The Effect of Prenatal Exposure to the Phytoestrogen Genistein on Sexual Differentiation in Rats (43832)

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Abstract. **Exposure to naturally occurring estrogens during critical periods of development can alter morphologic and physiologic markers of sexual differentiation. The current experiment characterizes the effects of *in utero* treatment with genistein, an isoflavonoid phytoestrogen, on birth weight, anogenital distance (AGD) at birth. GnRH stimulated luteinizing hormone (LH) secretion, volume of the sexually dimorphic nucleus in the preoptic area of the hypothalamus (SDN-POA), puberty onset, and vaginal cyclicity. Pregnant Charles River CD rats were injected sc daily on gestation day 16–20 with either 25,000 μg genistein (G25), 5,000 μg genistein (G5), 5 μg diethylstilbestrol (DES), 50 μg estradiol benzoate (E), or corn oil alone for controls. Birth weights and anogenital distance was taken and exposed progeny were subsequently used in two experiments. In Experiment 1 intra-atrial catheters were placed in adult castrated rats, GnRH was given iv, serial blood samples were drawn and sera were assayed for LH by radioimmunoassay (RIA). Brains obtained by subsequent decapitation were saved for histology. In Experiment 2, females were monitored for timing of vaginal opening as a marker of puberty onset, and vaginal smears were taken to monitor cyclicity. G25-treated females and DES- and E-treated animals of both sexes had decreased weights at birth compared with controls. G5- and E-treated animals of both sexes and DES males had smaller AGD than controls. No significant differences in pituitary responsiveness to GnRH were found among treatment groups. There was a nonsignificant decrease in SDN-POA volume in G5-treated females while DES- and E-treated females had increased SDN-POA volume compared with controls. G5-treated females had delayed puberty onset, and DES-treated females had atypical vaginal cycles in comparison with controls. The results confirm that prenatal exposure to estrogens in the environment can influence sexual differentiation. Our previous experiments have demonstrated that castrate female rats exposed as neonates to genistein have decreased pituitary responsiveness to GnRH challenge and enlarged SDN-POA volume in comparison with controls. Prenatal genistein at these dosages did not significantly alter these markers. However, genistein did mimic other estrogens' effects on AGD and birth weight and had a unique influence on puberty onset. Not only are genistein's effects different from other estrogens, but dosage and timing of exposure during development appear to be important factors in genistein's ability to modify these endpoints.**
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Estrogens and androgens play an important role in the sexual differentiation of animals. Evidence of hormonal influence is not only present in the reproductive tract but also can be seen in reproductive physiology and the central nervous system (CNS). Compounds such as diethylstilbestrol (DES) and the naturally occurring estrogens in plants, phytoestrogens, that mimic endogenous estrogens can also influence development of these characteristics.

Development of the reproductive tract and external genitalia is influenced by endogenous and exogenous hormones. Exogenous estrogen administered *in utero* caused external genital feminization, persistence of Mullerian structures, and atrophy of Wolffian structures in male rat fetuses and hyperstimulation of Mul-

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lerian structures in female rate fetuses (1). Normal variation in intrauterine exposure to androgens due to secretion from the testes of male siblings was found to lengthen the anogenital distance (AGD) (the length of tissue separating the anus and genitalla papilla) of neonatal females depending on the number of adjacent males *in utero*. This effect is additive, since females located between two male siblings had longer AGDs than females with only one adjacent male (2).

The perinatal environment has also been shown to affect neuroanatomy and neuroendocrinology. A morphological marker of sexual differentiation, the sexually dimorphic nucleus in the preoptic area of the hypothalamus (SDN-POA) which is normally larger in male rats than female rats is sensitive to hormonal influence during critical periods of development (3). Castration of neonatal males decreased SDN-POA size (3, 4) while exposure to aromatizable androgens or high-dose estrogens during the perinatal period resulted in increased SDN-POA in female rats (4, 5). Neuroendocrine effects included decreased adult basal and GnRH-stimulated luteinizing hormone (LH) secretion in female rats exposed to estrogen as neonates (6, 7, 8). In addition, exogenous estrogen exposure during the prenatal period delayed puberty onset in female guinea pigs in a dose-dependent fashion (9).

