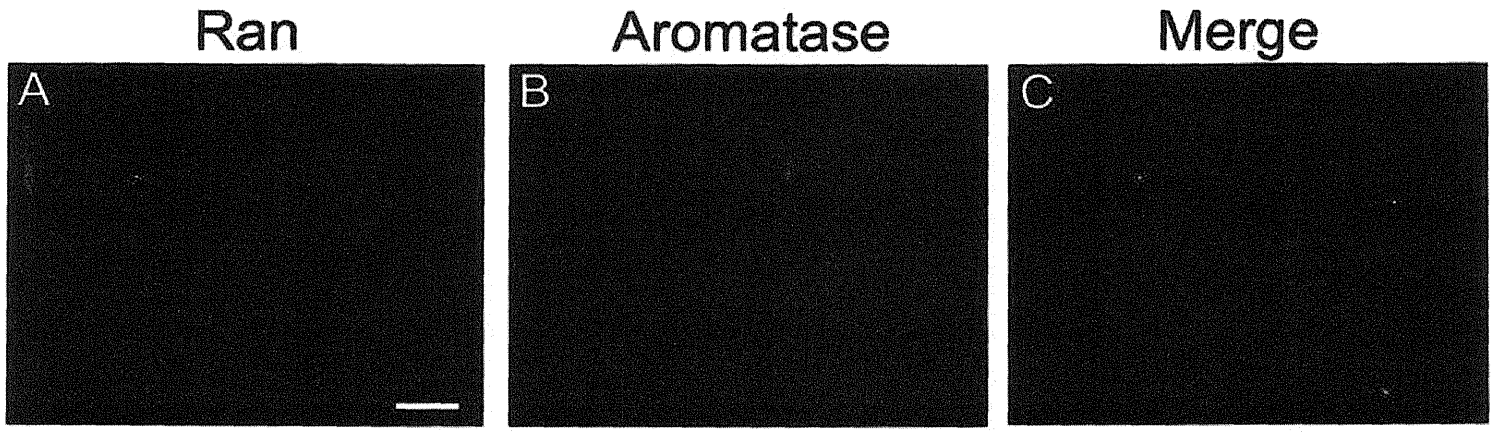
Figure 4


Ran
Aromatase
Merge
A
B
C

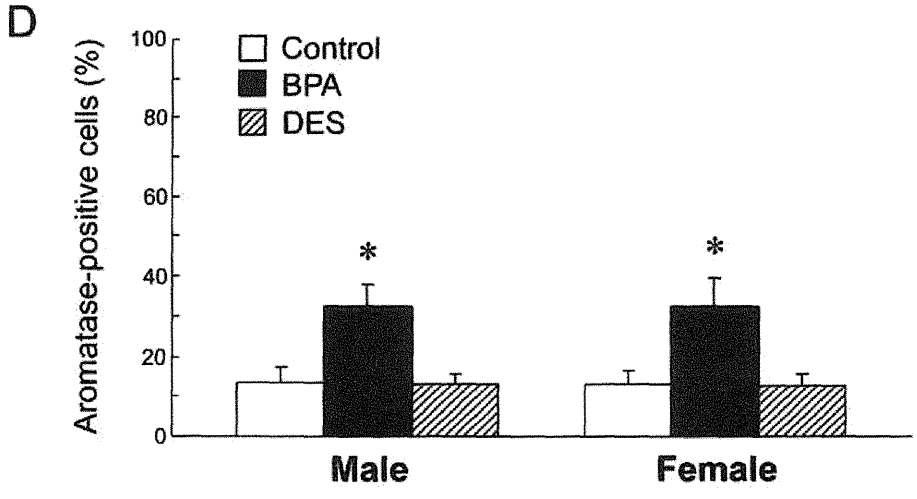

D
Aromatase-positive cells (%)
