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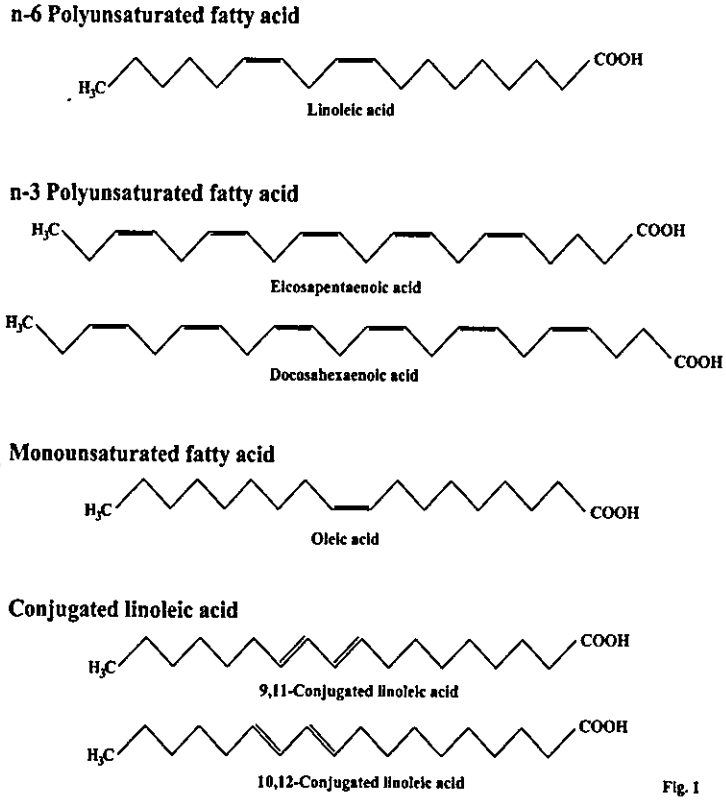

Fig. 1 Structures of n-6 and n-3 polyunsaturated fatty acids, monosaturated fatty acid, and conjugated linoleic acids that affect mammary carcinogenesis. The terms "n-6" and "n-3" describe the position of the double bond nearest to the terminal methyl group, and the terms "polyunsaturated" and "monosaturated" indicate the number of double bonds. The two double bonds in conjugated linoleic acid are at positions 9 and 11, or 10 and 12 (compare with parent linoleic acid), and each of the double bonds can be in the cis or trans configuration. The most common isomer in the diet is cis-9, trans-11.

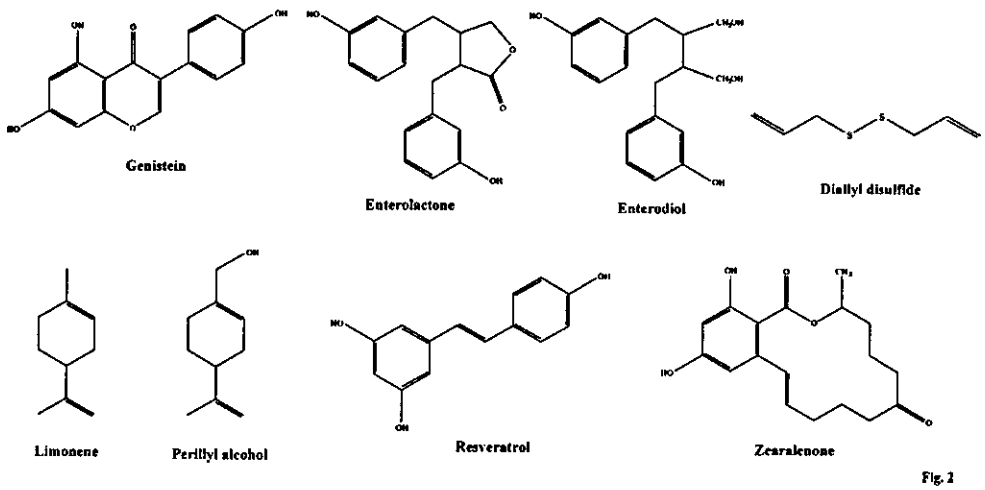

Fig. 2 Structures of chemicals derived from plants or produced by molds, that affect mammary carcinogenesis.

