Combined Effects of Dietary Phytoestrogen and Synthetic Endocrine-Active Compound on Reproductive Development in Sprague-Dawley Rats: Genistein and Methoxychlor

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Humans and wildlife are frequently exposed to mixtures of endocrine active-compounds (EAC). The objective of the present study was to investigate the potential of the phytoestrogen genistein to influence the reproductive developmental toxicity of the endocrine-active pesticide methoxychlor. Three levels of genistein (0, 300, or 800 ppm) and two levels of methoxychlor (0 or 800 ppm) were used in this study. Sprague-Dawley rats were exposed to the two compounds, either alone or in combinations, through dietary administration to dams during pregnancy and lactation and to the offspring directly after weaning. Both compounds, methoxychlor in particular, were associated with reduced body growth at 800 ppm, but pregnancy outcome was not affected by either treatment. An acceleration of vaginal opening (VO) in the exposed female offspring was the only observed effect of genistein at 300 ppm. Exposure to 800 ppm genistein or 800 ppm methoxychlor caused accelerated VO and also altered estrous cyclicity toward persistent estrus in the female offspring. The estrogenic responses to genistein and methoxychlor administered together were apparently accumulative of the effects associated with each compound alone. Methoxychlor, but not genistein, delayed preputial separation (PPS) in the male rats. When administered with methoxychlor, genistein at 800 ppm enhanced the effect of methoxychlor on delaying PPS. Genistein and methoxychlor treatment did not change gender-specific motor activity patterns in either sex. To explore possible mechanisms for interaction between the two compounds on development, we performed estrogen receptor (ER)- and androgen receptor (AR)-based in vitro transcriptional activation assays using genistein and the primary methoxychlor metabolite 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). While the in vitro assays supported the estrogenic effects of genistein and methoxychlor and the antiandrogenic effects of methoxychlor, the reactivity of these compounds with ERs α and β could not predict the greater in vivo estrogenic potency of methoxychlor over genistein; nor could the potentiation of the methoxychlor effect on PPS by genistein be predicted based on in vitro HPTE and genistein reactions with the AR. Data from this study indicate that phytoestrogens are capable of altering the toxicological behaviors of other EACs, and the interactions of these compounds may involve complexities that are difficult to predict based on their in vitro steroid receptor reactivities.

Key Words: methoxychlor; genistein; estrogen receptor; androgen receptor; reproductive development.

An increasing number of environmental and industrial compounds have been identified as endocrine-active, and exposure to these compounds raises a major health concern (Barlow et al., 1999; Colborn et al., 1993). Due to the widespread presence of endocrine-active compounds (EACs) in the environment, humans and animals may be exposed to various combinations of them under many circumstances. Among the classes of EAC drawing particular interest are the phytoestrogens, which are plant-derived chemical agents with estrogen-like activity commonly found in the diets of humans, wildlife, and experimental animals. The biological actions of the phytoestrogens are believed to have health implications to the populations that regularly consume them, while their existence in diets for experimental animals has also raised questions about their possible role in influencing studies probing the effects of test compounds (Brown and Setchell, 2001).

For instance, the popular NIH-07 rodent diet contains 12% soybean meal and 4% alfalfa meal, both of which are rich sources of phytoestrogens. Soy contains the isoflavones genistein and daidzein, and alfalfa contains coumestrol and formononetin (Franke et al., 1995, 1998). We have previously shown that the presence of phytoestrogens in NIH-07 diet has the potential to confound the outcome of experiments evaluating chemicals with weak estrogenic activities even though they did not by themselves cause significant developmental effect (Casanova et al., 1999). The objective of the present study was to investigate the potential of genistein to influence the reproductive developmental toxicity of the endocrine-active pesticide methoxychlor.

Methoxychlor (1,1,1-trichloro-2,2-bis(4-methoxyphenyl)-ethane, or methoxy-DDT) is used in the United States and some other countries for controlling insects on crops, livestock, and in animal feed and grain-storage bins. Household use of
methoxychlor includes applications in the garden and on pets (ATSDR, 2000). Methoxychlor is a known reproductive toxin with the capability of accelerating female pubertal development and delaying male pubertal development when given at high doses (Chapin et al., 1997; Gray et al., 1989).

Analyses previously performed in our laboratory indicated that the genistein concentration in NIH-07 is approximately 160 mg/kg and the daidzein concentration approximately 140 mg/kg (Casanova et al., 1999). Isoflavones other than genistein or daidzein may also be present in NIH-07 but are at much lower concentrations than genistein and daidzein (Franke et al., 1995). We selected two levels (300 and 800 ppm) of dietary genistein in the present study to probe potential interaction between the toxicities of genistein and methoxychlor. A custom-made soy- and alfalfa-free diet (SAFD) that we previously described (Casanova et al., 1999) was used as the base diet in this study. The 300 ppm genistein was used to approximate the estrogenic potency of the combined phytoestrogens expected in the NIH-07 diet. The 800 ppm of genistein was included based on our previous results that 1000 ppm genistein was capable of causing a significant estrogenic response in the immature rat (Casanova et al., 1999). We choose a level of 800 ppm methoxychlor in this study because it was close to the dietary dose of 1000 ppm shown to affect reproductive development in the rat (Harris et al., 1974). Our methoxychlor treatment was designed to produce reproductive endocrine toxicity in developing rats so that the responses could be used as reference end points to evaluate the potential influence of genistein.

In addition, to assess the potential cellular basis for interactions between the two compounds, we evaluated the combined effect of genistein and 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), the primary methoxychlor metabolite with steroid receptor reactivity, on activating the estrogen receptors (ERs) and the androgen receptor (AR). Our results in the present study indicated that coadministration of genistein and methoxychlor can result in biological interactions of the two compounds. Phytoestrogens at normal exposure levels have the potential to influence the toxicity of synthetic compounds, and the combined effects of EAC on reproductive development were difficult to predict based on in vitro reactions of such compounds with the steroid hormone receptors.

MATERIALS AND METHODS

Treatment Diets

Appropriate amounts of genistein (≥ 98% purity, INDOFINE, Somerville, NJ) and methoxychlor (≥ 95% purity, Sigma, St. Louis, MO) were blended with a base diet to form the treatment diets used in the present study. The base diet was a custom-prepared soy- and alfalfa-free diet (SAFD) (Zeigler Bros., Gardners, PA; formula code: 54120000) whose composition was previously described (Casanova et al., 1999). The uniformity of blending was verified through gas chromatography analyses. Each batch of diets was prepared to last approximately 4 weeks, and the prepared diets were stored at 5–10°C.

