図9 無処置9週齢SD雌雄ラットの強制水泳試験における無動時間

15分間水槽円柱(直径30cm 高さ60cm 水深 約40cm)に入れる
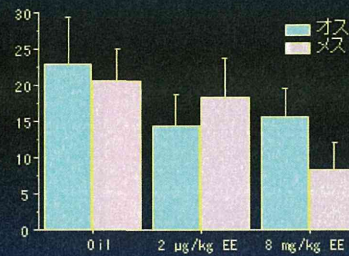
↓ 24時間後

5分間水槽に入れる

総無動時間を測定し、うつ様行動の指標とする

図10 受動回避学習試験装置

図11 無処置8週齢SD雌雄ラットの受動回避学習試験結果
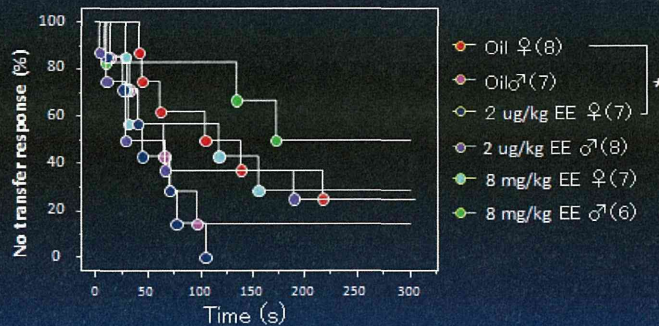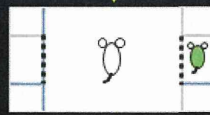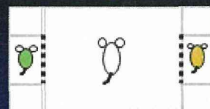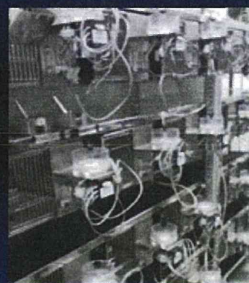
図12　社会性認知試験

個別飼育10日間　ハンドリング、フィールドへの馴化3日間

訓練試行
（社会性行動測定）

インターバル

保持試行
（社会性認知測定）

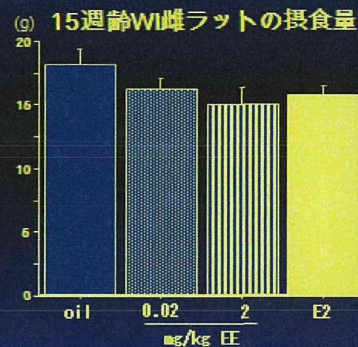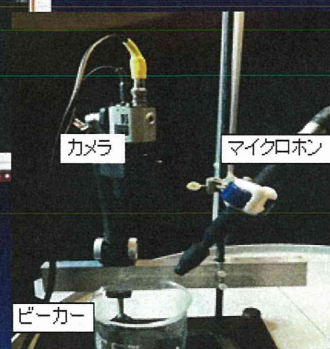行動を録画し、画像解析ソフト Ethovision XT8で動物の
鼻が相手ケージへ接触する回数と時間を解析する

図13　摂食量測定試験

(g)　15週齢WI雌ラットの摂食量

摂食量測定装置

oil　0.02　2　E2
mg/kg EE

図14　母子分離誘発啼鳴反応

超音波発声

カメラ　　マイクロホン

ビーカー

仔ラットを母獣から分離後、
すぐに防音箱内のビーカー
に入れ5分間測定した。鳴き
声はマイクロホンで集音して
アンプで増幅し、パソコンに
記録した。

Ⅲ. 研究成果の刊行に関する一覧表

雑誌

著者名	タイトル	雑誌名	管・号・ページ	年
Yoshida M, Takahashi M, Inoue K, Hayashi S, Maekawa A, Nishikawa A.	Delayed adverse effects of neonatal exposure to diethylstilbestrol and their dose dependency on female rats.	Tox. Pathol	39 (5): 823-834	2011
Taketa Y, Yoshida M, Inoue K, Takahashi M, Sakamoto Y, Watanabe G, Taya K, Yamate J, Nishikawa A.:.	Differential stimulation pathways of progesterone secretion from newly formed corpora lutea in rats treated with ethylene glycol monomethyl ether, sulpiride, or atrazine.	Toxicol Sci	121(2):267-78.	2011
Taketa Y, Yoshida M, Inoue K, Takahashi M, Sakamoto Y, Watanabe G, Taya K, Yamate J, Nishikawa A.	The newly formed corpora lutea of normal cycling rats exhibit drastic changes in steroidogenic and luteolytic gene expressions.	Exp Toxicol Pathol.	64(7-8):775-82.	2012
Takahashi M, Matsuo S, Inoue K, Tamura K, Irie K, Kodama Y, Yoshida M.	Development of an early induction model of medulloblastoma in Ptch1 heterozygous mice initiated with N-ethyl-N-nitrosourea	Cancer Sci.	103(12):2051-5	2012
Yoshida M, Katsuda S, Maekawa A.	Involvements of Estrogen Receptor, Proliferating Cell Nuclear	J Toxicol Pathol.	25(4):241-7	2012

	Antigen and p53 in Endometrial Adeno carcinoma Developm ent in Donryu Rats.	46		
Taketa Y, Inoue K, Takahashi M, Yamate J, Yoshida M.	Differential Morphological Effects in Rat Corpora Lutea among Ethylene Glycol	Toxicol Pathol.	In press	2012
Y. Horii, M. Kawaguchi (corresponding), R. Ohta, A. Hirano, G. Watanabe, N. Kato, T. Himi, and K. Taya.	Male hatano high-avoidance rats show high avoidance and high anxiety-like behaviors as compared with male low-avoidance rats.	Exp. Anim	Sep;61(5): 517–524	2012
Shirota M, Kaw ashima J, Ogaw a Y, Kamiie J, Yasuno K, Shi rota K, Yoshida M.	Delayed effects of si ngle neonatal subcut aneous exposure of l ow-dose 17α-ethynyl estradiol on reproduc tive function in fem ale rats.	Journal of Toxicological Sciences	37: 681-689	2012
Shirota M, Kaw ashima J, Naka mura T, Ogawa Y, Kamine J, Shirota K.	Vascular Hamartoma in the Uterus of a Female Sprague-Daw ley Rat with an Ep isode of Vaginal	Toxicol Pathol	In press	2013
Matsuo S, Takahashi M, Inoue K, Tamura K, Irie K, Kodama Y, Nishikawa A, Yoshida M.	Thickened area of external granular layer and Ki-67 positive focus are early events of medulloblastoma in Ptch1(+/-) mice.	Exp Toxicol Pathol.	In press	2013

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Ⅳ. 研究成果の刊行物

Delayed Adverse Effects of Neonatal Exposure to Diethylstilbestrol and Their Dose Dependency in Female Rats

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ABSTRACT

Neonatal exposure to estrogenic chemicals causes irreversible complex damage to the hypothalamus-pituitary-gonadal axis and reproductive system in females. Some lesions are noted after maturation as delayed adverse effects. We investigated the characteristics and dose dependence of delayed effects using female rats neonatally exposed to diethylstilbestrol (DES). Female Donryu rats were subcutaneously injected with a single dose of DES of 0 (control), 0.05, 1.5, 15, 150, or 1,500 µg/kg bw after birth. All except the lowest dose had estrogenic activity in a uterotrophic assay. All rats at 1500 µg/kg and some at 150 µg/kg showed abnormal morphologies in the genital tract, indicating they were androgenized before maturation. Although no morphological abnormalities were noted at 15 µg/kg or lower, onset of persistent estrus was significantly accelerated in the 1.5, 15, and 150 µg/kg groups with dose dependency, and the latest onset was from seventeen to twenty-one weeks of age at 1.5 µg/kg. The neonatal exposure to DES increased uterine adenocarcinoma development only at 150 µg/kg, although uterine anomalies were detected at 1,500 µg/kg. These results indicate that neonatal exposure to DES, which exerts estrogenic activity in vivo, induces delayed adverse effects in female rats in a dose-dependent manner. Early onset of persistent estrus appears to be the most sensitive parameter.

Keywords: delayed adverse effect; neonatal exposure; DES; dose dependence; estrous cycle; female rat.

INTRODUCTION

Damage to the hypothalamus–pituitary–gonadal axis is the most worrying issue with perinatal exposure to chemicals that have estrogenic activity, because changes caused during the developmental period can last throughout life. Effects of exposure may be qualitatively different from those experienced in adulthood in rodents as well as human beings (Takasugi et al. 1983; Newbold et al. 1999). Although species differences in neonatal effects are known—such as ovary-independent response to the vagina in mice but not rats (Katsuda et al. 2002; Takasugi et al. 1976) or adenomyosis induction by tamoxifen in mice (Parrott et al. 2001) but uterine cancer in rats (Carthlew et al. 2000)—many experimental data on perinatal exposure to estrogens using rodents are considered likely to be relevant to human beings (Herbst and Bern 1981).