We have previously shown that female rats exposed as neonates to the phytoestrogen genistein had increased SDN-POA volume with results similar to females exposed neonatally to DES. In addition, adult castrate female rats given low-dose genistein as neonates had increased pituitary responsiveness to GnRH while high-dose genistein exposure blunted GnRH-stimulated LH secretion (8).

Nothing is known about *in utero* effects of genistein. Since genistein had similar effects to DES exposure during the neonatal period, we hypothesized that genistein would also influence markers known to be sensitive to intrauterine estrogen exposure. To determine the effects of genistein *in utero,* pregnant rats were treated during gestation day 16–20 and the progeny were compared with rats exposed prenatally to the reference estrogens DES and estradiol benzoate (E). Markers studied included birth weight. AGD, GnRH-stimulated LH secretion, puberty onset, and vaginal cyclicity.

Materials and Methods

Time-mated pregnant rats of the Charles River CD strain (Raleigh, NC) were purchased prior to 16 days gestation and maintained in air-conditioned quarters with Purina Laboratory Chow (Ralston-Purina, St. Louis, MO), water *ad libitum,* and a 14:10-hr light: dark cycle, with lights on from 0500 to 1900 hr EST. On Day 16 through 20 of gestation, injections were given sc of either 25,000 μg genistein (Lot 44353; K and K laboratories, Division of ICN Biomedicals, Inc. Cleveland, OH) 5000 μg genistein, 5 μg DES (Lot #8836; Steraloids, Wilton, NH) 50 μg estradiol benzoate (1,3,5[10]-estratrien-33, 17B-diol 3 benzoate, Batch G160; Steraloids, Wilton, NH) or corn oil alone (Mazola 100% corn oil; Best Foods, CPC International Inc., Englewood, NJ). All injected doses were dissolved in 0.4 ml corn oil. Of note, two pregnant rats received sc injections of DES 5 μg on gestation day 15–20 and the resulting pups were used only in Experiment 2.

Day of delivery was defined as Day 1 if observed to occur prior to 1200 hr. All animals were weighed and the AGD was measured by vernier caliper on Day 1. All animals were identified by ink until Day 10, when ear marking was performed. Litters were then divided into two experimental groups.

In Experiment 1 animals were castrated under ketamine anesthesia (100 mg/kg body wt) and weaned on Day 21 of life. Animals were maintained in plexiglass cages. On Day 42 of life, right heart catheterization under ketamine anesthesia (100 mg/kg body wt) was performed. To control for the nonspecific effects of cannulation and fluid injection, animals were randomized to receive either saline alone (1 ml/kg) iv or GnRH (Factrel; Lot 3880235; Ayerst Laboratories, Inc., New York, NY) dissolved in saline (50 ng/kg body wt) iv 4 hr post cannulation. Blood samples of 0.3 ml volume were collected via the catheter immediately prior to and 5, 10, 15, and 30 min after injection of GnRH or saline. Blood volume was replaced with an equivalent volume of 10 U/ml heparinized saline each time. The sampling procedure was repeated 15 min after the last blood collection with animals receiving GnRH initially that had received saline injection and animals receiving saline that had initially received GnRH injection.

Blood samples were allowed to clot at room temperature and were centrifuged at approximately 1500*g* for 10 min. Sera were aspirated and frozen at −20°C for later radioimmunoassay (RIA).

Serum LH concentration were measured by double-antibody RIA with rat LH supplied by NIDDK and the National Hormone and Pituitary Program (University of Maryland School of Medicine, Baltimore, MD). Second antibody (sheep, anti-rabbit) was graciously supplied by L. Tyrey, PhD (Duke University Medical Center, Durham, NC). Aliquots of serum (50 μl or less) were assayed in duplicate and the means were expressed in terms of NIDDKD-rLH-RP-3.

Intra- and interassay coefficients of variation for the measurement of LH in three serum pools were 1.8% and 8.2%, respectively. The assay sensitivity was 0.48 ng/ml.