Table I. The 50% inhibitory concentration values of food chemicals with which breast-derived cell lines were incubated for 72 hours

Chemical	Cell line	Cell type	Estrogen receptor	IC_{50} (μM)	References
Eicosapentaenoic acid	MCF-7	Human breast carcinoma	+	562	26
	T-47D	Human breast carcinoma	+	234	
	KPL-1	Human breast carcinoma	+	669	
	MDA-MB-231	Human breast carcinoma	-	209	
	MKL-F	Human breast carcinoma	-	434	
Docosahexaenoic acid	KPL-1	Human breast carcinoma	+	270	28
Conjugated docosahexaenoic acid	KPL-1	Human breast carcinoma	+	97	28
Genistein	DD-762	Murine mammary tumor	-	7	40
	Sm-MT	Insectivora mammary tumor	-	86	
	MCF-7	Human breast carcinoma	+	274	
	MDA-MB-231	Human breast carcinoma	-	131	
	HBL-100	Human breast epithelial cell	-	100	
Enterolactone	MCF-7	Human breast carcinoma	+	82*	52
	MDA-MB-231	Human breast carcinoma	-	>100*	
Diallyl disulfide	MCF-7	Human breast carcinoma	+	4	64
	KPL-1	Human breast carcinoma	+	12	
	MDA-MB-231	Human breast carcinoma	-	2	
	MKL-F	Human breast carcinoma	-	18	
Perillyl alcohol	KPL-1	Human breast carcinoma	+	720	72
	MKL-F	Human breast carcinoma	-	993	
Resveratrol	MCF-7	Human breast carcinoma	+	137	81
	KPL-1	Human breast carcinoma	+	149	
	MKL-F	Human breast carcinoma	-	105	

*IC_{50} for 24 hours

Effects of Prepubertal Exposure to Xenoestrogen on Development of Estrogen Target Organs in Female CD-1 Mice

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Key words: Genistein, Resveratrol, Zearalenone, Zeranol, Bisphenol A, Diethylstilbestrol, CD-1 mouse, Prepubertal

Running title: Prepubertal xenoestrogen in mice

Abstract. *Background*: There have been no previous reports comparing the effects of prepubertal xenoestrogen exposure on development of the reproductive tract and mammary glands in female mice. Effects of genistein (GEN), resveratrol (RES), zearalenone (ZEA), zeranol (ZER), bisphenol A (BPA) and diethylstilbestrol (DES) were examined. *Materials and Methods*: Beginning at 15 days of age, female CD-1 mice were administered 4 daily subcutaneous injections of 10 mg/kg/day of GEN, RES, ZEA, ZER or BPA, or 10 μg/kg/day of DES dissolved in dimethylsulfoxide (DMSO), or DMSO vehicle. Vaginal opening was checked; estrous cyclicity was monitored from 5, 9 or 21 weeks of age for 21 consecutive days; and 6 animals per group were autopsied at 4, 8 and 24 weeks of age. *Results*: Prepubertal exposure to GEN, ZEA, ZER and DES (but not RES or BPA) accelerated puberty onset (vaginal opening). Vaginal smears indicated that all xenoestrogen-treated mice were cycling, but ZEA-, ZER- and DES-treated mice spent more time in estrus. At 4 weeks of age, absence of corpora lutea (anovulatory ovary) was observed in untreated controls (33%, 2/6) and the GEN (50%, 3/6) and RES (50%, 3/6), ZEA (100%, 6/6), ZER (100%, 6/6), BPA (83%, 5/6) and DES groups (100%, 6/6). At 8 weeks of age, absence of corpora lutea was observed in the ZEA (33%, 2/6) group. Corpora lutea were present in all mice sacrificed at 24 weeks of age. Groups that received prepubertal xenoestrogen injections exhibited no morphological abnormalities of the uterus and vagina, and exhibited mammary gland growth similar to that of the untreated controls at all time points. *Conclusions*: GEN, ZEA, ZER and DES (but not RES or BPA) caused early vaginal opening; mice exposed to ZEA, ZER or DES spent more time in estrus phase; and ZEA-treated mice had a longer period of anovulatory ovary than other xenoestrogen-treated mice; however, none of the xenoestrogens tested altered uterine or vaginal morphology or mammary gland growth.

Introduction

Xenoestrogens (chemicals with estrogenic activity) are endocrine-disrupting chemicals that include naturally occurring substances produced by plants (phytoestrogens) and molds (mycoestrogens) and man-made chemicals released into the environment (1). They may be ingested directly in plant material, in the tissues of animals that ingest xenoestrogen-producing plants or plants infected by xenoestrogen-producing molds, or in foodstuffs contaminated by release of xenoestrogens from polycarbonate plastic plates. Exposure to xenoestrogens during critical stages of growth can interfere with the development and differentiation of estrogen target organs (2). These effects can be severe, particularly in

prepubertal children, whose endogenous estrogen concentration is low (3). There is evidence that exposure of humans to the xenoestrogen diethylstilbestrol (DES; (E)-3,4-bis (4-hydroxyphenyl)-3-hexene) alters development of estrogen target organs. *In utero* exposure to DES as an antiabortive has been found to induce clear cell adenocarcinoma of the vagina in daughters after puberty (4). Although the DES-exposed daughters in that study had low risk of development of clear cell adenocarcinoma (<1%), DES is associated with increased frequency of benign reproductive tract dysfunction and structural abnormality. Many experiments using rodent models have shown strikingly similar abnormalities after exposure to DES in early life (5); mouse models have proven to be effective for examination of abnormalities in estrogen target organs.