100
80
60
40
20
0
Control
BPA
DES
*
*
Male
Female

Figure 5

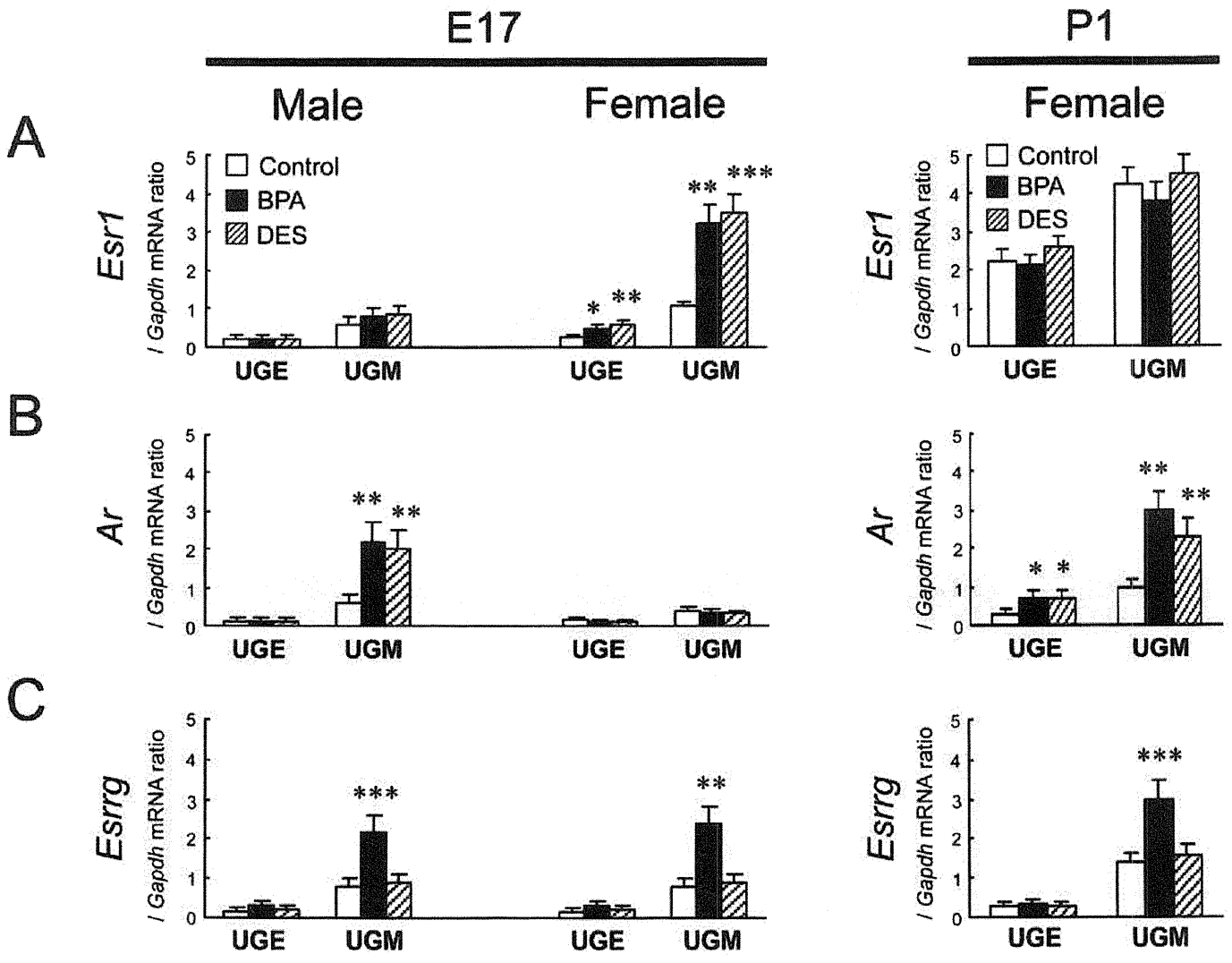
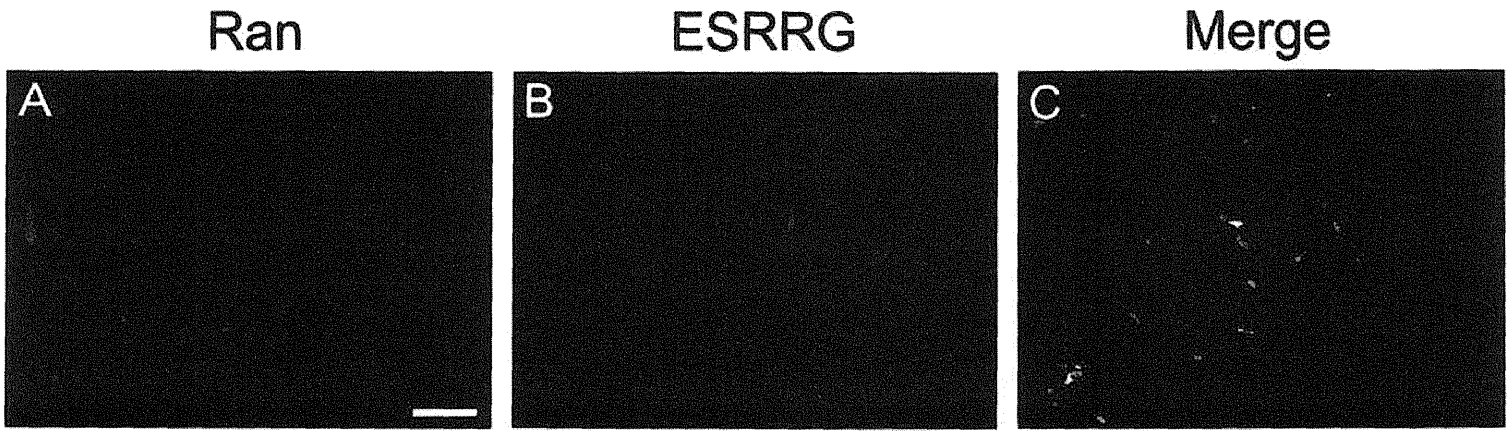

