Effects of Coumestrol and Equol on the Developing Reproductive Tract of the Rat

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Abstract. **The phytoestrogens, coumestrol and equol, are weakly estrogenic. Here, we have examined their ability to induce responses in the neonatal rat uterus. Potent estrogens such as diethylstilbestrol (DES) and 17β-estradiol which initially double uterine weight on postnatal Day (PND) 5 when given on PND 1–5 subsequently reduce both uterine growth and gland development at later ages. In this study, Sprague-Dawley pups were treated neonatally (PND 1–5) with various doses of coumestrol and equol, and sacrificed at different ages to determine alterations in biochemical and morphological endpoints. Other rats were injected with the same compounds during the critical period of gland genesis (PND 10–14) to examine their effects on gland development. At the 100 μg coumestrol dose, on PND 1–5, premature gland development and increased uterine weight were observed. However, at later ages, uterine weight was significantly lowered and there was a severe suppression in the estrogen receptor (ER) levels. Equol lowered uterine weight at the later ages but did not affect ER levels. When given on PND 10–14, both coumestrol and equol caused a dose-dependent inhibition of gland genesis though not as severe as either DES or tamoxifen. Coumestrol was about 10^3 more potent than equol as an estrogen and behaved much like DES with respect to its effects on uterine weight, glands, and ER levels. At the doses used in this study, equol failed to demonstrate either estrogenic or anti-estrogenic activity.** [P.S.E.B.M. 1995, Vol 208]

Investigations of the effects of phytoestrogens on the rodent reproductive tract have shown that coumestrol is 10^{-1} to 5×10^{-3} times and equol is 10^{-3}–10^{-5} times as potent as either estradiol (E_2) or diethylstilbestrol (DES) (1–3). Coumestrol-induced pathologies in the reproductive tracts of mice include persistent vaginal cornification, hemorrhagic ovarian follicles, and premature vaginal opening in neonatally treated animals and decreased ovulation rates and an increase in embryo degeneration in animals treated as adults (4–6). Despite the fact that equol is the primary metabolite excreted in the urine of humans and animals ingesting soy products, little is known about its potential toxicity in the developing reproductive tract (7).

Both estrogens and antiestrogens have a toxic effect on the developing reproductive tract of the neonatal rat (8–14). Exposure to DES on PND 1–5 causes early uterine weight gain and premature gland genesis; however, by PND 60 both uterine weights and gland numbers are reduced to levels below controls (8, 14). Tamoxifen (TAM), a triphenylethylene antiestrogen with partial estrogen agonist activity, given on PND 1–5, causes a reduction in uterine weight equivalent to that seen for E_2 and nearly abolishes gland development, while E_2 reduces gland numbers to 75% of controls at PND 26 (10, 13, 15). During the period of rapid uterine gland development (PND 10–14), estrogens cause a dose-dependent delay in the appearance of uterine glands which may be related to the maintenance of estrogen-induced luminal epithelial cell hy-

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pertrophy (LEH) (8, 9, 13, 14). Antiestrogens also cause substantial and long-lasting LEH and are potent inhibitors of uterine gland genesis (9, 10, 15).

In view of the several and varied developmentally toxic effects elicited by both weak and potent estrogen agonists and antagonists, we have examined uterine toxicity of phytoestrogens in the developing rat. In this paper, we report the effects of coumestrol and equol on uterine weight gain, LEH, gland genesis, and ER levels in neonatal rats. We also examine the persistence of these effects following neonatal dosing.

Materials and Methods

Offspring from date-mated Sprague-Dawley rats from the National Center for Toxicological Research (NCTR) breeding colony were culled according to sex and the females randomly distributed to foster dams within 24 hr of birth. Groups of pups were injected sc in the mid-dorsal region on PND 1–5 or 10–14 with 1, 10, or 100 μg of coumestrol; 10, 100, or 1000 μg of equol; or 10 μg of TAM or DES in sesame oil. Control animals were untreated. Rats treated with coumestrol and equol on PND 1–5 were sacrificed at various timepoints. Groups were sacrificed on PND 5, 10, 15, 20, and 25 to determine how persistent the effects of these compounds were on uterine weight and ER levels. Other groups were sacrificed on PND 5, 9, 26, and 60 to assess premature gland genesis and any long-term effects on either gland numbers or LEH. Finally, additional groups were sacrificed on PND 8, 9, and 10 to further define the temporal aspects of premature gland genesis, LEH alterations, and uterine weight changes and to compare them with a dose of DES known to elicit premature gland genesis. Animals injected on PND 10–14 were sacrificed on PND 14 to determine the effects of the phytoestrogens on uterine weight, gland number, and LEH when given during the critical period of gland genesis and to compare these compounds to doses of an estrogen (DES) and an antiestrogen (TAM) known to abolish gland genesis.