Perinatal exposure to estrogens or estrogenic compounds during critical period disrupts hypothalamus function, which results in lower gonadotropin (FSH and LH) production (Katsuda et al. 2000; Yoshida et al. 2002a). The disruption leads to many complicated abnormalities in the hypothalamus–pituitary–gonadal axis and the genital tract, and direct effects of estrogens on the genital system are also added. Although the abnormalities are dependent on exposure period, time, and dose, they are called androgenization and are commonly irreversible. Typical changes in affected females include lower gonadotropins, anovulation, persistent estrus,

abnormal organogenesis of the reproductive tract, abnormal expression of estrogen receptors in the uterus, or male-type sexual behavior. Testosterone is known to be converted to E2 by aromatase in the brain, and it has effects that are similar to those of estrogens. These abnormalities are usually detectable immediately after treatment or before puberty. Recently, effects of perinatal exposure that manifest after maturation have attracted attention (Adewale et al. 2009; Yoshida et al. 2002b). Such delayed effects of estrogenic chemicals were previously termed the "delayed anovulatory syndrome" (DAS) (MacLusky and Naftolin 1981), characterized by early onset of persistent estrus as determined by vaginal cytology (Ninomiya et al. 2007). Enhancement of tumor development may also result as well, as has been documented for uterine and vaginal neoplasms after perinatal exposure to diethylstilbestrol (DES) in mice (Newbold et al. 1990) as well as young women (Herbst and Bern 1981). Our previous study also showed increased uterine cancer development in rats with neonatal high-dose p-tert octylphenol (OP) (Yoshida et al. 2002b). Such effects are hypothesized to be caused by some disruption in the hypothalamus (MacLusky and Naftolin 1981), but precise mechanisms and associated characteristics other than persistent estrus (PE) remain to be determined. For risk assessment of chemicals, delayed adverse effects are of particular concern because they might be overlooked by short-term bioassays or developmental toxicity studies. Therefore, the present study was performed to clarify characteristics of delayed effects of neonatal exposure to DES at various doses on the female reproductive tract in rats using multiple retrospective indicators, including morphological parameters, estrous cyclicity, and

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uterine carcinogenesis. In the present study, we used Donryu rats, which characteristically show a regular four-day estrous cycle as young adults and a high rate of endometrial adenocarcinomas caused by ovarian hormone imbalance (Maekawa et al. 1999; Nagaoka et al. 1990; Nagaoka et al. 1994). To avoid accumulation of DES in the rat body with repeated treatment, single-dose administration was selected in the present study.

MATERIALS AND METHODS

Animals and Housing Conditions

Nonpregnant adult and pregnant female Crj:Donryu rats were purchased from Charles River Japan (Kanagawa, Japan), maintained in an air-conditioned animal room under constant conditions of 24°C ± 2°C and 55% ± 10% humidity with a twelve-hour light/dark cycle (light, 8:00 a.m.–8:00 p.m.; dark, 8:00 p.m.–8:00 a.m.), and housed three or four per cage. Commercial pellet diet (CRF-1, Oriental Yeast, Kanagawa) and drinking water were available ad libitum throughout the studies. Animal care and use followed the guidelines of laboratory animal welfare and use according to the Sasaki Institute Ethical Committee.

Chemicals

Diethylstilbestrol (CAS number 56-53-1, Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical, Osaka, Japan).

Experiment 1

Nonpregnant females underwent uterotrophic assays to examine estrogenic activity of the single doses applied in experiment 2 in vivo. Thirty-seven animals at three or four months of age were ovariectomized, and after complete castration was confirmed by vaginal smears, they were allocated to six groups. Three to eight animals per group were subcutaneously treated with a single dose of DES at 0 (control), 0.15, 1.5, 15, 150, or 1,500 µg/kg body weight in 0.1 mL/100 g body weight of DMSO. Twenty-four hours after treatment, all animals were euthanized under deep anesthesia and uterine weights were measured.

Experiment 2

Experiment 2 was conducted to characterize delayed effects of neonatal exposure to DES on the female reproductive tract and their dose dependence. Dams were assigned to one of six groups (six to eight per group) before delivery. Female offspring were treated with a single subcutaneous injection of DES at 0, 0.15, 1.5, 15, 150, or 1,500 µg/kg body weight in DMSO at 0.04 mL/10 g body weight in their back skin within twenty-four hours after birth. At postnatal days (PND) 14 and 21 (at weaning) and five weeks of age, three or four rats per group at each time point were examined to detect early effects on the female reproductive organs. The animals examined at five weeks of age were at estrous stage by vaginal smear.

Remaining animals were checked for clinical signs and body weights throughout their life. After vaginal opening (VO), which is a time point during puberty, all animals were checked for estrous cyclicity by vaginal smear every other week throughout the experiment. Additionally, to examine the effects of the neonatal exposure to DES on uterine carcinogenesis, fifteen to twenty-four rats per group were treated with a single injection of 20 mg/kg N-ethyl-N'-nitro-N-nitrosoguanidine (Nacalai Tesque, Kyoto, Japan) into the uterine horns via the vagina using a stainless steel catheter at seven weeks of age. This treatment is known to cause uterine endometrial adenocarinoma development in the uterine corpus of rats without carcinogenic effects in other sites, with no disruption of estrous cyclicity (Ando-Lu et al. 1994). All survivors were euthanized at ten months of age in the control and 15 µg/kg and higher treated groups, and at eight months of age in the 0.15 and 1.5 µg/kg groups.

Pathology

After complete necropsy of all rats in experiment 2, the ovaries, uterus, vagina, adrenals, liver, pituitary, thymus, brain, and sites with macroscopic abnormalities were fixed in 4% neutral buffered formaldehyde. All organs were routinely processed and stained with hematoxylin and eosin for histopathological examination. Number of uterine glands in the uterine horn were counted using four or five cross-sections (Katsuda et al. 2000). For the uterus at termination of eight or ten months of age, the main target organs of carcinogenic effects of DES—the upper, middle, and lower parts of each uterine horn and the cervix—were cut into three pieces each in cross-section. Preneoplastic or neoplastic lesions detected in each uterus were classified into three degrees of atypical hyperplasia (slight, moderate, or severe) and adenocarcinomas, according to the criteria described in our previous studies (Ando-Lu et al. 1994). Lesions composed of glandular-structured epithelial cells with atypia showing invasive proliferation to the muscle layer or serosa were diagnosed as endometrial adenocarcinomas.

Immunohistochemistry

Serial sections of the uterus, vagina, and ovary at PND14, PND21, and five weeks of age were stained immunohistochemically with the antibody of estrogen receptor (ER)-α (SC7027, Santa Cruz, CA, USA). The organs of three or four rats per group were analyzed, and immunoreactive complexes were detected by the avidin-biotin complex method (LSAB kit, Dako Japan, Kyoto, Japan) and visualized with 3-3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals, Osaka, Japan) as the chromogen. Immunohistochemical intensity of ERα expression in these organs was classified into four categories: 0, negative; 0.5, several positive cells in higher magnification field (40×); and 1.0, 2.0, or 3.0 represents about 10–30%, 30–50%, or 50% and higher positive cells in the high magnification, respectively.

TABLE 1.—Uterine weights in uterotrophic assay.

Group (µg/kg)	No. of rats	Body weight, mean ± SD (g)	Uterine weight, mean ± SD	
			Absolute (mg)	Relative (µg/kg BW × 100)
Control	8	317.2 ± 42.3*	195.8 ± 45.6	613.8 ± 74.1
DES 0.15	8	317.5 ± 42.4	192.8 ± 33.9	604.6 ± 37.3
DES 1.5	8	300.6 ± 37.9	259.0 ± 43.7*	858.8 ± 66.9**
DES 15	7	323.6 ± 66.5	331.7 ± 91.6**	1018.4 ± 138.9**
DES 150	3	317.0 ± 31.0	477.0 ± 26.0**	1519.0 ± 226.0**
DES 1500	3	350.0 ± 26.7	440.7 ± 59.0**	1262.0 ± 179.5**

Abbreviations: BW, body weight; DES, diethylstilbestrol; SD, standard deviation.
* Significantly different from control value at $p < .05$.
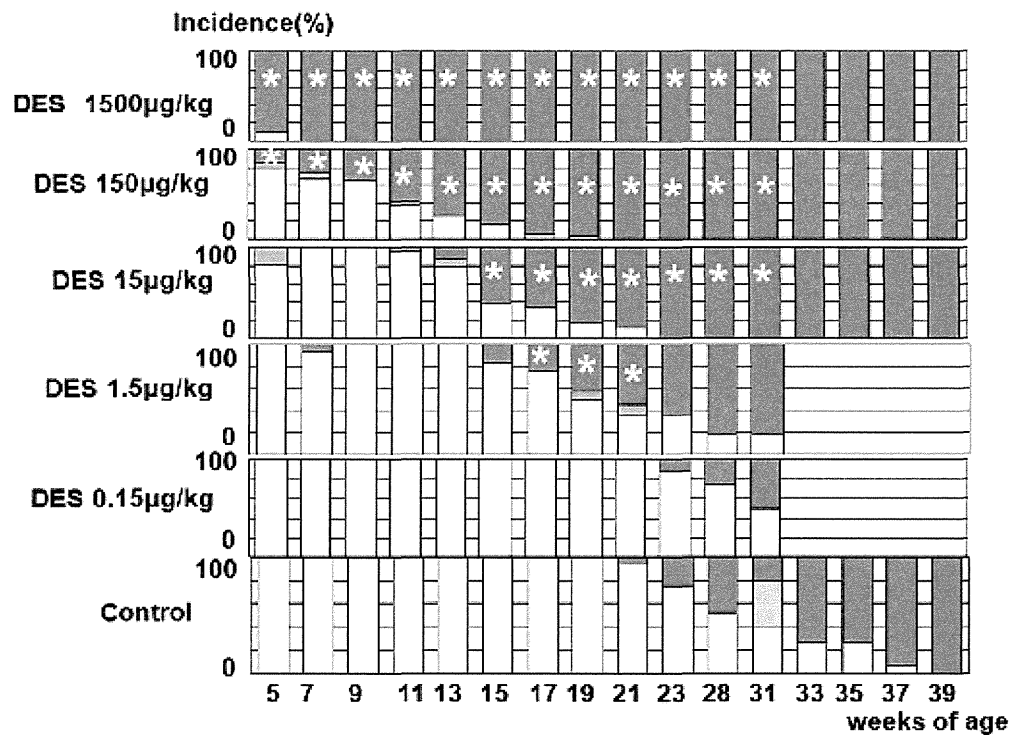** Significantly different from control value at $p < .01$.