E17
P1
Male
Female
Female
A
B
C
Control
BPA
DES
Esr1
Ar
Esrrg
/ Gapdh mRNA ratio
UGE
UGM

Figure 6


Ran
ESRRG
Merge
A
B
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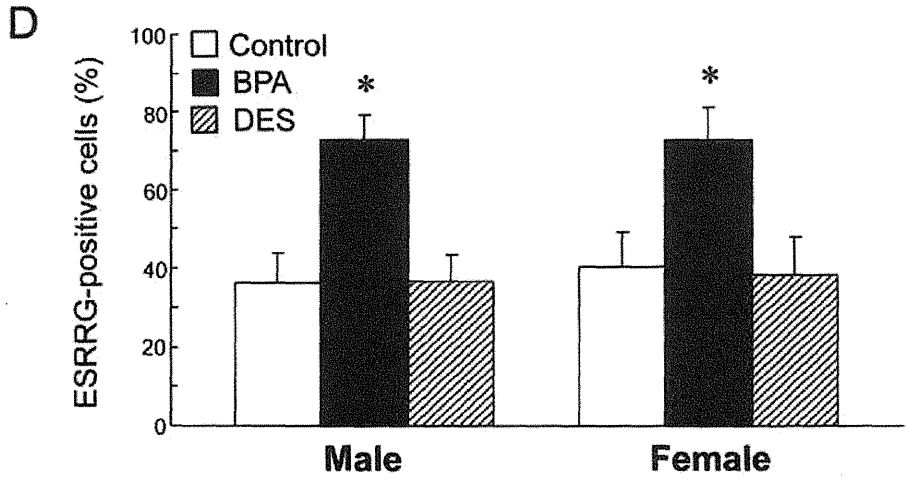

