FULL PAPER *Physiology*

Neonatal Exposure to 17α-Ethinyl Estradiol Affects Kisspeptin Expression and LH-Surge Level in Female Rats

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ABSTRACT. Contamination of estrogenic compounds disrupts endocrinological and neurological reproductive systems in animals. Neonatal exposure to 17α-ethinyl estradiol (EE) induced an abnormal estrous cycle at postnatal day (PND) 180, but not at PND90. We found that serum level of luteinizing hormone (LH) at the latter half of proestrus in EE-treated rats was lower than in the controls at PND90 when there was no significant difference on estrous cyclicity. Additionally, *kiss1* mRNA levels in the anteroventral periventricular nucleus-preoptic area (AVPV/POA) were lower in EE-treated rats than in the controls. The expression of GnRH precursor (*GNRH1*) mRNA in the AVPV/POA and that of LH beta subunit (*LHb*) mRNA in the pituitary were similar in the control- and EE-treated groups. Our results indicated that neonatal exposure to EE leads to reduced expression of *kiss1* mRNA in AVPV/POA and LH-surge, which is likely related to the delayed reproductive dysfunction seen in adult female rats.

KEY WORDS: 17α-ethinyl estradiol, AVPV/POA, endocrine disruptor, kisspeptin

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It has been approximately 20 years since the first World Wildlife Federation (WWF) Wingspread Conference focused on endocrine-disrupting chemicals (EDCs) [11]. EDCs are a broad class of synthetic and natural chemicals, most of which have estrogenic activity [11]. Because these chemicals mimic estrogens that regulate cell fate during embryonic development, exposure to these compounds during the fetal and perinatal periods leads to disruption of endocrinological, neurological and reproductive functions [24].

In rodents, the sexual differentiation of the brain occurs during the late embryonic and early postnatal periods [14]. In males, androgen secreted from the testis passes the Blood-Brain-Barrier (B-B-B) and reaches the brain, where it is converted to estradiol by the action of p450 aromatase enzymes [10]. The estradiol is essential for the normal sexual differentiation of the male brain. In females, the developing ovary secretes estradiol, but most of the circulating estradiol remains bound to α-fetoprotein and cannot pass the B-B-B [21]. Synthetic estrogenic compounds show the ability to escape from binding to α-fetoprotein. Therefore, exposure to synthetic estrogen compounds during the perinatal period may affect the sexual differentiation of the brain in female rodents.

Kisspeptin, a neuropeptide, plays key roles in determining the timing of puberty and regulation of the estrous cycle by stimulating the Hypothalamic-Pituitary-Gonadal axis (HPG axis) [15]. Kisspeptin is expressed in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) [27]. The number of kisspeptin neurons in the AVPV is substantially higher in females than in males, but their numbers in the ARC are not sexually dimorphic [2]. In female mice, the number of the kisspeptin neurons in the AVPV increases after birth and reaches adult levels at the time of puberty [4]. Because kisspeptin neurons express estrogen receptor α (ERα), it is thought that the increase in the number of kisspeptin neurons is regulated by estradiol secreted from the developing ovary. Conditional deletion of ERα from kisspeptin neurons resulted in an arrest in the pubertal maturation and a failure to acquire normal estrous cycle [20].

Kisspeptin acts through binding to the G protein-coupled receptor GPR54 [15]. This receptor is expressed in GnRH neurons, which are primarily located in the preoptic area (POA) and the ARC [15, 17]. Kisspeptin bound to the receptor in POA induces a GnRH-surge followed by an LH-surge [9]. Kisspeptin bound to its receptor in ARC controls the GnRH and LH pulses [17, 18]. Studies have shown that EDCs cause reproductive dysfunction by affecting the population of kisspeptin neurons [7, 26]. However, the effects of EDCs on kisspeptin neurons remain largely uncharacterized.

In this study, we used 17α-ethinyl estradiol (EE) as model EDC. Previous studies found that a one-time administration of EE (20 μg/kg) at postnatal day 1 (PND1) induced abnormal estrous cycle during PND171-190 [22]. To identify the potential changes in kisspeptin neuron following neonatal exposure to EE, we determined the serum levels of reproductive hormones and analyzed the gene expression in AVPV/POA, ARC and pituitary at PND90 before the appearance

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of the abnormal estrous cycle. Here, we show that neonatal EE exposure leads to a reduction in LH-surge and reduced expression of kisspeptin in AVPV/POA.

MATERIALS AND METHODS

Animals: Adult Wistar-Imamichi rats were maintained in an animal room under standard housing conditions with controlled lighting (lights on from 05 hr 00 to 19 hr 00), temperature (25 ± 2°C) and humidity (50 ± 10%). Animals were provided with a rat chow diet (MR-Breeder, Nosan Corporation, Yokohama, Japan) and tap water *ad libitum*. All animal experiments were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology, Japan.

Experimental design: Figure 1 illustrates the experimental design. The sex ratio of the newborn pups in each litter was adjusted to 6:3 (females: males). Female pups were given one of the following neonatal treatments: 1) sesame oil vehicle alone (control group), 2) EE at 20 μg/kg and 3) EE at 200 μg/kg. These treatments were administered within 24 hr of delivery, on PND0, by subcutaneous (S.C.) injection in the nape of the neck. Once vaginal opening occurred, a daily vaginal smear was collected from each rat and the cytological changes were monitored until PND90 (n=16 for each treatment). At approximately PND90, pups were euthanized at 11 hr 00 on the second diestrous day (D), at 11 hr 00 on the proestrous day (PE11), at 17 hr 00 on the proestrous day (PE17) and at 11 hr 00 on the estrous day (E), and the blood and brains were collected (n=4 for each time point). The blood samples were immediately centrifuged (1,500 × *g* for 15 min at 4°C), and the serum was stored at −20°C until use. The AVPV/POA, the ARC and the pituitary were dissected out from the brain, snap frozen in liquid nitrogen and stored −80°C until further use in RNA extraction. The AVPV/POA and the ARC regions were punched out with the help of a brain punch set (inner diameter of 1.0 mm; Stoeling Corporation., Wheat Lane, Wood Dale, IL, U.S.A. [12]) from the coronal section of the brain following the coordinates provided in the brain atlas (Paxinos and Watson atlas) [16].

Hormone assay: Serum concentrations of LH, follicle stimulating hormone (FSH) and prolactin were measured using a rat radioimmunoassay (RIA) kit (NIH, Bethesda, MD, U.S.A.). The iodinated preparations used were rat LH-I-7, rat FSH-I-7 and PRL-I-6. The antisera used were anti-rat LH-S-10, anti-rat FSH-S-11 and PRL-S-9, respectively. The results were expressed in terms of NIDDK rat LH-RP-3, FSH-RP-2 and PRL-RP3. The intra- and inter-assay coefficients of variations were; 2.7% and 22.08%; 7.1% and 22.75%; and 2.46% and 22.20% for LH, FSH and prolactin, respectively.

The serum concentrations of immunoreactive (ir-) inhibin were measured using the rabbit antiserum against bovine inhibin (TNDH-1) and the ^{125}I-labeled 32-kDa bovine inhibin. Results were expressed as the concentrations of 32-kDa bovine inhibin. The intra- and inter-assay coefficients of variation were 4.77% and 10.30%, respectively.

The serum concentrations of estradiol and testosterone were measured with the help of a double-antibody RIA system using ^{125}I-labeled radio ligands. Antisera against estradiol (GDN #244) and testosterone (GDN #250) were provided by Dr. G. D. Niswender (Animal Reproduction and Biotechnology, Colorado State University, Fort Collins, CO, U.S.A.). The intra- and inter-assay coefficients of variation were 5.47% and 18.40%, respectively for estradiol and 2.89% and 21.28%, respectively for testosterone.

Quantitative real-time PCR: Total RNA from each sample was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). Complementary DNA (cDNA) was synthesized using PrimeScript reverse transcriptase (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. Oligonucleotide primers were designed using the web-based Primer3 software and are listed in Supplementary Table 1. All polymerase chain reactions were performed using SYBR Premix Ex Taq™ (TaKaRa Bio). The relative expression level of each target mRNA was determined using the $2^{-\Delta\Delta CT}$ method. *GAPDH* or *β-Actin* was used as the endogenous control gene.

Statistical analysis: The data are presented as the mean ± SEM of values from three independent experiments. The level of significance was analyzed using one-way analysis of variance (ANOVA), followed by multiple range tests (Graph Pad Prism5). Differences with $P<0.05$ were considered statistically significant.

RESULTS

Effect of neonatal EE exposure on reproductive parameters: The changes in body weights are shown in Fig. 2a. The rats in all treatment groups grew normally, and there was no significant difference between the body weights of control and EE-treated groups. The timing of the vaginal opening, which is indicative of puberty, is compared between control and EE-treated groups in Fig. 2b. There was no significant difference between the timing of vaginal opening of the control and EE-treated groups. The time spent in each cycle day at PND90 is shown in Fig. 2c. There was no significant difference in the estrous cycles of all groups at PND90.

Effect of neonatal EE exposure on hormonal changes at PND90: The changes in the serum levels of LH, FSH, inhibin, prolactin, estradiol and testosterone are shown in Fig. 3. LH-surge was observed at PE17, and the peak levels were reduced in the EE-treated groups compared to control group. In 200 μg/kg EE-treated group, increases of FSH at diestrous and testosterone at PE11 were observed (Fig. 3b and 3e). There were no significant differences in the levels of other hormones between control and EE-treated groups.

Effect of neonatal EE exposure on hypothalamic gene expression at PND90: To examine the potential hypothalamic changes in EE-treated animals, the kisspeptin (*kiss1*), *GPR54*, *ERα* and *GNRH1* mRNA levels in the AVPV/POA and the ARC regions were quantified by quantitative real-time PCR. EE treatment reduced the levels of *kiss1* mRNA in the AVPV/POA at PE17 (Fig. 4a). In 200 μg/kg EE-treated group, decrease of GNRH1 mRNA expression at diestrous was observed (Fig. 4d). There were no significant differences in the mRNA levels of *GPR54* and *ERα* (Fig. 4b and

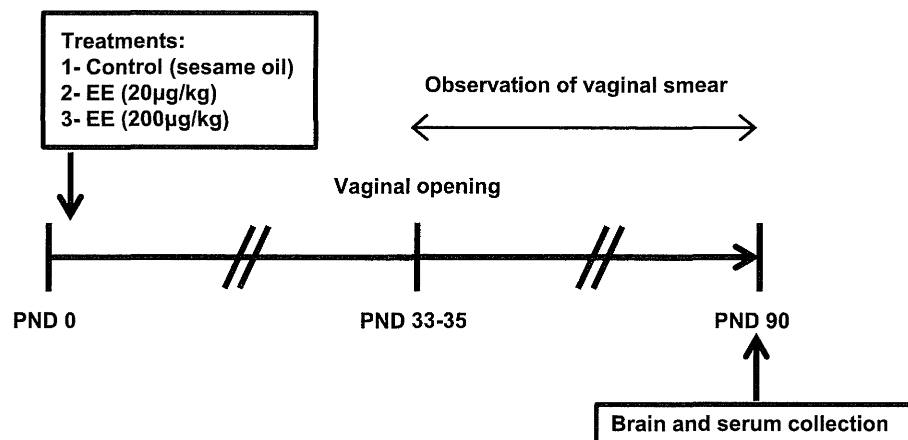

Fig. 1. Schematic representation of experimental protocol. PND, postnatal day.

