**in vivo** *19*: 487-494 (2005)

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

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**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. The 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; 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 the untreated controls (33%, 2/6) and the GEN (50%, 3/6), 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. Conclusion: 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 the estrus phase; ZEA-treated mice had a longer period of anovulatory ovary than other xenoestrogen-treated mice; however, none of the xenoestrogens tested altered the uterine or vaginal morphology or mammary gland growth.*

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*Key Words:* Genistein, resveratrol, zearalenone, zeranol, bisphenol A, diethylstilbestrol, CD-1 mouse, prepubertal.

Xenoestrogens (chemicals with estrogenic activity) are endocrine-disrupting chemicals that include naturally occurring substances produced by plants (phytoestrogens), 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 the development of estrogen target organs. *In utero* exposure to DES as an anti-abortive 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 was 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 the examination of abnormalities in estrogen target organs.

Naturally occurring and man-made chemicals that exhibit estrogenic biological 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 the *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 the 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 babies' 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. The adverse effects of estrogenic chemicals on the reproductive organs and mammary glands at crucial stages of development are a matter of concern. A recent study examined the 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, the 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 the reproductive organs and mammary glands.

In the present study, to compare the effects of early exposure to various xenoestrogens, prepubertal female mice were injected subcutaneously (*s.c.*) with one 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 doses of test chemicals used were based on our previous findings (14). The aim of this study was to evaluate the effects of prepubertal exposure to xenoestrogens on the development of the female reproductive system and mammary glands in CD-1 mice. Prepubertal exposure to any of several estrogenic chemicals induced early vaginal opening and disrupted the estrous cycle. However, permanent morphological alteration of the 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); RES, ZEA, BPA and DES were obtained from Sigma (St. Louis, MO, USA). 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, Japan) 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 animals were housed at 22±2˚C and 60±10% humidity, with a 12-h light/dark cycle. To avoid exposure to endocrine-disrupting chemicals, the mice were housed in standard mouse polyisopentene cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River, Yokohama, Japan) as bedding. To avoid exposure to dietary phytoestrogens, the 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 *s.c.* 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). The doses were adjusted daily according to body weight, to provide constant dose levels. The mice were weaned at 21 days of age. The 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 hematoxylin and eosin (HE). The 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 the 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, Japan.