Following the blood sampling, the animals were anesthetized for cannula removal and decapitated. The brains were promptly removed from the heads and

blocked. The block of tissue which included the optic chiasm and the hypothalamus was removed and placed in 10% formalin for a minimum of 2 weeks. Prior to frozen sectioning, the brains were placed in 30% sucrose and formalin solution for 48–72 hr. Sections were cut in the DeGroot plane (10) at 20 μm thickness, mounted on slides, and stained with cresyl violet acetate. The slides were coded such the investigator performing light microscopy was blinded to the identity of the sample. The SDN-POA, as described by Gorski (3) with confirmation of the surrounding structures according to the stereotaxic atlas of Pelligrino (11), was traced with the use of a camera lucida. The cross-sectional area was computed with the use of a digitalizing pad and planimetry computed software package (Sigmascan, Jandel Scientific, Corte Madera, CA). Volume of the SDN-POA was computed by adding together the traced areas and multiplying by the thickness of all sections.

In Experiment 2, animals were weaned on Day 21 of life. Females were separated from males and maintained in plexiglass cages as in Experiment 1. Females were monitored for day of vaginal opening. Subsequent to vaginal opening, female rats underwent daily vaginal cytology monitoring using staging criteria described by Everett (12). Smears were made by vaginal lavage with physiologic saline spread thinly on a clean glass slide. After fixation in 95% ethanol and removal of salt with deionized water, slides were then stained in 1% Toluidine Blue solution.

Individual birth weights, AGD measurements, SDN-POA volumes, and serum LH concentrations were all compared among treatment groups by one-way analysis of variance and Fisher's least significant difference test. Age at vaginal opening was compared among treatment groups by Kruskal-Wallis analyses and Mann-Whitney *U* tests. Vaginal smear cyclicity was compared qualitatively among groups.

Results

Disruption of Parturition and Resulting Progeny. Numbers of pregnant rats receiving treatments and numbers of viable progeny in each treatment group are recorded in Table I. Treatment of pregnant rats with DES and estradiol benzoate were associated with delayed parturition and increased rates of stillbirth and pup death before Day 10 of life, while these findings were not seen in animals treated with either dosages of genistein or corn oil treated animals. Pregnant rats were also treated with Estradiol Benzoate 100 μg and 500 μg during gestation day 16–20 but few viable pups were produced from these litters and these were not used in either experiment. Only one out of four mothers treated with estradiol benzoate 50 μg on gestation day 16–20 produced viable pups. Those animals receiving estradiol benzoate 50 μg *in utero* were used only in comparisons of birth weight, AGD, and SDN-POA.

Table I. Number of Pregnant Rats Receiving Treatment and Number of Progeny in Each Treatment Group

Treatment	Number of pregnant rats	Total number of pups	
		Males	Females
Corn Oil	4	25	19
G25	4	23	18
G5	4	26	27
DES	4	28	16
E	1	9	3

Birth Weights in Newborn Rats. Mean birth weights are presented in Figure 1. G25-treated females ($P < 0.05$) and DES- and E-treated animals of both sexes ($P < 0.01$) had smaller birth weights than corn oil-treated animals of the same sex. Of note is that four males in the DES group died prior to weighing and were not used in this analysis.

AGD in Newborn Rats. Mean AGD measured on Day 1 of life are presented in Figure 2. G5- and E-treated animals of both sexes and DES-treated males had significantly shorter AGD than corn oil-treated animals ($P < 0.01$ for all aforementioned groups).

GnRH-Stimulated Secretion in 42-Day Castrate Rats. Mean LH concentration measured initially and at 5-, 10-, 15-, and 30-min intervals post GnRH or saline administration in females of the G25, G5, DES,

