In Utero through Lactational Exposure to Ethinyl Estradiol Induces Cleft Phallus and Delayed Ovarian Dysfunction in the Offspring

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Most of the attention currently focused on endocrine-active chemicals is directed to their effects on the development of offspring exposed to them in utero or during the neonatal period. Pregnant Crl:CD(SD)IGS rats were given ethinyl estradiol (EE) orally in doses of 0.5–50 μg/kg/day from gestational day 7 to postnatal day 18, and their offspring were examined for its effects. Our previous study according to a similar protocol demonstrated the occurrence of cleft phallus in the female offspring exposed to 50 μg/kg of EE in utero and during the lactation period. The present study was designed to assess (1) the reproducibility of the induction of cleft phallus, (2) the fertility of female rats with cleft phallus, and (3) whether any delayed effects, possibly delayed anovulation, were induced. At 50 μg/kg cleft phallus was observed in almost all of the female offspring, and slight retardation of body weight gain was detected in both sexes. At 15–17 weeks of age the animals with cleft phallus could copulate and had fertility comparable to the control group. At 6 months of age, on the other hand, 6/8 of the female offspring at 50 μg/kg exhibited abnormal cyclicity, including persistent estrus, and histological examination revealed follicular cysts and absence of corpora lutea in the ovaries of the rats with persistent estrus. These findings are consistent with delayed anovulation syndrome. The results suggest that observation of cyclicity at 6 months old is able to detect possible delayed ovarian dysfunction induced by perinatal exposure to chemicals.

Key Words: ethinyl estradiol; delayed ovary dysfunction; cleft phallus

Numerous chemicals have been demonstrated to modify endocrine functions of humans and wildlife (Colborn et al., 1993; Danzo, 1997; McLachlan, 1993), and most of the attention that is currently focused on endocrine-active chemicals is being directed at their effects on the development of the offspring exposed to such chemicals in utero or during the neonatal period. An in utero and lactational exposure method has been employed to identify the developmental toxicities of various chemicals (Awoniyi et al., 1998; Moore et al., 2001; Mylchreest et al., 1998; Odum et al., 2002; You et al., 1998), and “in utero through lactational exposure” protocols have been proposed in the draft report by the United States Environmental Protection Agency as a method of detecting effects of prenatal and early postnatal exposure that would not be detected as a result of pubertal or adult exposure (Tyl and George, 2003).

In the previous study we conducted a preliminary trial of an “in utero through lactational exposure” method using ethinyl estradiol and discovered the occurrence of cleft phallus in only the female offspring at a dose at which no major adverse effects were induced (Sawaki et al., 2003). Excessive cleavage of the urethral slit, occasionally associated with insufficient raphe formation between the urethral orifice and vagina in female rats, is referred to as “cleft phallus.” Cleft phallus has been reported in female rats exposed in utero to 2,3,7,8-tetrachlorodibenzo-p-dioxin and an androgen, testosterone propionate (Gray et al., 1997; Wolf et al., 2002), as well to estrogens such as diethylstilbestrol (Henry and Miller, 1986; Henry et al., 1984). This means that cleft phallus is induced by disruption of the complicated process of the development of the external genitalia during the critical period of their morphogenesis, not simply by an estrogenic action. Although the mechanisms of the development of the external genitalia have not been well characterized, cleft phallus is considered to represent a mild form of female rodent hypospadias (Gray et al., 1997). In spite of the abnormality of the external genitalia, the female offspring exhibited normal cyclicity, and no gross or histopathological changes were detected in their internal reproductive tracts (Sawaki et al., 2003). This phenomenon led us to investigate whether the offspring with cleft phallus have normal reproductive performance.