FIGURE 1.—Estrous cycling in experiment 2. White, light grey, and dark grey columns, respectively, represent regular (normal) cycle, irregular cycles, and persistent estrus. Incidences are the percentages of animals showing each estrous stage per group. *, significantly different from the control value at $p < .05$ and $p < .01$, respectively.

mRNA Expression of Estrogen Receptors α and β and Progesterone Receptor (PR) in the ovary and uterus

At five weeks of age, total RNA samples obtained from samples of uterine tissues (about 100 mg) and unilateral ovary of three females at estrous phase in each group except the highest dose were isolated using RNeasy Midi Extraction Kit (QUIAGEN, Germany) and stored at −80°C until reverse transcriptase polymerase chain reaction analysis. Estrogen receptors α and β and glyceraldehyde 3-phosphate dehydrogenase

Age/organs/findings	Groups (μg/kg)					
	DES 0	DES0.15	DES1.5	DES15	DES150	DES1500
PND 14 (response to R3-4)						
Number of glands/section	4.3 ± 1.1	4.7 ± 1.5	3.1 ± 1.1	4.0 ± 1.8	4.2 ± 0.8	5.8 ± 0.6
Vagina: cornification	—[a]					+[b]
PND 21						
Ovary: small	—	—	—	—	—	+
5 weeks						
Ovary: atrophy with atretic follicles and no CL	—	—	—	—	+/—[c]	+
Uterus: intraluminal hyperplasia in the surface epithelium	—	—	—	—	—	+
Vagina: cornification	—	—	—	—	—	+

Abbreviations: CL, corpus luteum; DES, diethylstilbestrol; PND, postnatal day.
[a] No abnormalities detected in any animals.
[b] Detected in all animals.
[c] Detected in only some of the animals.

(GAPDH) mRNA transcription were examined using 400-ng aliquots of total RNA. The primers were synthesized and purified by Takara Bio Inc. (Shiga, Japan) based on previous reports (Azabo et al. 2000; Pillai et al., 2002). Expressed levels of ERα, ERβ and PR mRNA relative to GAPDH mRNA expression were compared among the control and the treated groups.

Statistical Analysis

Values for incidences were statistically analyzed using the Fisher exact probability test. Other data were assessed using analysis of variance (three groups) and t test (two groups), and post hoc comparisons between the treated and control groups were made with the Dunnett t test. Statistical significance was considered as $p < .05$.

RESULTS

Estrogenic Activity in Experiment 1

The results of uterotrophic assays are summarized in Table 1. Both the absolute and relative uterine weights were significantly increased at 1.5 μg/kg and above with dose dependence, except at the highest dose. The uterine weights at the lowest dose were comparable to those in the control group.

Clinical Observation in Life and Estrous Cyclicity in Experiment 2

No treatment-related clinical signs were observed at any time point. Body weight changes were comparable in all groups, including the controls (data not shown). The timing of VO was not different between the treated groups and control. The age at VO was 28.0 ± 1.0, 28.0 ± 0.0, 28.2 ± 0.4, 28.5 ± 0.5, 28.9 ± 1.2, 28.8 ± 1.3 in the 0, 0.15, 1.5, 15, 150, and 1,500 μg/kg groups, respectively.

Data for the estrous cycle are shown in Figure 1. At 1,500 μg/kg, most rats already showed PE at the start of

checking estrous cycles, and all were affected by seven weeks of age. At 150 μg/kg, a few females demonstrated PE at five weeks of age, and most by seventeen weeks of age. At 15 or 1.5 μg/kg, PE rates began to increase at thirteen or fifteen weeks of age, and most of them reached PE by nineteen or twenty-one weeks of age, respectively. In all these groups, the numbers of rats exhibiting PE were significantly increased compared to that of controls at fifteen to thirty-one weeks and seventeen to twenty-one weeks of age, respectively. At twenty-three weeks of age, PE began to increase in the control group, and most animals were affected by thirty-nine weeks of age. In the 0.15 μg/kg group, the lowest dose group, the onset of PE was similar to that in controls.

Morphological Changes up to Five Weeks of Age in Experiment 2

Changes in the ovary, uterus, or vagina at PND14, PND21, or five weeks of age are summarized in Table 2, and characteristic lesions are shown in Figures 2, 3, and 4, respectively. Although no abnormalities were detected in the ovary at PND14, those at 1,500 μg/kg were clearly small at PND21 (Figure 2). At five weeks of age, the ovaries of all rats at 1,500 μg/kg and some of rats at 150 μg/kg were macroscopically small in size and microscopically composed of cystic atretic follicles, and they lacked corpora lutea. No hyperplasia or hypertrophy of the interstitial cells was found in the ovaries of all treated groups. In the uterus, there were no differences until PND21, with normal gland genesis at PND14. At five weeks of age, the uteri at 150 μg/kg and higher doses showed intraluminal hyperplasia (Figure 3). The uteri in the other groups showed normal morphology at estrus. In the vagina, cornification was detected in the highest dose group at PND14 (Figure 4). At PND21, this change was recovered to several layered squamous cells similar to those of control animals. At five weeks of age, it was a feature in all rats at 1,500 and most of those in the 150 μg/kg group. The vaginas in the 15 μg/kg and lower groups showed morphology similar to those
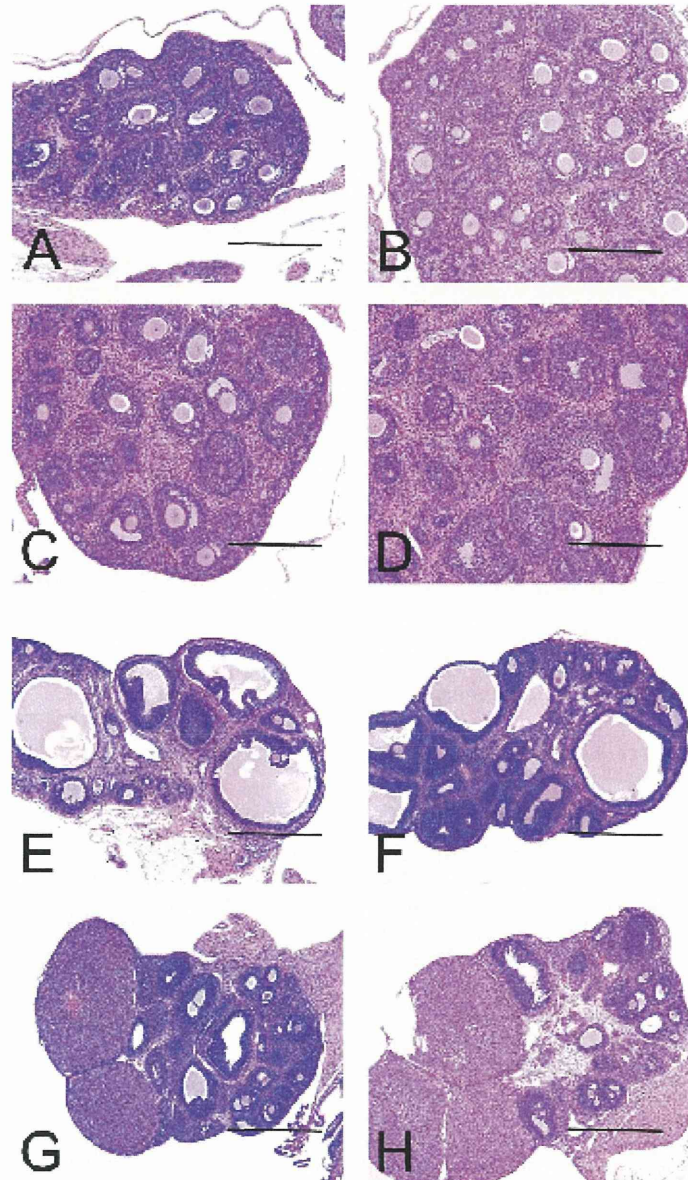
FIGURE 2.—Sequential morphological assessment of the ovaries. A–D and E–H illustrate changes at PND21 and five weeks of age (A, E; B, F; C, G; and D, H are for the 1,500, 150, 15, and 0 μg/kg group, respectively). At five weeks of age, the ovaries in A and B were obtained from animals demonstrating persistent estrus and that were given 1,500 and 150 μg/kg, and the remainder featured estrus with normal cyclicity. Hematoxylin and eosin stain. Scale bars are 1 mm and 2 mm in A–D and E–H, respectively.
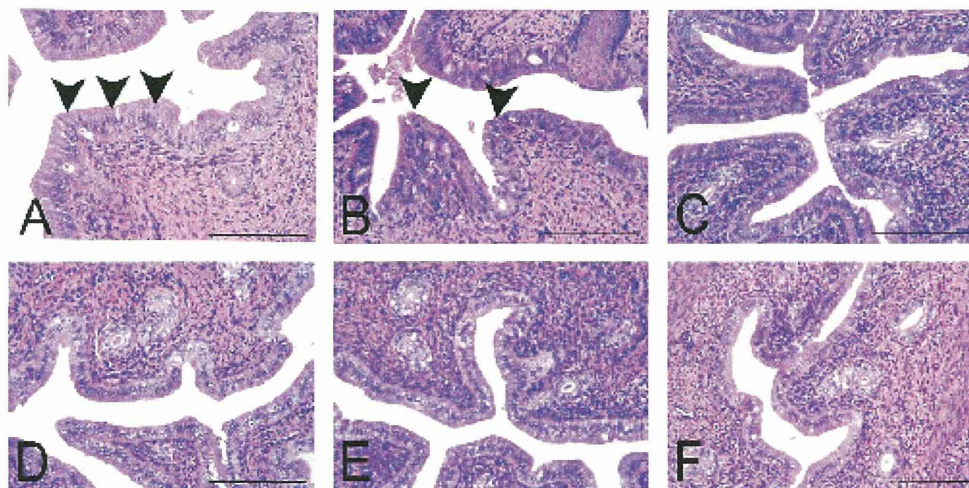
FIGURE 3.—Morphological changes in the uterus at five weeks of age. (A–F) The changes in the 1,500, 150, 15, 1.5, 0.15 and 0 µg/kg group, respectively. The uteri of A and B were obtained from animals demonstrating persistent estrus and that were given 1500 and 150 µg/kg, and the remainder featured estrus with normal cyclicity. (A) Arrowheads indicate high columnar epithelial cells in the luminal surface at 1,500 µg/kg. (B) Arrowheads indicate intraluminal hyperplasia in the luminal epithelium at 150 µg/kg. (C–F) A number of apoptotic cells in the luminal epithelium, which is a typical feature in the uterus at estrus. Hematoxylin and eosin stain. Scale bars are 200 µm.