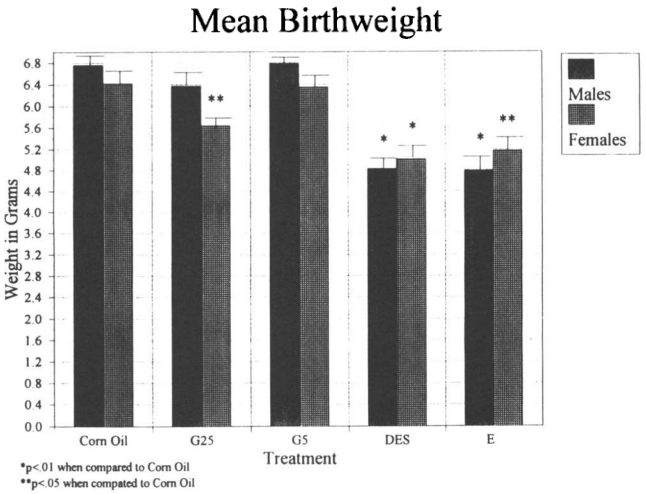

Figure 1. Mean birthweights in g ± SEM of rats exposed *in utero* on gestation day 16–20 to genistein 25,000 μg (G25), genistein 5000 μg (G5), diethylstilbestrol 5 μg (DES), estradiol 50 μg (E), or corn oil. Significantly decreased weights at birth were found in G25-treated females ($P < 0.05$), DES- and E-treated animals of both sexes ($P < 0.01$ for all DES animals and E males, $P < 0.05$ for E females). Number of animals within treatment groups were as follows: males—corn oil ($n = 25$), G25 ($n = 23$), G5 ($n = 26$), DES ($n = 24$), and E ($n = 9$); females—corn oil ($n = 19$), G25 ($n = 18$), G5 ($n = 27$), DES ($n = 16$), and E ($n = 3$).

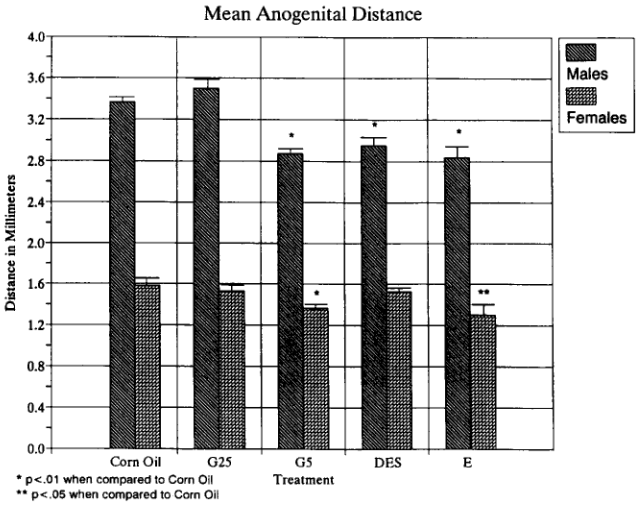

Figure 2. Mean anogenital distances (AGD) in millimeters ±SEM of rats exposed *in utero* on gestation day 16–20 to genistein 25,000 μg (G25), genistein 5,000 μg (G5), diethylstilbestrol 5 μg (DES), estradiol 50 μg (E), or corn oil. Significantly smaller AGD at birth were found in G5- and E-treated animals of both sexes and DES males ($P < 0.01$ for G5-treated males and females, E- and DES-treated males, $P < 0.05$ for DES females). Numbers of animals within each treatment group were as follows: males—corn oil ($n = 25$), G25 ($n = 23$), G5 ($n = 26$), DES ($n = 28$), and E ($n = 9$); females—corn oil ($n = 19$), G25 ($n = 18$), G5 ($n = 27$), DES ($n = 16$), and E ($n = 3$).

and corn oil treatment groups are presented in Figure 3 a–d. Other than the expected differences between GnRH and saline treatments within groups, concentration of LH at baseline, peak, or at any given time interval was not significantly different among treatment groups for either sex.

SDN-POA Volume in 42-Day Castrate rats. SDN-POA volume averages among treatment groups are presented in Figure 4 for females and Figure 5 for males. SDN-POA volume was significantly enlarged in DES- and E-treated females ($P < 0.01$) compared with corn oil treated females. There was an apparent but nonsignificant decrease in the SDN-POA volume of G5-treated females. Due to the high attrition rate of E-treated litters in the neonatal period, only three E-treated females were available for this part of the experiment. No significant differences were found among males of any treatment group.

Age at Vaginal Opening. Distribution of age at vaginal opening is presented in Figure 6. Corn oil-treated females were found to have vaginal opening consistently at 37 days (SD of 0). G5-treated females had significantly later onset of vaginal opening (mean, 38.8 days ± SD 1.32) than corn oil-treated females ($P < 0.01$). There was no significant differences in timing of vaginal opening among other treatment groups.