*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

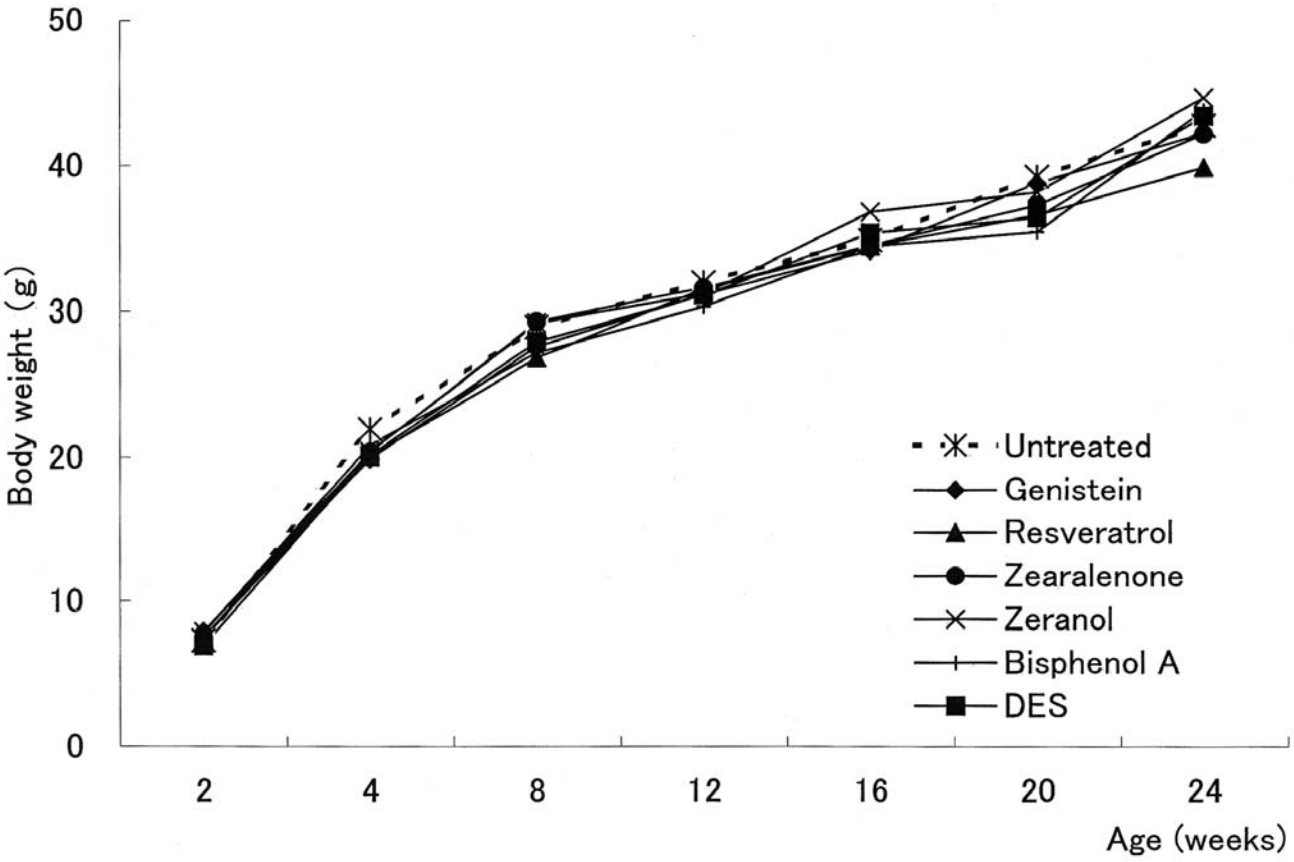


Figure 1. *Body weight gain in female CD-1 mice administered 4 daily injections of xenoestrogen beginning at 15 days of age.*

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 the test chemicals did not influence body weight gain, compared with untreated controls (Figure 1). At 24 weeks of age, the body weights of all groups were comparable.

*Vaginal opening.* The GEN, ZER, ZEA and DES groups exhibited accelerated timing of vaginal opening, compared with the untreated controls (Figure 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 the untreated controls.

*Reproductive tract structure*. Ovarian histology of the 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) (Figure 3). Corpora lutea were present in all mice sacrificed at 24 weeks of age. However, polyovular follicles were not observed in the 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