in the control group. In the other organs, no morphological features were different between treated groups and controls.

Immunohistochemical Intensity of ERα Expression in the Uterus, Vagina, and Ovary

The immunohistochemical expression of ERα in the uterus and vagina is summarized in Figure 5. The intensities were slightly higher in the uterus and vagina at PND14 and PND21 than those at five weeks of age. Estrogen receptor-α did not express in the granulosa cells, theca cells, or stromal cells of the ovary. Treatment-related changes were not detected in any other organs or at any examined time points.

Uterine Carcinogenicity and Histopathology at Termination

The incidences of atypical hyperplasias and endometrial adenocarcinomas are shown in Table 3. Values for both were lower in the highest dose group than in the controls. On the other hand, neoplastic lesions showed an increasing trend in the lower groups, and the incidence of adenocarcinomas and total multiplicity of atypical hyperplasias and adenocarcinomas were significantly higher in the 150 µg/kg group than those in the controls. In the 1.5 and 15 µg/kg groups, which were sacrificed after a shorter period than other groups, few atypical hyperplasias were

detected, and the incidence was similar to background data at the same age in this strain.

At termination, morphological abnormalities such as atrophic uterine horns, disappearance of lumina (Figure 6), or short and straight oviducts were detected in the highest dose group. Such abnormalities were not observed in other treated groups. As common changes in all groups including controls, most of the ovaries were atrophic with several cystic atretic follicles and few or no corpora lutea (Figure 6). Several primary follicles remained, with no obvious variation among the control and treated groups. The vaginal mucosa was similarly composed of several layered squamous cells in the control and treated groups. Neoplastic and non-neoplastic lesions were abundant in the treated and control groups, but their incidence and severity were comparable among the groups. Treatment-related morphological changes were not detected in the genital tracts.

mRNA Expression of the Ovary and Uterus at Five Weeks of Age

Data for mRNA expressions of ER α and β, progesterone receptor (PR), and GAPDH in the ovary are shown in Figure 7. Glyceraldehyde 3-phosphate dehydrogenase mRNA was constitutive in all animals. Although no differences in ERα or ERβ could be detected in the ovary or uterus, PR in the uterus demonstrated increased expression at the 150 µg/kg dose.
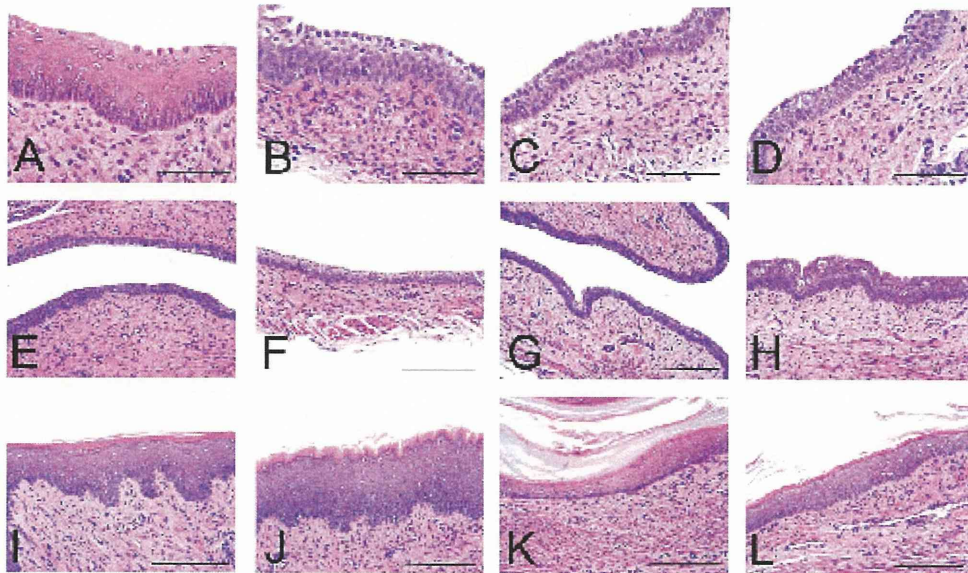
FIGURE 4.—Sequential morphological assessment of the vagina. A–D, E–H, and I–L illustrate changes at PND14, PND21, or five weeks of age (A, E, I; B, F, J; C, G, K, and D, H, L are for the 1,500, 150, 15, and 0 µg/kg groups, respectively). At five weeks of age, the vaginas of A and B were obtained from persistent estrus at 1,500 and 150 µg/kg, and the others were from rats at estrus with normal cyclicity. Hematoxylin and eosin stain. Scale bars are 200 µm and 400 µm in A–D and E–L, respectively.

DISCUSSION

The present study demonstrated clear, dose-dependent, delayed effects of a single neonatal treatment with DES at various doses on morphology in the female reproductive tract and estrous cyclicity in Donryu rats. In particular, earlier onset of PE was evident in all but the lowest dose group. Although timing of VO, which represents puberty, and uterine gland genesis was not affected, the ovarian morphology at five weeks of age at the highest dose indicated an abnormal status with anovulation and excess estrogen levels relative to progesterone (E:P ratio).

In addition, the cornification of vaginal mucosa and uterine intraluminal hyperplasia were consistent with those in rats (Katsuda et al. 2000) or mice (Bern et al. 1987; Yoshida et al. 1999) neonatally exposed to DES or high-dose p-tert octylphenol. The vaginal changes in the present study are considered to be ovary dependent events in rats (Katsuda et al. 2000), but not mice (Forsberg 1979; Takasugi et al. 1962). The present results clearly indicated that the animals at the highest dose were already affected before puberty. Perinatal exposures to high doses of estrogens are known to irreversibly disrupt steroid hormone receptor or related gene expression in the uterus (Nakamura et al. 2008; Yoshida et al. 2000; Yoshida et al. 2002a). The studies on ERα or ERβ mRNA as end points of neonatal exposure to estrogens were limited, whereas PR

and AR as well as IGF1 have been shown to be important (Weihua et al. 2002). The present study did not provide proof of the involvement of ERα in both mRNA and protein levels in the uterus. The increased expression in PR in the uterus at 150 µg/kg might be related to fewer corpora lutea, although mechanisms of the increase have not been determined.