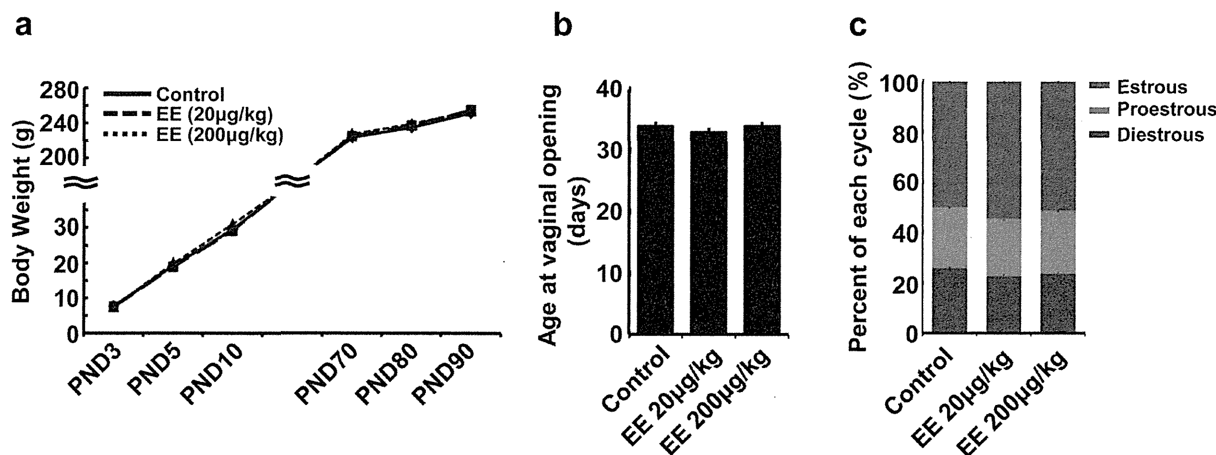

Fig. 2. Effect of neonatal EE exposure on body weight and reproductive parameters. (a) Body weight from PND3 to PND10 and from PND70 to PND90. PND, postnatal day. (b) Age at vaginal opening. (c) Percent of time spent in each cycle day during PND75-90. Rats were neonatally treated with sesame oil and with 2 concentrations of EE (20 μg/kg and 200 μg/kg). Estrous stage was determined by vaginal cytology. Data are presented as the mean ± SEM.

4c). Compared to controls, the expression of *kiss1* mRNA in the ARC of 200 μg/kg EE-treated group was lower at PE17 (Fig. 5a). However, there were no significant differences in the mRNA levels of *GPR54*, *ERα* and *GNRH1* between the ARCs of control and EE-treated groups (Fig. 5b, 5c and 5d).

Effect of neonatal EE exposure on pituitary gene expression at PND90: The mRNA levels of *LHb*, FSH beta subunit (*FSHb*), prolactin (*PRL*) and GnRH receptor (*GNRHR*) were determined by quantitative real-time PCR (Fig. 6). There were no significant differences in the mRNA expression levels of other genes between the control and the treatment groups.

DISCUSSION

It has been reported that the perinatal exposure to EDCs affects the HPG axis and the brain development related to sexual differentiation. Here, we report EE-induced changes that are potentially related to the delayed reproductive dysfunction. Neonatal EE exposure caused a reduction in the LH-surge level at PND90 when the animals showed a normal estrous cycle. In contrast, these animals showed abnormal estrous cycle at PND180. Reduced *kiss1* mRNA expression was also observed in the AVPV/POA of EE-treated animals. It has been reported that in males, no LH-surge occurred and the expression level of kisspeptin in the AVPV/POA was lower in males than females [1, 2]. Neonatal exposure of neonatal females to EE might induce the masculinization of the AVPV/POA region during the sexual differentiation of the brain, which is likely the reason for the reduced kisspeptin gene expression and the low LH-surge at PND90.

In this study, we administered EE (20 and 200 μg/kg) to pups subcutaneously at PND0. The EE has been widely used for oral contraception in women, and the pills contain 50 μg EE, corresponding to 1.0 μg/kg/day. The doses selected in this study were approximately 20–200 times higher than

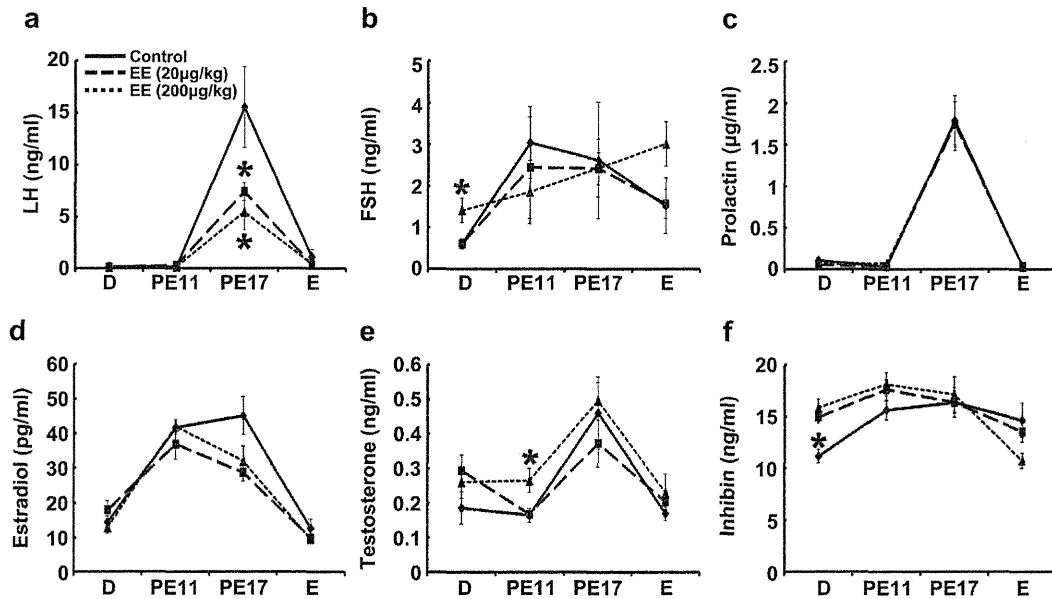

Fig. 3. Changes in serum level of LH (a), FSH (b), prolactin (c), estradiol-17β (d), testosterone (e) and inhibin (f) in neonatal EE treated rats. Bloods were collected at PND90 from animals treated with sesame oil and with 2 concentrations of EE (20 μg/kg and 200 μg/kg). Hormone level was measured by RIA. Each point represents mean ± SEM. Asterisk indicates a significant difference compared to the control ($P<0.05$). D, Diestrous PE11, Proestrous at 11 hr 00 PE17, Proestrous at 17 hr 00 E, Estrous.

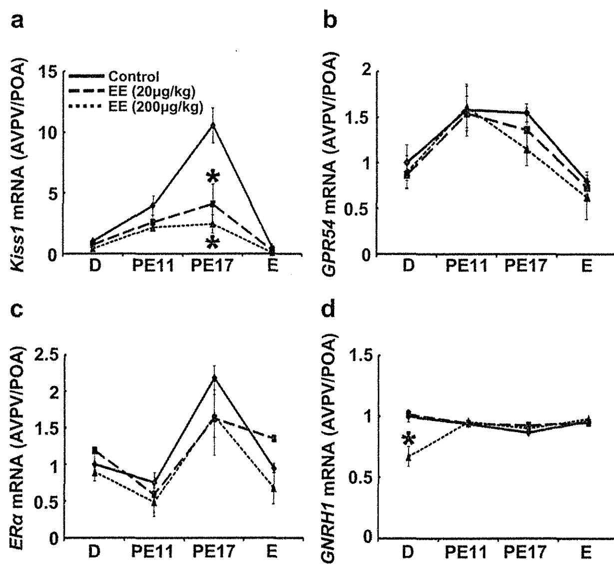

Fig. 4. Changes in *kiss1*(a), *GPR54* (b), *ERα* (c) and *GNRH1* (d) mRNA expression in AVPV/POA. Samples were collected at PND90 from animals treated with sesame oil and with two concentrations of EE (20 μg/kg and 200 μg/kg). mRNA expression level was analyzed by real-time PCR. Each point represents mean ± SEM. Asterisk indicates a significant difference compared to the control ($P<0.05$). D, Diestrous PE11, Proestrous at 11 hr 00 PE17, Proestrous at 17 hr 00 E, Estrous.

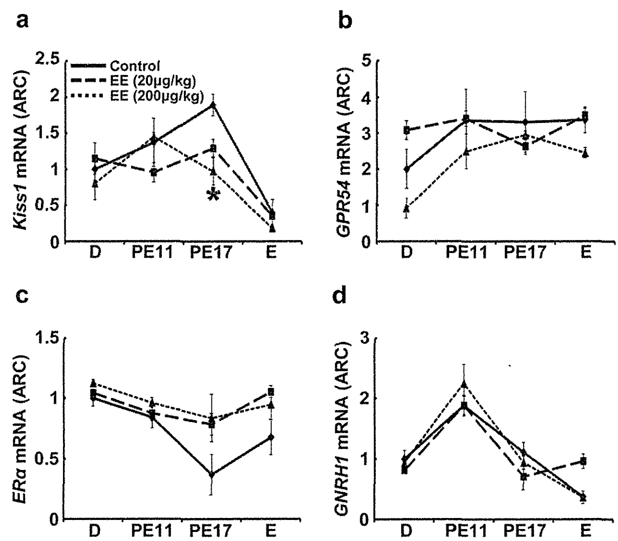

Fig. 5. Changes in *kiss1*(a), *GPR54* (b), *ERα* (c) and *GNRH1* (d) mRNA expression in ARC. Samples were collected at PND90 from animals treated with sesame oil and with 2 concentrations of EE (20 μg/kg and 200 μg/kg). mRNA expression level was analyzed by real-time PCR. Each point represents mean ± SEM. Asterisk indicates a significant difference compared to the control ($P<0.05$). D, Diestrous PE11, Proestrous at 11 hr 00 PE17, Proestrous at 17 hr 00 E, Estrous.

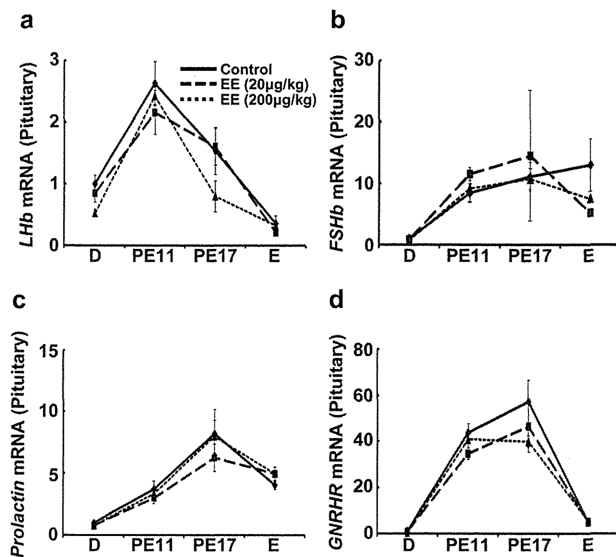

Fig. 6. Changes in *LHb* (a), *FSHb* (b), *prolactin* (c) and *GNRHR* (d) mRNA expression in pituitary. Samples were collected at PND90 from animals treated with sesame oil and with 2 concentrations of EE (20 μg/kg and 200 μg/kg). mRNA expression level was analyzed by real-time PCR. Each point represents mean ± SEM. Asterisk indicates a significant difference compared to the control ($P<0.05$). D, Diestrous PE11, Proestrous at 11 hr 00 PE17, Proestrous at 17 hr 00 E, Estrous.

exposure of women. In a previous study, EE (5–50 μg/kg/day) was administered to dams by oral gavage from gestational day (GD) 7 to PND22 [18]. The authors reported that there were no changes in the *kiss1* mRNA expression in the AVPV/POA of pups at PND50 [18]. However, the quantity of EE that reached the pup's circulation is not known. The lower chronic exposure was not sufficient to induce changes in *kiss1* gene expression. It is likely that the metabolism of EE in the mother's body, placenta and mammary gland might prevent EE from entering the pup's circulation. Therefore, the timing and method of administration should be prescribed for a comprehensive assessment of the effects of EE.

Estrogen regulates *kiss1* mRNA expression in kisspeptin neurons [15]. Two distinct subtypes of ER, ERα and ERβ, are known [21]. *ERα* is predominantly expressed in the uterus, mammary gland, testis, pituitary, liver and kidney, whereas *ERβ* is primarily expressed in the ovary and prostate [6]. Kisspeptin neurons highly express high levels of ERα, which is higher in females than in males [3, 16, 25]. Estrogen fails to stimulate *kiss1* mRNA expression in kisspeptin neurons of the ovariectomized ERα-knockout mice [28]. Thus, ERα mediates estrogen-induced *kiss1* gene expression [5]. Our results showed that the expression level of ERα in the AVPV/POA and the ARC of the control- and EE-treated groups was similar. Additionally, serum level of estrogen was unaffected by EE treatment. Taken together, the reduced expression of *kiss1* mRNA in the AVPV/POA of EE-treated animals may have been due to a functional impairment of signaling downstream of ER.