Naturally occurring and man-made chemicals that exhibit estrogenic biologic activity are widely distributed in the environment (1). Among the chemicals that exhibit such activity are genistein (GEN), resveratrol (RES), zearalenone (ZEA), zeranol (α-zearalanol; ZER) and bisphenol A (BPA). GEN (4', 5, 7-trihydroxy isoflavone) is a major component of soy-based foods. It is estimated that infants who consume a diet of soy-based formulas are exposed to 6 to 9 mg/kg/day of soy isoflavones, with GEN comprising more than 65% of these isoflavones by weight (6). It has been estimated that infants fed soy infant formula are exposed to GEN at a dose level of 4 mg/kg/day (7). The main sources of RES (trans-3, 4', 5-trihydroxystilbene) include grapes and red wine (8). A person who drinks one glass of red wine per day consumes ~0.02 mg/kg/day of RES (9). ZEA (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resocylic acid-lactone) is a mycotoxin synthesized by *Fusarium* mold, and is present as a natural contaminant in food as a result of infection of grain by *Fusarium* species. Human exposure to ZEA in the United States is ~0.1 mg/kg/day (10). ZER (6-6, 10-dihydroxyundecyl-β-resorcylic acid lactone) is a natural metabolic product of ZEA (11). In the United States, ZER and ZEA have been widely used to promote growth of livestock, due to their potent anabolic effects (12). BPA (4,4'-isopropylidenediphenol), an industrial chemical that exhibits estrogenic action, is a monomer used in the manufacture of many chemical products including the interior lining of food and beverage cans, dental sealants, and polycarbonate plastic products including baby bottles. Human exposure to environmental BPA is ~0.25 mg/kg/day (13). Developmental exposure to estrogenic chemicals induces morphological and functional abnormalities in estrogen target organs. Adverse effects of estrogenic chemicals on reproductive organs and mammary glands at crucial stages of development are a matter of concern. A recent study examined effects of prenatal exposure of mice to xenoestrogens at doses comparable to typical human exposure and doses 20 times greater than typical human exposure (14). However, effects of prepubertal exposure to these xenoestrogens have not been examined. There is a need to evaluate the effects of xenoestrogens at different stages of development, to clarify their effects on reproductive organs and mammary glands.

In the present study, to compare effects of early exposure to various xenoestrogens, prepubertal female mice were injected subcutaneously (sc) with 1 of several xenoestrogens once daily at a dose of 10 mg/kg/day for 4 consecutive days, beginning at 15 days of age. DES, a model xenoestrogen, was used as a positive estrogenic control, and was administered at doses approximately 1/1000 of those of the other xenoestrogens (10 μg/kg/day). The present doses of test chemicals were based on our previous findings (14). The aim of the present study was to evaluate effects of prepubertal exposure to xenoestrogens on development of the female reproductive system and mammary glands in CD-1 mice. We found that prepubertal exposure to any of several estrogenic chemicals induces early vaginal opening and disrupts the estrous cycle. However, permanent morphological alteration of reproductive organs was not observed, and mammary gland development was not affected.

Materials and Methods

Test chemicals. GEN was purchased from Fujicco (Kobe, Japan); ZER was purchased from Wako Pure Chemical (Osaka, Japan); and RES, ZEA, BPA and DES were obtained from Sigma (St. Louis, MO). The purity of all test chemicals was ≥99%. All chemicals arrived in powder form, and were kept at 0°C in the dark. Immediately before use, each chemical was dissolved in dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto), and stored at 4°C.

Animals. Fourteen-day-old outbred Crj:CD-1 (ICR) female mice (10 pups per nursing mother) were purchased from Charles River Japan (Atsugi). The room in which the animals were housed was kept at 22±2°C and 60±10% humidity, with a 12 hr light/dark cycle. To avoid exposure to endocrine-disrupting chemicals, mice were housed in standard mouse polyisopentene cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River, Yokohama) as bedding. To avoid exposure to dietary phytoestrogens, mice were fed a low-phytoestrogen diet (NIH-07 PLD; Oriental Yeast, Chiba, Japan); NIH standard dietary pellets (NIH-07 open formula) contain phytoestrogens from soy products and alfalfa (15). Water was supplied in polycarbonate bottles with rubber stoppers. Thus, exposure to known environmental endocrine-disrupting agents was minimized.