D
ESRRG-positive cells (%)
100
80
60
40
20
0
Control
BPA
DES
*
*
Male
Female

Figure 7

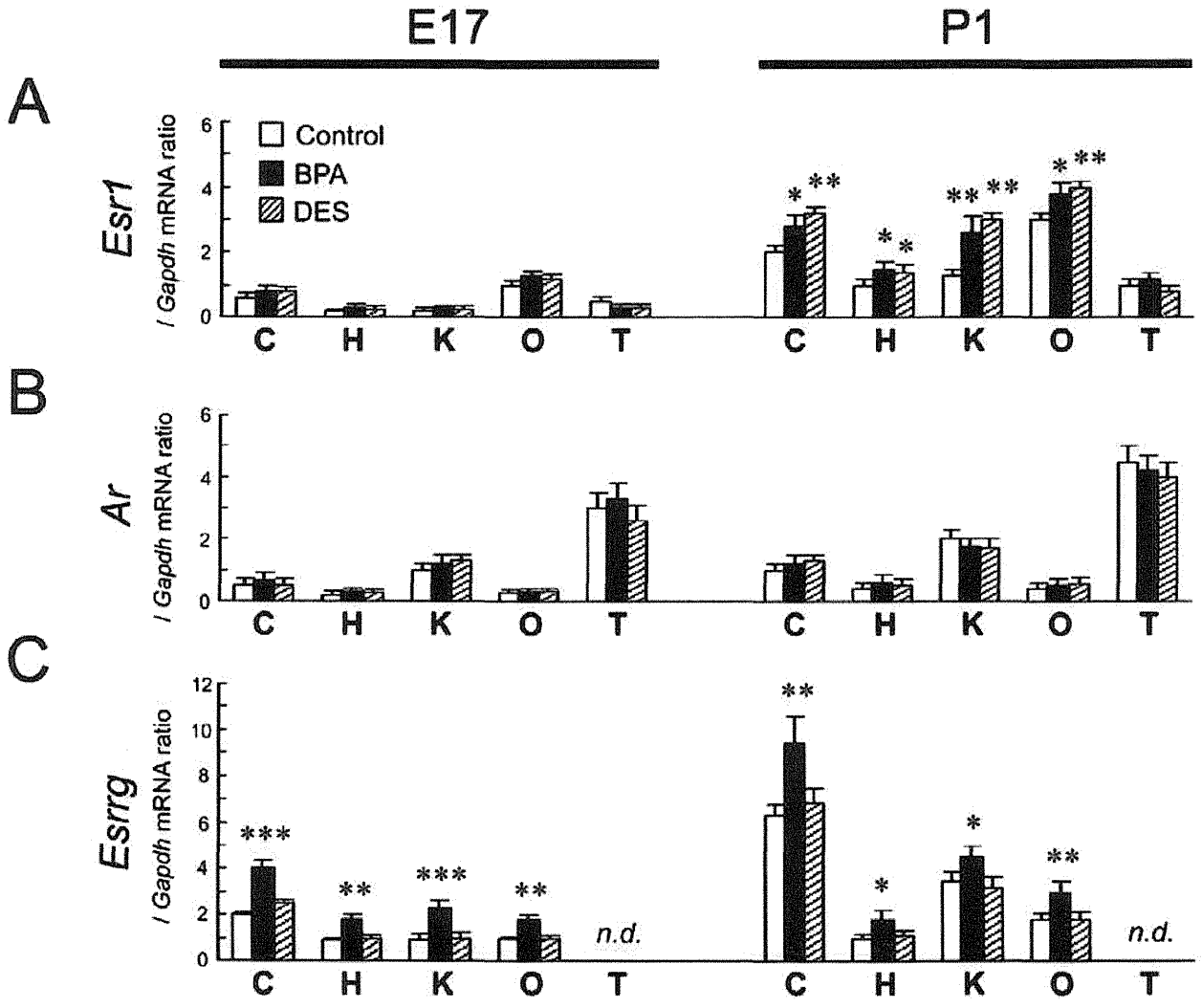
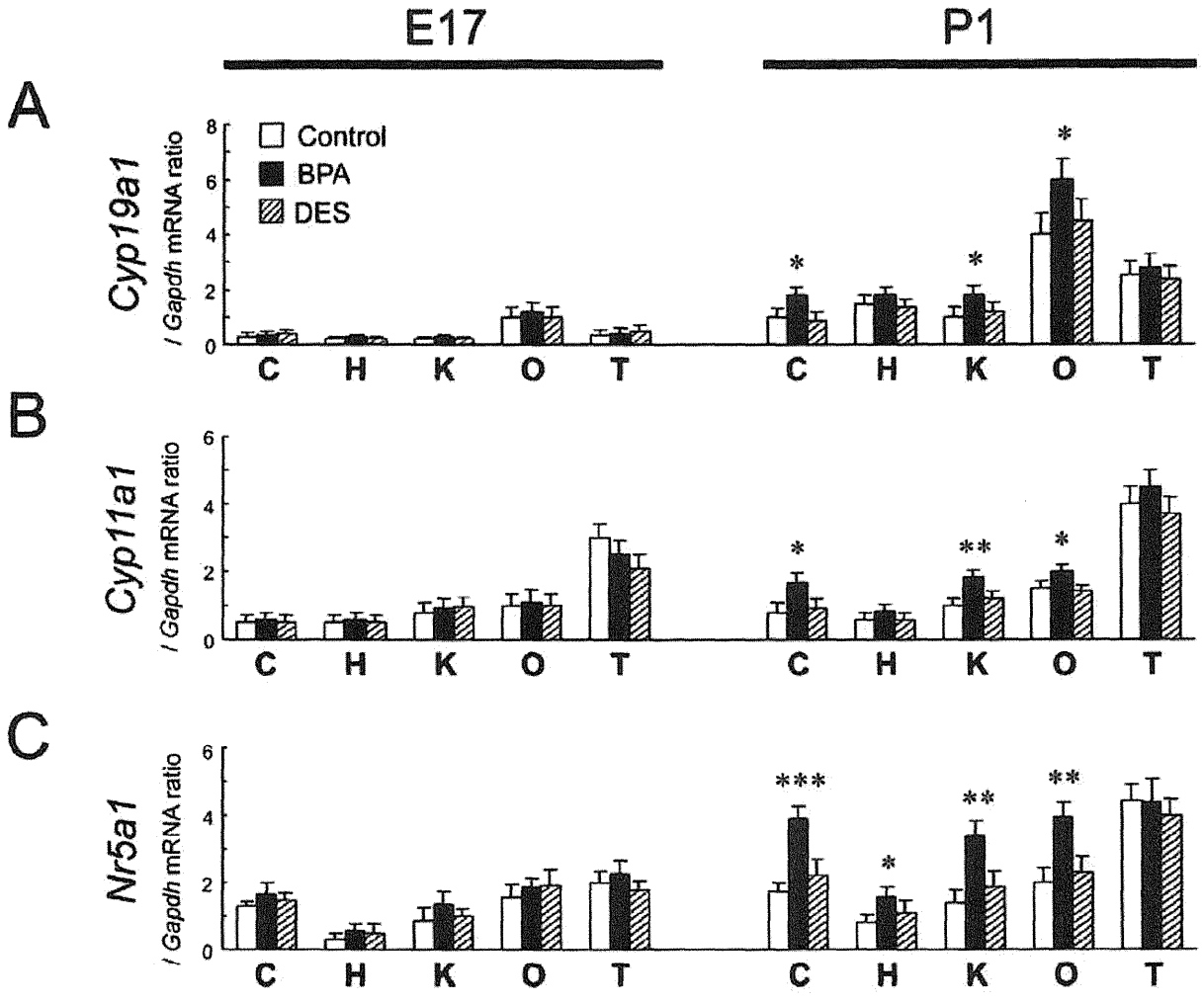