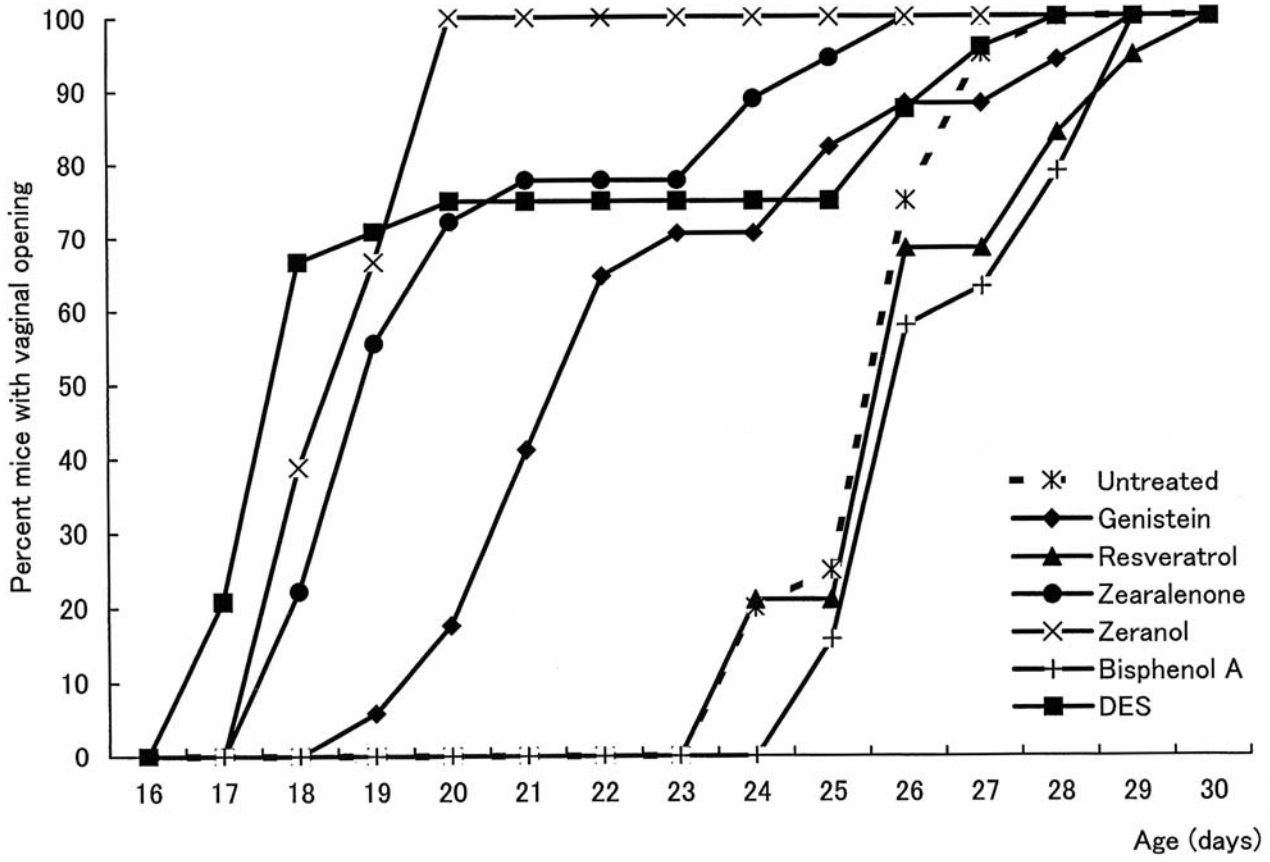


Figure 2. *Vaginal opening in xenoestrogen-treated mice and untreated controls. Mice treated prepubertally with GEN, ZEA, ZER or DES exhibited earlier vaginal opening.*

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 consisted of 17-24 mice.
*$p<0.01$, compared with untreated controls.

differentiation was unclear (Figure 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 (Figure 4b; Score 2); others exhibited somewhat greater alveolar development (Figure 4c; Score 3); while some exhibited complete lobulo-alveolar development (Figure 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 the mammary glands. Scores of mammary gland differentiation after prepubertal xenoestrogen exposure are summarized in Figure 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.

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.

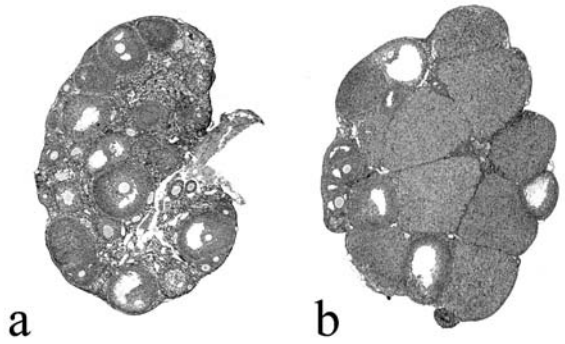


Figure 3. *Ovaries from 8-week-old CD-1 mice exposed prepubertally to zearalenone. a) Mouse without corpora lutea. b) Mouse with corpora lutea.*

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 caused earlier vaginal opening (18-21). Neonatal exposure to 0.4 mg/kg GEN or 0.04 mg/kg ZEA caused 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

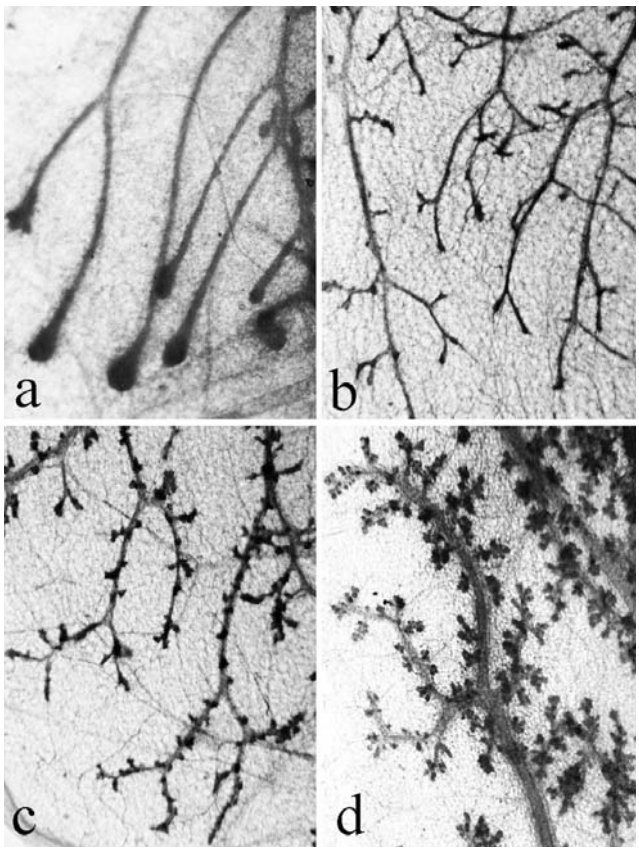


Figure 4. *Mammary glands from untreated control mice.*
*a) Note terminal end buds at the periphery and the lack of alveolar differentiation (Score 1).*
*b) Note small number of alveoli within poorly-developed duct (Score 2).*
*c) Note more advanced alveolar development, compared with b (Score 3).*
*d)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