The serum LH-surge levels decreased along with the reduction in the level of *kiss1* mRNA. Generally, kisspeptin controls LH levels through activating GnRH neuron. GnRH-surge released from the AVPV/POA stimulates pituitary gonadotroph to induce LH-surge without stimulating the transcription. GnRH-pulse generated from the ARC controls basal LH and FSH expression. The AVPV/POA and ARC contained GnRH neurons and we investigated the expression levels of *GNRH1* and *GPR54* mRNA, however, there were no significant differences in both regions. Furthermore, the expression of *LHb* and *GNRHR* mRNA remained unchanged in the pituitary. In 200 μg/kg EE-treated group, the serum FSH showed irregular changes during the estrus cycle, but the expression of *FSHb* mRNA did not change in the pituitary. It was reported that kisspeptin also elicits FSH secretion and intracerebroventricular administration of kisspeptin peptide stimulated FSH secretion in prepubertal and adult rats [23, 27]. These observations provided the possibility that the decrease in LH-surge and irregular FSH might be linked to the weaken GnRH neuron activity and/or reduced GnRH secretion via down-expressed kisspeptin in the AVPV/POA and ARC of EE-treated animals.

In conclusion, we showed that neonatal exposure to EE leads to reduced kisspeptin expression in the AVPV/POA and reduced LH-surge, which is likely involved in the delayed reproductive dysfunction in the adult animals. Previous studies have found that when compared to young rats exhibiting normal estrous cycle, middle-aged rats with persistent estrous had low LH-surge and lower percentage of *kiss1* mRNA-positive neurons with c-fos immunoreactivity in the AVPV [8, 13, 19, 29]. Since there are some similarities on reproductive phenotypes between neonatal EE exposure animals and middle-aged animals, neonatal EE administration may be useful for generating animal models to investigate the physiological and molecular mechanisms of reproductive aging. Further studies are needed to identify the mechanisms responsible for the suppression of kisspeptin expression following exposure to EE.

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REFERENCES

1. Bai, Y., Chang, F., Zhou, R., Jin, P. P., Matsumoto, H., Sokabe, M. and Chen, L. 2011. Increase of anteroventral periventricular kisspeptin neurons and generation of E2-induced LH-surge systems in male rats exposed perinatally to environmental dose of bisphenol-A. *Endocrinology* **152**: 1562–1571. [Medline] [CrossRef]
2. Bateman, H. L. and Patisaul, H. B. 2008. Disrupted female reproductive physiology following neonatal exposure to phytoestrogens or estrogen specific ligands is associated with decreased GnRH activation and kisspeptin fiber density in the hypothalamus. *Neurotoxicology* **29**: 988–997. [Medline] [CrossRef]
3. Cao, J. and Patisaul, H. B. 2011. Sexually dimorphic expression of hypothalamic estrogen receptors α and β and kiss1 in neonatal

male and female rats. *J. Comp. Neurol.* **519**: 2954–2977. [Medline] [CrossRef]

4. Clarkson, J. and Herbison, A. E. 2006. Postnatal development of kisspeptin neurons in mouse hypothalamus: Sexual dimorphism and prejections to gonadotropin-releasing hormone neurons. *Endocrinology* **147**: 5817–5825. [Medline] [CrossRef]
5. Clarkson, J., Boon, W. C., Simpson, E. R. and Herbison, A. E. 2009. Postnatal development of an estradiol-kisspeptin positive feedback mechanism implicated in puberty onset. *Endocrinology* **150**: 3214–3220. [Medline] [CrossRef]
6. Couse, J. F. and Korach, K. S. 1999. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr. Rev.* **20**: 358–417. [Medline] [CrossRef]
7. Dickerson, S. M., Cunningham, S. L., Patisaul, H. B. P., Woller, M. J. and Gore, A. C. 2011. Endocrine disruption of brain sexual differentiation by developmental PCB exposure. *Endocrinology* **152**: 581–594. [Medline] [CrossRef]
8. Downs, J. L. and Wise, P. M. 2009. The role of the brain in female reproductive aging. *Mol. Cell. Endocrinol.* **299**: 32–38. [Medline] [CrossRef]
9. García-Galiano, D., Pinilla, L. and Sempere, M. T. 2012. Sex steroids and the control of the kiss1 system: Developmental roles and major regulatory actions. *J. Neuroendocrinol.* **24**: 22–33. [Medline] [CrossRef]
10. Gore, A. C. 2008. Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. *Front. Neuroendocrinol.* **29**: 358–374. [Medline] [CrossRef]
11. Hotchkiss, A. K., Rider, C. V., Blystone, C. R., Wilson, V. S., Harting, P. C., Ankley, G. T., Foster, P. M., Gray, C. L. and Gray, L. E. 2008. Fifteen years after "wingspread"-environmental endocrine disrupters and human and wildlife health: Where we are today and where we need to go. *Toxicol. Sci.* **105**: 235–259. [Medline] [CrossRef]
12. Horii, Y., Dalpatadu, S. L., Soga, T., Ohta, R., Watanabe, G., Taya, K. and Parhar, I. S. 2013. Estrogenic regulation of *kiss1* mRNA variants in Hatano rats. *Gen. Comp. Endocrinol.* **181**: 246–253. [Medline] [CrossRef]
13. Ishii M. N., Matsumoto, K., Matsui H., Seki, N., Matsumoto, H., Ishikawa, K., Hatani, F., Watanabe, G. and Taya, K. 2013. Reduced responsiveness of kisspeptin neurons to estrogenic positive feedback associated with age-relate disappearance of LH surge in middle- age female rats. *Gen. Comp. Endocrinol.* **193**: 121–129. [Medline] [CrossRef]
14. Juraska, J. M., Sisk, C. L. and Doncarlos, L. L. 2013. Sexual differentiation of the adolescent rodent brain: Hormonal influences and developmental mechanisms. *Horm. Behav.* **64**: 203–210. [Medline] [CrossRef]
15. Kauffman, A. S., Gottsch, M. L., Roa, Byquist, A. C., Crown, A. and Clifton, D. K. 2007. Sexual differentiation of *kiss1* gene expression in the brain of the rat. *Endocrinology* **148**: 1774–1783. [Medline] [CrossRef]
16. Li, X. F., Kinsey-Jones, J. S., Cheng, Y., Konx, A. M. I., Lin, Y. and Petrou, N. A. 2009. Kisspeptin signalling in the hypothalamic arcuate nucleus regulates GnRH pulse generator frequency in the rat. *PLoS ONE* **4**. [Medline] [CrossRef]
17. Maeda, K., Ohkura, S., Uenoyama, Y., Wakabayashi, Y., Oka, Y., Tsukamura, H. and Okamura, H. 2010. Neurobiological mechanisms underlying GnRH pulse generation by the hypothalamus. *Brain Res.* **1364**: 103–115. [Medline] [CrossRef]
18. Matt, D. W., Glison, M. P., Sales, T. E., Krieg, R. J., Kerbeshian, M. C., Veldhuis, D. and Evans, W. S. 1998. Characterization of attenuated proestrous luteinizing hormone surges in middle-aged rats by deconvolution analysis. *Biol. Reprod.* **59**: 1477–1482. [Medline] [CrossRef]
19. Mayer, C., Martinez, M. A., Dubois, S. L., Wolfe, A., Radovick, S., Boehm, U. and Levine, J. E. 2010. Timing and completion of puberty in female mice depend on estrogen receptor α-signaling in kisspeptin neurons. *Proc. Natl. Acad. Sci. U.S.A.* **107**: 22693–22698. [Medline] [CrossRef]
20. De Mees, C., Laes, J. F., Bakker, J., Smitz, J., Hennuy, B. and Vooren, P. V. 2006. Alpha-fetoprotein controls female fertility and prenatal development of the gonadotropin-releasing hormone pathway through an antiestrogenic action. *Mol. Cell. Biol.* **26**: 2012–2018. [Medline] [CrossRef]
21. Mueller, S. O. and Korach, K. S. 2001. Estrogen receptors and endocrine diseases: lessons from estrogen receptor knockout mice. *Curr. Opin. Pharmacol.* **1**: 613–619. [Medline] [CrossRef]
22. Nozawa, K., Nagaoka, K., Zhang, H., Usuda, K., Okazaki, S., Taya, K. and Watanabe, G. 2014. Neonatal exposure to 17α-ethynyl estradiol affect ovarian gene expression and causes reproductive dysfunction in female rats. *Reprod. Toxicol.* (in press). [CrossRef]
23. Navarro, V. M., Castellano, J. M., Fernandez-Fernandez, R., Tovar, S., Roa, J., Mayen, A., Barreiro, M. L., Casanueva, F. F., Aguilar, E., Dieguez, C., Pinilla, L. and Tena-Sempere, M. 2005. Effects of KISS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. *Endocrinology* **146**: 1689–1697. [Medline] [CrossRef]
24. Ohta, R., Ohmukai, H., Marumo, H., Shindo, T., Nagata, T. and Ono, H. 2012. Delayed reproductive dysfunction in female rats induced by early life exposure to low- dose diethylstilbestrol. *Reprod. Toxicol.* **34**: 323–330. [Medline] [CrossRef]
25. Okamura, H., Yamamura, T. and Wakabayashi, Y. 2013. Kisspeptin as a master player in the central control of reproduction in mammals: An overview of kisspeptin research in domestic animals. *Anim. Sci. J.* **84**: 369–381. [Medline] [CrossRef]
26. Overgaard, A., Holst, K., Mandrup, K. R., Boberg, J., Christiansen, S., Jacobsen, P. R., Hass, U. and Mikkelsen, J. D. 2013. The effect of perinatal exposure to ethinyl oestradiol or a mixture of endocrine disrupting pesticides on kisspeptin neurons in the rat hypothalamus. *Neurotoxicology* **37**: 154–162. [Medline] [CrossRef]
27. Pinilla, L., Aguilar, E., Dieguez, C., Millar, R. P. and Sepere, T. 2012. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol. Rev.* **92**: 1235–1316. [Medline] [CrossRef]
28. Smith, J. T., Cunningham, M. J., Rissman, E. F., Clifton, D. K. and Steiner, R. A. 2005. Regulation of *kiss1* gene expression in the brain of the female mouse. *Endocrinology* **146**: 3686–3692. [Medline] [CrossRef]
29. Zhang, J., Yang, L., Lin, N., Pan, X., Zhu, Y. and Chen, X. 2014. Aging- related changes in the RP3V kisspeptin neurons predate the reduced activation of GnRH neurons during the early reproductive decline in female mice. *Neurobiol. Aging* **35**: 655–668. [Medline] [CrossRef]

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Original Article

Effects of pyperonyl butoxide on the female reproductive tract in rats

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ABSTRACT — This study was investigated the effects of piperonyl butoxide (PBO) on the female reproductive tract. Female Crj:Donryu rats were fed a basal diet containing 5,000, 10,000 or 20,000 ppm PBO for 28 days, and compared with food-restricted rats of comparable body weights to those in the PBO 10,000 or 20,000 ppm groups. Although treatment with 20,000 ppm PBO for 28 days depressed body weight gain, the abnormal estrous cyclicity, mainly prolonged diestrus, was also induced by the PBO treatment which was not correlated with body weight change. 20,000 ppm PBO treatment markedly decreased uterine weights and slightly decreased ovarian weights. 10,000 and 20,000 ppm PBO treatment increased liver weights. These cycle and organ weight changes were linked to atrophic uterus and increased atretic follicles in the ovary. In hormone assays, PBO at both doses reduced serum E2 levels, but did not affect corticosterone levels. An anti-uterotrophic assay showed a slight but significant decrease in absolute uterine weight and a reduction of endometrial epithelium height in the 20,000 ppm group. PBO was positive in an ER α antagonist reporter gene assay, although the activity was much weaker than that of 4-hydroxytamoxifen. These results indicate that high-dose PBO treatment directly induces atrophic changes in the female reproductive tract in rats, and these effects are likely the result of a hypoestrogenic state and the anti-estrogenic activity of PBO.