Three levels of genistein (nominal 0, 300, and 800 mg/kg diet or ppm) and two levels of methoxychlor (nominal 0 and 800 ppm) were used in six different combinations. The treatment groups and their test chemical contents were designated as follows: control (no genistein and no methoxychlor, i.e., base SAFD diet), 800 M (800 ppm methoxychlor, no genistein), 300 G (300 ppm genistein, no methoxychlor), 300 G + 800 M (300 ppm genistein and 800 ppm methoxychlor), 800 G (800 ppm genistein, no methoxychlor), and 800 G + 800 M (800 ppm genistein and 800 ppm methoxychlor).

Animals and Treatment

Time-mated, young-adult female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) and delivered to CIIT Centers for Health Research (CIIT) on gestation day (GD) 0, which was defined as the day of sperm-positive. The animals were randomized into six groups (n = eight per group) and were provided immediately with the treatment diets as described above.

The pregnant dams were housed individually in plastic cages with dry cellulose bedding (Shepherd Specialty Papers, Kalamazoo, MI) and were provided with deionized water ad libitum. The animal room was maintained within a temperature range of 22–25°C and relative humidity of 50 ± 10% with 12-h light cycles (7:00 –19:00). Body weight and food consumption of each dam were recorded on a twice-weekly schedule.

Offspring were housed with their respective dams until postnatal day (PND) 21, at which time they were weaned and housed up to four same-sex littersmates per cage. The offspring rats continued to be fed with the respective maternal diets throughout the study. The number of rats per cage was adjusted around PND 55 so that up to two same-sex littersmates were housed in each cage. The body weight of the offspring was measured at birth, once a week during lactation, and on approximately PND 30, 55, and 100.

Necropsy and Measurements

Pregnancy outcome. Following parturition, litter size and sex composition of each litter, and weight and anogenital distance (AGD) of each pup were determined on PND 1 (day of birth defined as PND 0) as previously described (You et al., 1998). Dams were killed on PND 22 by CO₂ asphyxiation, and the number of implantation sites was determined by using 0.5% ammonium sulfide staining of the uteri.

Necropsies. On PND 22, one male and one female pup per litter were killed by CO₂ asphyxiation. Brain, liver, testis, ventral prostate, and uterus were dissected, weighed, and fixed in neutral-buffered formalin for pathological evaluation (see below). On approximately PND 110, adult male and female offspring (mostly three male pups per litter with only one or two pups in a few litters and one female pup per litter) were killed via decapitation. Organs weighed at necropsy were the testis, epididymis, seminal vesicles, and ventral prostate in the male; ovary and uterus in the female; and pituitary gland, adrenal gland, liver, and brain of both sexes. Both sides of bilateral organs were weighed, and their average was used for data analysis and result presentation.

Vaginal opening and preputial separation. Starting on PND 25 until completion, all the remaining female offspring (range, 1–9 per litter; average, 5.0 per litter) were examined daily (between 9:00 A.M. and 12:00 P.M.) for vaginal opening (VO). Daily vaginal lavage smear was performed for approximately 2 weeks once VO was observed. The collected slides were then read in the same manner as for the adult rats (see below). Starting on PND 35 until completion, all the remaining males (range, 1–8 per litter; average, 4.4 per litter) were examined daily (between 9:00 A.M. and 12:00 P.M.) for PPS. The body weights at which either VO or PPS occurred were also recorded.

 Estrous cyclicity. Two adult female offspring randomly selected from each litter (six to eight litters per dietary group) were used to determine estrous cyclicity. On approximately PND 90, half the rats (one in each litter) were removed from their treatment diets and were instead given SAFD for 30 days. The second rat in each litter remained on its treatment diet. Starting on approximately PND 120 and for the duration of 3 weeks, daily vaginal lavage was performed on all the female rats, and vaginal smear samples were
collected from lavage fluid obtained by flushing the vagina with phosphate-buffered saline via a medicine dropper. The vaginal smear slides were determined to be in the following estrous stages according to cytology of the vaginal smears: metestrus (M), a dense appearance of nucleated epithelial cells, secretary matrix, and leukocytes; diestrus (D), predominance of leukocytes and a few scattered cornified epithelial cells; proestrus (P), predominance of round nucleated epithelial cells that may be dispersed or clumped; or estrus (E), all cornified cells. A number of rats showing a mixing of nucleated epithelial cells and cornified cells were classified as PE due to their similarities to both proestrus and estrus stages. All the vaginal smear slides were evaluated by one individual.

### Neurobehavioral Evaluation

Locomotor activity is a sexually dimorphic behavior in rats, with females demonstrating a higher level of running wheel activity, open field exploration, and other forms of spontaneous motor activity than males (Shulkin, 1999). Likewise, amphetamine-induced locomotor activity is more prominent in female rats (Forgie and Stewart, 1993, 1994). Changes in sexual differentiation may be reflected through changes in levels of motor activities. In addition, estrogenic action is thought to play a protective role in the central nervous system against certain insults (Wise et al., 2001). In this study, we evaluated the potential of genistein and methoxychlor to alter sex-dependent motor activity. Motor activity was measured in one male and one female from each litter on PND 64 – 65 (n = six to eight litters/treatment group). Each animal received an intraperitoneal (ip) injection of saline (1 ml/kg) immediately (∼10 min) prior to testing on PND 64. On the following day (PND 65), each animal received an ip injection of d-amphetamine (1 mg/kg) in saline (1 ml/kg) 10–20 min prior to assessment of spontaneous locomotor activity. Spontaneous motor activity was measured during 10 intervals of 6 min each for a total of 60 min using an automated cage rack photobeam activity system (San Diego Instruments, San Diego, CA). Each animal was placed into an individual, clear polycarbonate cage, 45.7 × 23.5 × 20.3 cm, with four photobeams spaced 11 cm apart on either side of the cage. Photobeams were positioned approximately 5.7 cm above the cage floor. Motor activity testing was completed during the light phase of the animal’s diurnal cycle. White noise levels of 68.8 ± 0.9 dBA and room illumination of approximately 2.5 ± 0.4 foot-candles were maintained in the laboratory during motor activity testing. White noise was generated with a white noise generator (Coulbourn Instruments, Allentown, PA). Data from the first 6-min interval for the saline-treated animals was inadvertently lost due to a computer programming error. Mean and standard deviation values were calculated for total motor activity for each 6-min interval during the 60-min measurement period.

### Pathology Evaluations

Testes, prostate, pituitary gland, and adrenal gland from adult male offspring were evaluated histologically. Unilateral testis samples were collected from two littersmates per litter (seven to eight rats per treatment group). Prostate and pituitary and unilateral adrenal gland tissues were also collected from one of the two male littersmates and prepared for light microscopic examination. A similar sampling scheme was used for the female offspring with a single ovary from each of the two selected animals. All tissues were fixed in 10% neutral-buffered formalin for 24 h, paraffin-embedded and microtomed into 5-μm sections, and stained with hematoxylin and eosin for light microscopic evaluation.