Several animals at 150 µg/kg revealed similar PE status and morphology in the female reproductive tract to those in the highest group, indicating that these animals at 150 µg/kg were also affected before puberty. Besides, clear abnormalities including morphological or gene expressions were not detected in the remainder of the 150 µg/kg group and in all rats in the 15 and 1.5 µg/kg groups until five weeks of age. Therefore, the earlier onset of PE in these animals was considered to be a delayed effect. In addition, the onset showed clear dose dependency. The early onset of PE has already been reported as an indicator of DAS (Mobbs et al. 1985), but there are limited data on dose dependency (Bern et al. 1987). Recently, Ninomiya et al. (2007) reported early onset PE with dose dependency in Sprague-Dawley rats exposed to DES at doses of 1–100 µg/rat (approximately equivalent to 17–1,700 µg/kg if the body weights are expected to be 5–6 g at PND0), but not 0.1 µg/rat (approximately equivalent to 1.7 µg/kg) until 150 days of age. Although diversity exists at lower doses, their results showing delayed effect are in line with our data. The
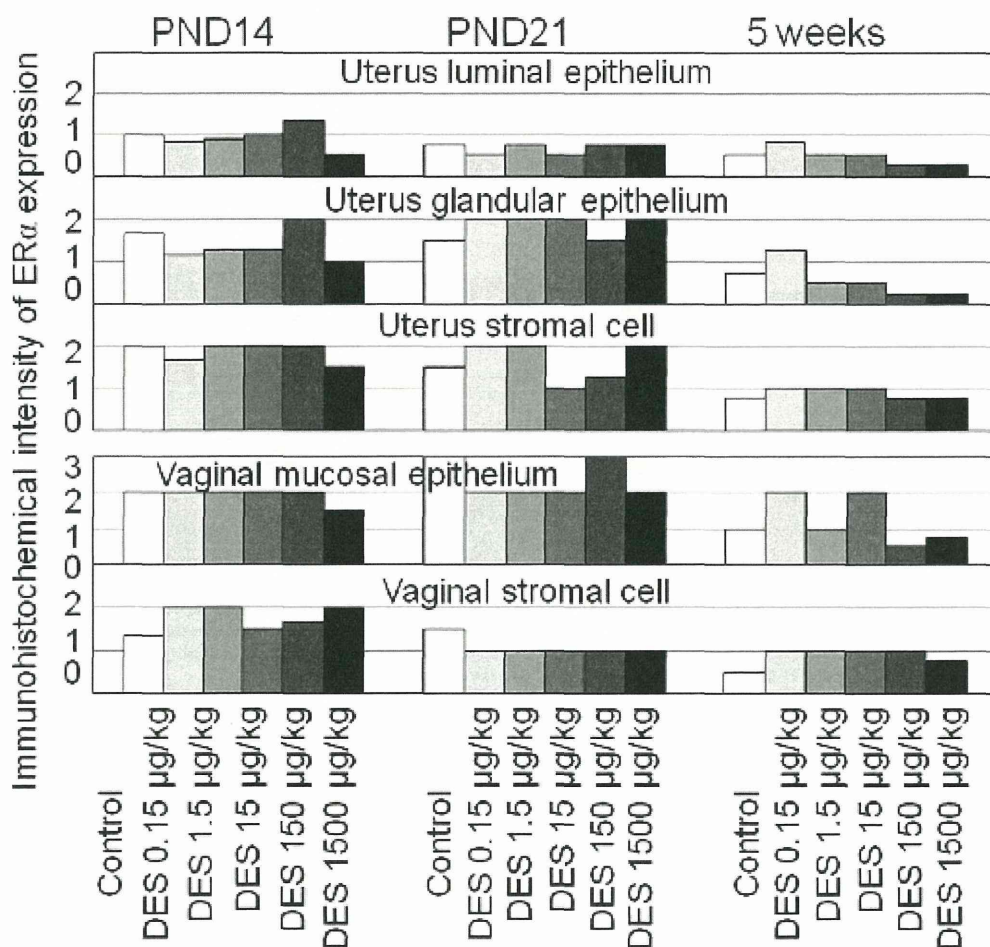
FIGURE 5.—Immunohistochemical intensity of ERα expression in the uterus and vagina at PND14, PND21, and five weeks of age. Treatment-related changes were not detected in any organs. The intensities of these organs at PND14 and PND21 were similar, but overall, expression had declined at five weeks of age.

estrous cyclicity follows a similar tendency until eight months of age in the 0.15 μg/kg and control groups. Although the lower two groups were terminated two months earlier than the other groups, most of the control rats (80%) reached persistent estrus at thirty-three weeks of age. Thus, the lowest dose would not be likely to affect the estrous cycle after eight months of age.

The results of the uterotrophic assay demonstrated that the single treatments with DES at 1.5 μg/kg and higher were inducible doses of estrogenic activity in vivo with dose dependency. Interestingly, our results demonstrated that the doses showing

estrogenic activity in vivo corresponded to the inducible doses of early onset of PE. These results suggest that test substances with estrogenic activity in vivo may induce delayed effects. A concern that twenty-four hours after a single treatment is too soon to detect any estrogenic activity in uterotrophic assay might exist, because uterine weights are usually measured twenty-four hours after a three-day treatment with a test substance in a uterotrophic assay (OECD 2007). However, a previous study that demonstrated, using radioautography, that DES di [^{35}S] sulfate injected intraperitoneally at 1 mg/rat

TABLE 3.—Incidence (%) and multiplicity of uterine proliferative lesions in rats neonatally exposed to diethylstilbestrol.

| Group | No. of rats | No lesions | Atypical hyperplasias | | | Adenocarcinomas | Total incidence of proliferating lesions[a] | Multiplicity[b] |
			Slight	Moderate	Severe			
Control	16	35.7	28.6	42.9	7.1	14.3	64.3	1.44 ± 0.53
DES 15 µg/kg	15	0	46.7	53.3	20.0	20.0	100.0	1.60 ± 0.63
DES 150 µg/kg	24	8.4	12.5	54.2	29.2	50.0*	91.6	3.83 ± 0.87*
DES 1500 µg/kg	17	52.9	17.6	23.5	23.5	5.9	47.1	1.29 ± 0.59

Abbreviation: DES, diethylstilbestrol.
* Significantly difference from control value, $p < .05$.
[a] The incidence of rats bearing atypical hyperplasias or adenocarcinomas (%).
[b] The average number of proliferating lesions per rat bearing these lesions.
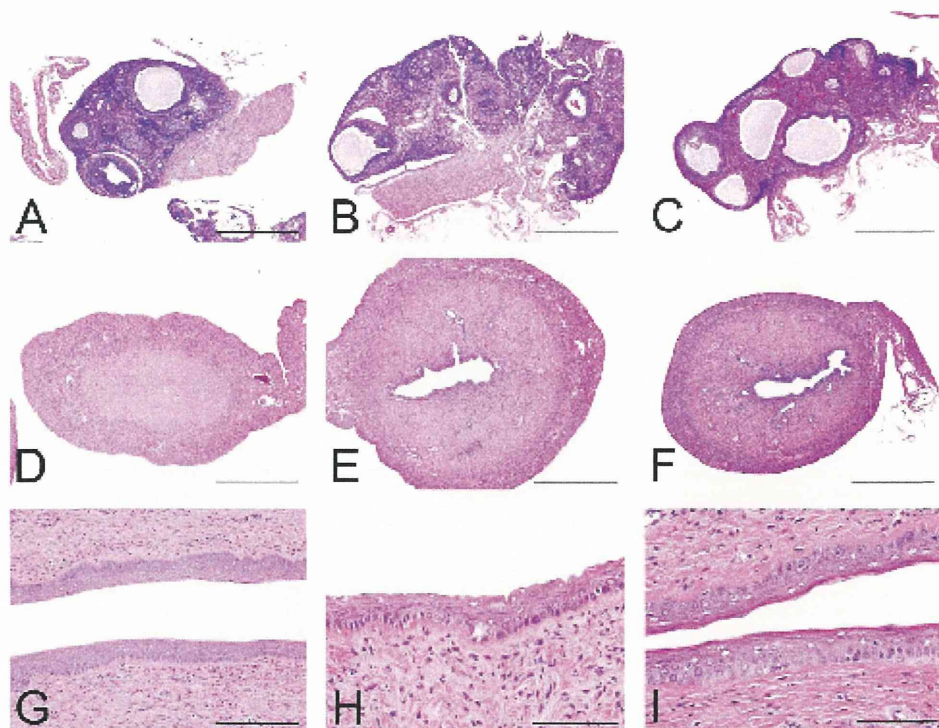
FIGURE 6.—Morphological changes at the termination. A–C, D–F, and G–I are the ovaries, uteri, and vaginas, respectively. (A, D, G) 1,500 µg/kg; (B, E, H) 15µg/kg; and (C, F, I) 0 µg/kg. Hematoxylin and eosin stain. Scale bars are 1 mm, 2 mm, 400 µm, and 200 µm in A–C, D–F, G, and H–I, respectively.

distributed in the whole body within twenty-four hours (Barford et al. 1977) could be support that the concern could be excluded.