Experimental procedures. Beginning at 15 days of age, female mice were given 4 daily sc injections of 10 mg/kg/day of GEN, RES, ZEA, ZER or BPA, 10 μg/kg/day of DES, or the DMSO vehicle alone (untreated control). Doses were adjusted daily according to body weight, to provide constant dose levels. The mice were weaned at 21 days of age. Timing of vaginal opening was recorded; vaginal smears were taken for 21 consecutive days beginning at 5, 9 and 21 weeks of age; and the estrous cycle was monitored. In each group, body weight was recorded every week. At 4, 8, 12 and 24 weeks of age, 6 randomly selected mice from each group were weighed, anesthetized, sacrificed by cervical dislocation, and autopsied. At sacrifice, the ovaries, uterus, vagina and the inguinal mammary glands from one side were fixed in 10% neutral buffered formalin. Mid-uterine transverse segments, vaginal transverse segments, the center of each ovary, and inguinal mammary glands were sectioned (thickness, 4 μm) and stained with hematoxilin and eosin (HE). Ovaries were analyzed histologically for the presence or absence of the corpus luteum and polyovular follicles (16). The inguinal mammary glands from the remaining side were processed for whole-mount preparation, and the degree of growth and differentiation was evaluated. The degree of differentiation of inguinal mammary glands was assigned a score ranging from 1 to 4, using previously reported criteria (14), as follows: Score 1, little differentiation, terminal end buds (TEBs) in the periphery with lateral buds but no alveolar development; Score 2, small number of alveoli in poorly developed ductal tree; Score 3, intermediate development of alveolar structure; Score 4, high degree of development, and lobulo-alveolar formation in the gland. Our experimental protocol was approved by the Animal Experimentation Committee, Kansai Medical University.

Statistical analysis. All data were expressed as mean±S.E. After assurance of homogeneity of variance, analysis was performed using the non-repeated measure ANOVA parametric test or Kruskal-Wallis non-parametric test. If the p value of these pre-tests was <0.05, post-hoc analysis was performed using Fisher's protected least significant difference test. Differences between groups were considered significant if the p value was <0.05.

Results

Body weight gain in female CD-1 mice. Prepubertal exposure to test chemicals did not influence body weight gain, compared with untreated controls (Fig. 1). At 24 weeks of age, body weights of all groups were comparable.

Vaginal opening. The GEN, ZER, ZEA, and DES groups exhibited accelerated timing of vaginal opening, compared with untreated controls (Fig. 2). Vaginal opening was accelerated

by 3 to 7 days in the GEN, ZER, ZEA and DES groups ($p<0.01$, respectively) (Table I). In contrast, the RES and BPA groups were similar to the untreated group.

Estrous cycle. All untreated controls exhibited a regular cycle during 5-8, 9-12 and 21-24 weeks of age (Table II). Although vaginal cycling was observed in all xenoestrogen-treated mice, the time spent in the estrus phase was significantly longer in the ZEA, ZER and DES groups than in untreated controls.

Reproductive tract structure. Ovarian histology of mice sacrificed at 4 weeks of age revealed absence of corpora lutea in untreated controls (2/6) and the GEN, RES, ZEA, ZER, BPA and DES groups (3/6, 3/6, 6/6, 6/6, 5/6 and 6/6, respectively). At 8 weeks of age, absence of corpora lutea was only observed in the ZEA group (2/6) (Fig. 3). Corpora lutea were present in all mice sacrificed at 24 weeks of age. However, polyovular follicles were not observed in xenoestrogen-treated mice or untreated controls. The test chemicals produced no morphological abnormalities in the uterus or vaginal epithelium.

Mammary gland development. In untreated control mice, growth of the mammary ductal tree progressed normally with increasing age. In untreated controls at 4 weeks of age, TEBs were observed at the periphery, but alveolar differentiation was unclear (Fig. 4a; Score 1). At 8 and 24 weeks of age, alveoli and lobuli were observed in the untreated controls, but the degree of development varied somewhat among the animals. In some animals, the mammary glands were relatively poorly differentiated, with few alveoli (Fig. 4b; Score 2); others exhibited somewhat greater alveolar development (Fig. 4c; Score 3); and some exhibited complete lobulo-alveolar development (Fig. 4d; Score 4). At 4, 8 and 24 weeks of age, none of the xenoestrogen-treated mice exhibited adverse effects on growth or differentiation of mammary glands. Scores of mammary gland differentiation after prepubertal xenoestrogen exposure are summarized in Fig. 5.