For ER determinations, all animals were injected with 10 μg DES 1 hr before sacrifice to maximize the ER binding in the nuclear fraction (16). Following sacrifice, the uteri were pooled and then homogenized in cold TE buffer (10 m*M* Tris, 1.5 n*M* EDTA, pH 7.4) at a concentration of 30 mg/ml of buffer. The homogenates were processed as described previously with nuclear ER levels being determined by the [^{3}H]-E_2 exchange assay (12).

For measurement of gland number and LEH, the uteri were carefully removed, stripped of connecting mesentery and fixed in 10% neutral buffered formalin. They were then processed using standard histological procedures, stained with hematoxylin and eosin, and sectioned at 4 μm. At least six sections per animal were scored for uterine glands and measurements of LEH (13).

Coumestrol and equol were purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA). DES was obtained from Research Plus Steroid Laboratories, Inc. (Denville, NJ). TAM was a gift of Stuart Pharmaceuticals (Wilmington, DE). [^{3}H]-E_2 (sp act 92–115 Ci/mMol) used in the receptor exchange assay was purchased from Dupont NEN Products (Boston, MA). All other chemicals were laboratory grade.

Statistical analyses were done using a two-way analysis of variance (ANOVA) and are presented as means ± SEM with a level of significance of $P \leq 0.05$ (significance was determined by Duncan's multiple range test).

Results

Following dosing on PND 1–5, both the 10 and 100 μg dose of coumestrol elicited significant uterine weight gain on PND 5; however, by PND 10, only the 100 μg dose group had uterine weight above controls (Fig. 1A). At PND 10, 20, and 25 for the 10 μg coumestrol group and PND 20 and 25 for the 100 μg coumestrol and equol groups, uterine weights were significantly lower than controls (Fig. 1A). However, by PND 60 there was no significant difference between the treatment groups and the controls (data not shown). Relative to control level, the 10-μg dose of coumestrol given on PND 1–5 caused a significant reduction in the uterine ER level by PND 10 which was still reduced on PND 25 (Fig. 1B); ER levels remained constant from PND 10 to 25. The 100-μg dose of coumestrol significantly reduced ER levels relative to control uteri on PND 5–25, although the levels observed on PND 15–25 were double that seen on PND 5 and 10 (Fig. 1B). Equol (100 μg) had no effect on ER levels at any age.

Only the 100 μg coumestrol dose had a significant impact on uterine morphology. On PND 5, coumestrol more than doubled the LEH, which had not dropped by PND 8 although there was a slight but significant increase in gland number (Fig. 2 and 3, B and C). Even though the gland number continued to increase on PND 9, no further increase was seen on PND 10 despite a continued decrease in LEH. This differs from the effect elicited by 10 μg DES which caused slight but significant increase in gland number (equivalent to that of coumestrol) on PND 8, and a further increase to Day 10 (Fig. 3B). While equol (100 μg) did not elicit premature gland genesis on PND 8–10 (Fig. 3B), LEH was significantly higher than in controls on PND 9 and 10 (Fig. 3C). Equol also had no effect on uterine weight, whereas the 100-μg dose of coumestrol and the 10-μg dose of DES both elicited a significant increase