The study described in this report was similar to the in utero through lactational study of exposure to ethinyl estradiol reported previously (Sawaki et al., 2003) and was conducted to confirm the reproducibility of the formation of cleft phallus as well as to evaluate the reproductive performance of the offspring with cleft phallus. In addition, some of the offspring were sacrificed at weaning or at 6 months of age. Sacrifice at 6 months of age was performed in view of the possibility of the occurrence of “delayed anovulation syndrome,” a term pro-

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posed by Gorski (Gorski, 1968) to describe the condition of female rats neonatally exposed to low doses of testosterone propionate that retain the ability to ovulate for a time after puberty but eventually become anovulatory (Arai, 1971). Delayed anovulation induced by neonatal exposure to the estrogenic compound p-octylphenol has been documented (Yoshida et al., 2002).

MATERIALS AND METHODS

Chemicals. Ethinyl estradiol (EE, lot No. KSF1601, purity 99.0%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and olive oil (Fujimi Pharmaceutical Co., Ltd., Osaka, Japan) was used as the vehicle. The physical stability of EE was assessed with a Fourier transform infrared spectrophotometer (FTS-135, Nippon Bio-Rad Laboratories K.K., Tokyo, Japan), and the stability, homogeneity, and concentration of each EE suspension prepared for administration were confirmed by HPLC.

Animals and experimental design. Timed-pregnant Crj: CD® (SD) IGS BR rats (Charles River Japan Inc., Shiga, Japan) were used in this study. They were nulliparous, 11 weeks old, and at gestational day (GD) 1 on arrival (vaginal plugs noted = GD 0). On GD 6, 10 dams each were randomly assigned to four experimental groups by a body-weight-stratified randomization method to minimize the variation in body weights between the groups, and EE 0.5, 5, or 50 µg/kg/day or vehicle alone, was administered to them by gavage from GD 7 to postnatal day 18 (day 0 = delivery day). Their body weight at the time they were divided into groups ranged from 237.5 g to 304.7 g. Our previous study according to a similar protocol demonstrated cleft phallus in the female offspring at 50 µg/kg/day (Sawaki et al., 2003).

The pups were kept in polycarbonate pens (280 W × 440 D × 150 H mm, Tokiwa Kagaku Kikai Co., Ltd., Tokyo, Japan) with the dams until weaning. Bedding was SUNFLAKE® supplied by Charles River Japan Inc (Atsugi, Japan). To maximize the uniformity of growth rates, at 4 days of age the litters were culled randomly to four male and four female pups per dam. When the number of pups of either sex was insufficient to obtain four offspring of each sex, litter size was adjusted to eight by excluding pups of the sex with animals in surplus but adjustment was not made when the number of pups per dam was less than eight. Pups were weaned at 19 days of age, that is, on the day after the final dose. Before weaning the pups in each group were randomly assigned to three subgroups: a subgroup sacrificed at weaning, a subgroup sacrificed at 18–20 weeks of age subjected to caesarean section to evaluate the reproductive performance of the offspring (caesarean section subgroup) and a subgroup sacrificed at 6 months of age to examine the animals for delayed toxicities (6-month-sacrifice subgroup). The reproductive organs of the subgroup sacrificed at weaning were collected for a gene expression study, but the results will not be reported here. The numbers of animals in each group are shown in Table 1.

After weaning, offspring were housed individually in stainless steel wire-mesh cages (260 mm W × 380 mm D × 180 mm H, Tokiwa Kagaku Kikai Co., Ltd., Tokyo, Japan) in a controlled environment of 12 h light/12 h darkness (lights on at 0700 h), 23 ± 2°C and 50 ± 10% humidity with 10–15 air exchanges per h. The rats were fed an MF diet (certified pellet rodent diet, Oriental Yeast Co., Tokyo, Japan) and given free access to tap water. All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by the Japanese Association for Laboratory Animal Science. At sacrifice, animals were euthanized by exsanguination from the femoral arteries under deep anesthesia using diethyl ether. The culled pups were euthanized only by deep anesthesia.