Vaginal Smear Cytology. Length and regularity of cycles from vaginal opening until after 90 days of age were compared among treatment groups. Corn oil, G5, and G25 animals consistently exhibited estrous cycles usually between 3 and 5 days. DES animals treated on gestation Day 15–20 were used in comparison of vaginal smear cyclicity. Three of the four DES-treated animals had marked cycle irregularity ranging from 2 to 12 days in length with a mean cycle length of 4.9 days. Prolonged cycles in DES were characterized by prolonged intervals of diestrus.

Discussion

The results of this study demonstrated that exposure to genistein *in utero* can influence markers known to be sensitive to estrogens. To our knowledge, this is the first demonstration of the effects of *in utero* exposure to the phytoestrogen genistein on hormone-sensitive characteristics. The effects of prenatal genistein are different from those associated with neonatal treatment with genistein, suggesting that timing of exposure or maternal and placental influences are important in the development of these characteristics. In addition, responses to genistein differ at low and high dosages possibly secondary to estrogenic agonist versus antagonist actions or estrogenic versus non-estrogenic metabolite effects. Finally, genistein effects differ from both DES and E, indicating that not all estrogens act alike and that each compound has its own profile of activity.

DES and estradiol were selected as comparison agents to genistein because of their known estrogenic properties. Genistein did not appear to adversely affect pregnancy, delivery, or survival in the neonatal period. However, there were some difficulties obtaining sufficient quantities of pups surviving past delivery and Day 10 of life in the litters exposed to DES and Estradiol *in utero*. Animals that were treated with estradiol benzoate at dosages of 100 μg and 500 μg had few pups surviving the perinatal period and were not used in these experiments. Cesarean sections were performed on some of the DES-treated pregnant rats who had not delivered by gestation Day 23, the typical length of gestation in rats, and evidence of fetal resorption was found. Similar results of prolonged delivery and increased perinatal mortality in pregnant rats treated with DES near gestation Day 18 were found by Zimmerman *et al.* who attributed uterine contraction failure to a concomitant depression in maternal steroid hormone levels. The relative incidence of stillbirth was reduced in their study by cesarean delivery indicating that DES induced higher pup mortality partly by disruption of labor (13).

Intrauterine androgen exposure is known to be associated with lengthened AGD. Likewise, AGD shortening in G-5, DES- and E-treated males is consistent with previous reports of "feminization" of external genitalia in male rats secondary to prenatal exposure (1). While estrogens mimic the actions of aromatizable androgens in masculinizing the CNS, at the external genitalia estrogens have the opposite effects of andro-