Key words: Piperonyl butoxide, Female reproductive tract, Anti-uterotrophic assay, Reporter gene assay

INTRODUCTION

Piperonyl butoxide (PBO), α-[2-(2-butoxyethoxy) ethoxy]-4,5-methylenedioxy-2-propyltoluene, is a pesticide synergist that is widely used in combination with pyrethrins and synthetic pyrethroids. PBO acts as a synergist through acute inhibition of cytochrome P450, thereby reducing pesticide metabolism in the insect (Franklin, 1972). Sub-acute or chronic exposure to PBO in rodents, however, induces liver hypertrophy and increases cytochrome P450 activity (Butler *et al.*, 1998; Philips *et al.*, 1997). Furthermore, previous studies have shown that dietary exposure to 2.4% PBO also affects the female reproductive tract of F344 rats, resulting in decreased ovarian weight (Takahashi *et al.*, 1994) and uterine atrophy (Fujitani *et al.*, 1992). However, the mechanism by which these effects are induced by PBO remains unclear.

High-dose exposure to PBO is also reported to cause suppression of body weight gain in rats (Philips *et al.*, 1997; Fujitani *et al.*, 1992), which may potentially confound studies assessing its effects on reproduction. Feeding restriction with resultant body weight reduction affects reproductive function and reduces fertility in rats (Chapin *et al.*, 1993; Terry *et al.*, 2005). Therefore it is unclear whether the reproductive effects of PBO treatment are primary or secondary. In general, atrophy of the female reproductive tract induced by a chemical compound is caused by disruption of the hypothalamus-pituitary axis, ovarian dysfunction, and/or anti-estrogenic effects of the compound.

In the present study, we clarified the effects of PBO on the female reproductive tract in rats. We first focused on the question of whether the effects of PBO on the female

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reproductive tract are secondary to body weight change. We compared the effects of PBO on estrous cyclicity, reproductive organ weight, and serum hormone levels in PBO-treated rats and those with similar body weights induced by food restriction. To evaluate other possible causes of the effects of PBO on the female reproductive tract, we assessed the anti-estrogenic potential of PBO using both an anti-uterotrophic assay and an estrogen reporter gene assay.

MATERIALS AND METHODS

Chemicals

Piperonyl butoxide (PBO, CAS No. 51-03-6) and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 17β-estradiol (E2, CAS No. 50-28-2) was purchased from Sigma Aldrich (St. Louis, MO, USA). PBO was mixed with the powdered basal diet, CRF-1 (Oriental Yeast Co., Tokyo, Japan). Test diets were prepared every 2 weeks, and stored at 4°C before use.

Animals and housing conditions

Female Crj:Donryu rats bred in our laboratory were used in the present study. This rat strain has a regular four-day estrous cycle. They received the basal diet (CRF-1, Oriental Yeast) and tap water *ad libitum*, and were housed 2-3 per plastic cage with sterilized softwood chips as bedding in a barrier-maintained animal room, conditioned at 24 ± 1°C and 55 ± 5% humidity with a 12-hr light/dark cycle. All experimental protocols and animal use were reviewed and approved by the Animal Care and Utilization Committee of the National Institute of Health Science.

Experiment 1 (28-day feeding study of PBO)

6-week-old female rats were fed diets containing either 0, 5,000, 10,000 and 20,000 ppm of PBO for 28 days (10 animals/group). The PBO doses were determined based on previous reports that uterine atrophy was induced by 2.4% PBO treatment (Fujitani *et al.*, 1992), and effects on body weight gain were observed with 2% PBO treatment (Muguruma *et al.*, 2007). The rats were observed daily for clinical signs and mortality, and body weight was recorded twice a week. The amounts of supplied and residual diet were weighed twice a week in order to calculate the average daily consumption of PBO. To investigate the effect on weight gain, we included two food-restricted (FR) groups with weight changes comparable to those observed in the PBO 10,000 and 20,000 ppm groups (FR-1 and FR-2 respectively, each with eight animals). The animals were weighed daily and fed a weight-based basal diet in the mornings (FR-1: 13.3 g/day average, FR-2: 9.3 g/day average). Estrous cyclicity was checked by vaginal cytology in all animals in the morning (9:00-10:00) throughout the treatment period. To compare the effect on serum hormone levels and the morphological findings of the female reproductive tract between the control and other groups, the animals were euthanized by decapitation at proestrus (high E2 level) and diestrus. If the rats were acyclic or predominantly diestrus, these animals were sacrificed at these stages. Table 1 shows the number of animals at each of estrous stages at autopsy. After the treatment period, all rats were euthanized by decapitation, and trunk blood was collected. Serum samples were stored at -80°C until the hormonal assay. Serum concentrations of estradiol-17β (E2) and progesterone (P4) were determined by double-antibody radioimmunoassay (RIA) systems using ^{125}I-labeled radioligands, as described previously (Taya *et al.*, 1985). Serum concentrations of follicle stimulated hormone (FSH), luteinizing hormone (LH) and prolactin (PRL) were measured using National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) radioimmunoassay kits for rat FSH, LH and PRL (NIAMDD, NIH, Bethesda, MD, USA) (Taya *et al.*, 1983). The serum concentration of immunoreactive inhibin-α was analyzed using a rabbit anti-serum against bovine inhibin (TNDH-1) and ^{125}I-labelled 32-kDa bovine inhibin, as described previously (Hamada *et al.*, 1989). After the macroscopic examination, the ova-

Table 1. Number of animals at each of estrous stages at autopsy (experiment 1)

	Pyperonyl butoxide in the diet (ppm)				FR-1	FR-2
	0	5,000	10,000	20,000		
No. of examined animals	10	10	10	10	8	8
Estrous stages						
Proestrus	5	5	6	0	3	4
Diestrus	5	5	4	10	5	4

ries, uterus, vagina, adrenals, pituitary, thymus and liver were removed and the weight of each was recorded. Immediately after weighing, the organs were fixed in 10% neutral buffered formalin, and routinely processed for paraffin embedding, sectioned and stained with hematoxylin and eosin (HE). For analysis of ovarian histopathology, ovaries were transversally halved at dissection at the point with the greatest cross-sectional area, and both sections were examined. For uterine histopathology, both the uterine horns and uterine cervix were examined in one section each. Morphological classification of the female reproductive tract at each estrous stage was in accordance with previous reports (Westwood, 2008; Yuan and Foley, 2002; Yoshida *et al.*, 2009).

Experiment 2 (Anti-uterotrophic assay)

Female rats were ovariectomized at 15 weeks of age, and randomly allocated to five treatment groups (5 animals/group). PBO treatment began 2 weeks postoperatively at the following doses: 0 (negative control; NC), 5,000, 10,000 and 20,000 ppm PBO in the diet and daily subcutaneous injection of E2 in DMSO at a dose of 1 µg/kg concurrently for 2 weeks. E2 was dissolved in DMSO to allow for dosing at 1 ml/kg body weight. The rats in the vehicle control (VC) group were fed the basal diet and were injected with DMSO in the same manner as the treated groups. Animals were weighed daily and food consumption was measured twice a week. After the treatment period, all animals were euthanized, and the wet and blotted uterine weights recorded. The uteri were fixed in 10% neutral buffered formalin, routinely processed for paraffin embedding, sectioned and stained with HE for histopathologic examination. The luminal epithelial cell height in the uterine horn was measured at 5 different locations per animal using an image analyzer, Image J (National Institutes of Health, Bethesda, Maryland, USA).

Experiment 3 (Reporter gene assay)

To assess for anti-estrogenic activity *in vitro*, a reporter gene assay employing human estrogen receptor (ERα) was performed by Chemicals Evaluation and Research Institute (Tokyo, Japan), as previously reported (Takesyoshi *et al.*, 2002a, 2002b).

Statistical analysis

Differences in final body weight, absolute and relative organ weights, serum hormone level and uterine epithelial cell height were examined. These data were analyzed using Bartlett's test or the F test. When variances were homogeneous, Dunnett's multiple comparison test or Student's t test was performed. Steel's multiple comparison test or Aspin-Welch t test was employed, when variances were not homogenous. The levels of significance were set at $P < 0.05$ and 0.01.

RESULTS

Experiment 1 (28-day feeding study of PBO)

Body weight

Fig.1 shows the body weight curves for the female rats treated with PBO. Weight loss occurred within the first seven days in the 20,000 ppm group, and within the first three days in the 10,000 ppm group. Both groups gradually gained weight thereafter. Body weight reduction in the FR-2 group was noted several days later than that in the 20,000 ppm group, and the reduction continued until day 17. Overall, the sequential changes in body weight in the FR-1 and FR-2 groups were similar to those in the matched PBO-treated groups. In the 5,000 ppm group, weight gain was slightly reduced during the study period. Final body weights of the 5,000, 10,000 and 20,000 ppm, FR-1, and FR-2 groups were 91, 87, 67, 87 and 68% of those in the control group, respectively.

Food consumption and chemical intake

The food consumption in each of the PBO treated groups was lower than that in the control group in the first 3 days; however, the difference was no longer evident from days 7-28 days (Fig. 2). The actual PBO intake of the 5,000, 10,000 and 20,000 ppm groups was 468.8, 1104.0 and 2394.8 mg/kg per day, respectively (Table 2).

Estrous cyclicity

All rats in the control group showed a regular 4-day estrous cycle throughout the treatment period (Fig. 3A). Five rats in the 20,000 ppm group showed prolonged die-

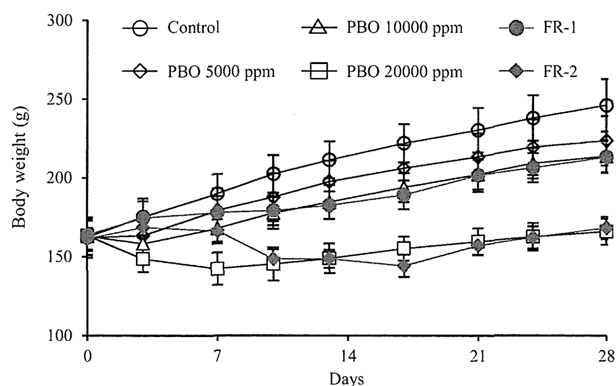


Fig. 1. Body weight curves in experiment 1 (mean ± S.D.).

Table 2. Achieves dose of PBO (experiment 1).

	Pyperonyl butoxide in diet (ppm)		
	5,000	10,000	20,000
No. of animals	10	10	10
Intake of PBO (mg/kg bw/day)	468.76 ± 83.01[a)]	1103.97 ± 205.94	2394.80 ± 446.01

[a)]Mean ± S.D.

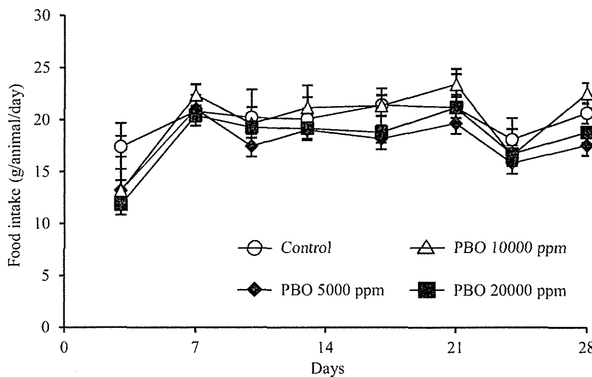


Fig. 2. Daily food intake in experiment 1 (mean ± S.D.).

strus, and the remaining rats in the same group cycled irregularly within 5 to 7 days of treatment initiation. The rats' estrous cyclicity remained abnormal for the duration of the experiment (Fig. 3B). In the FR-2 group, prolonged diestrus or cycle irregularity appeared on day 14, coincidental with the reduction in body weight. Estrous cyclicity normalized once body weight gain recovered to increase (Fig. 3C). The 5,000 and 10,000 ppm and FR-1 groups exhibited no estrous cycle disruption over the entire study period (data not shown).

Organ weights

Table 3 shows the organ weights of the PBO and FR groups. The most marked change in organ weight was the decrease in uterine weight at diestrus with 20,000 ppm PBO treatment. This group had uterine weights that were one-third the weights in the control group. In the FR-2 group, absolute uterine weights were slightly decreased, but relative uterine weights were slightly increased at both proestrus and diestrus. Absolute and relative weights of the ovaries and pituitary were slightly decreased in the 20,000 ppm group, but not in the FR2 group. Liver weights were significantly increased in the 10,000 and 20,000 ppm groups, but decreased in the FR2 group. Absolute but not relative weights of the adrenals and thymus were decreased in the PBO and FR groups.