Endpoints. The general and nursing states and body weight of all dams were monitored daily. The gestational period of each dam was calculated. On the day of delivery, the numbers of live newborns and stillborns and the sex ratio of the pups were recorded, and the pups were inspected for external anomalies. The dams were euthanized and necropsied on the day after weaning. The number of implantation sites in each uterus was counted by immersing the dissected uteri in ammonium sulfate solution, and the birth rate was calculated (number of newborns/ number of implantation sites). The duration of pregnancy, numbers of live newborns and of stillborns, and the sex ratios of the live newborns were also recorded.

The general condition of the pups was observed daily. Body weight was measured at 0, 4, 7, 12, 15, and 19 days of age and at weekly intervals thereafter until sacrifice. The females were examined for vaginal opening daily from 28 days of age, and the males for preputial separation from 35 days of age until they occurred. Ano-genital distance was measured at 4 days of age. The estrous cycle of the female offspring was evaluated based on vaginal cytology performed for at least 14 days starting at 13 weeks in the caesarean section subgroup and for at least 20 days prior to sacrifice in the 6-month-sacrifice subgroup. Following criteria were used for determination of cyclicity: cyclicity of 0.01 mm (Digimatic Caliper CD-15CP, Mitsutoyo Corp., Kanagawa, Japan) were measured morphometrically with calipers having a reproducible precision of 0.01 mm (Digimatic Caliper CD-15CP, Mitsutoyo Corp., Kanagawa, Japan). Morphometry included the length of the urethral slit across top of the phallus and the vertical distance between the tip of the phallus and the urethral orifice.

Statistical analyses. Body weight, organ weight, ano-genital distance, and the morphometric data were analyzed by the Bartlett’s test for homogeneity of

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Dams and Their Offspring after Weaning</strong></td>
</tr>
<tr>
<td><strong>Dose of EE (µg/kg/day)</strong></td>
</tr>
<tr>
<td><strong>Dams</strong></td>
</tr>
<tr>
<td><strong>Weanlings (male/female)</strong></td>
</tr>
<tr>
<td><strong>Castration after weaning</strong></td>
</tr>
<tr>
<td><strong>Castration after 36 months</strong></td>
</tr>
<tr>
<td><strong>Castration at 6 months</strong></td>
</tr>
</tbody>
</table>

*Note. EE, ethinyl estradiol; VC, vehicle control.
*Not reported in this paper.
variance. If the variances were homogeneous at the 5% level of significance, one-way analysis of variance was performed, and if it showed a significant difference, the difference between the control group and each of the experimental groups was analyzed by Dunnett’s test. If the variances were not homogeneous, the Kruskal-Wallis test was used, and if it showed a significant difference, the difference between the control group and each of the experimental groups was analyzed by the nonparametric Dunnett’s test. The pregnancy period of the dams, age data at preputial separation and vaginal opening, estrus cyclicity data, and the numbers of corpora lutea, implantation sites, and live fetuses of the offspring were analyzed by the Kruskal-Wallis test, and if there was a significant difference, the difference between the control group and each of the experimental groups was analyzed by the nonparametric Dunnett’s test. Fisher’s exact test was applied to the sex ratios of live newborns or fetuses and all other parameters that were represented as ratios.

Analyses were performed using the mean litter values prior to weaning and those of individual pups after weaning. A $p$ value of 0.05 was adopted as the level of significance.

### RESULTS

Dams that delivered live newborns exhibited no abnormal findings during pregnancy or the lactational period, and no abnormal findings were detected at necropsy. The duration of pregnancy ranged from 21 to 23 days, and there was no difference between the vehicle control and EE-treated animals.

The numbers of implantation sites in the EE-treated dams, the durations of pregnancy (days, mean ± SD) for VC, 21.8 ± 0.4; for 0.5, 22.1 ± 0.4. Number of implantation sites for VC, 134 [13.4 ± 1.6]; for 0.5, 112 [14.0 ± 1.7]. EE, ethinyl estradiol; VC, vehicle control. Value in brackets represents mean ± SD.