### Statistics

All values were expressed as means ± standard deviations when appropriate. When littersmates were included in a measurement, the data presented are group means, and their standard deviation was derived from litter averages. The primary statistical tool used in data analysis was a full two-way analysis of variance (ANOVA) model with genistein and methoxychlor each as a treatment factor. When the analysis involved a time series of data, such as in the case of the body weight of the dam and offspring at different time points, a repeated-measures ANOVA approach was adopted. When organ weights of the dams and the offspring in different treatment groups were analyzed, the corresponding body weights were included as covariate.

In the cases of pup body weight, AGD, PPS, VO, estrous cycle stages, and adult male offspring reproductive organ weights that involved measurements of littersmates, a nesting term with random effect was included in the statistical models to accommodate possible litter effects in the data. When the methoxychlor term was significant in an analysis, a treatment effect due to methoxychlor was concluded without further post hoc analysis because there were only two levels. When the genistein term became positive, however, a t-test was used to compare individual pairs between the 0, 300-ppm, and 800-ppm dose levels, and a Bonferroni correction was used to adjust the significance values with respect to multiple pairwise comparisons.

Motor activity data were analyzed using a repeated-measures analysis with dietary exposure as a between-groups variable (MANOVA). Because significant gender effects were observed, separate analyses of total motor activity were performed for each gender. Paired student t-tests were used to evaluate whether amphetamine exposure increased mean total locomotor activity when compared with each individual animal’s baseline activity observed following saline pretreatment. In all cases, the level of statistical significance was set at p < 0.05. All statistical procedures were performed with either the JMP or SAS statistical software packages (SAS Institute, Cary, NC).

### Receptor-Dependent Transactivation Assays

To understand the reactivity of genistein or methoxychlor with the sex steroid receptors in the presence of each other, we conducted AR-, ERα-, and ERβ-dependent transcriptional activation assays using genistein and HPTE. The experiments were performed based on procedures previously described (Gaido et al., 2000). Briefly, HepG2 human hepatoma cells were transfected with expression plasmids for human ERα, ERβ, and AR. The activities of ERα and ERβ were detected through the expression of a cotransfected estrogen-responsive complement 3-luciferase reporter plasmid, and a constitutively active cytomegalovirus (CMV)-β-galactosidase reporter plasmid was used for transfection control. The AR construct used an androgen-responsive MMTV-luciferase as the reporter with the CMV-β-galactosidase as transfection control. Transfected cells were treated for 24 h with various concentrations of genistein (>97%, Gibco BRL, Rockville, MD) and HPTE. The luciferase and β-galactosidase activities were determined in cell lysate preparations.

## RESULTS

### Level of Exposure and Effects on Body Weight

Both methoxychlor and genistein reduced the feed consumption for the pregnant dams during gestation (p < 0.01 for both treatments; two-way ANOVA, repeated measures; Table 1). The genistein effect occurred at the dose level of 800 ppm but not at 300 ppm. Methoxychlor (800 ppm) was more potent than genistein in reducing feed intake. The effect of 800 ppm methoxychlor (comparing the control and the 800 M group) was 20–34% reduction and for 800 ppm genistein was 9–21% reduction during the period from the beginning of gestation to the end of lactation. When administered together (as in groups 300 G + 800 M and 800 G + 800 M), the effects of the two compounds in reducing the feed intake appeared to be accumulative. The feed intake of the offspring in both sexes was also affected by both methoxychlor and genistein (p < 0.01 in both sexes with both compounds; two-way ANOVA, repeated measures). While the male consumed larger amounts of feed...
Feed Consumption, Body Weight, and Estimated Dose Levels of Dams and Male and Female Offspring Exposed to Genistein and Methoxychlor

### Table 1

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<tr>
<td>800 ppm</td>
<td>16.6 ± 1.5</td>
<td>23.5 ± 2.8</td>
<td>22.4 ± 3.4</td>
<td>29.5 ± 6.2</td>
<td>13.7 ± 0.8</td>
<td>30.3 ± 1.9</td>
<td>29.8 ± 2.7</td>
<td>11.7 ± 0.6</td>
<td>19.6 ± 2.3</td>
<td>18.5 ± 1.7</td>
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<td>300 ppm</td>
<td>10.3 ± 1.9</td>
<td>15.4 ± 1.9</td>
<td>18.0 ± 2.8</td>
<td>21.1 ± 4.4</td>
<td>13.0 ± 1.3</td>
<td>23.4 ± 1.6</td>
<td>23.4 ± 2.5</td>
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<td>300 G + 800 M</td>
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<td>19.9 ± 2.5</td>
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<td>12.9 ± 1.5</td>
<td>26.7 ± 3.8</td>
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<td>18.5 ± 1.2</td>
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<td>14.6 ± 0.9</td>
<td>13.9 ± 1.3</td>
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**Note.** Data are mean ± SD (n = eight litters for all groups); litter means were used for the offspring data.

*Feed consumption for the offspring was calculated for individual rats based on group consumption data. The offspring were group-housed up to four same-sex litters per cage from PND 21 to 55, and up to two same-sex litters per cage from PND 55 and on. Both genistein and methoxychlor reduced feed consumption in pregnant dams, male offspring, and female offspring (p < 0.05, two-way ANOVA, repeated measures). The genistein effect on the dam was effective only at the 800-ppm dose level but was effective on the male and female pups at both 300 and 800 ppm. The offspring data were analyzed with litters nested.

*Genistein (p < 0.05) but not methoxychlor (p < 0.07) reduced the body weight of the dams. The body weight of both male and female offspring was affected by 800 ppm methoxychlor and 800 ppm genistein (p < 0.01, two-way ANOVA, repeated measures).

*The dose level was calculated based on average group body weight and feed consumption for each litter in the treatment groups with their respective chemical contents. The presence of genistein did not affect the dose level of methoxychlor except for the male offspring during PND 28–31 (between 300 and 800 ppm); the presence of methoxychlor affected the dose level of genistein at 300 ppm for the male offspring (during PND 28–31) and for the female offspring (at both genistein dose levels during PND 55–58 and at 300 ppm genistein during PND 97–100; p < 0.05 in all cases, repeated measures and one-way ANOVA).

than the female, both sexes responded to treatment in a similar manner, i.e., the reduction of feed intake was seen at the adult stages but not at the prepubertal stage, and the suppressive effect of the two compounds on the feed intake was greater than each compound alone.