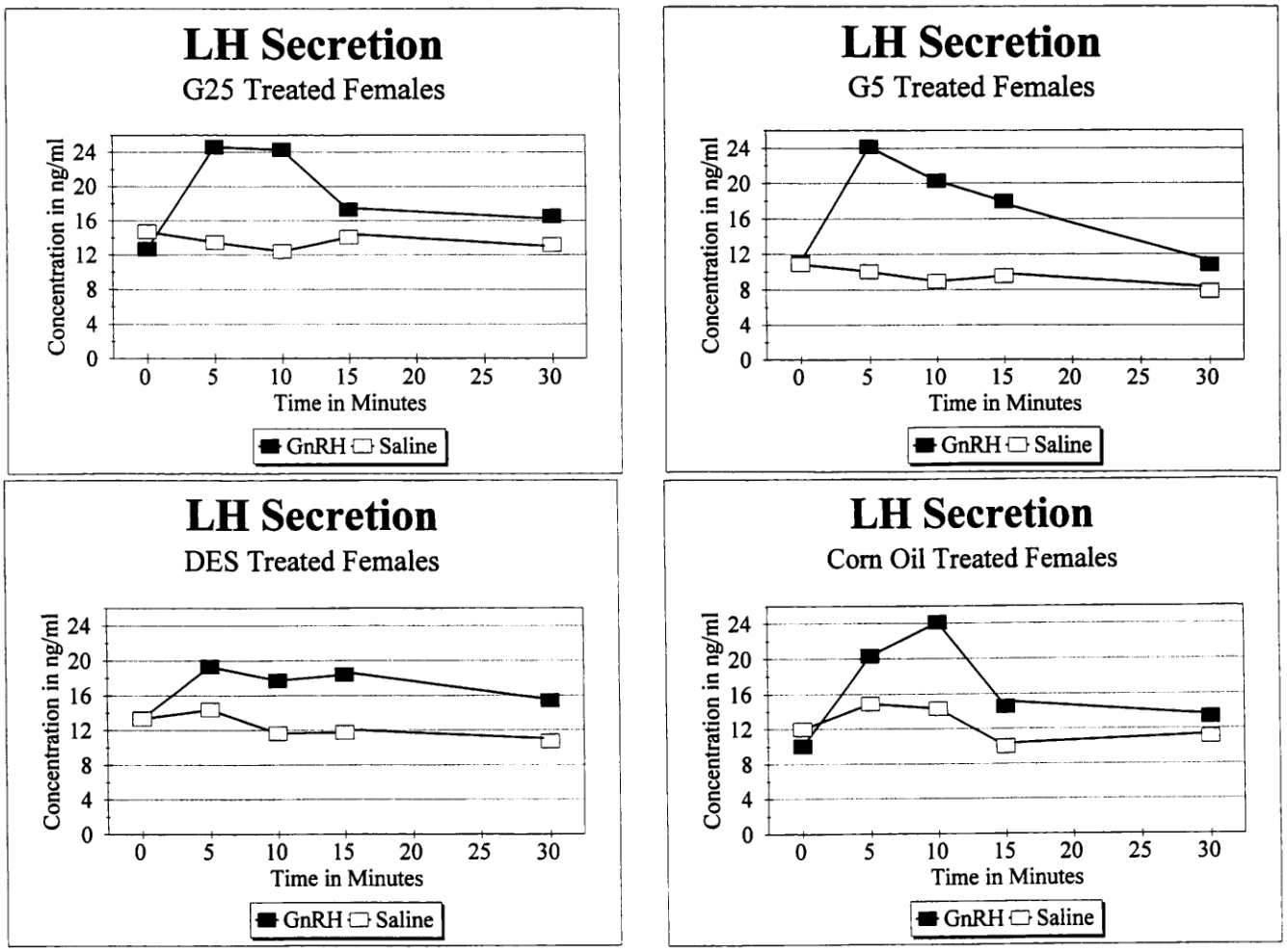

Figure 3. Mean serum LH secretion in ng/ml over time in female rats exposed *in utero* on gestation day 16–20 to (A) genistein 25,000 μg (G25), (B) genistein 5000 μg (G5), (C) diethylstilbestrol 5 μg (DES), or (D) corn oil. Animals were given either GnRH or saline via intraatrial catheters at $t = 0$. No significant differences were found among treatment groups. Number of animals within each treatment group were as follows: G25 ($n = 9$), G5 ($n = 13$), DES ($n = 10$), and corn oil ($n = 7$).

gens. In addition, overall intrauterine growth as characterized by birth weight was decreased in female rats treated with higher dosages of genistein along with E and DES exposed animals of both sexes. This indicates that the shortened AGD length in the affected males are independent of the overall growth inhibition seen in both sexes.

Our data on pituitary responsiveness to GnRH stimulation in genistein exposed rats demonstrates how both dosage and timing are important factors with respect to the effects of an estrogen. Adult rats treated as adults with 100 μg/kg body wt iv genistein had blunted GnRH-induced LH secretion when compared with control animals (14). Later experiments demonstrated that neonatal treatment with 1000 μg of subcutaneous genistein during Day 1–10 of life had blunted GnRH-stimulated LH secretion, while neonatal treatment with genistein dosages of 100 μg enhanced GnRH-induced secretion (6). There was a lack of demonstrable differences in basal LH levels or GnRH-stimulated secretion among any of the treatment groups in this study. It is possible that pituitary function development in rats may occur later than the prenatal period and is not consistently influenced by *in utero* exposure to estrogenic compounds. However, prenatal exposure to estrogens have been shown in this experiment to influence both indirect markers of pituitary function, vaginal opening, and vaginal cyclicity. An alternative explanation is that treatment dosages were not sufficient to induce consistent change GnRH-stimulated LH secretion.