Discussion

In the present study, prepubertal exposure to xenoestrogens (phytoestrogens, mycoestrogens and industrial chemicals) produced various degrees of functional and structural alteration in the reproductive tract of female CD-1 mice; mammary gland growth and differentiation were unaffected. In our previous studies, prenatal exposure to GEN, RES, ZEA, BPA or DES accelerated body weight gain at 16 weeks of age (14). In the present study, all xenoestrogen-treated groups exhibited body weight gain that was comparable to that of the untreated controls.

Prepubertal exposure of adult rats to estrogen has previously been shown to accelerate vaginal opening and disrupt estrous cyclicity (17). In rats, prepubertal exposure to 30 mg/kg GEN, 100 mg/kg RES, 10 mg/kg ZEA or 0.1 mg/kg ZER causes earlier vaginal opening (18-21). Neonatal exposure to 0.4 mg/kg GEN or 0.04 mg/kg ZEA causes earlier vaginal opening in mice (22). In the present study, prepubertal exposure of mice to GEN, ZEA, ZER or DES caused significantly earlier vaginal opening (which is consistent with previous xenoestrogen studies), whereas RES and BPA had no such effect. Thus, RES and BPA appear to be less estrogenic than GEN, ZEA, ZER and DES.

In a previous study, exposure of female mice to DES during their first 5 days of life decreased the frequency of presence of corpora lutea at 13 months of age (23). In another study, prenatal exposure of female mice to 10 mg/kg BPA significantly reduced the frequency of presence of corpora lutea at 30 days of age, but 91% of the BPA-treated mice exhibited a normal estrous cycle at 41 to 70 days of age, and all were fertile at 90 days of age; suggesting transient delay of ovulation (24). In the present study, at 4 weeks of age, absence of corpora lutea was observed in several xenoestrogen-treated mice and untreated controls. However, at 8 weeks of age, corpora lutea were present in all groups other than the ZEA-treated mice (2/6); at 24 weeks of age, corpora lutea were present in all groups. ZER has previously been

found to have greater estrogenic potential than ZEA (25). However, in the present study, injections of ZER produced a longer duration of absence of corpora lutea (anovulatory ovary) than ZEA treatment. In a previous study, among mice exposed to ZEA prenatally, ovaries without corpora lutea were observed at 4, 8, 12 and 16 weeks of age (83%, 5/6; 100%, 6/6; 83%, 5/6; 33%, 2/6, respectively), with the frequency of absence of corpora lutea decreasing with increasing age (14). In humans, absence of corpora lutea is the most frequent cause of female infertility (26). Polyovular follicles have previously been observed in mice exposed neonatally to DES or genistein (27, 28). However, in the present study, polyovular follicles were not observed in any of the xenoestrogen-treated mice.

Neonatal administration of 40 mg/kg GEN has previously been shown to induce permanent estrus in female mouse pups (7); 4 mg/kg GEN caused no such effects. Perinatal or prepubertal exposure of rats to RES causes estrous cycle abnormalities (29), and causes an increase in the percentage of time spent in the estrus phase (19). Prenatal or neonatal administration of ZEA to rats and mice induces abnormal vaginal cyclicity by prolonging estrus (30, 31); irregular estrous cycle with prolonged estrus precedes persistent estrus (32). Prenatal and neonatal administration of estrogenic chemicals to female animals can abolish luteinizing hormone (LH) surges (33). The persistent estrus induced in animals exposed to ZEA may be the result of an inability to produce an LH surge. Exposure of mice to the estrogenic chemical coumestrol throughout their entire lactation period produces an acyclic condition in early adulthood, resembling premature anovulatory syndrome (34). In the present study, ZEA, ZER and DES increased the length of the estrous cycle by prolonging estrus phase.

Neonatal exposure of mice to DES causes squamous metaplasia of the uterine gland later in life (23). Treatment of mice with DES (1 μg/kg/day) or GEN (50 mg/kg/day) at 1 to 5 days of age has been shown to cause considerable numbers of uterine adenocarcinomas at 18 months of age (35). In the present study, morphological changes in the uterus and vagina were not observed in all xenoestrogen-treated mice; carcinogenic response was not observed in reproductive organs during the present 24-week observation period. Longer observation may be necessary to accurately assess carcinogenecity of xenoestrogens in reproductive organs.