Pathology findings

Macroscopically, marked uterine atrophy was noted in the 20,000 ppm group. Hepatic enlargement was observed in the 10,000 and 20,000 ppm groups. These changes correlated with the changes in the organ weights and his-

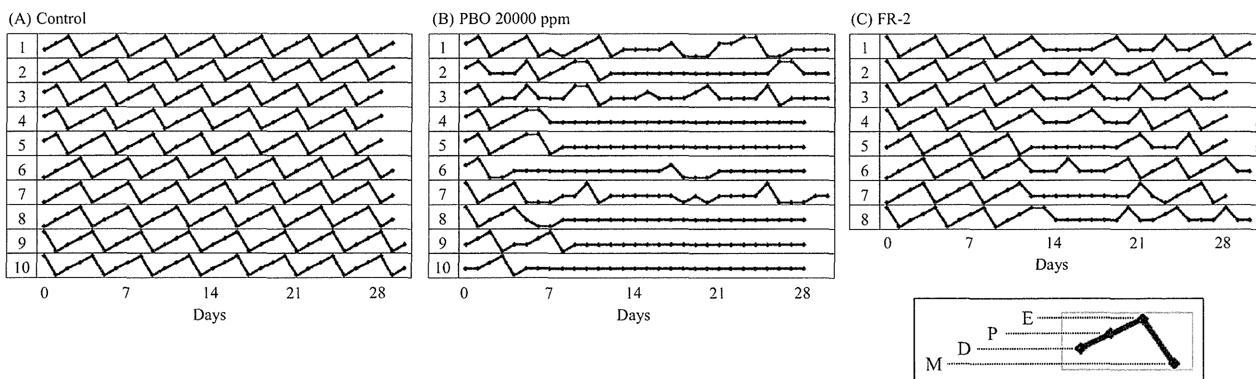


Fig. 3. Estrous cyclicity in experiment 1. (A) control group, (B) 20,000 ppm group, (C) FR-2 group. The plot for each animal is a four-level depiction of the estrus cycle: data points indicate each estrus stage illustrated in the right column (M: metestrus, D: diestrus, P: proestrus, E: estrus).

Table 3. Final body weight and organ weights (experiment 1).

	Pyperonyl butoxide in the diet (ppm)				FR-1	FR-2
	0	5,000	10,000	20,000		
No. of examined animals	10	10	10	10	8	8
Final B.W.(g)	246.6 ± 16.6[a)]	223.9 ± 16.5**	213.4 ± 10.2**	165.8 ± 9.0**	246.6 ± 16.6**	223.9 ± 16.5**
Uterus						
Proestrus						
Absolute (g)	0.658 ± 0.020 (5)	0.647 ± 0.070 (5)	0.599 ± 0.068 (6)	NE	0.632 ± 0.042 (3)	0.547 ± 0.085** (4)
Relative (g/100g B.W.)	0.267 ± 0.019 (5)	0.283 ± 0.029 (5)	0.279 ± 0.017 (6)	NE	0.298 ± 0.020 (3)	0.320 ± 0.037* (4)
Diestrus						
Absolute (g)	0.506 ± 0.031 (5)	0.491 ± 0.035 (5)	0.446 ± 0.010 (4)	0.171 ± 0.043** (10)	0.553 ± 0.075 (5)	0.420 ± 0.043 (4)
Relative (g/100g B.W.)	0.207 ± 0.022 (5)	0.225 ± 0.019 (5)	0.210 ± 0.005 (4)	0.103 ± 0.026** (10)	0.252 ± 0.032* (5)	0.247 ± 0.020 (4)
Ovary						
Absolute (g)	0.093 ± 0.004	0.090 ± 0.009	0.081 ± 0.011**	0.051 ± 0.009**	0.091 ± 0.013	0.069 ± 0.013**
Relative (g/100g B.W.)	0.038 ± 0.003	0.040 ± 0.006	0.038 ± 0.005	0.031 ± 0.004**	0.042 ± 0.006	0.040 ± 0.008
Pituitary						
Absolute (g)	0.014 ± 0.001	0.012 ± 0.002**	0.011 ± 0.001**	0.007 ± 0.002**	0.012 ± 0.002**	0.011 ± 0.001**
Relative (g/100g B.W.)	0.006 ± 0.000	0.005 ± 0.001	0.005 ± 0.000	0.004 ± 0.001**	0.005 ± 0.001	0.006 ± 0.001
Adrenals						
Absolute (g)	0.065 ± 0.008	0.065 ± 0.004	0.063 ± 0.008	0.048 ± 0.007**	0.063 ± 0.007	0.050 ± 0.009**
Relative (g/100g B.W.)	0.027 ± 0.003	0.029 ± 0.002	0.030 ± 0.004	0.029 ± 0.003	0.029 ± 0.003	0.030 ± 0.005
Thymus						
Absolute (g)	0.533 ± 0.089	0.435 ± 0.045	0.409 ± 0.059**	0.327 ± 0.061**	0.481 ± 0.082	0.358 ± 0.082**
Relative (g/100g B.W.)	0.216 ± 0.027	0.194 ± 0.016	0.192 ± 0.029	0.197 ± 0.032	0.222 ± 0.033	0.210 ± 0.045
Liver						
Absolute (g)	10.879 ± 1.105	10.637 ± 0.925	12.105 ± 1.087*	11.387 ± 0.931	8.126 ± 0.754**	6.238 ± 0.581**
Relative (g/100g B.W.)	4.414 ± 0.365	4.749 ± 0.135	5.670 ± 0.378**	6.864 ± 0.388**	3.751 ± 0.272**	3.665 ± 0.196**

[a)]Mean ± S.D.
*, ** : Significantly different from the control group at $P < 0.05$ and 0.01, respectively.
NE : Not examined
Values in parentheses indicate the number of animals examined.

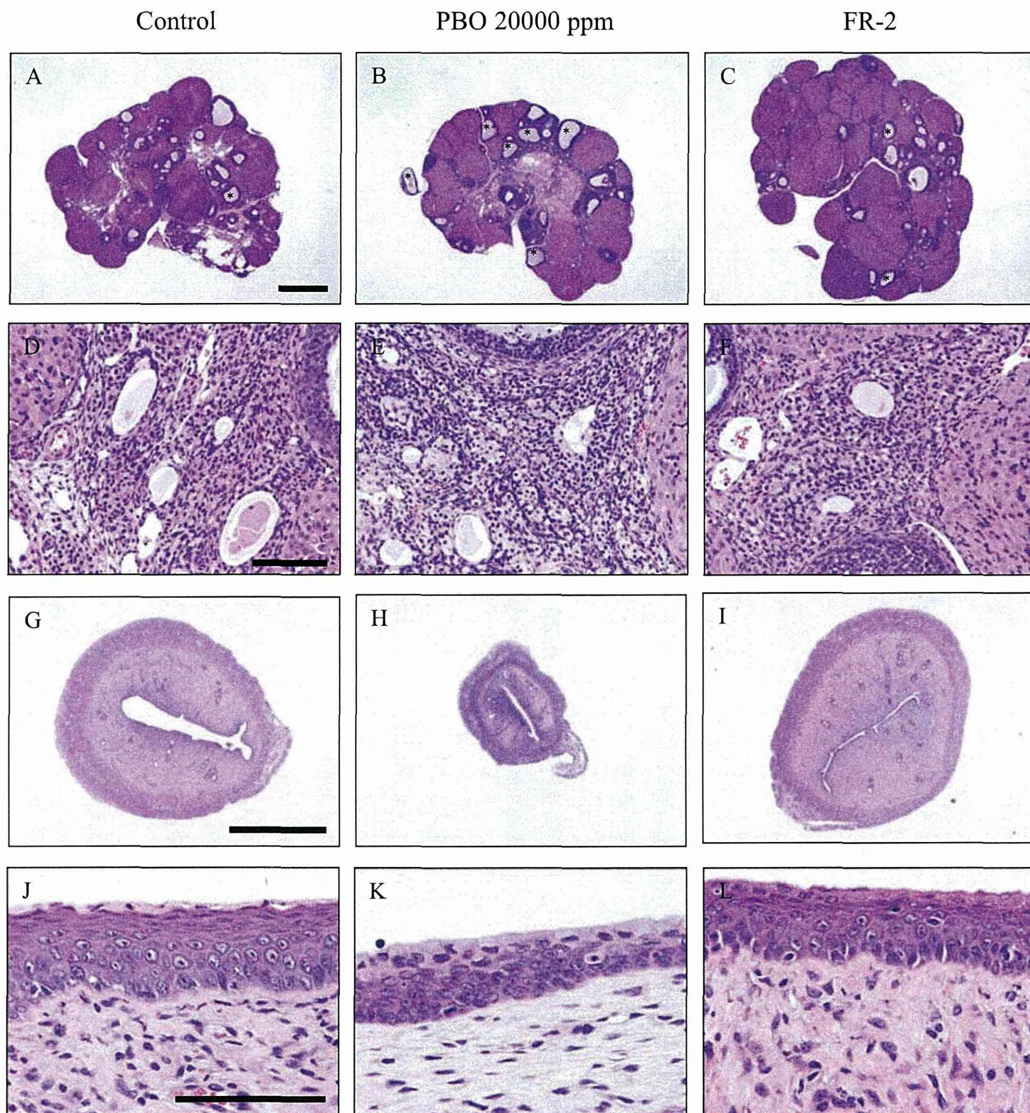


Fig. 4. Representative histopathology of the ovary, uterus and vagina at diestrus in experiment 1. Control ovary (A, D), 20,000 ppm group with numerous large, atretic follicles (*), decrease in newly formed corpora lutea and vacuolation of interstitial cells (B, E), and FR-2 without abnormality (C, F). Uterus of the control group (G), the 20,000 ppm group showing atrophy (H), FR-2 group showing no abnormalities (I). Vagina of the control group (J), the 20,000 ppm group showing thin and mucinous degeneration of the vaginal epithelium (K), and the FR-2 group showing no abnormalities (L). Bars = 1 mm (A, B, C, G, H, I), and 100 μm (D, E, F, J, K, L).

topathological findings.

Microscopic changes noted in the 20,000 ppm group are summarized in Fig. 4 and Table 4. The most pronounced was the severe atrophy of the uterus (Fig. 4H) with thinning and mucinous degeneration in the vaginal epithelium visible at a higher magnification (Fig. 4K). The ovaries in this group had numerous, large, atretic follicles with the most recent formed corpora lutea (Fig. 4B) and vacuolation of the interstitial cells (Fig. 4E). In the 5,000 and 10,000 ppm groups and the FR groups (Fig. 4C, F, I, L), there were no changes in the female reproductive tract. Centrilobular hypertrophy of the hepatocytes was observed in the 10,000 and 20,000 ppm groups (Table 4). No abnormalities were detected in the

Table 4. Incidence and grading of histopathologic findings (experiment 1).

Organ and findings	Piperonyl butoxide in the diet (ppm)				FR-1	FR-2
	0	5,000	10,000	20,000		
Ovary						
Increase in atretic follicle	0/10	0/10	0/10	5/10 (2)	0/8	0/8
Vacuolation, interstitial cell	0/10	0/10	0/10	10/10 (1-2)	0/8	0/8
Decrease/lack in newly formed corpora lutea	0/10	0/10	0/10	9/10 (1-2)	0/8	0/8
Uterus						
Atrophy	0/10	0/10	0/10	10/10 (4)	0/8	0/8
Vagina						
Mucinous degeneration	0/10	0/10	0/10	8/10 (1-2)	0/8	0/8
Thinning in the vaginal epithelium	0/10	0/10	0/10	10/10 (1-2)	0/8	0/8
Liver						
Hepatocellular hypertrophy, centrilobular	0/10	0/10	10/10 (1-2)	10/10 (2-3)	0/8	0/8

Incidence is given as total observations/ number of animals examined. Values in parentheses indicate grading or grading range as subjective severity scores : 1 = minimal, 2 = mild, 3 = moderate, 4= severe.

other organs in any of the groups.

Serum hormone levels

The serum E2 levels at diestrus in the 10,000 and 20,000 ppm groups were significantly lower than that of the control group (Fig. 5A). The serum LH levels at diestrus in the 20,000 ppm group were significantly higher than those in the control group (Fig. 5D). The serum inhibin levels at proestrus in the 10,000 ppm group were significantly lower than those of the control group (Fig. 5F). In the FR groups, the most notable change was an increase in corticosterone levels (Fig. 5G), and serum P4 and LH levels at proestrus were significantly higher and the serum FSH levels at both proestrus and diestrus were significantly lower than those of the control group (Fig. 5B, C, D). There was no significant difference in the PRL levels (Fig. 5E).