<table>
<thead>
<tr>
<th>Period</th>
<th>Litters</th>
<th>VC (No. of dams = 10)</th>
<th>0.5 (No. of dams = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Number of stillbirths</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Number of live newborns at birth</td>
<td>62 [6.2 ± 2.5]</td>
<td>60 [6.0 ± 2.9]</td>
</tr>
<tr>
<td></td>
<td>Sex ratio of live newborns at birth (male/female)</td>
<td>103.3 (62/60)</td>
<td>62 (100)</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Number of dead</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>Number of live newborns on day 4</td>
<td>62 (100)</td>
<td>60 (100)</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Number of dead</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Number of live newborns after culling</td>
<td>40 [4.0 ± 1.1]</td>
<td>40 [4.0 ± 1.1]</td>
</tr>
<tr>
<td></td>
<td>Number of weanings</td>
<td>40 (100)</td>
<td>40 (100)</td>
</tr>
<tr>
<td></td>
<td>Period III (after weaning)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Number of live animals</td>
<td>40 (100)</td>
<td>40 (100)</td>
</tr>
</tbody>
</table>

**Note.** Number of dams that delivered live newborns for VC, 10 (percentage of no. of dams 100); for 0.5, 8 (percentage of number of dams100). Duration of pregnancy (days, mean ± SD) for VC, 21.8 ± 0.4; for 0.5, 22.1 ± 0.4. Number of implantation sites for VC, 134 [13.4 ± 1.6]; for 0.5, 112 [14.0 ± 1.7]. EE, ethinyl estradiol; VC, vehicle control. Value in brackets represents mean ± SD.

- Value in parentheses represents the percentage of the number of implantation sites.
- Value in parentheses represents the percentage of the number of live newborns at birth.
- Value in parentheses represents the percentage of the number of live newborns just after culling.
- Value in parentheses represents the percentage of the number of weanlings.
phallus. The extent of the cleft phallus in the female rats was assessed quantitatively by measuring the length of the urethral slit across top of the phallus and the vertical distance between the tip of the phallus and the urethral orifice. The results showed that both measurements were significantly larger in the 50 μg/kg group than in the control group (Table 5). Cleft phallus was not grossly clear at the age of weaning, and morphometry of the external genitalia did not disclose the presence of the phallus. The extent of the cleft phallus in the female rats was 

| TABLE 3 |
| Postnatal Courses of Dams and Their Litters for EE 5 and 50 μg/kg/day |

<table>
<thead>
<tr>
<th>Litters</th>
<th>VC (No. of dams = 10)</th>
<th>0.5 (No. of dams = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Period I (from birth to day 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of stillborns</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Number of live newborns at birth</td>
<td>66 [6.6 ± 1.5]</td>
<td>61 [6.0 ± 1.9]</td>
</tr>
<tr>
<td>Sex ratio of live newborns at birth (male/female)</td>
<td>108.2 (66/61)</td>
<td></td>
</tr>
<tr>
<td>Number of dead</td>
<td>0 (0.0)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Number of live newborns on day 4</td>
<td>66 (100)</td>
<td>60 (98.3)</td>
</tr>
<tr>
<td>Period II (from day 4–19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of live newborns just after culling</td>
<td>40 [4.0 ± 0.0]</td>
<td>40 [4.0 ± 0.0]</td>
</tr>
<tr>
<td>Number of dead</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Number of weanlings</td>
<td>40 (100)</td>
<td>40 (100)</td>
</tr>
<tr>
<td>Period III (after weaning)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of dead</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Number of live animals</td>
<td>40 (100)</td>
<td>40 (100)</td>
</tr>
</tbody>
</table>

Note. Number of dams delivered live newborns for VC, 10 (percentage of the no. of dams 100); for 0.5, 9 (percentage of the no. of dams 100). Duration of pregnancy (days, mean ± SD) for VC, 21.8 ± 0.4; for 0.5, 22.0 ± 0.5. Number of implantation sites for VC, 134 [13.4 ± 1.3]; for 0.5, 130 [14.4 ± 1.7]. EE, ethinyl estradiol. Value in brackets represents mean ± SD.