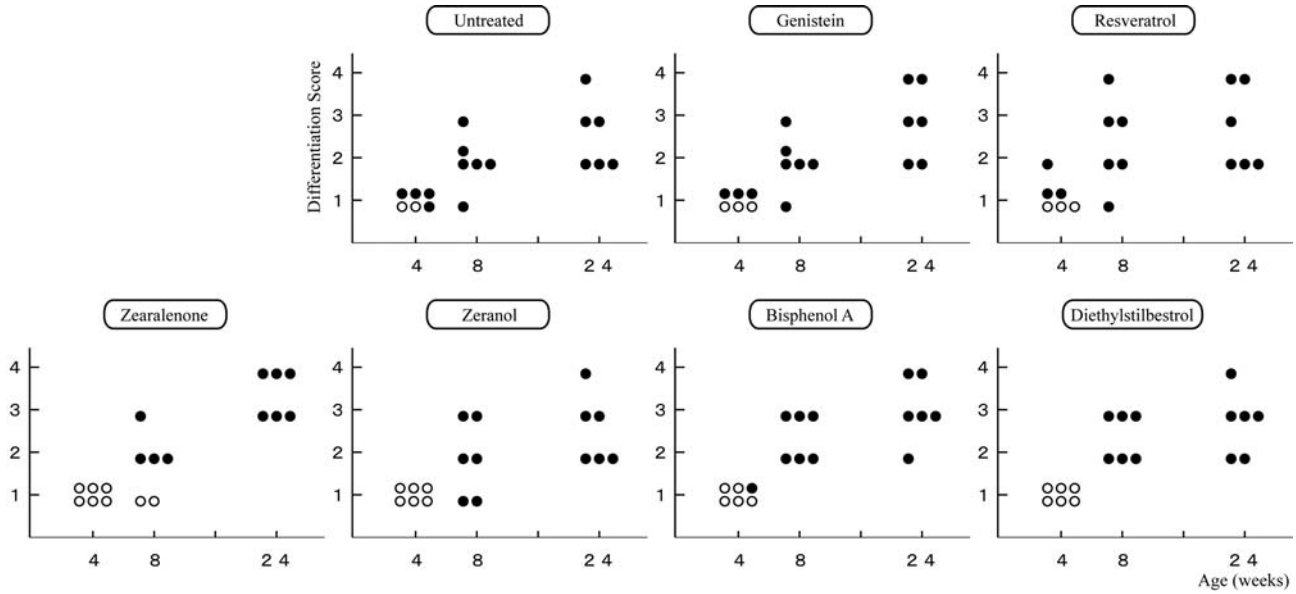


Figure 5. *Degree of mammary gland development in female CD-1 mice administered 4 daily injections of xenoestrogen beginning at 15 days of age (●, mouse with corpora lutea; ○, mouse without corpora lutea).*

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 caused estrous cycle abnormalities (29), and an increase in the percentage of time spent in the estrus phase (19). Prenatal or neonatal administration of ZEA to rats and mice induced abnormal vaginal cyclicity by prolonging estrus (30, 31); irregular estrous cycle with prolonged estrus preceded 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 produced 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 the estrus phase.

Neonatal exposure of mice to DES caused 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; no carcinogenic response was observed in the reproductive organs during the present 24-week observation period. Longer observation may be necessary to accurately assess the carcinogenecity of xenoestrogens in reproductive organs.

Neonatal treatment of mice with DES decreased the frequency of presence of corpora lutea, and induced 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 accelerated 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.

## Acknowledgements

The authors thank Ms. T. Akamatsu for her technical assistance and Ms. Y. Yoshida for preparing the manuscript. This study was supported in part by a Health and Labor Sciences Research Grants for Research on Risk of Chemical Substances, from the Ministry of Health, Labor and Welfare, Japan, and by a Grant-in-Aid for Scientific Research (C) (16790766) and Grant-in-Aid for Young Scientists (B) (16510047) from the Japan Society for the Promotion of Science.

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*Received December 9, 2004*
*Accepted February 14, 2005*