Neonatal treatment of mice with DES decreases the frequency of presence of corpora lutea, and induces a castrate-like morphology in mammary glands (23). Prenatal treatment of mice with ZEA has been shown to induce retardation of mammary gland growth and absence of corpora lutea (14); the mammary glands consisted only of the major duct system with dilated ducts that exhibited a beaded appearance and were filled with secreted fluid. In contrast, gestational exposure to BPA or ZEA accelerates mammary gland growth in female mice with intact ovaries (14). In one study, prenatal and neonatal exposure to GEN at levels comparable to or greater than typical human exposure had no effect on mammary gland morphology in pubertal female mice (36). In the present study, prepubertal exposure to xenoestrogen produced transient anovulatory ovaries (at 4 and 8 weeks of age), but mammary gland growth later in life was not affected.

In conclusion, the present data show that prepubertal exposure to xenoestrogens at doses greater than typical human exposure induced various degrees of functional alteration and structural changes in the estrogen target organs. Further study is needed to clarify the consequences for humans of possible side-effects of xenoestrogens contained in food, and to determine the dose levels that produce harmful effects.

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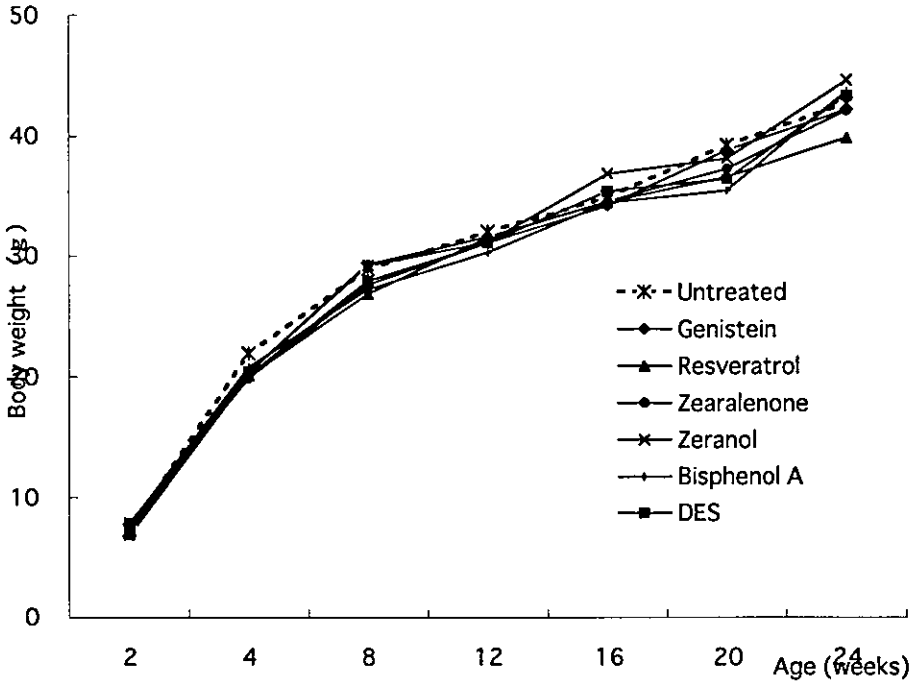

Figure 1. Body weight gain in female CD-1 mice administered 4 daily injections of xenoestrogen beginning at 15 days of age.