with persistent estrus revealed the absence of corpora lutea and follicular cysts (Fig. 3). The endometrial epithelium of their uterus had become columnar, and the vaginal epithelium was keratinized. Mucinous change and keratinization of the epithelium was seen in the vagina of one animal with irregular cyclicity. The offspring in the vehicle control group, on the other hand, did not show any abnormal cyclicity. There were only two animals with normal cyclicity in the 50 μg/kg group, and their gross and histopathological examination of the ovary, uterus, and vagina revealed normal findings, the same as those in the vehicle control group. However, 1/7 of the females in the 0.5 μg/kg group showed persistent estrus and pathological changes similar to those in the 50 μg/kg group with persistent estrus. No abnormal histological findings were detected in the hypophysis in any of the animals.

No abnormalities were observed in the male rats in either subgroup at necropsy. No marked changes were noted in the organ weights of either sex (Table 8).

**DISCUSSION**

Pregnant Crj: CD (SD) IGS rats were given EE orally from GD 7 to PND 18, and their offspring were examined for its effects. Our previous study with a similar protocol demonstrated the occurrence of cleft phallus in the female offspring...
exposed to 50 µg/kg of EE in utero and during the lactational period (Sawaki et al., 2003). This study was designed to clarify (1) the reproducibility of the induction of cleft phallus, (2) the fertility of the female rats with cleft phallus, and (3) whether any delayed effects, e.g., delayed anovulation, were induced.

In the 50 µg/kg group cleft phallus was observed in almost all of female offspring, and slight retardation of body weight gain was noted in both sexes. These results are consistent with our previous preliminary study (Sawaki et al., 2003). In normal female, the urethral slit, at which urethral orifice opens, takes its position at the tip of the phallus. Cleft phallus denotes excessive cleavage of the urethral slit. Occasionally it is accompanied by insufficient raphe formation between the urethral slit and the vagina (Fig. 2). Recent studies on the development of the external genitalia by Haraguchi and colleagues have demonstrated that the fibroblast growth factor gene and Sonic Hedgehog gene play a crucial role in the organogenesis of mouse external genitalia (Haraguchi et al., 2000, 2001). However, the molecular mechanisms underlying the development of the external genitalia, especially at the stages mediated by sex hormones, are not well understood. Nevertheless, cleft phallus in female rats has been shown to be a sensitive endpoint of the effects of estrogen in in utero through lactational exposure protocol, although cleft phallus is not induced by estrogenic chemicals alone (Gray et al., 1997; Wolf et al., 2002).

At 15–17 weeks of age the animals with cleft phallus were able to copulate and possessed fertility comparable to that in the vehicle control group (Table 6). This finding is not surprising because the abnormalities of their external genitalia were mild, did not interfere with coitus, and cyclicity was normal. Although there were 2/15 animals with irregular cyclicity in the 5 µg/kg group and 1/16 animals in the 50 µg/kg group, these findings were not statistically significant and could not be unequivocally concluded to be a result of EE exposure. On the other hand, clear evidence of the effects of EE was noted at 6