Experiment 2 (Anti-uterotrophic assay)

Body weight

The body weights in the 5,000, 10,000 and 20,000 ppm groups were reduced during the treatment period (Fig. 6). Final body weights in the 5,000, 10,000 and 20,000 ppm groups were about 95, 91 and 83% of the corresponding body weights at day 0.

Food consumption and chemical intake

The food consumption in the 10,000 and 20,000 ppm groups was lower than that in the NC group in the first 3 days (Fig. 7). The actual PBO intake in the 5,000, 10,000 and 20,000 ppm group was 283.9, 543.8 and 1342.1 mg/kg per day, respectively (Table 5). The chemical intake in each treatment group was about half of what was consumed by the matched treatment group in experiment 1.

Uterine weight

Both the wet and blotted absolute uterine weights in the 10,000 and 20,000ppm groups were significantly lower than those of the NC group (Fig. 8A). Relative uterine weights, however, showed a trend towards increase given the body weight reduction in these groups (Fig. 8B).

Measurement of uterine epithelial cell height

Histopathological measurement demonstrated that the uterine epithelial cell height in the 20,000 ppm group was significantly lower than that of the NC group (Fig. 9A, B).

Experiment 3 (Reporter gene assay)

Fig. 10 shows concentration response curve for the estrogen receptor antagonist activity of PBO and 4-hydroxytamoxifen (4OH-TAM), the active metabolite of tamoxifen, a known estrogen receptor antagonist. The IC50 was the concentration at which the transcription activity of 25pM E2 was reduced by 50%, in the concentration range showing more than 80% cell survival. The IC50 of PBO (2.24×10^{-5} M) was much higher than that of 4OH-TAM (3.11×10^{-10} M).

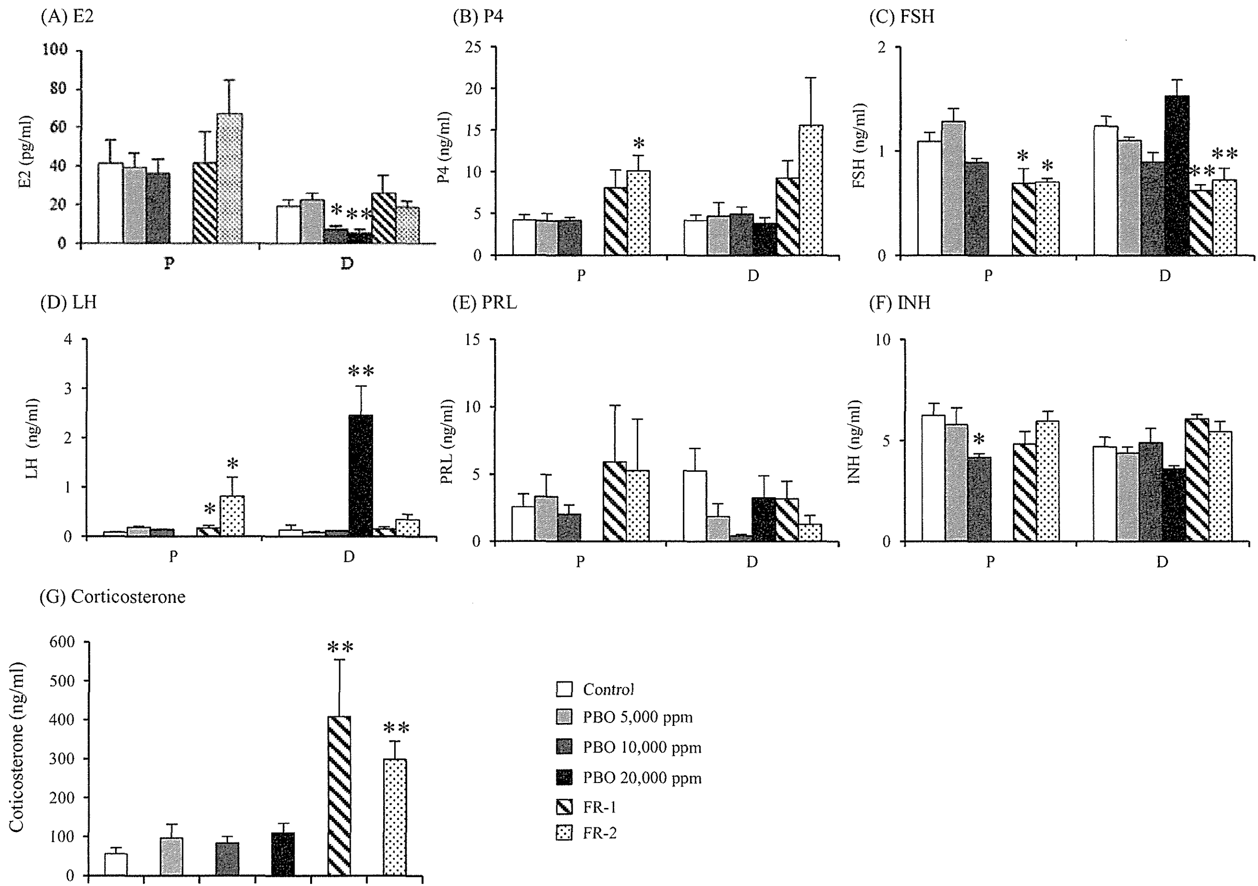


Fig. 5. Serum hormone levels in experiment 1. (A) 17β-estradiol (E2), (B) Progesterone (P4), (C) FSH, (D) LH , (E) Prolactin (PRL), (F) Inhibin α (INH) concentration at each estrus stage (P: proestrus, D: diestrus). (G) Mean serum corticosterone concentration. Data are represented as mean ± S.E.M. *, **: Significantly different from the 0 ppm group at $P < 0.05$ and 0.01, respectively.

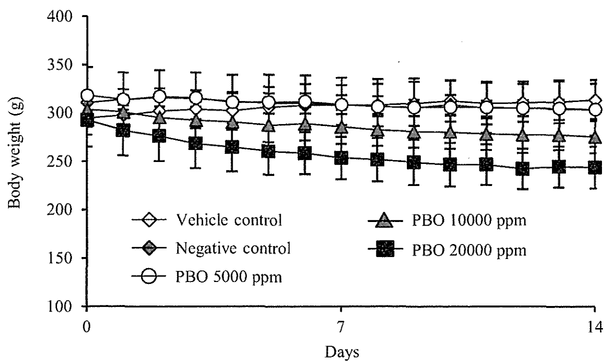


Fig. 6. Body weight curves in experiment 2 (mean ± S.D.).

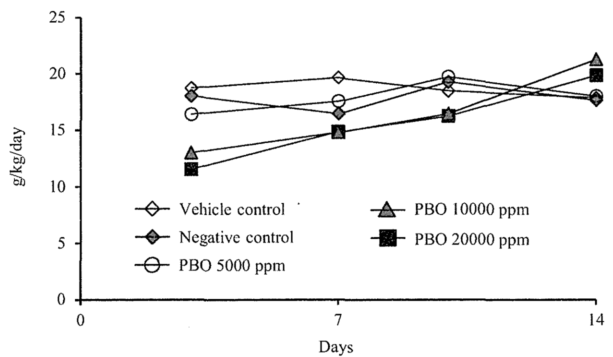


Fig. 7. Daily food intake in experiment 2 (mean ± S.D.).

Table 5. Achieves dose of PBO (experiment 2).

	Pyperonyl butoxide in diet (ppm)		
	5,000	10,000	20,000
	E2 1μg/kg	E2 1μg/kg	E2 1μg/kg
No. of animals	5	5	5
Intake of PBO (mg/kg bw/day)	283.93 ± 17.22[a)]	543.79 ± 75.34	1342.06 ± 225.68

[a)]Mean ± S.D.

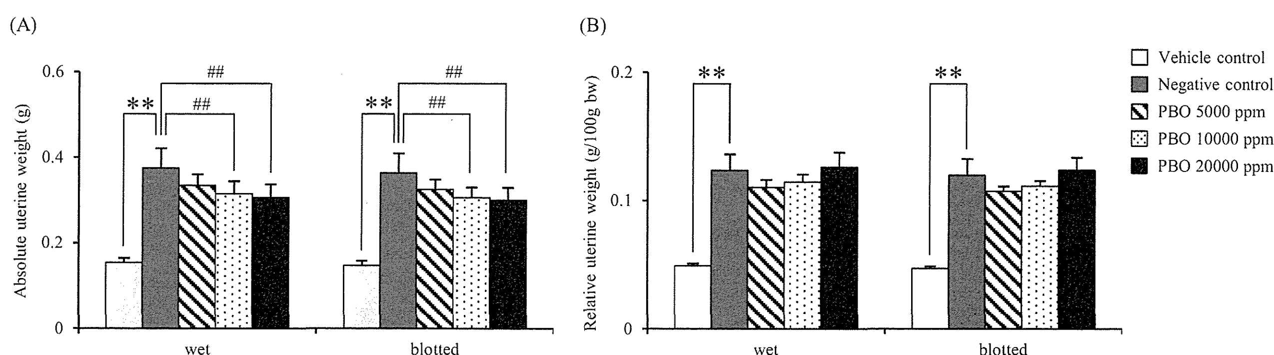


Fig. 8. Wet and blotted uterine weights in experiment 2. Data are represented absolute (A) and relative (B) uterine weights (mean ± S.D.), ** ; significantly different from the vehicle control group at $P < 0.01$, #, ## ; significantly different from the negative control group at $P < 0.05$, 0.01, respectively.

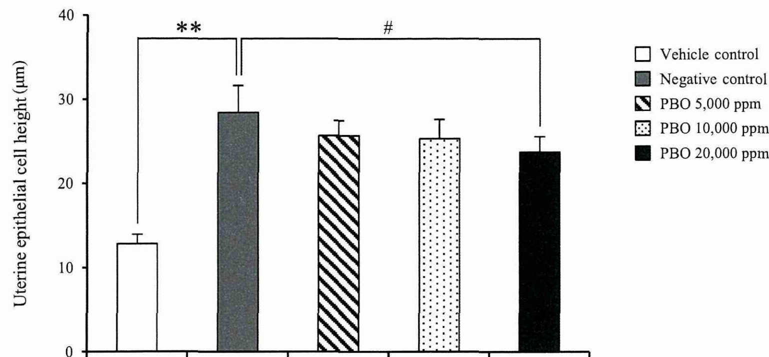
DISCUSSION

The present study investigated the effects of PBO on the female reproductive tract, focusing on the possibility that these are nonspecific effects related to the suppression of body weight gain by PBO. The most notable changes induced by PBO were estrous cycle disruption, uterine atrophy, and lowered serum E2 levels. Furthermore, *in vivo* and *in vitro* assays demonstrated that PBO has weak anti-estrogenic activity.

Estrous cycle disruption was present in both the PBO 20,000 ppm group and the FR-2 group in experiment 1. A previous report showed that rapid body weight reduction was responsible for the increased estrous cycle length in rats subjected to FR (Chapin *et al.*, 1993). However, the present study showed that the PBO group differed in the duration of the estrous cycle abnormalities from food restricted rats in the previous report and in the FR-2 group. The estrous cycles of the FR-2 group normalized once body weight gain recovering to increase, but prolonged diestrus continued in the 20,000 ppm PBO group even after body weight gain recovered to increase. These results suggest that prolonged diestrus in the PBO 20,000 ppm group may have resulted from something other than reduced body weight. This idea was further supported by the significant decrease in the relative weights of the reproductive organs in the PBO 20,000 ppm group and differences in the patterns of reproductive hormone levels between the PBO and FR groups. Elevated corticosterone is a known stress response induced by hunger and limited access to food (Kasanen *et al.*, 2009). High-dose PBO treatment did not affect corticosterone levels whereas the FR groups showed increased corticosterone levels. These results indicate that the reproductive effects of reduced body weight in the FR group may be related to stress, whereas this indicator of stress was not present in the PBO group under the present study conditions.