The body weights of the pregnant dams and the male and female offspring were closely correlated with their respective feed intake levels (Table 1). The body weights of the pregnant dams were significantly reduced in the methoxychlor-fed groups (p < 0.01; two-way ANOVA, repeated measures) but not in the genistein-fed group (p = 0.07). When the six treatment groups were compared for their difference in mean body weights by one-way ANOVA, the difference became significant starting at gestation days 3-7 and was persistent afterward.

The genistein and methoxychlor dose levels were calculated based on feed consumption over periods of 3–4 days at various developmental stages (Table 1). While the levels of exposure were relatively consistent for the pregnant dams, the dose levels were much higher for the offspring at the prepubertal
stage, when the ratio of feed intake to body weight ratio was high, than at adult stages, when this ratio gradually decreased. The presence of methoxychlor affected the levels of genistein intake only in the female offspring at 300 ppm genistein ($p < 0.01$; repeated measures ANOVA). The reason that the presence of methoxychlor had a limited effect on genistein intake levels was that the effects of methoxychlor on both food consumption reduction and growth inhibition were proportional, so that the body weight-based dose level did not vary drastically from methoxychlor- and nonmethoxychlor-treated rats. The presence of genistein did not affect intake levels of methoxychlor except for the male offspring during the PND day 28-31 period ($p < 0.01$, one-way ANOVA).

**Pregnancy Outcome**

Dams in all treatment groups completed pregnancy (Table 2). Neither methoxychlor nor genistein affected the number of implantation sites, as observed at necropsies of the dams. Comparison of litter sizes and the number of implantation sites for each dam indicated that embryo loss was minimal. The methoxychlor-treated dams had virtually the same levels of embryo loss (averaging less than one per litter with or without methoxychlor in the diet), whereas the genistein-treated dams had a trend ($p = 0.067$) to numeric decrease in embryo loss (1.64, 0.45, and 0.38 per litter for the 0, 300-ppm, and 800-ppm genistein groups, respectively). This slight downward trend in embryo loss was matched by a modest increase in litter sizes for the genistein-treated groups. The mean litter sizes were 10.6, 11.7, and 12.4 for the 0, 300-ppm, and 800-ppm genistein groups ($p = 0.051$), with the difference between the 0 and 800-ppm groups being significantly different ($p = 0.034$). The body weights of the male newborns were not affected by either treatment (Table 2; $p$ values were 0.17 and 0.06 for the genistein and methoxychlor treatment, respectively, two-way ANOVA). Both methoxychlor and genistein significantly decreased the body weight of the female newborns ($p < 0.01$ for both factors), with the effect of both the 300 and 800 ppm genistein being significant ($p < 0.01$). The interaction term of the two-way ANOVA for treatment effect on body weight was significant for both the male and the female ($p < 0.05$, male; < 0.01, female). This interaction was likely due to reduced susceptibility in body weight reduction to methoxychlor in the 800-ppm genistein groups. Meanwhile, the body weight of the dams at the end of lactation (PND 21) was also affected significantly by both genistein and methoxychlor ($p < 0.01$; data not shown); genistein exposure caused a significant difference in the body weights between groups 300 and 800 ppm ($p < 0.01$). The AGD at birth (Fig. 1), an indicator of masculinization during *in utero* development, was highly correlated with body weight in the males as expected ($p < 0.01$ for the

![Image](324x116 to 535x260)

**FIG. 1.** Anogenital distance of male and female pups on PND 1 following exposure to maternally administered genistein and methoxychlor throughout pregnancy. No statistically significant treatment effect was observed.
covariate term), but not in the females. There was no treatment-associated significant effect in male or female AGD.

**Prepubertal Growth**

A prominent effect of methoxychlor and genistein on the developing rats was a decrease in rate of body growth (Table 3). On PND 21, the body weights of both male and female offspring rats were lower in the methoxychlor- and 800 ppm genistein-treated groups ($p < 0.05$ and 0.01 for genistein and methoxychlor, respectively). The effect on the females was more severe than on the males, and methoxychlor affected body weight to a greater degree than genistein. The genistein effect was only seen at the 800-ppm level for both males and females ($p < 0.05$ and 0.01 for males and females, respectively). The 800 ppm methoxychlor alone reduced the body weights in a degree similar to the 800 ppm genistein treatment (approximately 15% reduction in both cases). A significant interaction between the two compounds was also seen in the body weight of male rats ($p < 0.05$) but not in the females. This significant interaction in the males was due to the fact that the combined effect of the two compounds was substantially smaller than the sum of the effect caused by the two compounds administered individually.

Male organ weights, including the liver, brain, ventral prostate, and testis, were not significantly affected by the treatment at PND 21, although liver and brain weights were highly correlated to body weight ($p < 0.01$). Similarly, the treatment did not affect the liver or brain organ weight in the female at PND 21. However, the mean uterus weights were more than doubled in the methoxychlor-treated rats compared with the nonmethoxychlor-treated rats ($p < 0.01$), whereas the genistein treatment did not result in a significant effect in this regard. This lack of genistein effect on uterus weight was unexpected, as we found previously that exposure to 1000 ppm genistein resulted in increased uterus weights (Casanova et al., 1999).

**Pubertal Sexual Development**

PPS was delayed in methoxychlor-treated offspring rats ($p < 0.01$) (Fig. 2). The means for groups with or without the presence of methoxychlor were 41.2 and 43.6 days, respectively (and were 40.9 and 43.8 when least square means were adjusted for body weight). Genistein by itself did not change the age of PPS when analyzed without covariate. However, a significant genistein effect was detected when body weight was used as a covariate ($p < 0.05$); the significant difference was seen at the 800-ppm ($p < 0.01$) but not the 300-ppm level.

<table>
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<tr>
<th>Diet</th>
<th>Male Body wt (g)</th>
<th>Male Liver (g)</th>
<th>Male Brain (g)</th>
<th>Male Ventral prostate (g)</th>
<th>Male Testis (g)</th>
<th>Female Body wt (g)</th>
<th>Female Liver (g)</th>
<th>Female Brain (g)</th>
<th>Female Uterus (g)</th>
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</tr>
<tr>
<td>300 G</td>
<td>51.0 ± 8.5</td>
<td>2.62 ± 0.69</td>
<td>1.53 ± 0.09</td>
<td>0.048 ± 0.021</td>
<td>0.150 ± 0.051</td>
<td>48.0 ± 6.3</td>
<td>2.30 ± 0.48</td>
<td>1.45 ± 0.07</td>
<td>0.049 ± 0.027</td>
</tr>
<tr>
<td>300 G + 800 M</td>
<td>38.7 ± 4.9</td>
<td>1.77 ± 0.30</td>
<td>1.44 ± 0.07</td>
<td>0.043 ± 0.025</td>
<td>0.113 ± 0.039</td>
<td>38.3 ± 3.1</td>
<td>1.70 ± 0.25</td>
<td>1.43 ± 0.09</td>
<td>0.089 ± 0.036</td>
</tr>
<tr>
<td>800 G</td>
<td>42.7 ± 3.2</td>
<td>2.02 ± 0.31</td>
<td>1.46 ± 0.07</td>
<td>0.052 ± 0.028</td>
<td>0.139 ± 0.049</td>
<td>41.5 ± 3.4</td>
<td>2.05 ± 0.19</td>
<td>1.39 ± 0.11</td>
<td>0.049 ± 0.022</td>
</tr>
<tr>
<td>800 G + 800 M</td>
<td>41.0 ± 5.0</td>
<td>1.96 ± 0.41</td>
<td>1.49 ± 0.06</td>
<td>0.047 ± 0.033</td>
<td>0.114 ± 0.013</td>
<td>38.5 ± 2.5</td>
<td>1.82 ± 0.34</td>
<td>1.42 ± 0.34</td>
<td>0.110 ± 0.026</td>
</tr>
</tbody>
</table>