It is well known that neonatal exposure to DES can induce malformations in the female reproductive tract not only rodents (Newbold et al. 1983; Rothschild et al. 1987–88), but also in
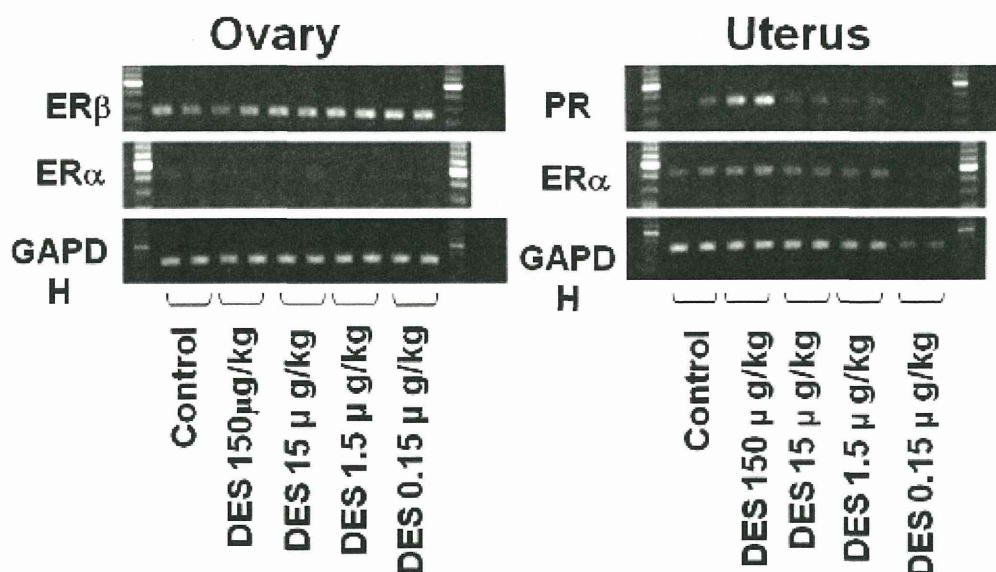
FIGURE 7.—mRNA Expression of estrogen receptors α and β and progesterone receptor in the ovary and uterus in the 150, 15, 1.5, 0.15 and 1.5 µg/kg and control groups at five weeks of age. The ovaries and uteri at 150 µg/kg were obtained from animals in persistent estrus, and others are from rats at estrus with normal cyclicity.

human beings as DES daughters (Jeffries et al. 1984). The present results of uterine carcinogenesis did not show a dose dependency. The reason is likely to be related to the extreme atrophy without the endometrial epithelium apparent in the uteri at the highest dose at termination, because the epithelial cells are the histogenesis of endometrial adenocarinomas. The increase of endometrial adenocarcinoma development at 150 µg/kg of DES observed here is in line with previous studies of neonatal treatment with estrogenic compounds such as DES, tamoxifen, or high-dose p-tert octylphenol (Carthew et al. 2000; Newbold et al. 1990; Yoshida et al. 2002b). Prolonged exposure to excess estrogen relative to progesterone in the blood (increased E:P ratio), which is reflected as PE in a vaginal smear, is an important cause of uterine cancer development in Donryu rats, a high-yield strain of uterine endometrial cancers (Ando-Lu et al. 1994; Nagaoka et al. 1990; Vollmer 2003; Yoshida et al. 2004). Excess estrogens also play a crucial role in cancer of the uterine corpus in other strains of rats (Deerberg and Kaspareit 1987) or mice, as well as women (World Health Organization Classification of Tumours 2003), although androgen or prolactin might influence uterine cancer development in rodents (Nanternet et al. 2005; Yoshida et al. 2009). Our results described above show that early onset of PE might be the most sensitive indicator of delayed effects by neonatal exposure to estrogenic compounds rather than susceptibility

to induction of preneoplastic and neoplastic lesions and other morphological changes.

It is still unclear what mechanism is crucial for the early onset of PE. MacLusky and Naftolin (1981) first reported such effects as DAS, and they hypothesized that disruption of the hypothalamus might be the trigger. There is evidence of involvement of loss of noradrenergic neuronal responsiveness in rats (Lookingland and Barraclough 1982) and that DAS is caused by age-related neuroendocrine function rather than ovarian impairment (Mobbs et al. 1985). Recently, Adewale et al. (2009) reported gonadotropin-releasing hormone (GnRH) release following neonatal estrogen exposure to be normal in the organum vasculosum of the lamina terminals in rats displaying early onset of irregular cycles, including PE. One present focus is on the newly discovered neuropeptide in the hypothalamus, kisspeptin, a regulator of gonadtropin secretion via GnRH secretion (Messager et al. 2005), because it plays pivotal roles in the onset of puberty (de Roux et al. 2003; Seminara et al. 2003), sex differentiation (Kauffman et al. 2007), and reproductive activities such as estrous cycling (Adachi et al. 2007) and ovulation (Clarkson and Herbison 2009; Uenoyama et al. 2009). It has been established that neonatal exposure to estrogens or estrogenic compounds decreased hypothalamic Kiss 1 mRNA levels or stimulation of GnRH neurons by kisspeptins, resulting in

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impairment of female reproductive functions (Bateman and Patisaul 2008; Navarro et al. 2009). Kisspeptin and its signaling pathway in GnRH neurons might be anticipated as key factors in delayed effects.

In conclusion, neonatal exposure to a single dose of DES, which shows estrogenic activity in vivo, induces delayed adverse effects on female rats in a dose-dependent manner. Detection of early onset of PE by vaginal smear appears to be the most sensitive and useful parameter to detect such effects, though a wait of over four months is necessary for significance to become apparent. Its appearance, however, can be considered adverse in rats as well as human beings, because it is irreversible and increases the risk to development of uterine corpus cancer.

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Differential Stimulation Pathways of Progesterone Secretion from Newly Formed Corpora Lutea in Rats Treated with Ethylene Glycol Monomethyl Ether, Sulpiride, or Atrazine

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Ethylene glycol monomethyl ether (EGME), sulpiride, and atrazine are known ovarian toxicants, which increase progesterone (P4) secretion and induce luteal cell hypertrophy following repeated administration. The aim of this study was to define the pathways by which these compounds exerted their effects on the ovary and hypothalamic-pituitary-gonadal (HPG) axis. In the ovary, changes in the steroidogenic activity of new and old corpora lutea (CL) were addressed. EGME (300 mg/kg), sulpiride (100 mg/kg), or atrazine (300 mg/kg) were orally given daily for four times from proestrus to diestrus in normal cycling rats. Treatment with all chemicals significantly increased serum P4 levels, and EGME as well as sulpiride induced increases in prolactin (PRL) levels. In new CL, at both the gene and the protein levels, all three chemicals upregulated the following steroidogenic factors: scavenger receptor class B type I, steroidogenic acute regulatory protein, P450 cholesterol side-chain cleavage, and 3β-hydroxysteroid dehydrogenase (HSD) and downregulated the luteolytic gene, 20α-HSD. Coadministration of EGME and bromocriptine, a D2 agonist, completely inhibited PRL but not P4 secretion. Additionally, steroidogenic factor expression levels were upregulated, and 20α-HSD level was downregulated in new CL. These results suggest that EGME both directly and indirectly stimulates P4 production in luteal cells, whereas sulpiride elevates P4 through activation of PRL secretion in the pituitary. Atrazine may directly activate new CL by stimulating steroidogenic factor expressions. The present study suggests that multiple pathways mediate the effects of EGME, sulpiride, and atrazine on the HPG axis and luteal P4 production in female rats in vivo.

Key Words: progesterone; newly formed corpora lutea; ethylene glycol monomethyl ether; sulpiride; atrazine; laser microdissection.

Numerous drugs and chemicals have been found to interfere with reproductive function in female experimental animals (Yuan and Foley, 2002). The female reproductive organs are regulated across the estrous cycle by the hypothalamic-pituitary-gonadal (HPG) system through a complex feedback loops. These feedback mechanisms are perturbed by ovarian toxicants including ethylene glycol monomethyl ether (EGME), sulpiride, and atrazine. In rodents, repeated exposure to ovarian toxicants produces identifiable histopathological changes in the reproductive tract as well as abnormal hormone secretion and disrupted estrous cycles (Sanbuissho et al., 2009).

EGME, sulpiride, and atrazine induce luteal hypertrophy following repeated administration (Davis et al., 1997; Dodo et al., 2009; Shibayama et al., 2009; Yuan and Foley, 2002). EGME, which is widely used in various industrial products such as detergents, adversely affects both the male and the female reproductive systems (Johanson, 2000; Welsch, 2005). In females, EGME and its active metabolite, 2-methoxy acetic acid, induce the hypersecretion of progesterone (P4) from luteal cells both in vivo and in vitro (Almekinder et al., 1997; Davis et al., 1997). Sulpiride, a dopamine D2 antagonist, is used clinically as an atypical antipsychotic drug (Lacruz et al., 2000). In rats, D2 antagonists block the inhibitory effect of dopamine on prolactin (PRL) release, which results in the preservation of functional corpora lutea (CL) and produces a pseudopregnant state (Rehm et al., 2007). Atrazine, a chlorotriazine herbicide, is a potent endocrine disruptor, which promotes mammary tumor growth in female rats (Eldridge et al., 1994) and alters the central nervous system regulation of the reproductive system in mammals (Eldridge et al., 1999). Recently, atrazine has been shown to inhibit the release of gonadotropin-releasing hormone and diminish the estrogen-induced surge of luteinizing hormone (LH) and PRL in rats (Cooper et al., 2000, 2007). Other studies indicate that atrazine also stimulates steroidogenesis and increases serum concentrations of P4 and corticosterone in female rats (Fraites et al., 2009). Effects of atrazine on the HPG

axis have been previously reported; however, its direct effects on the ovary are not completely understood.

