinatal treatment, coumestrol-treated females continued to cycle normally until 270 days, when the majority of all animals were in persistent estrous.

Male Sexual Behavior. Figure 1 shows that coumestrol-treated males exhibited a number of deficits in sexual behavior. The number of mounts per test was significantly lower in the coumestrol-treated males than in control males. The latency to the first mount was significantly lower in coumestrol-treated males. The rate of ejaculations also was significantly lower in coumestrol-treated males. Three of the four coumestrol-treated males, in fact, failed to ejaculate at all. In contrast, none of the control males failed to ejaculate during the 30-min test. The latency to the first ejaculation was significantly longer in the coumestrol-treated males. This effect was due primarily to the three males who did not ejaculate and were assigned a conservative estimate of a minimal latency of 30 min. The one male who did ejaculate did not differ from the control males in either ejaculatory or mount latency.

In contrast, the chow treatment appeared to have few effects on male sexual behavior. Ejaculatory rate and latencies to the first mount and first ejaculation were similar to the values of control males. Mount rates of chow-treated males, however, were intermediate between the values of control and coumestrol-treated males.

Indices of Androgenic Status. Table IV illustrates measures of androgenic status obtained at the close of behavior testing (7 months) and at sacrifice at 11 months. Neither testicular weights nor plasma testosterone levels differed among treatment groups.

Table I. Effects of Neonatal Phytoestrogen Treatments on Vaginal Opening

Treatment	Individuals	Weight (g)	Age (days)
21-Day[a]			
Control	11	134.3 ± 1.2	35.5 ± 1.2
Coumestrol	12	103.5 ± 5.1[b]	35.2 ± 0.9
10-Day			
Control	14	114.9 ± 2.7	35.3 ± 1.0
Chow	9	105.0 ± 2.9[c]	34.1 ± 2.7
Coumestrol	4	109.4 ± 3.0	35.5 ± 0.6

[a] Data for 21-day treatments adapted from Whitten et al. Biol Reprod **49**:1117–1121, 1993.
[b] $P < 0.01$.
[c] $P < 0.05$.

Discussion

These data provide evidence that phytoestrogen diets can alter neuroendocrine development in lactationally exposed female and male rats. In females, exposure to coumestrol throughout the period of lactation produced growth suppression and an acyclic condition in early adulthood resembling the premature anovulatory syndrome. When the period of treatment was restricted to the first 10 PNDs, however, no effects on vaginal cyclicity were seen. Thus, it would appear that a prolonged period of exposure to coumestrol is required to produce the organizational effects that ultimately result in the anovulatory syndrome. Although the first 10 PNDs are thought to be the period of greatest responsiveness to the organizing effects of estrogen, there is evidence that estrogen can exert similar effects in pubertal (19) and adult life (20). Our previous data suggest that coumestrol may parallel steroid estrogens in this respect, since postweaning treatment with coumestrol results in an increased incidence of irregular cycles by 131 days (17).

The limited exposure period produced more marked effects in males, resulting in transitory reductions in body weight in weanling males and deficits in sexual behavior in adult males. The effects on male sexual behavior included a reduction in mount and ejaculation frequency and a prolongation of the latencies to mount and ejaculate. Testicular weights and plasma testosterone levels did not differ among treatment groups, suggesting that the deficits in male sexual behavior were not due to deficits in adult gonadal

Table II. Effects of Phytoestrogen Treatments on Body Weight

Treatment	Females			Males		
	n	4 Weeks	6 Weeks	*n*	4 Weeks	6 Weeks
Control	9	51.8 ± 1.3	108.6 ± 2.3	5	55.2 ± 1.0	131.7 ± 5.8
Chow	14	54.6 ± 1.2	112.2 ± 1.7	11	54.5 ± 3.6	123.8 ± 3.1
Coumestrol	4	48.0 ± 1.9	107.0 ± 3.7	4	40.8 ± 1.1[a]	115.9 ± 3.2

[a] $P < 0.05$.

Table III. Effects of Postnatal Phytoestrogen Treatments on the Onset of Constant Estrus

Treatment	Percentage of females in constant estrus		
	132 days	200 days	270 days
21-Day[a]			
Control	9.1	—	—
Coumestrol	83.3[b]	—	—
10-Day			
Control	0.0	22.2	60.0
Chow	0.0	21.4	64.8
Coumestrol	0.0	25.0	100.0

[a] Data adapted from Whitten et al. Biol Reprod **49**:1117–1121, 1993.
[b] $P < 0.01$.

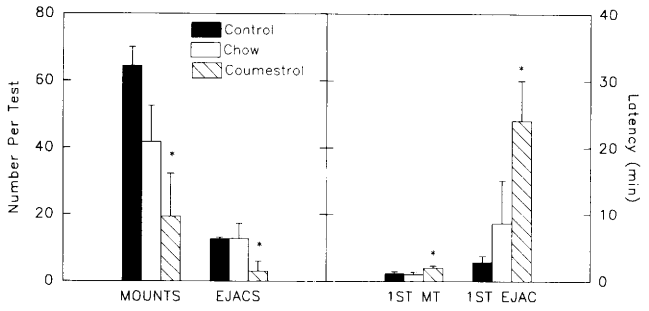

Figure 1. Effects of phytoestrogen diets on male sexual behavior. Bars indicate the mean and SEM of mounts and ejaculations per test (left panel) and latencies to the first mount and first ejaculation (right panel). *Significantly different from control group, $P < 0.05$.

Table IV. Indices of Androgenic Status in Phytoestrogen-Treated Males

Treatment	Testes weight (g)	Testosterone (ng/ml)	
		7 months	11 months
Control	4.5 ± 0.2	0.8 ± 0.2	2.0 ± 0.6
Chow	4.2 ± 0.2	0.8 ± 0.2	1.3 ± 0.4
Coumestrol	4.2 ± 0.1	0.6 ± 0.2	0.9 ± 0.3

function. These deficits resembled the demasculinizing effects of acute perinatal estrogen treatment (21, 22).

Few effects of chow treatment were observed. However, significant differences from controls were apparent for weight at vaginal opening in females, and mount rate for chow-treated males was intermediate between that of control and that of coumestrol-treated groups. Although these effects suggest that the phytoestrogens present in chow diets may be sufficient to influence sexual development, the differences between these effects and those of the coumestrol diet illustrate the importance of examining the action of each phytoestrogen separately.

Further studies will be needed to clarify whether the differences in the effectiveness of the 10-day treatment for males and females represent sex differences in responsiveness to phytoestrogens (with males more vulnerable) or differences in time or degree of responsiveness of CNS functions (with sexual behavior more responsive than gonadotropin function). However, these investigations do show that phytoestrogens can influence the development of both males and females, and argue for more thorough investigation of the organizational actions of these chemicals.

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