**Note.** Data are means ± SD (n = seven to eight litters for each treatment group). One male and one female pup were sampled from each litter (n = 7–8). Testis weight was analyzed and is presented as the average of both sides.

*Both compounds reduced male and female body weight significantly ($p < 0.05$ for genistein at 800 ppm and $p < 0.01$ for methoxychlor for both sexes, two-way ANOVA); a modest but significant interaction between the two compounds in affecting the male body weight was detected ($p = 0.044$).

*Liver and brain weights in male and liver weights in female rats were highly correlated with their respective body weights ($p < 0.01$, two-way ANOVA with body weight as covariant); the weights of ventral prostate and testis in the male were not affected by either genistein or methoxychlor.

*Methoxychlor significantly increased uterine weight ($p < 0.01$, two-way ANOVA).
When analyzed according to the presence or absence of methoxychlor, control, 300 G, and 800 G showed very similar mean ages of PPS; however, the mean ages of PPS for 800 M, control, 300 G, and 800 G were significantly different than group 800 M ($p < 0.01$). These results indicated that genistein alone was not effective in delaying PPS, but enhanced the potency of methoxychlor in delaying male pubertal development when coadministered with methoxychlor.

VO in the females was monitored starting from postnatal 25 days, respectively ($p < 0.05$), two-way ANOVA with nested litters). When body weight was included as covariate, the organ weights of the testis and epididymis but not the ventral prostate were found to be reduced by methoxychlor ($p < 0.01$). Genistein did not significantly reduce the reproductive organ weights. Neither methoxychlor nor genistein affected the organ weights of males.

### TABLE 4

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body wt (g)$^a$</th>
<th>Ventral prostate (g)</th>
<th>Testis (g)$^b$</th>
<th>Epididymis (g)$^c$</th>
<th>Liver (g)</th>
<th>Brain (g)</th>
<th>Pituitary gland (mg)$^d$</th>
<th>Adrenal gland (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>569 ± 34</td>
<td>0.619 ± 0.084</td>
<td>1.86 ± 0.10</td>
<td>0.702 ± 0.066</td>
<td>23.3 ± 5.5</td>
<td>2.02 ± 0.10</td>
<td>12.3 ± 4.0</td>
<td>33.7 ± 3.9</td>
</tr>
<tr>
<td>800 M</td>
<td>399 ± 26</td>
<td>0.435 ± 0.076</td>
<td>1.63 ± 0.14</td>
<td>0.595 ± 0.047</td>
<td>16.5 ± 2.6</td>
<td>2.03 ± 0.13</td>
<td>12.9 ± 3.5</td>
<td>32.0 ± 2.8</td>
</tr>
<tr>
<td>300 G</td>
<td>563 ± 54</td>
<td>0.588 ± 0.073</td>
<td>1.81 ± 0.14</td>
<td>0.692 ± 0.063</td>
<td>22.1 ± 3.2</td>
<td>2.04 ± 0.11</td>
<td>11.3 ± 3.4</td>
<td>36.3 ± 4.0</td>
</tr>
<tr>
<td>300 G + 800 M</td>
<td>366 ± 34</td>
<td>0.384 ± 0.071</td>
<td>1.47 ± 0.13</td>
<td>0.531 ± 0.054</td>
<td>13.6 ± 1.6</td>
<td>1.94 ± 0.14</td>
<td>10.4 ± 3.6</td>
<td>31.1 ± 6.3</td>
</tr>
<tr>
<td>800 G</td>
<td>514 ± 35</td>
<td>0.602 ± 0.081</td>
<td>1.83 ± 0.11</td>
<td>0.684 ± 0.030</td>
<td>20.7 ± 3.4</td>
<td>2.04 ± 0.06</td>
<td>16.0 ± 3.5</td>
<td>31.4 ± 7.4</td>
</tr>
<tr>
<td>800 G + 800 M</td>
<td>373 ± 20</td>
<td>0.394 ± 0.053</td>
<td>1.49 ± 0.02</td>
<td>0.541 ± 0.036</td>
<td>13.4 ± 1.1</td>
<td>1.95 ± 0.16</td>
<td>12.3 ± 3.4</td>
<td>34.5 ± 6.2</td>
</tr>
</tbody>
</table>

*Note.* Data are means ± SD. Testis, epididymis, and adrenal weights were analyzed and are presented as the average of both sides. Body weight and organ weight of ventral prostate, testis, and epididymis are presented as means and standard deviations of litter average ($n = three$ for each litter; $n = six$ to eight litters per treatment group). The liver, brain, pituitary gland, and adrenal were sampled from one rat per litter.

$^a$Body weight was significantly reduced by both methoxychlor and 800 ppm genistein ($p < 0.01$ and 0.05, respectively, two-way ANOVA with nested litters).

$^b$Organ weight of testis and epididymis, but not ventral prostate, was significantly reduced by methoxychlor treatment ($p < 0.05$, two-way ANOVA with nested litters and body weight as covariant).

$^c$Pituitary weight in the 800-ppm genistein group was significantly greater than that of the control and 300-ppm genistein group ($p < 0.05$, two-way ANOVA and t-test with Bonferroni correction).
No treatment effect in regard to organ weights was associated with the two compounds (two-way ANOVA with body weight as covariant).

Genistein by itself did not cause an effect at this age, but its interaction with methoxychlor was significant (\( p < 0.05 \), two-way ANOVA), and the interactive term of the two factors in reducing body weight was also significant (\( p = 0.03 \)). The genistein effect was only significant at the 800-ppm level (\( p < 0.05 \), t-test with Bonferroni correction). No treatment effect in regard to organ weights was associated with the two compounds (two-way ANOVA with body weight as covariant).