In normal rat ovaries, CL are formed and regress over several estrous cycles. Consequently, at any one time, the rat ovary will contain CL at various stages of development and regression. The CL are classified into two main types: those which are newly formed by the current ovulation (new CL) and CL remaining from prior estrous cycles (old CL) (Bowen and Keyes, 2000). Each type is morphologically distinguishable at each estrous stage (Yoshida et al., 2009). A number of key factors within the HPG axis are related to P4 biosynthesis in the CL. Scavenger receptor class B type I (SR-BI), steroidogenic acute regulatory protein (StAR), P450 cholesterol side-chain cleavage (P450scc), and 3β-hydroxysteroid dehydrogenase (HSD) are critical for the uptake, synthesis, and transport of cholesterol and the processing of cholesterol to P4 in luteal cells (Stocco, 2001; Stocco et al., 2007). P4 secretion from the CL in rodents is regulated by the balance between synthesis and catabolism. Briefly, it depends not only on the amount of P4 synthesized but also on the expression of the enzyme 20α-HSD that catabolizes P4 into the inactive progestin, 20α-dihydroprogesterone (20α-DHP). Once 20α-HSD is expressed in the CL, P4 secretion declines and 20α-DHP becomes the major steroid secreted by luteal cells (Stocco et al., 2000). This process is equivalent to the functional regression of the CL. Thus, changes in 20α-HSD activity and the level of its inducible prostaglandin F2α (PGF2α) are considered to be the important factors of luteolysis (Stocco et al., 2007). This pathway may also be the target of agents, which block luteal regression, including the previously mentioned ovarian toxicants.

EGME, sulpiride, and atrazine all target luteal cells with a resulting increase in serum P4; because their chemical structure and properties are completely different, it is speculated that their toxicological mechanisms in CL were different among them, but their mechanisms in vivo have not been elucidated. The present study was performed to clarify how these agents target the luteal cells and affect the HPG axis in vivo. This study focused on changes in the gene expression levels of steroidogenic factors (SR-BI, StAR, P450scc, and 3β-HSD) and luteolytic factors (20α-HSD and PGF2α receptor [PGF2α-R]) in new and old CL, which were isolated with laser microdissection (LMD). In addition, serum hormone assays and a morphometric analysis of immunohistochemical staining intensities of steroidogenic factors were also performed. For EGME, its direct luteal effect was examined under a PRL inhibitory state by coadministration of EGME and the D2 agonist bromocriptine (BRC).

MATERIALS AND METHODS

Animals. Female 6-week-old Sprague-Dawley (Crl:CD) rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were maintained at 23–25°C and a relative humidity of 50–60% with a 12 h light cycle. Commercial rodent chow (CRF-1; Oriental Yeast Co., Ltd, Tokyo,

Japan) and drinking water were available *ad libitum*. Estrous cycle stage was determined each morning by vaginal smear. Only animals displaying 4-day estrous cycles were included in the experiments. The animal protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Chemicals and reagents. EGME was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and was dissolved in water for injection. Sulpiride and atrazine were purchased from Wako Pure Chemical Industries, Ltd and Tokyo Chemical Industry (Tokyo, Japan), respectively, and were suspended in 0.5% wt/vol methyl cellulose 400 solution (Wako Pure Chemical Industries, Ltd). BRC was purchased from Sigma-Aldrich (St Louis, MO) and was dissolved in 70% ethanol diluted with saline. Control animals received vehicle solutions at each experiment.

Experiment 1. To confirm the histopathological changes of luteal cell hypertrophy following treatment with EGME, sulpiride, and atrazine, the animals were gavaged once per day for 2 weeks (Supplementary fig. 1A). The doses used for the study were as follows: EGME (300 mg/kg), sulpiride (100 mg/kg), and atrazine (300 mg/kg). Effective doses on the ovary and dosing period were selected based on the relevant literatures (Davis et al., 1997; Dodo et al., 2009; Ishii et al., 2009; Shibayama et al., 2009). After treatment, the animals were euthanized in the morning (n = 5–8 per group). The ovary and other reproductive and endocrine organs (uterus, vagina, pituitary, adrenal, thyroid, and mammary gland) were fixed in 4% paraformaldehyde and 10% vol neutral-buffered formalin, respectively, and routinely processed with hematoxylin and eosin (HE) staining.

Experiment 2. Because the repeated administration of EGME, sulpiride, or atrazine would disrupt the estrous cycle within 1 week, short-term treatment was required for clearly distinguishing the luteal effects of these chemicals on new CL from those on old CL in normal cycling animals. The animals were treated with these chemicals by gavage for four times from proestrus to diestrus, once per day in the morning (Supplementary fig. 1B). The doses were the same as for experiment 1. Between 1000 and 1100 h of the fourth day of treatment, when all animals were in diestrus, they were euthanized by decapitation after 4 h (EGME and sulpiride) or 1 h (atrazine) following the last treatment (n = 4–7 per group). The necropsy time points were chosen based on previously reported pharmacokinetic data for these agents (Fraites et al., 2009; Hays et al., 2000; Laws et al., 2009; McMullin et al., 2007; Yamada et al., 1990). The serum was stored at −30°C until use in the hormone assays. For LMD, the left ovaries were rapidly removed, embedded in optimal cutting temperature (OCT) compound, and frozen with liquid nitrogen. The right ovaries were fixed in 4% paraformaldehyde and serially sectioned. Sections were then stained with HE or used for immunohistochemical studies.

Experiment 3. To evaluate the luteal effects of EGME in the absence of a PRL effect, the animals were treated with EGME (300 mg/kg) by gavage, BRC (2 mg/kg) by sc injection, or EGME and BRC once per day from proestrus to diestrus as the same procedure of experiment 2 (Supplementary fig. 1C). Effective dose of BRC was selected based on the relevant literature (Bridges and Ronsheim, 1990). On the fourth day of treatment, when all animals were in diestrus, they were euthanized by decapitation between 1000 and 1100 h after 4 h following the last treatment (n = 4–5 per group). The serum hormone assays, gene expression analysis of CL with LMD, and immunohistochemical examination of CL were performed in the same manner as for experiment 2, omitting fixation of ovary in 10% vol neutral-buffered formalin instead of 4% paraformaldehyde.

Hormone assays. The serum concentrations of P4, estradiol-17β (E2), PRL, LH, and follicle-stimulating hormone (FSH) were determined using double-antibody radioimmunoassay and ^{125}I-labeled radioligands. P4 and E2 were measured as described by Taya et al. (1985). National Institute of Diabetes and Digestive and Kidney Disease radioimmunoassay kits were employed for rat PRL, LH, and FSH (National Institute of Arthritis, Metabolism and Digestive Diseases [NIAMDD], National Institutes of Health [NIH], Bethesda, MD) (Taya et al., 1985).

LMD of new and old CL. The OCT-embedded frozen ovaries were sectioned onto membrane-based LMD slides (Leica Microsystems, Wetzlar, Germany) and fixed in 75% ethanol. The sections were then stained with 1% toluidine blue solution, dehydrated, and air-dried. New CL (CL formed by the current ovulation) and old CL (CL remaining from prior estrous cycles) were captured using a Leica LMD6000 LMD system (Leica Microsystems).

Extraction of total RNA and reverse transcription. Laser-captured tissues were pooled in lysis buffer, and RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Residual genomic DNA was removed with an RNase-free DNase Set (Qiagen). The concentration and purity of RNA were checked using a spectrophotometer (NanoDrop ND-1000, Thermo Fischer Scientific Inc., Waltham, MA) and stored at −80°C until analysis. For complementary DNA synthesis, reverse transcription (RT) was performed with the Sensiscript RT Kit (Qiagen) following the manufacturer's instructions.

Real-time quantitative PCR. Messenger RNA (mRNA) levels of SR-BI, StAR, P450scc, 3β-HSD, 20α-HSD, PGF2α-R, PRL receptor (PRL-R) (Long and Short forms), steroidogenic factor 1 (SF-1), nuclear receptor 5A2 (NR5A2), acetyl-coenzyme A acetyltransferase 1 (ACAT-1), and hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Table 1) were analyzed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The probes and primers of SF-1 (Rn00584298_m1) and ACAT-1 (Rn00579605_m1) were prepared with TaqMan gene expression assays (Applied Biosystems). To compare mRNA levels among samples,

mRNA levels for each gene were normalized to a housekeeping gene, HPRT, using the standard curve method.