### TABLE 4
Ano-Genital Distance and Sexual Maturation Markers of Offspring

<table>
<thead>
<tr>
<th>Dose of EE (µg/kg/day)</th>
<th>Male</th>
<th></th>
<th>Female</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGD at 4 days old (mm)</td>
<td>Age of PPS completion (Days)</td>
<td>AGD at 4 days old (mm)</td>
<td>Age of VO onset (Days)</td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>4.75 (10)</td>
<td>± 0.38</td>
<td>41.2 (27)</td>
<td>± 1.3</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>4.86 (7)</td>
<td>± 0.24</td>
<td>41.0 (21)</td>
<td>± 1.3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.76 (9)</td>
<td>± 0.31</td>
<td>41.9 (27)</td>
<td>± 1.6</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>4.50 (8)</td>
<td>± 0.28</td>
<td>42.1 (23)</td>
<td>± 1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.00 (10)</td>
<td>± 0.13</td>
<td>34.2 (26)</td>
<td>± 2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.12 (7)</td>
<td>± 0.14</td>
<td>34.5 (21)</td>
<td>± 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.09 (9)</td>
<td>± 0.21</td>
<td>34.3 (26)</td>
<td>± 2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.09 (8)</td>
<td>± 0.22</td>
<td>33.3 (24)</td>
<td>± 1.5</td>
<td></td>
</tr>
</tbody>
</table>

**Note.** EE, ethinyl estradiol; VC, vehicle control; AGD, ano-genital distance; PPS, preputial separation; VO, vaginal opening. Values in parentheses indicate number of animals examined. Mean AGD was calculated on litter-basis and mean PPS and VO were on pup-number-basis.
months of age. In the 50 μg/kg group 6/8 of the female offspring showed abnormal cyclicity, including persistent estrus in 4/8 of the animals. Histopathologically, follicular cysts and absence of corpora lutea were observed in the ovaries of the rats with persistent estrus (Fig. 3). The histopathological changes of the uterus and vagina observed in this study were known to be accompanied with the changes of the ovarian function (Biegel et al., 1998). These conditions, namely, delayed appearance of abnormal cyclicity and the pathologic changes in the ovary, are consistent with delayed anovulation syndrome (Arai, 1971; Hendricks et al., 1977). Estrogen has been well documented to affect ovarian function and cyclicity (Biegel et al., 1998). Anovulation syndrome induced by neonatal exposure to estradiol benzoate was first documented by

FIG. 2. Photographs of the external genitalia of offspring sacrificed at 6 months of age. (A): vehicle control showing normal appearance; (B): 50 μg/kg of ethinyl estradiol. Cleft phallus. Excessive cleavage of urethral slit and insufficient raphe formation are evident. Inset: “V” marks indicate urethral slit. Shaded areas indicate normal raphe (A) or insufficient raphe formation (B).
Gorski (1963). However, few studies have shown that perinatal exposure to estrogen causes “delayed” anovulation. A recent study has demonstrated that neonatal exposure to an estrogenic compound, p-t-octylphenol, induces delayed anovulation syndrome (Yoshida et al., 2002).

Abnormal cyclicity, including persistent estrus, with accompanying follicular cysts and decreased number of corpora lutea is observed in aged rats (Chern et al., 2000; Gore et al., 2000; Greaves, 1990). A lowering of the incidence of regular cyclicity in the rat becomes apparent at the age of 6–9 months (Anzalone et al., 2001) or 1 year (Gore et al., 2000). In the present study one female in the 0.5 μg/kg group showed persistent estrus and pathological changes similar to those in the 50 μg/kg group with persistent estrus. This is considered to have been a spontaneous occurrence, because it was not dose-dependent and 6 months may be the age when the incidence of abnormal cyclicity begins to increase in SD IGS rats, although no background information is available.

The significance of the delayed anovulation syndrome-like phenomenon observed in the present study is not clear in terms of the hazard assessment. Swanson suggested that delayed anovulation syndrome represents acceleration of premature aging (Swanson et al., 1964). The process of reproductive senescence in humans and rats seems to differ, because follicular cysts and persistent estrus are frequently seen in the rat, whereas decrease in oocytes in the ovary is predominant in humans (Zapantis and Santro, 2002), and the removal of ovarian negative feedback is thought to result in alteration of the neuroendocrine system (Gore et al., 2000). On the other hand, it is still unclear whether the hormonal changes causing abnormal cyclicity in the rat are due primarily to deficits in neuroendocrine function, altered ovarian function, or both (Anzalone et al., 2001). Chern et al. suggested that the attenuated proestrous progesterone surge from the ovary in middle-aged rats, which is considered to be related to the facilitation and maintenance of the magnitude of the LH surge, is due to the attenuated preovulatory LH surge rather than decreased ovarian sensitivity to LH (Chern et al., 2000). Gore and colleagues demonstrated that the attenuation of the preovulatory gonadotropin-releasing hormone and subsequent LH surge occurs prior to reproductive failure (Gore et al., 2000). Norepinephrine and epinephrine in the hypothalamus also play a critical part in the