The pituitary weight in the 800-ppm genistein group was significantly greater than that of the control and the 300 G group (\( p < 0.01 \)). The genistein effect was only significant at the 800-ppm level (\( p < 0.05 \), t-test with Bonferroni correction). No significant effect of genistein in altering the length of stages in the estrous cycles in pubertal rats was observed.

In the adult female offspring, feeding the methoxychlor- and genistein-containing diets was associated with marked changes in estrous cyclicity (Fig. 5). Similar to the observations in pubertal rats, methoxychlor prolonged the EP phase and decreased the M and D phases of the estrous cycles at PND 120 (\( p < 0.01 \) in all cases). In contrast with the pubertal stage, genistein treatment increased the estrus portions of the cycles in the nonmethoxychlor groups (300 G and 800 G) and decreased the portions of M and D in the methoxychlor-fed groups (800 M, 300 G + 800 M, and 800 G + 800 M; \( p < 0.01 \) in all cases). In regard to discontinuation of the treatment diets, no statistical difference could be found in any stage of the cycle between the rats continuously fed with the treatment diets and the ones whose treatment diet ended 4 weeks prior to the beginning of data collection on vaginal cytology (t-test). These

**TABLE 5**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body wt (g)</th>
<th>Uterus (mg)</th>
<th>Ovary (mg)</th>
<th>Brain (g)</th>
<th>Pituitary gland (mg)</th>
<th>Liver (g)</th>
<th>Adrenal gland (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>302 ± 32</td>
<td>526 ± 117</td>
<td>57.0 ± 17.1</td>
<td>1.81 ± 0.13</td>
<td>47.0 ± 28.6</td>
<td>10.87 ± 1.81</td>
<td>29.2 ± 10.6</td>
</tr>
<tr>
<td>800 M</td>
<td>254 ± 46</td>
<td>489 ± 106</td>
<td>45.8 ± 13.7</td>
<td>1.81 ± 0.11</td>
<td>51.7 ± 42.5</td>
<td>9.61 ± 1.41</td>
<td>30.8 ± 4.8</td>
</tr>
<tr>
<td>300 G</td>
<td>312 ± 22</td>
<td>612 ± 196</td>
<td>58.8 ± 8.4</td>
<td>1.90 ± 0.08</td>
<td>42.7 ± 28.3</td>
<td>11.63 ± 1.00</td>
<td>37.3 ± 6.6</td>
</tr>
<tr>
<td>300 G + 800 M</td>
<td>202 ± 14</td>
<td>447 ± 224</td>
<td>36.4 ± 8.0</td>
<td>1.77 ± 0.08</td>
<td>47.2 ± 31.2</td>
<td>7.48 ± 0.48</td>
<td>32.1 ± 7.9</td>
</tr>
<tr>
<td>800 G</td>
<td>278 ± 26</td>
<td>514 ± 160</td>
<td>59.6 ± 11.0</td>
<td>1.81 ± 0.13</td>
<td>41.1 ± 34.5</td>
<td>10.27 ± 1.04</td>
<td>34.4 ± 6.2</td>
</tr>
<tr>
<td>800 G + 800 M</td>
<td>211 ± 21</td>
<td>443 ± 135</td>
<td>38.0 ± 5.0</td>
<td>1.81 ± 0.04</td>
<td>55.2 ± 25.4</td>
<td>7.72 ± 1.03</td>
<td>27.8 ± 6.8</td>
</tr>
</tbody>
</table>

Note. Data are means ± SD. One female offspring rat per litter was sampled to collect data (\( n = 6 \) to 8 per diet group). Ovary and adrenal weights were analyzed and are presented as average of both sides. A two-way analysis of variance was used for body weight analysis. A two-way analysis of covariance with body weight as the covariate was used for the analyses of organ weight of the uterus, ovary, brain, pituitary gland, liver, and adrenal gland.

The body weight was reduced by both genistein and methoxychlor (\( p < 0.05 \) and 0.01, respectively, two-way ANOVA), and the interactive term of the two factors in reducing body weight was also significant (\( p = 0.05 \)). The genistein effect was only significant at the 800-ppm level (\( p < 0.05 \), t-test with Bonferroni correction). No treatment effect in regard to organ weights was associated with the two compounds (two-way ANOVA with body weight as covariant).

**Estrous Cyclicity**

Vaginal smear samples were collected from each female offspring for 14 days immediately following identification of VO. Stages of proestrus (P), estrus (E), metaestrus (M), and diestrus (D) were identified. We also included a stage of proestrus-estrus (PE) to represent cytological characteristics similar to both proestrus and estrus stages (Fig. 4). Methoxychlor treatment increased the proportion of time that the animals experienced estrus (E and PE stages) (\( p < 0.01 \); two-way ANOVA with litter nested) and decreased the proportion of time that the animals were in diestrus (\( p < 0.01 \)). No significant effect of genistein in altering the length of stages in the estrous cycles in pubertal rats was observed.

In the adult female offspring, feeding the methoxychlor- and genistein-containing diets was associated with marked changes in estrous cyclicity (Fig. 5). Similar to the observations in pubertal rats, methoxychlor prolonged the EP phase and decreased the M and D phases of the estrous cycles at PND 120 (\( p < 0.01 \) in all cases). In contrast with the pubertal stage, genistein treatment increased the estrus portions of the cycles in the nonmethoxychlor groups (300 G and 800 G) and decreased the portions of M and D in the methoxychlor-fed groups (800 M, 300 G + 800 M, and 800 G + 800 M; \( p < 0.01 \) in all cases). In regard to discontinuation of the treatment diets, no statistical difference could be found in any stage of the cycle between the rats continuously fed with the treatment diets and the ones whose treatment diet ended 4 weeks prior to the beginning of data collection on vaginal cytology (t-test).
results suggest that treatment-caused alterations in estrous cycling regulation may be irreversible.

Pathological Evaluations

Sections of adrenal gland, liver, pituitary gland, and testis tissues from male animals of this study had no significant histological changes associated with treatment. In females, microscopic lesions were limited to the ovarian tissues in methoxychlor-treated animals (800 M, 300 G + 800 M, and 800 G + 800 M). These changes consisted of various degrees of ovarian atrophy and cyst formation. Ovaries were characterized by the presence of multiple large antral and early cystic follicles and a paucity of corpora lutea (Fig. 6A). Groups of numerous small and primordial follicles were aggregated within the ovary of these animals (Fig. 6B).