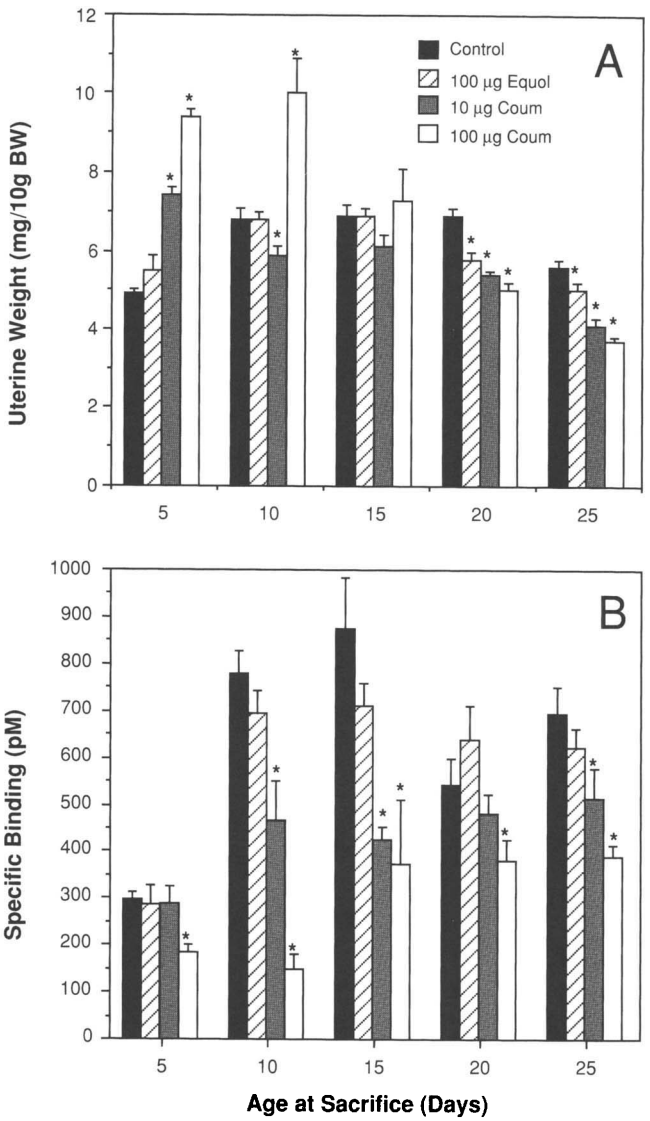

Figure 1. Uterine weight (A) and ER levels (B) in untreated rat pups or pups injected on PND 1–5 with 100 µg equol, 10 µg coumestrol, or 100 µg coumestrol and sacrificed on PND 5, 10, 15, 20, and 25. The data are presented as means ± SEM with $n \geq 18$ (A) or $n \geq 3$. Asterisks indicate significant differences at $P \leq 0.05$.

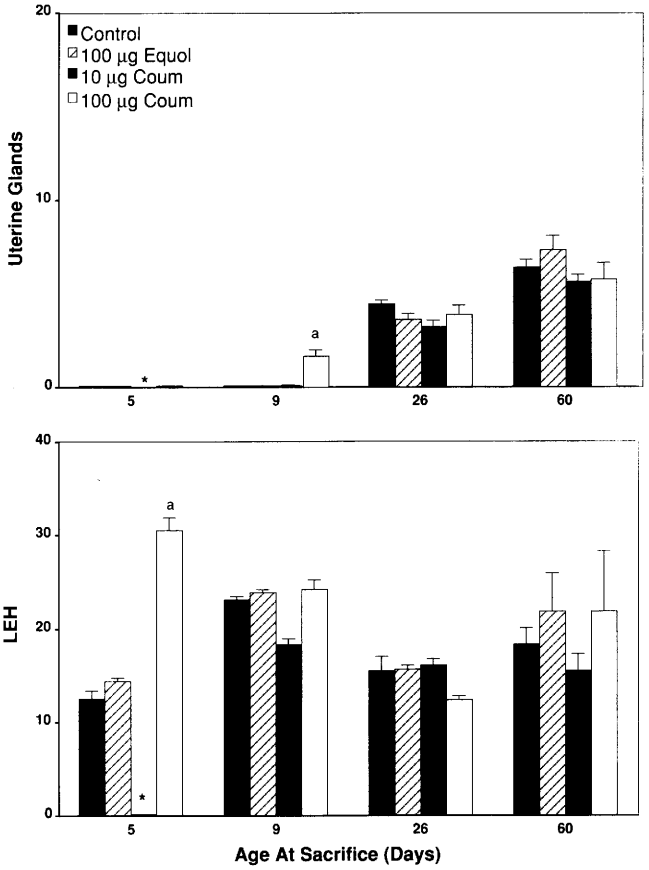

Figure 2. Gland number (glands/uterine section) (A) and LEH (B) in untreated pups or pups injected PND 1–5 with 100 µg equol, 10 µg coumestrol, or 100 µg coumestrol and sacrificed on PND 5, 9, 26, and 60. The data are presented as means ± SEM with $n \geq 6$. Asterisks indicate that measurements were not made at this dose. Values significantly different from controls are indicated by the letter *a* at $P \leq 0.05$.

in uterine weight on PND 8–10 with the coumestrol group significantly higher than the DES group (Fig. 3A).