### TABLE 5
Morphology and Morphometry of External Genitalia of Female Offspring by Dose of EE (μg/kg/day)

<table>
<thead>
<tr>
<th></th>
<th>VC</th>
<th>0.5</th>
<th>5</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caesarean section subgroup</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals examined</td>
<td>19</td>
<td>14</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Length of urethral slit across top of phallus (mm)</td>
<td>1.10 ± 0.19</td>
<td>1.13 ± 0.21</td>
<td>1.17 ± 0.27</td>
<td>1.36 ± 0.33*</td>
</tr>
<tr>
<td>Vertical distance between tip of phallus and urethral orifice (mm)</td>
<td>1.16 ± 0.16</td>
<td>1.13 ± 0.18</td>
<td>1.12 ± 0.24</td>
<td>1.72 ± 0.34**</td>
</tr>
<tr>
<td>6-month-sacrifice subgroup</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals examined</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Size of urethral slit (mm)</td>
<td>1.08 ± 0.13</td>
<td>1.05 ± 0.27</td>
<td>1.21 ± 0.19</td>
<td>1.54 ± 0.26**</td>
</tr>
<tr>
<td>Distance between tip of phallus and urethral orifice (mm)</td>
<td>1.14 ± 0.12</td>
<td>1.18 ± 0.24</td>
<td>1.31 ± 0.19</td>
<td>1.88 ± 0.26**</td>
</tr>
</tbody>
</table>

Note. EE, ethinyl estradiol; VC, vehicle control. Significantly different from VC at *p < 0.05, **p < 0.01.

### TABLE 6
Reproductive Performance of Female Offspring in Caesarean Section Subgroup by Dose of EE (μg/kg/day)

<table>
<thead>
<tr>
<th></th>
<th>VC</th>
<th>0.5</th>
<th>5</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats mated</td>
<td>19</td>
<td>14</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Number of rats with normal cyclicity</td>
<td>19</td>
<td>14</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Number of rats with irregular cyclicity</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Sex cycle of rats with normal cyclicity (days, mean ± SD)</td>
<td>4.2 ± 0.3</td>
<td>4.2 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>Copulation index (% copulating rats/mated rats)</td>
<td>100.0</td>
<td>100.0</td>
<td>94.1</td>
<td>100.0</td>
</tr>
<tr>
<td>Fertility index (% fertilized rats/copulating rats)</td>
<td>94.7</td>
<td>92.9</td>
<td>81.3</td>
<td>93.8</td>
</tr>
<tr>
<td>Number of corpora lutea (mean ± SD)</td>
<td>16.4 ± 1.9</td>
<td>14.2 ± 3.6</td>
<td>17.0 ± 2.2</td>
<td>16.8 ± 3.2</td>
</tr>
<tr>
<td>Number of implantation sites (mean ± SD)</td>
<td>15.3 ± 2.6</td>
<td>13.9 ± 4.3</td>
<td>16.0 ± 2.0</td>
<td>14.0 ± 2.5</td>
</tr>
<tr>
<td>Number of live fetuses (mean ± SD)</td>
<td>14.4 ± 2.4</td>
<td>13.0 ± 4.0</td>
<td>15.1 ± 2.0</td>
<td>13.5 ± 2.6</td>
</tr>
<tr>
<td>Sex ratio (% male/female)</td>
<td>104.7 (133/127)</td>
<td>138.0 (98/71)</td>
<td>133.3 (112/84)</td>
<td>78.3 (83/106)</td>
</tr>
</tbody>
</table>