E17
P1
A
B
C
Esr1
Ar
Esrrg
/ Gapdh mRNA ratio
Control
BPA
DES
C H K O T
n.d.

Figure 8


E17
P1
A
B
C
Control
BPA
DES
Cyp19a1
Cyp11a1
Nr5a1
/ Gapdh mRNA ratio
C H K O T
C H K O T

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Delayed reproductive dysfunction in female rats induced by early life exposure to low-dose diethylstilbestrol

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ABSTRACT

A one-lifespan test was carried out to establish a test protocol for endocrine-disrupting chemicals (EDCs). Diethylstilbestrol was administered by oral gavage to neonatal rats at doses of 0.05, 0.5 and 5 μg/kg/day for 5 days after birth. Abnormal estrous cycles were observed throughout the study in all females from the 5 μg/kg group, and in 40% from the 0.5 μg/kg group from 24 weeks of age. The conception rate of 12-week-old females in the 5 μg/kg group was 0%, and that of the 23-week-old females in the 0.5 μg/kg group was 33.3%. No effect of DES was observed at the first parturition in any group, except for the 5 μg/kg group. However, litter size was significantly reduced in the 0.5 μg/kg group at the second parturition. These results indicated that a prolonged period of observation of reproductive function is necessary to determine EDCs reliably.