Histopathological changes induced by high-dose PBO treatment, including severe uterine atrophy, vaginal thinning with mucification, and ovarian changes such as an increased number of atretic follicles and decreased numbers of recent corpora lutea, were typical of the hypoestrogenic or anti-estrogenic state resulting in anovulation. These changes were not observed in the FR-2 group. Our hormone measurements in experiment 1 revealed that both the 10,000 and 20,000 ppm PBO groups were in a

(A)



(B)

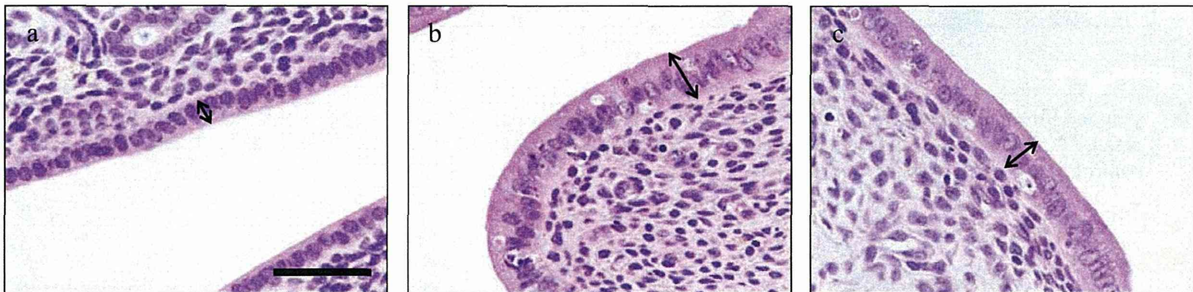


Fig. 9. Histopathological examination of uterus in experiment 2. (A) Uterine epithelial cell height (mean ± S.D.), **; significantly different from the vehicle control group at $P < 0.01$, #; significantly different from the negative control group at $P < 0.01$. (B) Histopathology of uterine epithelial cells. (a) vehicle control group, (b) negative control group, (c) 20,000 ppm group. Double-headed arrows indicate uterine epithelial cell heights. Bars = 50μm.

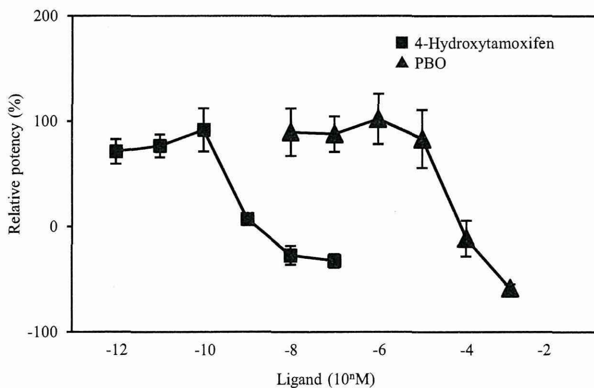


Fig. 10. Concentration response curve of the ER α reporter gene assay in experiment 3 (mean ± S.D.).

hypoestrogenic state at diestrus when E2 levels are normally gradually increasing, indicating that high-dose PBO treatment may induce a hypoestrogenic state although a clear mechanism was not determined in the present study. E2 is metabolized by cytochrome P450s (CYPs) in the liver and other organs and tissues (Dannan *et al.*, 1986; Hammond *et al.*, 1997; Zhu and Conney, 1998; Yoshida *et al.*, 2004). PBO causes liver hypertrophy and induces CYP 1A and 2B mRNA expression (Muguruma *et al.*, 2006), which may affect E2 metabolism. In contrast, treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an aryl hydrocarbon receptor (AhR) agonist, induces CYP1A and 1B mRNA expression without affecting E2 levels (Shiverick and Muther, 1982; DeVito *et al.*, 1992). Another possible mechanism of hypoestrogenism is the

inhibition of steroidogenesis in the ovary; this possibility was not investigated in the present study. Further analysis is needed to fully elucidate the effects of PBO on E2 metabolism in the liver and steroidogenesis in the ovary.

The anti-estrogenic effects of PBO are another possible cause of estrous cycle disruption and atrophic changes (Tsujioka *et al.*, 2009). A reporter gene assay indicated that PBO has anti-estrogenic effects, but they were much weaker than those of the estrogen receptor antagonist 4OH-TAM. In anti-uterotrophic study (experiment 2), the significant decrease in absolute uterine weight induced by E2 and PBO treatment groups was likely due to the weak anti-estrogenic effects of PBO, because the relative uterine weight was not decreased due to body weight depression in the PBO group. In addition, the statistically significant decrease in the heights of uterine epithelial cells in the 20,000 ppm group compared with the E2 only group also supports the idea that PBO has anti-estrogenic effects, because uterine epithelial cell height is a well-established indicator of estrogenicity (Cho *et al.*, 2003; Diel *et al.*, 2002).

In conclusion, high-dose PBO treatment resulted in marked uterine atrophy, vaginal atrophy, and ovarian changes resulting in anovulatory status in rats. These changes were independent of body weight depression, and may result from a hypoestrogenic state and anti-estrogenic effects of PBO. However, the levels of PBO used in the present study were very high. Acceptable daily intake (ADI) of PBO was set at 0.2 mg/kg body weight by JMPR in 1995 (FAO, 2011), so margin of exposure is approximately 5,000 fold. Therefore, these effects are not considered to be relevant to humans at exposure levels present in the environment.

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REFERENCES

Butler, W.H., Gabriel, K.L., Osimitz, T.G. and Preiss, F.J. (1998): Oncogenicity studies of piperonyl butoxide in rats and mice. Hum. Exp. Toxicol., **17**, 323-330.

Chapin, R.E., Gulati, D.K., Barnes, L.H and Teague, J.L. (1993): The effects of feed restriction on reproductive function in Sprague-Dawley rats. Fundam. Appl. Toxicol., **20**, 23-29.

Cho, S.D., Kim, J.H., Kim, D.Y., Lee, Y.S. and Kang, K.S. (2003): Pre-validation study for OECD enhanced test guideline 407 protocol by gavage for 4 weeks using propylthiouracil and tamoxifen. Toxicol. Lett., **144**, 195-204.

Dannan, G.A., Porubek, D.J., Nelson, S.D., Waxman, D.J. and Guencerich, E.P. (1986): 17 β-estradiol 2- and 4-hydroxylation catalyzed by rat hepatic cytochrome P-450: roles of individual forms, inductive effects, developmental patterns, and alterations by gonadectomy and hormone replacement. Endocrinology, **118**, 1952-1960.

DeVito, M.J., Thomas, T., Martin, E., Umbreit, T.H. and Gallo, M.A. (1992): Antiestrogenic action of 2,3,7,8-tetrachlorodibenzo-p-dioxin: tissue-specific regulation of estrogen receptor in CD1 mice. Toxicol. Appl. Pharmacol., **113**, 284-292.

Diel, P., Schmidt, S. and Vollmer, G. (2002): *In vivo* test systems for the quantitative and qualitative analysis of the biological activity of phytoestrogens. J. Chromatogr. B Anal. Technol. Biomed. Life Sci., **777**, 191-202.

Food and Agriculture Organization of the United Nations (FAO) (2011): FAO specifications and evaluations for agricultural pesticides. http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/ Specs/piperonyl_butoxide_2011.pdf

Franklin, M.R. (1972): Inhibition of hepatic oxidative xenobiotic metabolism by piperonyl butoxide. Biochemical Pharmacology, **21**, 3287-3299.

Fujitani, T., Ando, H., Fujitani, K., Ikeda, T., Kojima, A., Kubo, Y., Ogata, A., Oishi, S., Takahashi, H., Takahashi, O. and Yoneyama, M. (1992): Sub-acute toxicity of piperonyl butoxide in F344 rats. Toxicology, **72**, 291-298.

Hamada, T., Watanabe, G., Kokuho, T., Taya, K., Sakamoto, S., Hasegawa, Y., Miyamoto, K. and Igarashi, M. (1989): Radioimmunoassay of inhibin in various mammals. J. Endocrinol., **122**, 697-704.

Hammond, D.K., Zhu, B.T., Wang, M.Y., Ricci, M.J. and Liehr, J.G. (1997): Cytochrome P450 metabolism of estradiol in hamster liver and kidney. Toxicol. Appl. Pharmacol., **145**, 54-60.

Kasanen, I.H., Inhilä, K.J., Vainio, O.M., Kiviniemi, V.V., Hau, J., Scheinin, M., Mering, S.M. and Nevalainen, T.O. (2009): The diet board: welfare impacts of a novel method of dietary restriction in laboratory rats. Lab. Anim., **43**, 215-223.

Muguruma, M., Nishimura, J., Jin, M., Kashida, Y., Moto, M., Takahashi, M., Yokouchi, Y. and Mitsumori, K. (2006): Molecular pathological analysis for determining the possible mechanism of piperonyl butoxide-induced hepatocarcinogenesis in mice. Toxicology, **228**, 178-187.

Muguruma, M., Unami, A., Kanki, M., Kuroiwa, Y., Nishimura, J., Dewa, Y., Umemura, T., Oishi, Y. and Mitsumori, K. (2007): Possible involvement of oxidative stress in piperonyl butoxide induced hepatocarcinogenesis in rats. Toxicology, **236**, 61-75.

Phillips, J.C., Price, R.L., Cunninghame, M.E., Osimitz, T.G., Cockburn, A., Gabriel, K.L., Preiss, F.J., Butler, W.H. and Lake, B.G. (1997): Effect of piperonyl butoxide on cell replication and xenobiotic metabolism in the livers of CD-1 mice and F344 rats. Fundam. Appl. Toxicol., **38**, 64-74.

Shiverick, K.T. and Muther, T.F. (1982): Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on serum concentrations and the uterotrophic action of exogenous estrone in rats. Toxicol. Appl.

Pharmacol., **65**, 170-176.
Takahashi, O., Oishi, S., Fujitani, T., Tanaka, T. and Yoneyama, M. (1994): Chronic toxicity studies of piperonyl butoxide in F344 rats: induction of hepatocellular carcinoma. Fundam. Appl. Toxicol., **22**, 293-303.
Takeyoshi, M., Yamasaki, K., Sawaki, M., Nakai, M., Noda, S. and Takatsuki, M. (2002a): The efficacy of endocrine disruptor screening tests in detecting anti-estrogenic effects downstream of receptor-ligand interactions. Toxicol. Lett., **126**, 91-98.
Takeyoshi, M., Kuga, N. and Yamasaki, K. (2002b): Development of a high-performance reporter plasmid for detection of chemicals with androgenic activity. Arch. Toxicol., **77**, 274-279.
Taya, K., Mizokawa, T., Matsui, T. and Sakamoto, S. (1983): Induction of superovulation in prepubertal female rats by anterior pituitary transplants. J. Reprod. Fertil., **69**, 265-270.
Taya, K., Watanabe, G. and Sasamoto, S. (1985): Radioimmunoassay for progesterone, testosterone and estradiol-17β using 125I-iodohistamine radioligands. Jpn. J. Anim. Reprod., **31**,186-197.
Terry, K.K., Chatman, L.A., Foley, G.L., Kadyszewski, E., Fleeman, T.L., Hurtt, M.E. and Chapin, R.E. (2005): Effects of Feed Restriction on Fertility in Female Rats. Birth. Defects Res. B Dev. Reprod. Toxicol., **74**, 431-441.
Tsujioka, S., Ban, Y., Wise, L.D., Tsuchiya, T., Sato, T., Matsue, K., Ikeda, T., Sasaki, M. and Nishikibe, M. (2009): Collaborative work on evaluation of ovarian toxicity. 3) Effects of 2- or 4-week repeated-dose toxicity and fertility studies with tamoxifen in female rats. J. Toxicol. Sci., **34**, SP43-SP51.
Westwood, F.R. (2008): The female rat reproductive cycle: A practical histological guide to staging. Toxicol. Pathol., **36**, 375-384.
Yoshida, M., Katashima, S., Ando, J., Tanaka, T., Uematsu, F., Nakae, D. and Maekawa, A. (2004): Dietary indole-3-carbinol promotes endometrial adenocarcinoma development in rats initiated with N-ethyl-N'-nitro-N-nitrosoguanidine, with induction of cytochrome P450s in the liver and consequent modulation of estrogen metabolism. Carcinogeneisis, **25**, 2257-2264.
Yoshida, M., Sanbuissyo, A., Hisada, S., Takahashi, M., Ohno, Y. and Nishikawa, A. (2009): Morphological characterization of the ovary under normal cycling in rats and its viewpoints of ovarian toxicity detection. J. Toxicol. Sci., **34**, **Suppl. 1**, SP189-SP97.
Yuan, Y.D. and Foley, G.L. (2002): Female Reproductive System. In Handbook of Toxicologic Pathology. Second Edition (Haschek, W.M., Rousseaux, C.G., Walling, M.A. eds.), pp847-894. Academic Press, San-Diego.
Zhu, B.T. and Conney, A.H. (1998): Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis, **19**, 1-27.