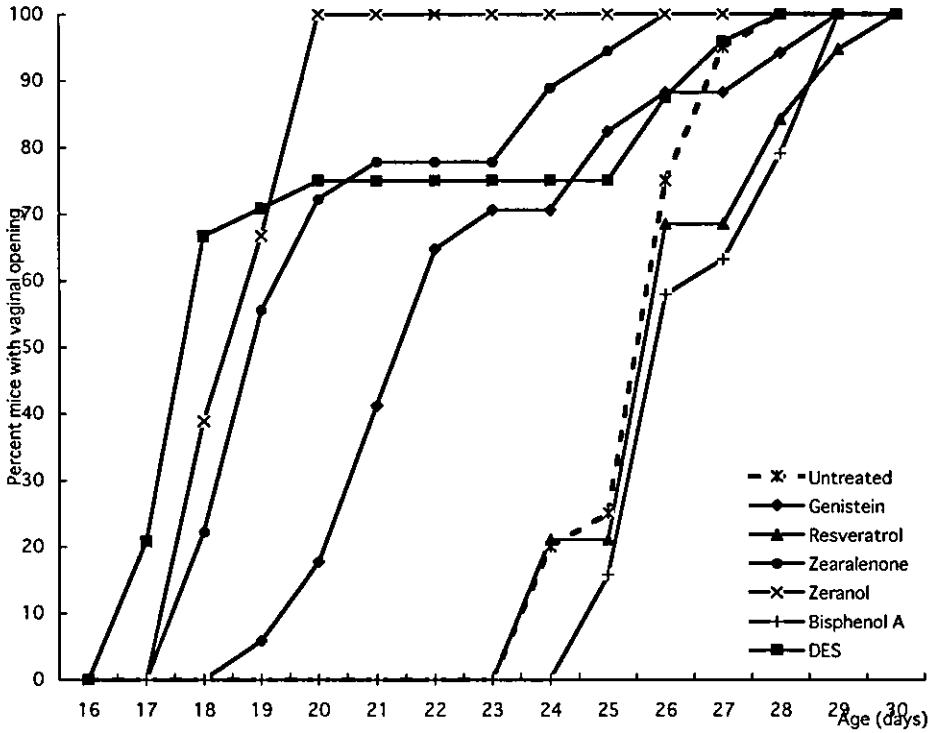

Figure 2. Vaginal opening in xenoestrogen-treated mice and untreated controls. Mice treated prepubertally with GEN, ZEA, ZER or DES exhibited earlier vaginal opening.

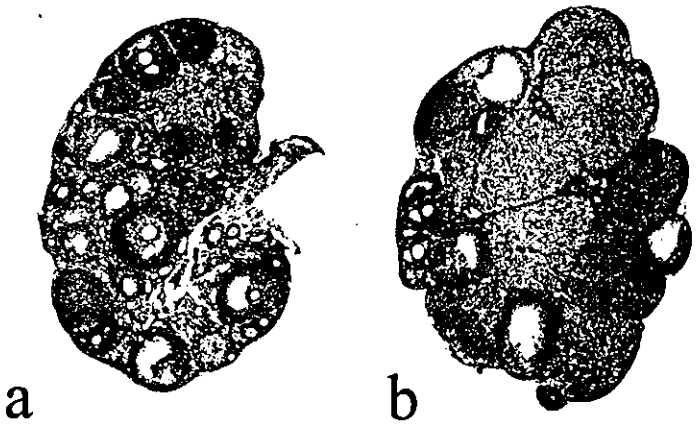

Figure 3. Ovaries from 8-week-old CD-1 mice exposed prepubertally to zearalenone. a.Mouse without corpora lutea. b. Mouse with corpora lutea.

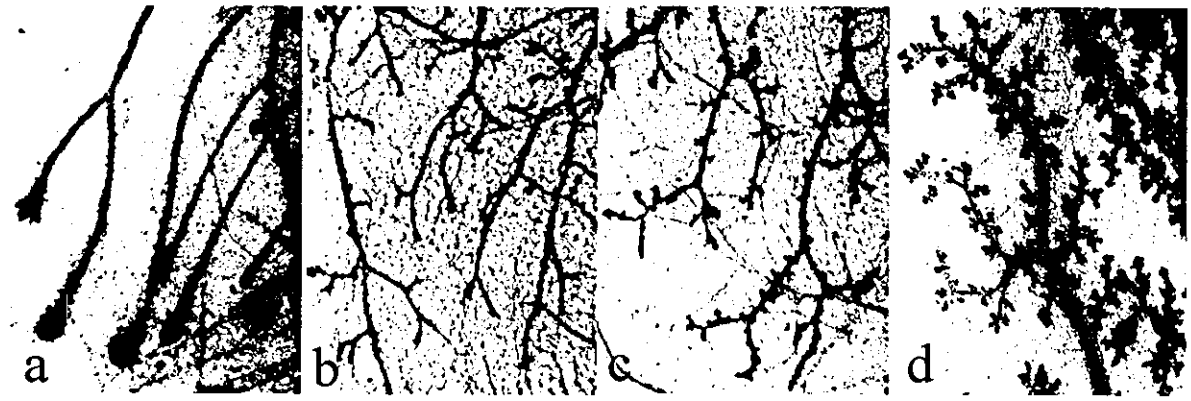

Figure 4. Mammary glands from untreated control mice. a. Note terminal end buds at the periphery and the lack of alveolar differentiation (Score 1). b. Note small number of alveoli within poorly developed duct (Score 2). c. Note more advanced alveolar development, compared with b (Score 3). d. Note prominent lobulo-alveolar development (Score 4).