1. Introduction

There are many chemicals in the environment that have hormonal activity. It has been suggested that some of them might cause serious problems in humans and wildlife. These chemicals are called endocrine-disrupting chemicals (EDCs). At present, one of the focuses of EDC research is to develop a definitive protocol for testing the endocrine-disruption properties of chemicals. In silico screening, *in vitro* screening and *in vivo* screening (uterotrophic assay, Hershberger assay) have been developed as test protocols by the Organization of Economic Cooperation and Development (OECD) to distinguish endocrine active compounds from non-active compounds [1]. However, a definitive test protocol for EDCs has yet to be established. Although a multi-generation reproductive toxicity study has been recommended as a definitive test protocol, only the reproductive toxicity of EDCs can be determined with this test protocol. It is considered that EDCs induce not only reproductive toxicity but also endocrine-associated neurotoxicity and immunotoxicity [2,3]. Recently, the substitution of two-generation reproductive toxicity study by extended one-generation reproductive toxicity study has been proposed by the OECD to evaluate specific life stages not covered by other types of toxicity studies and to test for effects that may occur as a result of pre- and postnatal chemical exposure. Therefore, a one-lifespan test was devised as a definitive test protocol of EDCs, instead of the multi-generation reproductive toxicity test [4]. The one-lifespan test focused mainly on delayed abnormalities in the estrous cycle of female offspring induced by intrauterine or lactation exposure to chemicals. Sawaki et al. [5] reported that delayed ovarian dysfunction appeared, despite normal sexual maturation, upon exposure to ethinyl estradiol that exceeded the physiological range at critical periods in fetus or neonate of female rat.

As it was suggested that the fetus and neonate are more sensitive to EDCs than pubertal or adult rodents [6], maternal administration was usually selected for regular reproductive toxicity study of EDCs. However, gestational and lactational administrations through a dam make the amount of chemical exposure to fetus and offspring uncertain. Therefore, dosing of pups was selected for this study according to previous study in our laboratory [7,8]. An oral route instead of a subcutaneous route was selected for this study because an exact amount of chemicals can thus be administered and the loss of dosing caused by maternal licking can be avoided.

In this study, diethylstilbestrol (DES), which is a synthetic non-steroidal estrogen, was administered to Sprague-Dawley (SD) rats during the neonatal period, and then these animals were subjected to examination of reproductive toxicology from the developmental period, through maturation, to the aging period. In particular, the estrous cycles of females were observed over a long period, from 8 weeks to 49 weeks of age, and females were subjected to mating three times, that is, at 12, 23 and 34 weeks of age. In addition, the survival periods of these treated rats were determined.

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2. Materials and methods

2.1. Animals and housing

Male and female Crl:CD(SD) rats were purchased from Charles River Japan Laboratories at 8 weeks of age. SD rats are generally used in reproductive toxicology, and their background data are available in our laboratory [9,10]. For example, we know that abnormal estrous cycles induced by aging occur earlier in SD rats than in Wistar rats [11].

The animals were kept individually in metal cages with a metal meshed floor (220 mm (w) × 270 mm (d) × 190 mm (h)) in an animal room maintained at a room temperature of 22–25 °C, a relative humidity of 50–65%, and 12-h lighting (7:00–19:00 lighting). Feed (CE-2 pellet feed, CLEA Japan) and tap water were available ad libitum. The diet, CE-2, contained 25.2% protein, 4.4% fat and 50.2% carbohydrate. CE-2 is a standard rodent diet, and includes soybean or white fish meal as a source of protein. From gestational day (GD) 10 through postnatal day (PD) 10, female rats were housed individually in aluminum cages (350 mm (w) × 400 mm (d) × 190 mm (h)) with Paper Clean (SLC Japan). All the operations of the experiment were performed in accordance with the Guidelines for Animal Experiments in Hatano Research Institute, FDSC.