Immunohistochemistry. The ovarian sections were deparaffinized, treated with 90% methanol containing 3% H_2O_2 for 10 min at room temperature, heated in a 0.01M citric acid buffer (pH − 7.0) at 95°C for 5 min, and incubated with antibodies of SR-BI (NB400-104; Novus Biologicals, Littleton, CO), StAR (sc-25806; Santa Cruz, CA), and P450scc (AB1244; Millipore Corporation, Temecula, CA) overnight at 4°C. For 3β-HSD (sc-30821; Santa Cruz) immunostaining, there was no antigen retrieval treatment in experiment 2, heating in a citric acid buffer at 95°C for 10 min was performed in experiment 3. The sections were then incubated with the secondary antibody (HISTOFINE SIMPLESTAIN MAX-PO, Nichirei Bioscience, Tokyo, Japan) and were visualized with 3,3'-diaminobenzidine and counterstaining with hematoxylin.

Morphometric analysis of immunohistochemical staining intensity. Immunohistochemical staining intensities of SR-BI, StAR, P450scc, and 3β-HSD in new and old CL were quantified using ImageJ software (NIH, http://rsbweb.nih.gov/ij/). Pictures obtained from different areas (five areas per animal) of new and old CL ($n = 4$–5) were taken using a ×40 objective and a digital camera system. Digital images were processed by subtracting the light background, splitting the blue channel to remove the hematoxylin staining, followed by adjustment of the density threshold (SR-BI, StAR, and P450scc; 0-210, 3β-HSD; 0-220). The pixel intensity was measured and compared by calculating the integrated density, which was the product of the area and the mean gray value.

TABLE 1
Primers and Probes Used for Real-Time PCR Analysis

| Gene		Primer and probe	GenBank accession No.
SR-BI	Forward	5'-CCGAATCCTCACTGGAATTCTTC-3'	NM_031541
	Reverse	5'-CGAACACCCTTGATTCCTGGTA-3'	
	Probe	5'-VIC-AAGCCTGCAGATCTATGA-MGB-3'	
StAR	Forward	5'-GGGAGAGTGGAACCCAAATGT-3'	NM_031558
	Reverse	5'-CATGGGTGATGACTGTGTCTTTTC-3'	
	Probe	5'-VIC-AAGGAAATCAAGGTCCTGAAG-MGB-3'	
P450scc	Forward	5'-TCTCCTCTACCAACAGTCCTCGAT-3'	BC089100
	Reverse	5'-TGGTACAGGTTTATCCAACCATTG-3'	
	Probe	5'-VIC-CTTCAATGAGATCCCTTC-MGB-3'	
3β-HSD	Forward	5'-GCCCAACTCCTACAAGAAGATCAT-3'	L17138
	Reverse	5'-CTCGGCCATCTTTTTGCTGTATG-3'	
	Probe	5'-VIC-ATGTGCTTTCATGATGCTCT-MGB-3'	
20α-HSD	Forward	5'-TTTCAATGAGGAGAGAATCAGAGAGA-3'	L32601
	Reverse	5'-CCATGTCATCTGAAGCCAACTG-3'	
	Probe	5'-VIC-CCTGCAGGTCTTTGAT-MGB-3'	
PGF2α-R	Forward	5'-CTCTGGCTTGTGCCCACTTT-3'	U47287
	Reverse	5'-CCGATGCACCTCTCAATGG-3'	
	Probe	5'-VIC-CCTGGGCAGTACGATG-MGB-3'	
PRL-R (Long form)	Forward	5'-CTGGGCAGTGGCTTTGAAG-3'	M57668
	Reverse	5'-CCAAGGCACTCAGCAGCTCT-3'	NM_012630
	Probe	5'-FAM-ATCTTTCCACCAGTTCCTGGGCCAAAAATA-MGB-3'	
PRL-R (Short form)	Forward	5'-CTGGGCAGTGGCTTTGAAG-3'	M57668
	Reverse	5'-AAGGGCCAGGTACAGATCCA-3'	NM_012630
	Probe	5'-FAM-ATCTTTCCACCAGTTCCTGGGCCAAAAATA-MGB-3'	
NR5A2	Forward	5'-TTCGGGCCAATGTACAAGAGA-3'	NM_021742
	Reverse	5'-GGCTCGAATGAGGGCTTTCT-3'	
	Probe	5'-VIC-ACAGGGCCTTGAAAC-MGB-3'	
HPRT	Forward	5'-GCCGACCGGTTCTGTCAT-3'	X62085
	Reverse	5'-GGTCATAACCTGGTTCATCATCAC-3'	
	Probe	5'-FAM-CAGTCCCAGCGTCGTG-TAMRA-3'	
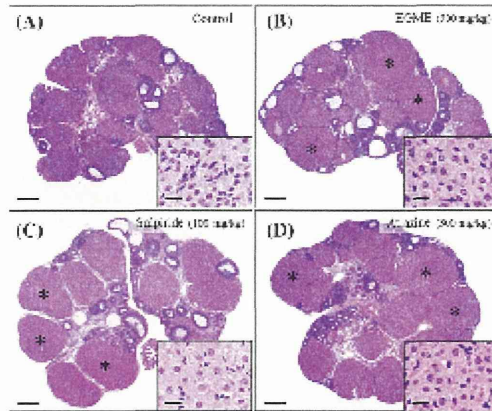
FIG. 1. Luteal cell hypertrophy induced by the 2-week administration of EGME, sulpiride, or atrazine. Luteal cells become hypertrophied with abundant eosinophilic cytoplasm following EGME (B), sulpiride (C), or atrazine (D) treatment compared with respective control CL at the diestrus stage (A). Asterisks indicate hypertrophied CL. Bars represent 500 μm, and bars in the inset images represent 20 μm.

Statistical analysis. All data are presented as the mean ± SEM. Differences in hormone assays, mRNA level in new and old CL, and immunohistochemical staining intensity were evaluated by Student's or Welch's t-test. $p < 0.05$ was considered statistically significant.

RESULTS

Experiment 1

Luteal cell hypertrophy induced by a 2-week treatment of EGME, sulpiride, or atrazine. The animals treated with EGME, sulpiride, or atrazine showed abnormal estrous cycle including persistent diestrus as determined by vaginal cytology within 2 weeks. Luteal cell hypertrophy and an increased number of large, atretic follicles were observed on histology

in all treatment groups (Figs. 1B–D). New CL were rarely detected. The hypertrophic CL contained round to polygonal luteal cells with abundant eosinophilic and sometimes well-vacuolated cytoplasm. Few apoptotic cells were observed. In the control females, the ovaries at diestrus contained new CL characterized by foamy basophilic luteal cells and old CL with some vacuolated eosinophilic luteal cells and numerous fibrous cells (Fig. 1A). With respect to other reproductive and endocrine organs, mucinous degeneration of the vagina occurred in all treatment groups, and hyperplasia of the mammary gland was found in all sulpiride-treated and some EGME-treated animals, being most pronounced in the former group (data not shown).

Experiment 2

Alterations of serum hormone concentrations. The serum P4 level was significantly higher in all treatment groups compared with the respective control groups at diestrus (2.1-, 4.2-, and 6.3-fold in EGME, sulpiride, and atrazine groups, respectively) (Fig. 2A). In the EGME- and sulpiride-treated groups, the PRL level was significantly higher (4.3- and 12.1-fold in EGME and sulpiride groups, respectively) (Fig. 2B). Other hormone levels (E2, LH, and FSH) were within basal levels and not changed in all groups (data not shown).

Changes of steroidogenic and luteolytic gene expression levels in new and old CL. New CL of the EGME and sulpiride treatment groups showed significantly higher expression levels of SR-BI, StAR, P450scc, and 3β-HSD mRNA ($p < 0.01$ or 0.05) and lower levels of 20α-HSD mRNA ($p < 0.05$) than controls at diestrus (Figs. 3A–E). The PGF2α-R mRNA level in new CL of the EGME group was also lower than in the control (Fig. 3F). Atrazine-treated new CL showed a significantly higher level of SR-BI mRNA ($p < 0.05$). Of the other steroidogenic factors, StAR, P450scc, and 3β-HSD had higher mRNA expression levels and the luteolytic factor, 20α-HSD, had a lower mRNA level (Figs. 3A–E). In the old CL, the 3β-HSD and PGF2α-R mRNA levels were significantly increased by EGME treatment ($p < 0.05$) (Fig. 3D).
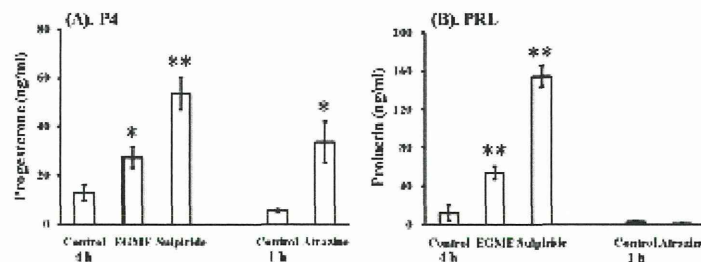

FIG. 2. Serum hormone levels following four daily treatments with EGME, sulpiride, or atrazine. Data represent serum P4 (A) and PRL (B) levels (mean ± SEM). Animals were euthanized at 4 h (EGME and sulpiride) or 1 h (atrazine) after the last dose ($n = 4–5$). Double asterisks ($p < 0.01$) and asterisk ($p < 0.05$) indicate significant differences as compared with the controls.