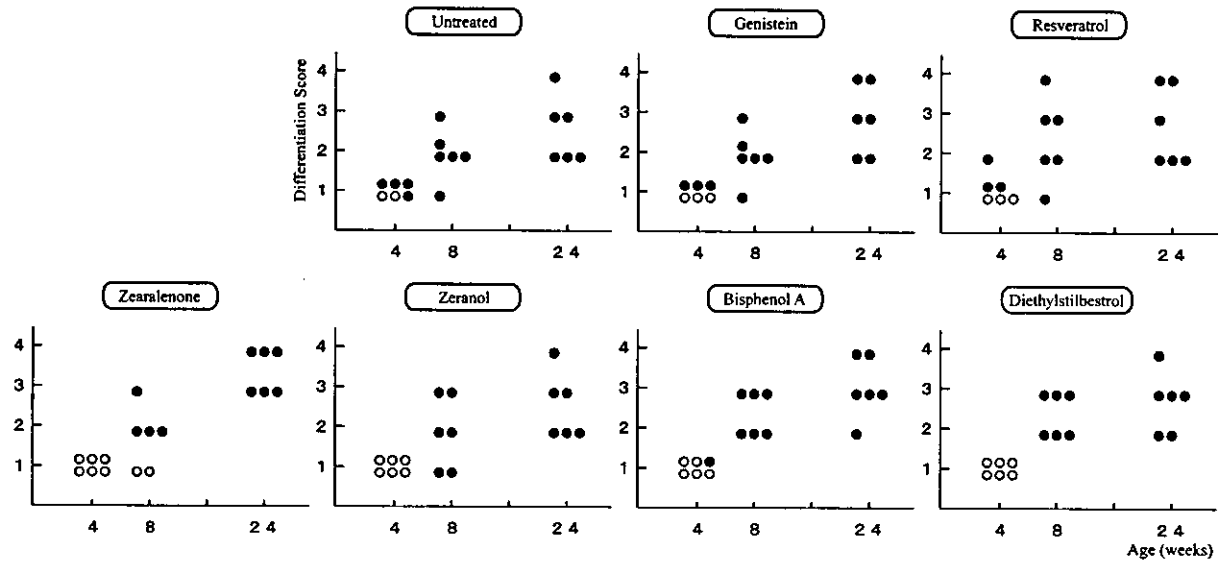

Figure 5. Degree of mammary gland development in female CD-1 mice administered 4 daily injections of xenoestrogen beginning at 15 days of age (● , mouse with corpora lutea; ○ , mouse without corpora lutea).

Table I. Mean age at vaginal opening in female CD-1 mice exposed to xenoestrogen (4 daily injections, beginning at 15 days of age)

Test chemical	Dose	Vaginal opening (days)
Untreated	-	25.9 ± 0.3
Genistein	10 mg/kg x4	22.8 ± 0.7*
Resveratrol	10 mg/kg x4	26.4 ± 0.4
Zearalenone	10 mg/kg x4	20.3 ± 0.6*
Zeranol	10 mg/kg x4	18.9 ± 0.2*
Bisphenol A	10 mg/kg x4	26.8 ± 0.3
Diethylstilbestrol	10 μg/kg x4	20.1 ± 0.8*

Values represent mean ± SE.
Each group consists of 17-24 mice.
*$p<0.01$, compared with untreated controls.

Table II. Estrous cycle alteration in female CD-1 mice exposed to xenoestrogen (4 daily injections, beginning at 15 days of age)

Chemical	Dose	Age 5-8		9-12		21-24 (weeks)	
		One cycle length	Day spent in estrus	One cycle length	Day spent in estrus	One cycle length	Day spent in estrus
Untreated	-	5.4 ± 0.3	1.0 ± 0.0	5.9 ± 0.4	1.0 ± 0.1	6.0 ± 0.4	1.1 ± 0.1
Genistein	10 mg/kg x4	5.2 ± 0.3	1.1 ± 0.0	6.0 ± 0.3	1.2 ± 0.1	6.3 ± 0.3	1.1 ± 0.1
Resveratrol	10 mg/kg x4	6.0 ± 0.3	1.0 ± 0.0	5.7 ± 0.2	1.0 ± 0.1	6.1 ± 0.4	1.2 ± 0.1
Zearalenone	10 mg/kg x4	4.8 ± 0.4	2.8 ± 0.4**	6.5 ± 0.4	2.9 ± 0.3**	6.7 ± 0.4	2.9 ± 0.4**
Zeranol	10 mg/kg x4	5.8 ± 0.3	3.2 ± 0.4**	6.8 ± 0.5	2.0 ± 0.2**	6.2 ± 0.5	1.2 ± 0.1
Bisphenol A	10 mg/kg x4	5.7 ± 0.3	1.1 ± 0.0	5.3 ± 0.2	1.0 ± 0.0	5.2 ± 0.2	1.0 ± 0.0
Diethylstilbestrol	10 μg/kg x4	6.1 ± 0.3	1.9 ± 0.2*	6.1 ± 0.3	2.1 ± 0.2**	6.0 ± 0.2	1.7 ± 0.2*

Values represent mean ± SE (days) of ≥6 mice.
*$p<0.05$, **$p<0.01$ compared with untreated controls.