Neurobehavioral Testing

Motor activity data for the female and male rats are presented in Figures 7A and 7B, respectively. For clarity, error bars have not been presented in these figures; however, the coefficient of variation for saline-treated rats ranged from 19 to 187% with a mean (± SD) value of 98 ± 57%. For amphetamine-treated rats the coefficient of variation ranged from 7 to 54% with a mean (± SD) value of 29 ± 11%. A statistically significant, gender-related effect on overall motor activity was
observed following either saline ($p = 0.009$) or amphetamine ($p < 0.001$) treatment. Female rats displayed a higher amount of motor activity during each test session when compared with their male littermates. Locomotor activity was increased in both male and female rats given amphetamine when compared to their activity following saline pretreatment ($p < 0.001$, paired student t-test). No statistically significant, treatment-related effects on overall motor activity were observed during either motor activity test session.

**Interactions of Genistein and Methoxychlor with the Estrogen Receptors and Androgen Receptor**

In the HepG2 cell transfection experiments, genistein alone activated ERβ in a dose-dependent manner. The primary estrogenic metabolite of methoxychlor HPTE shifted the genistein dose-response curve to the right (Fig. 8A), showing an ERβ antagonist property. Genistein also activated ERα (Fig. 8B). Coadministration of HPTE with genistein resulted in additional estrogenic activity at ERα; however, at high concentrations ($3 \times 10^{-6} \text{ M}$), HPTE tended to attenuate the effect of genistein on ERα (Fig. 8B). In the AR-activation assay, HPTE shifted the dose-response curve of dihydrotestosterone (DHT) activation of AR to the right, showing an AR antagonist property as previously reported (Gaido et al., 1999); see Fig. 9A. The presence of genistein did not change the antagonist behavior of HPTE at the AR (Fig. 9B). The dissociation equilibrium constant ($\rho K_b$) for HPTE was $6.42 \pm 0.17$ without genistein and was $6.64 \pm 0.17$ with genistein.

**DISCUSSION**

The influence of phytoestrogens on the toxicological actions of synthetic endocrine-active compounds (EAC) is important in understanding the potential health impact of both classes of compounds. This study evaluated the potential for interaction between the phytoestrogen genistein and the pesticide methoxychlor on reproductive development in the rat. As expected, both compounds were endocrine-active, as indicated by their ability to alter sexual and reproductive development of exposed male and female rats.

Both genistein and methoxychlor potentially have multiple sites of action. Both compounds interact with ERα as well as ERβ. Genistein is more potent than methoxychlor in activating the ERs (Kuiper et al., 1998; Legler et al., 1999). Methoxychlor preferentially activates ERα, while genistein preferentially activates ERβ (Kuiper et al., 1998). A major metabolite of methoxychlor, HPTE, is an ERα agonist and ERβ antagonist (Gaido et al., 1999). In addition, HPTE is an AR antagonist and an inhibitor for the steroidogenic enzyme cholesterol side-chain cleavage enzyme (P450sscc; Akingbemi et al., 2000; Gaido et al., 1999). Genistein is also known to be an inhibitor of tyrosine kinase, which is believed to be important in transducing the estrogenic signals (Koroma and de Juan, 1997). These receptor and enzyme reactive properties may not only be the basis for the endocrine activities of two compounds acting individually but may also constitute potential mechanisms for one compound to influence the biological effects of the other.

**Estrogenic Actions of Methoxychlor and Genistein**

The most prominent effect of genistein and methoxychlor demonstrated in our study was related to their estrogenic activity. Both compounds were effective in accelerating VO in female rats. Genistein was effective in this regard at the 300-ppm as well as the 800-ppm dose level. The calculated intake of genistein for the offspring rats ranged approximately from 45 mg/kg/day during the prepubertal growth stage to 15 mg/kg/day during the postpubertal adult age. In comparison, infant
intake of isoflavones from soy-containing formulas is estimated to be between 5.7 and 11.9 mg/kg/day (Setchell et al., 1998). Methoxychlor was more effective than genistein in accelerating VO. For example, rats given 800-ppm methoxychlor reached 100% VO by PND 27, whereas rats fed the 800-ppm genistein diet did not achieve 100% VO positive until PND 35.

A greater estrogenic potency of methoxychlor than genistein was also reflected in the change in vaginal estrous cycles. The percentage of days in estrus (E, including the PE phase) shifted toward higher values in the order of 800 M > 800 G > control in both pubertal and adult female offspring, indicating an increasing degree of persistent estrus. The differences between control and 300 G and between 800 M and 300 G were minimal; thus the 300 ppm genistein was not effective in this regard. In addition, treatment effects on vaginal estrous cyclicity were persistent. The one-month discontinuation of treatment did not result in any recovery towards normal estrous cycles in the affected groups, suggesting that methoxychlor treatment results in long-lasting changes in the hypothalamus-pituitary-gonad regulatory circuits. However, the longer-term reversibility of these estrogenic effects following termination of treatment is unknown. As methoxychlor is excreted rapidly, with approximately 100% elimination through feces and urine within 48 h after oral dosing to mice (ATSDR, 2000), the estrous cycle effects were unlikely due to direct action of methoxychlor residue in the body.
The difference in the \textit{in vivo} estrogenic potencies of these two compounds was in contrast to their potencies in interacting with the ER-based \textit{in vitro} experiments. Ligand-dependent transcriptional activation assays rank genistein as more potent than methoxychlor in activating the ER\(\alpha\) and ER\(\beta\) (Kuiper et al., 1998; Legler et al., 1999) and in causing estrogen-responsive gene expressions in cultured cells (Jorgensen et al., 2000). The present study clearly demonstrated that methoxychlor was more potent than genistein in causing estrogenic responses in the whole animal (i.e., advancing VO and causing persistent estrus). The methoxychlor metabolite HPTE is known to be more estrogenic than the parent compound (Cummings, 1997). The EC50 value for HPTE to activate ER\(\alpha\) in an \textit{in vitro} transcriptional activation assay was 51 nM (Gaido et al., 1999), which was similar to that of genistein at 31 nM (Casanova et al., 1999). Genistein was more potent at ER\(\beta\), with an EC50 value of 4.9 nM (Casanova et al., 1999), than HPTE, which displays antagonist activity at ER\(\beta\) (Gaido et al., 1999). Overall, the differences between the ER reactivities of genistein and methoxychlor do not explain the \textit{in vivo} potency differences of the two compounds. While the \textit{in vitro} potency was more likely indicative of receptor reactivities of the administered compounds, the \textit{in vivo} potencies of the compounds are influenced by their pharmacokinetics and metabolism. Genistein is extensively metabolized to glucuronide conjugates and other metabolites (Piskula, 2000), which likely do not maintain the same level of reactivity as the parent compound at the ERs. In addition, elimination of genistein from dosed animals is rapid. When injected iv at 25 mg/kg, genistein plasma concentration decreased from 30.8 to 0.036 \(\mug/ml\) during 3 to 150 min postinjection in the mouse (Supko and Phillips, 1995).