A hormone sensitive period for differentiation of the SDN-POA in rats had been described to occur between gestation Day 16 and Day 5 of life (15, 16). SDN-POA volume increases associated with prenatal treatment with DES and E found in this experiment were consistent with these studies. Unlike our previous studies of neonatal genistein exposure that demonstrated increased SDN-POA volume in females (6, 8), prenatal genistein treatment did not cause any significant changes in SDN-POA volume. There was only a suggestion of a decreased SDN-POA volume with

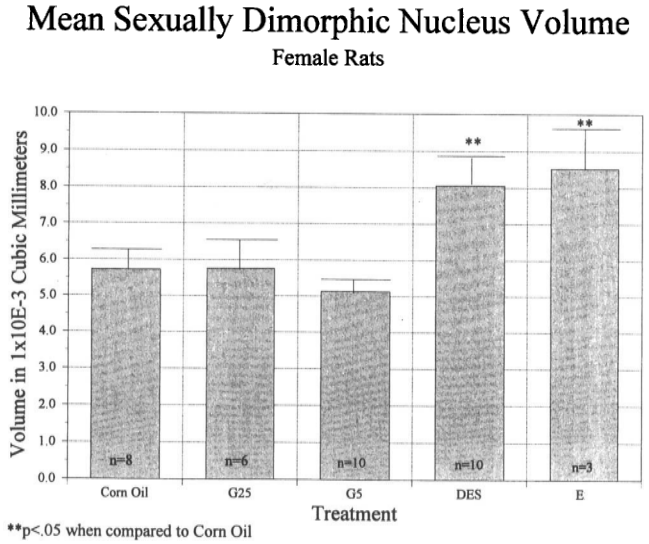

Figure 4. Mean volumes of the sexually dimorphic nucleus in the preoptic area (SDN-POA) of female rats exposed *in utero* on gestation day 16–20 to genistein 25,000 μg (G25), genistein 5000 μg (G5), diethylstilbestrol 5 μg (DES), estradiol 50 μg (E), or corn oil. SDN-POA volumes were significantly increased in DES- and E-treated females ($P < 0.05$) when compared with corn oil treated females.

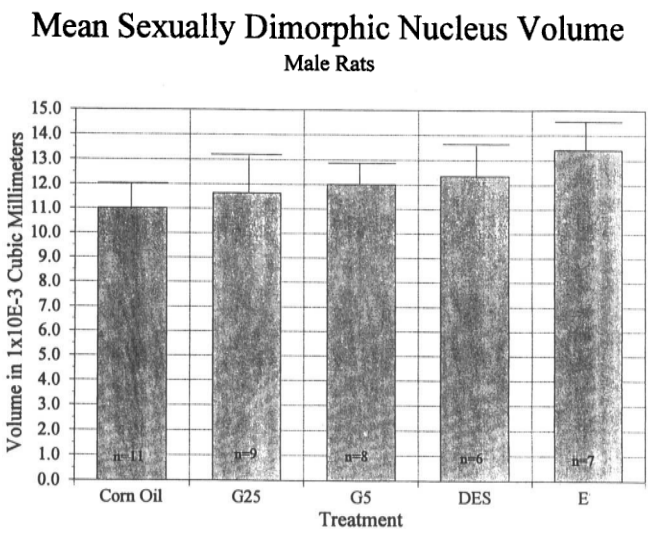

Figure 5. Mean volumes of the sexually dimorphic nucleus in the preoptic area (SDN-POA) of male rats exposed *in utero* on gestation day 16–20 to genistein 25,000 μg (G25), genistein 5000 μg (G5), diethylstilbestrol 5 μg (DES), estradiol 50 μg (E) or corn oil. There were no significant differences among treatment groups.

low-dose genistein, but this apparent difference did not attain statistical significance. If further studies demonstrate a reduction of the SDN-POA volume, then a low-dose antiestrogenic effect of genistein might be implied. It is difficult to assess whether high-dose genistein's relative inactivity in influencing most of the markers studied was due to differences in maternal metabolism or placental transport, some alteration of bioavailability due to concentration in the corn oil vehicle or expression of mixed agonist-antagonist