When given on PND 10–14, the 10-µg and 100-µg doses of coumestrol doubled uterine weight (Fig. 4A). The 10-µg dose of the antiestrogen TAM caused a slight but significant increase in uterine weight while the same dose of DES tripled uterine weight (Fig. 4A). Although equol had no effect on uterine weight (Fig. 4A), there was a dose-dependent decrease in gland number (Fig. 4B). Coumestrol also exhibited a reduction in glands, but there was no difference between the two higher doses (Fig. 4B). Coumestrol appeared to be 10–100 times more potent than equol with respect to inhibition of gland genesis, although the maximum reduction (25% of controls) was the same for both compounds (Fig. 4B). Glands were essentially abolished by the 10-µg doses of DES and TAM, which also caused identical increases in LEH (Fig. 4, B and C). Compared with controls, the 10- and 100-µg doses of coumestrol doubled LEH, although both were significantly lower than TAM or DES (Fig. 4C). LEH was not increased by any dose of equol (Fig. 4C). With the exception of TAM, the effect of all treatments on LEH was very similar to that seen for uterine weight (Fig. 4, A and C).

Discussion

Coumestrol is clearly estrogenic, although its potency depends on the endpoint measured and the time and conditions under which it is measured. Comparison of normalized uterine weight on PND 10 in this study shows that the 10-µg dose of DES falls between the 10- and 100-µg doses of coumestrol when given on PND 1–5. However, we have shown earlier that DES is about 400 times more potent than coumestrol when given on PND 1–5 and measured on PND 5 (3). Measurement of premature uterine gland genesis on PND 9

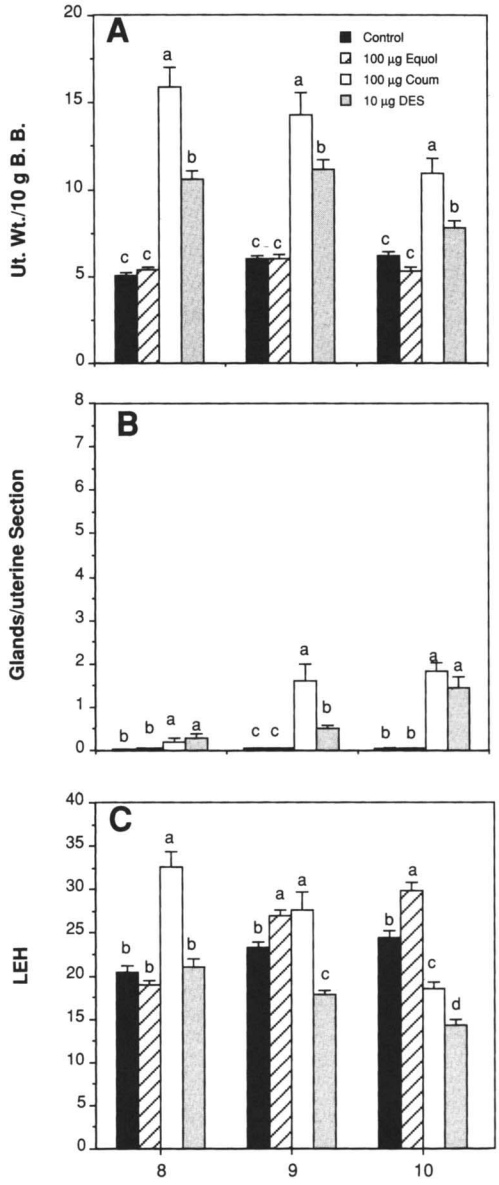

Figure 3. Temporal response for (A) uterine weight, (B) gland number, and (C) LEH in rat pups either untreated or injected with 10 μg DES, 100 μg coumestrol, or 100 μg equol on PND 1–5 and sacrificed on PND 8, 9, and 10. The data are presented as means ± SEM with $n \geq 7$. Values with different letters are statistically different from each other at $P \leq 0.05$.