Note. EE, ethinyl estradiol; VC, vehicle control. Value in parentheses represents total number of male/total number of female.
maintenance of normal cyclicity by stimulating the release of gonadotropin-releasing hormone (Temel et al., 2002). On the other hand, it has been reported that young hemiovariectomized rats show a decrease in the magnitude of the LH surge that occurs spontaneously in middle age, suggesting that reproductive aging is associated with a reduced ovarian follicular reserve (Anzalone et al., 2001). Nevertheless, the ovarian dysfunction observed in this study probably represents reproductive senescence or a defect in the hypothalamic-pituitary-ovarian axis. Further experiments are necessary to elucidate the mechanisms of reproductive senescence in humans and rats, particularly the differences between them, in order to estimate the impact of the delayed anovulation induced by endocrine-active compounds in the rat.

In conclusion, cleft phallus was observed in the females in the group exposed to 50 μg/kg, a dose that did not induce major adverse effects. Although their fertility was confirmed at the age of 15–17 weeks, later examination disclosed loss of reproductive cyclicity in pre-middle age. These findings suggest that observation of the cyclicity at 6 months of age is able to detect possible delayed ovarian dysfunction induced by perinatal exposure to chemicals including endocrine-active

### TABLE 7
Cyclicity and Gross and Histopathological Findings of Female Offspring Sacrificed at 6 Months of Age

<table>
<thead>
<tr>
<th>Dose of EE (μg/kg/day)</th>
<th>Cyclicity</th>
<th>Sex cycle (Days)</th>
<th>Gross pathological findings</th>
<th>Histopathological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td>Normal (8/8)</td>
<td>4.1 ± 0.2</td>
<td>No.abnormalities detected (8/8)</td>
<td>No.abnormalities detected (8/8)</td>
</tr>
<tr>
<td></td>
<td>Normal (6/7)</td>
<td>4.2 ± 0.4</td>
<td>No.abnormalities detected (6/7)</td>
<td>No.abnormalities detected (6/7)</td>
</tr>
<tr>
<td></td>
<td>Persistent estrus (1/7)</td>
<td>Uterus: Watery contents in lumen (1/7)</td>
<td>Hypophysis: Enlargement (1/7)</td>
<td>Uterus: Columnar endometrial epithelium (1/7)</td>
</tr>
<tr>
<td>5</td>
<td>Normal (9/9)</td>
<td>4.1 ± 0.3</td>
<td>No.abnormalities detected (8/9)</td>
<td>No.abnormalities detected (8/9)</td>
</tr>
<tr>
<td></td>
<td>Uterus: Watery contents in lumen (1/9)#</td>
<td>Uterus: Columnar endometrial epithelium (1/9)#</td>
<td>Vagina: Keratinization of epithelium (1/9)#</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Irregular cyclicity (2/8)</td>
<td>No.abnormalities detected (2/8)</td>
<td>Vagina: mucinous change and keratinization of epithelium (1/8)</td>
<td></td>
</tr>
</tbody>
</table>

| Note. EE, ethinyl estradiol; VC, vehicle control. |
| One animal unwillingly sacrificed at estrus stage because of a vaginal smear diagnosis error. |

FIG. 3. Loupe images (A) and (C) and photomicrographs (B) and (D) of the ovaries of offspring sacrificed at 6 months of age. (A) and (B): vehicle control showing normal appearance. Layer of granulosa cells (arrowheads) and formation of corpora lutea (arrows) are evident. (C) and (D): 50 μg/kg of ethinyl estradiol with persistent estrus. Dilatation of follicles, and thinning of follicular cell layers (*) that are typical findings of follicular cysts, and absence of corpora lutea are observed. (A) and (C): bar = 1 mm; (B) and (D): bar = 0.2 mm.
compounds, whereas such conditions as delayed anovulation syndrome were not foreseen in the current reproductive toxicity test protocols.

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