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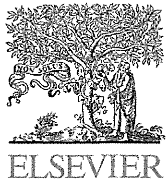

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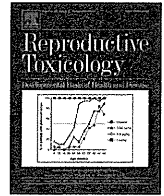

Delayed effects of neonatal exposure to 17alpha-ethynylestradiol on the estrous cycle and uterine carcinogenesis in Wistar Hannover GALAS rats

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A B S T R A C T

We investigated the delayed effects of neonatal exposure to 17α-ethynylestradiol (EE) on the female reproductive tract using Wistar Hannover GALAS rats. Female pups received single injections of EE (0, 0.02, 0.2, 2, 20, or 200 μg/kg) within 24 h after birth and estrous cyclicity was observed until 10 months of age. All animals were treated at 9 weeks of age with the uterine carcinogen, *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine. Although the vaginal opening was not affected, abnormal cycles were significantly increased from 0.2 μg/kg. Persistent estrus was prominent and the incidence increased age- and dose-dependently. Severity of atypical hyperplasia of the uterus tended to increase from 2 μg/kg. In these groups, serum progesterone level was lowered relative to estradiol level. In conclusion, estrous cyclicity was a sensitive indicator reflecting delayed effects on the female reproductive tract. Early onset of anovulation leading to prolonged estrogen exposure might be a risk factor for uterine carcinogenesis.

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1. Introduction

Many chemicals, especially those with estrogenic activity, are able to disrupt the programming of endocrine signaling pathways established during development and cause irreversible complex damage to the hypothalamus-pituitary-gonadal (HPG) axis and reproductive system in females [1,2]. In rodents, the sensitive period spans from late embryonic to early postnatal age, and is defined as the critical window of brain sex differentiation [3]. The altered programming can result in numerous adverse consequences in estrogen-target tissues, and some effects, such as increased carcinogenic risk and impaired reproductive function, are apparent after maturation as delayed adverse effects [2,4,5]. In human it is widely known that females exposed *in utero* to the synthetic estrogen, diethylstilbestrol (DES), commonly referred to as "DES daughters", have increased risks of vaginal cancer after puberty [6,7].

For risk assessment of chemicals, the delayed adverse effects have become a serious issue because delayed adverse effects might be overlooked by existing reproductive toxicity or developmental toxicity studies required by regulatory authorities due to limited observation periods. In addition, the mechanisms underlying the occurrence of delayed adverse effects remain unknown, thus toxicologic indicators applicable for risk assessment are needed. Previously, we examined the delayed effects of neonatal exposure to DES on the female reproductive tract using Donryu rats, and demonstrated that detection of the early onset of persistent estrus by vaginal smear appears to be the most sensitive and useful parameter [2]. In the present study, to confirm the characteristics of delayed adverse effects and identify the available indicators for evaluation, the long-term effects of neonatal exposure to 17α-ethynylestradiol (EE) at various doses on the female reproductive tract, such as estrous cyclicity and uterine carcinogenesis, were examined. Wistar Hannover GALAS rats were used to verify whether there is a strain difference in delayed effects. We selected EE for the current study because EE is more rapidly excreted than DES and does not bind to α-fetoprotein in neonatal blood, thus limiting the exposure time to the neonatal period [8]. *In vivo* kinetics of EE was also measured.

2. Materials and methods

2.1. Animals

Pregnant Wistar Hannover GALAS rats were obtained from CLEA Japan, Inc. (Tokyo, Japan) at gestational day 14 for experiments 1 ($n = 13$) and 2 ($n = 47$). The rats

Abbreviations: DES, diethylstilbestrol; EE, 17α-ethynylestradiol; ENNG, N-ethyl-N′-nitro-N-nitrosoguanidine; E2, estradiol-17β; FSH, follicle-stimulating hormone; HPG, hypothalamus-pituitary-gonadal; LH, lutenizing hormone; PND, postnatal day; PRL, prolactin; P4, progesterone.

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were housed individually in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature, 24 ± 1 °C; relative humidity, 55 ± 5%; 12-h light/dark cycle) with a basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and tap water available *ad libitum*. CRF-1 is a standard diet including soy protein and is known to contain relatively low level of estrogens.

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences (Japan).

2.2. Chemicals

EE was purchased from Sigma (CAS No. 57-63-6; St. Louis, MO, USA) with purity > 98%. EE was stirred in a small amount of sesame oil overnight, then used after dilution. *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG) was obtained from Nacalai Tesque (CAS No. 4245-77-6; Kyoto, Japan).

2.3. Experiment 1 (uterotrophic assay using immature rats)

To confirm the *in vivo* estrogenic activity of the EE doses used in experiment 2, we performed uterotrophic assays using immature rats. Sixty female pups at 21 days of age were allocated to 12 groups, each consisting of 5 animals from different dams. EE (0, 0.02, 0.2, 2, 20, or 200 μg/kg of body weight) was dissolved in sesame oil and subcutaneously injected on 1 or 3 consecutive days. The uterotrophic assay with dosing for 3 days has been established as a standard protocol for the detection of estrogenic activity *in vivo* [9]. Additionally, we set a single injection group in accordance with experiment 2. The animals were sacrificed by bleeding from the abdominal vein under deep isoflurane anesthesia approximately 24 h after the final injection. At necropsy, after measurement of the body weight, the uteri were carefully dissected to cut off adherent fat and mesentery. The body of the uterus was cut just above the junction with the cervix and at the junction of the uterine horns with the ovaries, and the tissue was softly wiped to remove outer fluid and weighed (wet weight). Then, the uterine horn was punctured to release fluid inside and weighed (blotted weight). After that, the relative uterine weight was calculated.

2.4. Experiment 2

Dams were assigned to 6 groups (7–9 dams/group) before delivery. All of the pups received a single subcutaneous injection of EE (0, 0.02, 0.2, 2, 20, or 200 μg/kg of body weight) dissolved in sesame oil within 24 h after birth. Litters were culled randomly to preserve 8 pups, with a female predominance on postnatal day (PND) 3. On PND 21, the offspring were weaned, and 24 female rats per group were housed 3 per cage and maintained until 10 months of age. From PND 25, we checked vaginal opening every day. After that, all animals were observed for estrous cyclicity by vaginal smear for 5 consecutive days every other week throughout the experiment. The decision of the cycle pattern was made with every 5-day observations. Regular 4- or 5-day cycles were determined as normal cycles, and other patterns were judged to be abnormal cycles. In particular, the animals showing proestrus and estrus continuously for 5 days were designated as persistent estrus. Additionally, to examine the effects of neonatal exposure to EE on uterine carcinogenesis, all rats were treated with a single injection of ENNG (20 mg/kg) into the uterine horns *via* the vagina using a stainless steel catheter at 9 weeks of age. This treatment is based on medium-term carcinogenicity bioassays, which were established to detect modifying effects on tumor development in a short term [10,11]. ENNG is known to cause endometrial adenocarcinoma development in the uterine corpus of rats in a short time without carcinogenic effects in other sites with no disruption of estrous cyclicity [12]. Observations regarding clinical signs, body weight, and mortality were made throughout the experimental period. At 10 months of age (44 week-old), all surviving rats were autopsied at estrus or persistent estrus. The animals were decapitated, blood samples were collected for hormone assays, and the ovaries and uteri were removed and weighed. We excluded 2 animals per group that underwent transcardial perfusion from blood sampling and measurement of organ weights. The vagina, adrenal glands, liver, pituitary, thymus, brain, mammary glands, thyroid, and sites with macroscopic abnormalities were also resected from each animal. These procedures of autopsy including decapitation and blood collection were conducted in a separate room from the animal room at 10:00–12:00. All organs were fixed in 10% neutral buffered formalin. Tissues were routinely processed and stained with hematoxylin and eosin for histopathologic examination.

2.5. Measurement of the EE level

The *in vivo* kinetics of EE in neonatal rats were examined using male pups that received a single subcutaneous injection of EE (200 μg/kg) within 24 h after birth. The entire body (minus the injection site), brain and liver were collected 1, 2, 4, and 24 h after injection and stored at −80 °C. Pooled samples of the brains and livers from three rats were used. The concentrations of EE were measured at Japan Food Research Laboratories (Osaka, Japan) by LC–MS/MS (detection limit, 0.02 ppm).

2.6. Histopathologic assessment of proliferative lesions in the uterus

The uteri *in toto* were cut in cross-section at 5 mm intervals, and histologically assessed in the upper, middle, and lower parts of the uterine horn and the cervix. Preneoplastic or neoplastic lesions were classified into three degrees of atypical hyperplasia (slight, moderate, or severe) and adenocarcinomas according to a previous study [2]. Lesions composed of glandular-structured epithelial cells with atypia showing invasive proliferation to the muscle layer or serosa were diagnosed as endometrial adenocarcinomas.

Table 1
EE level in neonatal rats that received 200 μg/kg subcutaneously.

Organ (ppm)	Time after EE injection (h)			
	1	2	4	24
Whole body	0.096	0.095	0.100	–
Brain[a]	0.029	0.042	0.059	–
Liver[a]	0.093	0.099	0.210	0.003

[a] Organs from 3 animals were pooled.
– Under the detection limit (0.002 ppm).

2.7. Hormone assays

Serum samples obtained after decapitation were stored at −80 °C until assay. The serum concentration of follicle-stimulating hormone (FSH), luteinizing hormone (LH), inhibin, estradiol-17β (E2), progesterone (P4), and prolactin (PRL) were determined using double-antibody radioimmunoassays and ^{125}I-labeled radio-ligands. National Digestive and Kidney Disease (NIDDK) radioimmunoassay kits were used for rat FSH, LH, and PRL (NIAMDD, NIH, Bethesda, MD, USA) with anti-rat LH-S-11, anti-rat FSH-S-11 and anti-rat PRL-S-9 sera, as described previously [13]. P4 and E2 were measured using the anti-sera against P4 (GDN 337) [14] and E2 (GDN 244) [15] as described by Taya et al. [16] with minor changes of tracers, *i.e.* iodine-125 labeled tracers of estradiol and progesterone (MP Biomedicals, LLC, OH, USA, 07138226 and 07170126, respectively). Iodinated 32-kDa bovine inhibin and a rabbit antibody against bovine inhibin (TNDH-1) were used for measurement of immunoreactive serum inhibin, as described previously [17].

2.8. Statistical analysis

Following Bartlett's test, variance in data for uterine weights in the uterotrophic assay, days of vaginal opening, body and organ weights, multiplicity of uterine hyperplasia, and hormone assays were compared with the 0 μg/kg group by one-way analysis of variance or the Kruskal–Wallis test. When statistically significant differences were detected, Dunnett's multiple comparison test was employed for comparison between the 0 μg/kg group and the treatment groups. The incidence of histopathologic findings was compared using Fisher's exact probability test. In these test, the level of significance was set at 0.05.

3. Results

3.1. Uterotrophic assay

There were no intergroup differences in body weight at necropsy (data not shown). The wet and blotted weights of the uteri in the single-dose groups were significantly increased from 0.02 μg/kg (Fig. 1A). After 3 days of treatment, a significant increase was found from 0.02 μg/kg in the blotted weight and 0.2, 2, and 20 μg/kg in the wet weights (Fig. 1B). Thus, it was confirmed that a single injection of EE (≥0.02 μg/kg) has *in vivo* estrogenic activity.

3.2. In vivo kinetics of EE in neonatal rats

The concentration of EE in the whole bodies, livers and brains of neonatal rats was detected 1 h after injection, and reached a peak at 4 h (Table 1). Twenty-four hours after injection, the level of EE was markedly decreased to the near detection limit or less. The time of exposure to EE was shown to be limited to several hours on PND 0-1.

3.3. Clinical observation in life and estrous cyclicity in experiment 2

Before weaning, no abnormalities or deaths related to EE treatment were demonstrated, and the body weight gain was similar among the groups (data not shown). Also, growth and development