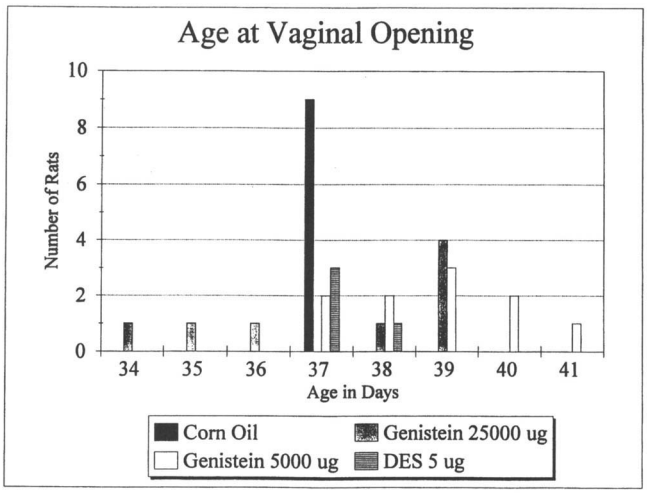

Figure 6. Frequency histogram of age in days at vaginal opening, a marker of puberty onset, of female rats exposed *in utero* on gestation day 16–20 to genistein 25,000 μg (G25), genistein 5000 μg (G5), or corn oil. Those animals treated with diethylstilbestrol 5 μg (DES) were exposed on gestation day 15–20. G5-treated females had later onset of vaginal opening when compared with corn oil controls.

effects. Of note is that while the SDN-POA volumes in male rats were found to be normally three to five times larger than those found in females (3), SDN-POA volumes in control animals in this experiment were consistent with those of gonadectomized animals in studies by Rhees *et al.* (16).

Low-dose genistein was the only treatment that delayed puberty marked by vaginal opening in comparison to the corn oil treatment group in this study. Previous research appears to be contradictory concerning the effect of perinatal estrogen exposure on puberty onset. Increased estrogen production by the ovary at puberty under the control of the hypothalamo-hypophysial axis is believed to initiate vaginal opening. While neonatal estrogen exposure had been shown to cause precocious onset of puberty in mice (17), prenatal estrogen and testosterone exposure delayed onset of puberty in guinea pigs (18, 19). These latter studies suggest that testosterone *in utero* influences neuroendocrine mechanisms regulating puberty onset in part via conversion to estrogen. Prenatal exposure to estrogens (including genistein) may also cause a delay in puberty onset by an inhibitory or androgenizing effect on the hypothalamo-hypophysial axis.

Genistein was not found to significantly disrupt vaginal smear cyclicity in this experiment. Persistent vaginal estrus or cornification, anovulation, and infertility during adulthood were shown to be characteristic responses to androgen and estrogen exposure in rodents during critical periods of neuroendocrine differentiation (less than 5 to 10 days of age) (19). Exposure to the phytoestrogen coumestrol also induced persistent vaginal estrus (20). Only DES-treated animals had

significantly disrupted cycles in our study, further highlighting the differences among estrogens. In addition, cycles in these DES-treated females were characterized by long periods of diestrus rather than estrus. There may be some differences in the type of ovulation disruption seen in this experiment. An alternative explanation is that the onset of persistent estrus may occur later in the rat's life cycle. The sampling period in this experiment may have been too short to detect this change.

One of the variables not controlled in this experiment was the amount of phytoestrogens present in the laboratory chow. The concentrations of phytoestrogens (including genistein) and how they varied between batches were not analyzed. In addition, the relative amounts of laboratory chow consumed by each pregnant rat and the resultant pups were not recorded. It is unclear how much impact this baseline exposure to phytoestrogens had on the resulting measures.

Differences in action were found among genistein, DES, and E in this study. Prenatal exposures to genistein also did not always have the same effects on pituitary responsiveness and SDN-POA volume as those associated with neonatal exposures demonstrated in our earlier (6, 7, 8, 14) studies. Dosage of genistein was an important factor with respect to the effects seen in our present and previous developmental studies. Genistein may have estrogen agonist and antagonist effects at high and low dosages and this relationship may not be simple or unidirectional. While this study suggests these effects are complex, genistein influences estrogen-dependent aspects of fetal and neonatal development by modifying both morphologic and neuroendocrine endpoints.

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