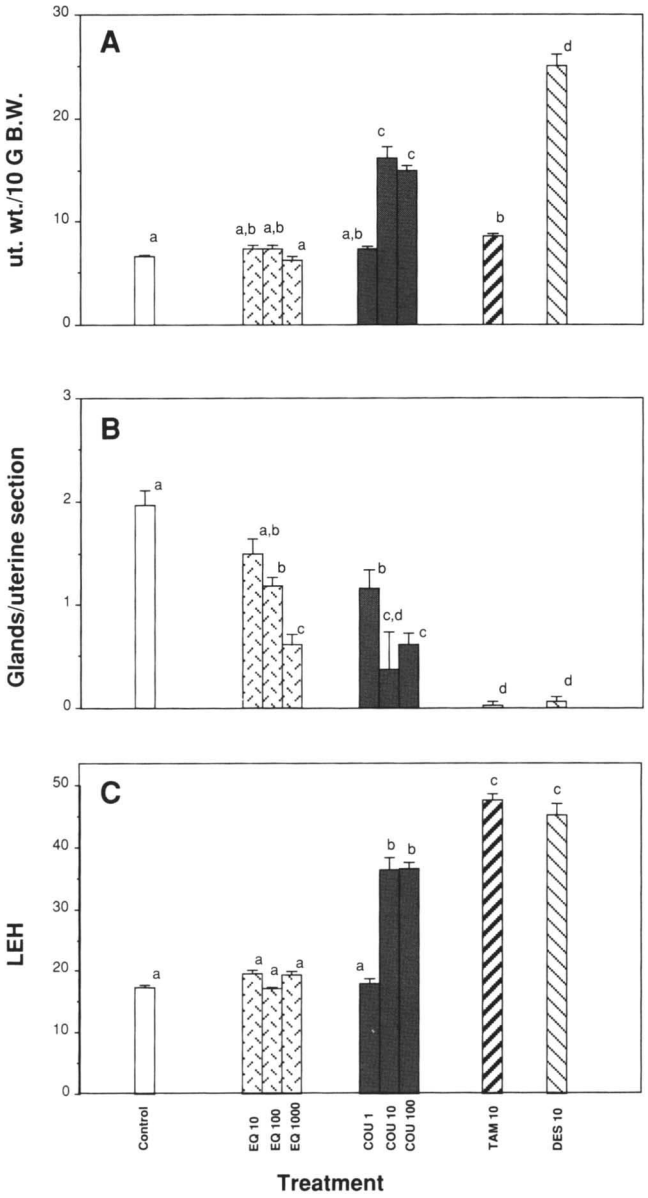

Figure 4. Dose response for uterine weight, gland number, and LEH in rat pups injected on PND 10–14 and sacrificed on PND 14. Treatments were untreated controls; 10, 100, or 1000 μg equol; 1, 10, or 100 μg coumestrol, or 10 μg TAM or DES. The data are presented as means ± SEM with $n \geq 7$. Values with different letters are statistically different from each other at $P \leq 0.05$.

and 10 also shows that DES is less than 10 times more potent than coumestrol. These data would suggest a long half-life for coumestrol which is supported by a recent report in the literature (17). It is interesting that 10 μg DES given on PND 1–5 results in a severe reduction in gland number (40% of controls) on PND 60 (14), while 100 μg coumestrol does not cause any reduction in gland number on PND 60. This may be a result of potency differences between DES and coumestrol, or it may indicate that these compounds interact differently with other growth factors or that they act differently at the genomic level. The latter possibilities are currently under investigation. Comparison of the effects of coumestrol given on PND 10–14 on uterine growth and morphology with the effects of DES and TAM given on the same days again demonstrates that coumestrol is a weak estrogen although its potency is uncertain.

As with DES (8), there is no apparent correlation between uterine growth and ER levels following neonatal administration of either phytoestrogen. Coumestrol initially causes an uterine weight increase while severely reducing ER levels. However, by PND 20, uterine weight is significantly lower than control and

ER levels have increased although still below the control levels. Equol significantly lowers uterine weight on PND 20 and 25 but does not affect ER levels. The data presented in this study would suggest that there are no long-term adverse effects following neonatal exposure to the concentrations of phytoestrogens used as are seen for 10 μg DES (14).

The preponderance of the data presented in this study suggests that equol does not act entirely as either an estrogen or an antiestrogen. When given on PND 1–5, equol lowers uterine weight (as does DES) on PND 20 and 25 and causes a significant increase in LEH on PND 9 and 10. Treatment with equol on PND 10–14 has no effect on uterine weight or LEH at any dose. DES (10 μg) given on PND 10–14 caused a significant increase in both uterine weight and LEH, while TAM (10 μg) had no effect on uterine weight and significantly increased LEH. Lastly, equol causes a dose-dependent decrease in gland number although not nearly as severe as either DES or TAM. We have reported earlier that equol had an estrogenic potency 10^{-5} times that of DES (3). It would seem, therefore, that if equol is an estrogen, it is an extremely weak one.

This study has characterized several aspects of the developmental toxicity of the phytoestrogens, coumestrol and equol. Coumestrol exhibits properties generally found with estrogens while equol possesses a pattern of developmental outcomes that is not shared by either estrogens or antiestrogens. The findings with equol require more detailed studies of its pharmacological and toxicological properties to understand its mechanism of action.

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