Both compounds at the 800-ppm level inhibited body weight gain, and the effect of methoxychlor was more severe than genistein in this regard. While retardation in body growth is often indicative of systemic toxicity, the estrogenicity of the compounds might have contributed to reduction in feed intake due to the appetite-repressing action of estrogen (Wade, 1975). In addition, palatability of methoxychlor could also be a factor in the reduction of feed intake.

\textbf{Antiandrogenicity and Other Effects}

In the present study, methoxychlor treatment delayed the age of PPS an average of 2.5 days. Gray et al. (1989) demonstrated that daily gavage of methoxychlor from weaning to PND 45 delayed PPS for 3–4 days at the dose of 100 mg/kg. Treatment with this compound at higher concentrations caused even more severe delays (Gray et al., 1999). The dietary concentration of methoxychlor used in the present study resulted in a calculated dose of approximately 130 mg/kg/day during the prepubertal period (PND 28–31), 100 mg/kg/day during puberty (around PND 40), and approximately 70 mg/kg during the postpubertal period (PND 55–58). The magnitude of PPS delay in this study was comparable to the study by Gray et al. (1989), but was much smaller than an 8-day delay reported by Chapin et al. (1997) that was caused by methoxychlor at 50 mg/kg/day given via gavage to pregnant and lactating dams from GD 14 to PND 7 and then to the pups directly from PND 7 to 42.

While delayed onset of PPS and reduced organ weights for epididymis and testis in the adult male rats are evidence for the antiandrogenicity of methoxychlor, neither Chapin et al. (1997) nor we in the present study observed an effect of this compound on AGD, an androgenic response in newborn pups. This lack of effect on AGD is unlike some other weak environmental antiandrogens such as DDE and vinclozolin, which cause demasculinizing effects in the newborn male pups at high doses, as indicated by reduced AGD (Kelce et al., 1998; You et al., 1998). This apparent lack of antiandrogenic efficacy at the fetal stage may be due to the fact that methoxychlor itself has little ability to antagonize physiological ligands of AR (Gaido et al., 2000), and the antiandrogenicity of methoxychlor is a consequence of its metabolism to HPTE. Conversion of methoxychlor to the antiandrogenic HPTE requires demethylation reactions mediated by cytochrome P450 enzymes (ATSDR, 2000), whose expression in the fetus may not be sufficient to ensure the production of HPTE at a significant rate.

Estrogens are able to exert protection to the central nervous system against certain neurodegenerative processes and neurotoxicological insults (Garcia-Segura et al., 2001; Yu and Wagner, 1994). Speculation has arisen regarding the ability of environmental estrogens to affect an animal’s susceptibility to neurotoxicants (Gursoy et al., 2001). Motor activity is known to be sexually dimorphic, and ovarian steroids play a major role in shaping this gender difference (Van Hartesveldt and Joyce, 1986). Gray et al. (1988) demonstrated that exposure of female Long-Evans hooded rats to methoxychlor (400 mg/kg/day) from PND 22 through PND 58 was associated with increased running wheel activity. This finding was also observed in ovariectomized rats, suggesting that methoxychlor exposure could maintain this estrogen-dependent behavior. In the present study, developmental exposure to methoxychlor did not affect locomotor activity in male or female offspring. Our findings with genistein were similar to those reported by Flynn and coworkers (2000) showing that developmental exposure of pregnant Sprague-Dawley rats to genistein at maternally toxic levels (1250 ppm) did not alter either running wheel or open field activity in their prepubertal or postpubertal offspring. In addition, we did not find that exposure to either genistein or methoxychlor significantly affected the animals’ response to the CNS-stimulating effects of amphetamine. Our negative results must be viewed with some caution, however, as locomotor activity is known to be highly variable. Our group sizes, designed primarily for studying reproductive development, were smaller than those used in most behavioral studies (MacPhail et al., 1989; Maurissen and Mattsson, 1989), where the variations tend to be greater.
**Interactions between Genistein and Methoxychlor**

The estrogenic responses to genistein and methoxychlor were highly consistent in the measurements of VO and estrous cyclicity. Overall, the estrogenic actions of the two compounds, when given in combination, were apparently greater in their magnitude than the effects associated with each compound alone, at least for a genistein dose above 300 ppm. In the case of VO, although our data collection did not start early enough to include full time courses for the methoxychlor-containing diet groups, the differences in the proportions of VO-positive rats in 800 M, 300 G + 800 M, and 800 G + 800 M on PND 25 nonetheless indicated an accumulation of the effect associated with the two compounds.

A different type of interaction was observed in the measurement of pubertal sexual development in male rats. Genistein did not alter the age of PPS by itself but increased the PPS-delaying potency of methoxychlor. This is an interaction different in nature from the estrogenic effects of the two compounds. The results from the ERα-, ERβ-, and AR-dependent transcriptional assays supported the estrogenic actions of genistein and HPTE and the antiandrogenic action of HPTE. We did not, however, observe a potentiation by genistein of the antiandrogenicity of HPTE in the AR-dependent transcriptional activation assay. At present, we do not know what the mechanistic basis is for genistein to influence this response, nor do we know whether similar interaction may be ensued between genistein and other antiandrogenic compounds.

**Summary**

Coadministration of dietary genistein and methoxychlor to developing rats in the present study resulted in greater estrogenic effects than either compound alone. Genistein also potentiated an effect of methoxychlor in delaying PPS even though genistein alone was not effective in this regard. Our data on steroid hormone receptor-dependent transcriptional activation by genistein and HPTE supported the estrogenic action associated with genistein and methoxychlor and the antiandrogenic action associated with methoxychlor on sexual and reproductive development in the offspring rats. However, the in vitro receptor activation data could not explain the greater estrogenic action of methoxychlor than genistein and the potentiation of methoxychlor antiandrogenicity by genistein. These results indicate that the interplay between phytoestrogens and synthetic EACs can be highly complex, and factors other than reactivity with sex hormone receptors may be responsible for some of the biological effects of these compounds.

While serving our purpose of providing reference end points, the high dose of methoxychlor used in the present study clearly does not represent levels of environmental exposure for humans and wildlife. The nature of interactions between such endocrine-active compounds at low doses may differ from our observations in the present study. What we believe to be the case, however, is that dietary phytoestrogens do possess the ability to affect endogenous endocrine signals at dose levels relevant to human and animal exposure. Consequently, the presence of phytoestrogens in the diet of experimental animals needs to be fully considered as part of the experimental conditions, particularly when such experiments are aimed at evaluating endocrine properties of test compounds.

**ACKNOWLEDGMENTS**

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**REFERENCES**


ATSDR (2000). Toxicological Profile for Methoxychlor (update; draft for public comments). Agency for Toxic Substances and Disease Registry, Atlanta, GA.