2.2. Dose administration

Mating of rats was conducted at 11 weeks of age, and copulated females were divided into 4 groups of more than 12 animals per group. Neonates delivered spontaneously and were checked for sex and external malformations on PD 0. Five male and female pups without any abnormalities were selected from each litter and tattooed on the limbs for identification. These selected neonates were orally administered daily from PD 1 to PD 5 using a micro-syringe connected with a catheter as described previously [12]. The doses of DES were set at 0 (vehicle only), 0.05, 0.5 and 5 μg/kg/day in this study on the basis of results of a 3-day oral uterotrophic assay of DES using ovariectomized mice, in which increased uterine weight was observed in the 5 μg/kg/day or greater DES groups. The DES (Lot No. 123K0687, Sigma–Aldrich) was dissolved in a few drops of ethanol and then diluted with corn oil (Lot No. PKJ7877, Wako Pure Chemical) to prepare the doses.

The dosing volume was set at 10 ml/kg body weight. At the end of the treatment period, the number of neonates was adjusted to 4 by sex for each litter, and weaned on PD 21.

2.3. Body weight, sexual maturation, estrous cycle and mating

Body weights of neonates were measured on PDs 1–5, and on PDs 7, 14 and 21. After weaning, body weights of offspring were measured once a week, from 3 to 10 weeks of age; every two weeks, from 10 to 26 weeks of age; and then once every 4 weeks, after 26 weeks and up to 101 weeks of age.

As an index of sexual maturation, the vaginal opening of all females and the preputial separation of all males were checked daily from PD 25 and PD 35, respectively. Daily vaginal smear was collected every two weeks, from 8 to 49 weeks of age, from two females (Cohorts C and D) in each litter. Two males and two females (Cohorts A and B) in each litter were subjected to mating. Males and females were mated 1:1 within groups, avoiding brother–sister mating. Mating was carried out for as long as two weeks, at 12, 23 and 34 weeks of age. Vaginal smear was collected until copulation, which was confirmed by the presence of sperm or internal vaginal plug. If copulation was not observed in a pair, re-mating was performed with other pairs. Furthermore, treated males that did not impregnate the copulated treated females were also re-mated with intact SD females for up to two weeks. In males, further mating was carried out with intact SD females at 56 and 68 weeks of age. The inseminated females delivered spontaneously, and gestational days, number of pups and weights of pups were recorded at birth. F2 pups were removed at PD 4.

2.4. Behavioral test

At 24 and 48 weeks of age, one male and one female (Cohort C) from each litter were tested in a shuttle-box (TK-401L, Unicom Inc.) in order to determine avoidance learning ability as described previously [13]. For each trial, a 3-s conditioning stimulus (CS), comprising a buzzer and a lamp, was followed by a 3-s unconditioned stimulus, comprising the CS plus a 1.0 mA scrambled shock delivered through the floor grid. The number of avoidance responses, in which animals moved to the other side during the CS, was recorded. Sixty conditioning trials separated by 30-s intertrial intervals were given daily on 3 consecutive days.

2.5. Necropsy and immune response of males

One male (Cohort D) from each litter was subjected to necropsy at 26 weeks of age. Another male (Cohort C) from each litter was subjected to necropsy at 52 weeks of age. Prior to the necropsy, these males were given a single intravenous injection of 0.7 ml of 1% sheep red blood cells (SRBC) 4 days before blood sampling (i.e., 7 days before necropsy). Blood samples were collected from the tail vein and then the serum was separated and stored at −80 °C until determination of anti-SRBC-IgM. The SRBC membrane was purified according to the method of Temple et al. [14]. ELISA was conducted with immunoplates (Nunc, Roskilde, Denmark) that had been coated with SRBC membrane [15]. At necropsy, these males were exsanguinated under sodium pentobarbital anesthesia. The weights of the brain, pituitary, thyroid gland, liver, spleen, kidney, adrenal gland, testis, epididymis, ventral prostate and seminal vesicle (including coagulating gland) were measured. The caudal epididymis was homogenized in water by sonication for assessment of the total sperm concentration as described previously [16]. The decapsulated testis was homogenized in water by sonication and used for the testicular sperm head counts [16]. Samples from homogenates of the caudal epididymis and testis were stained with Ident Stain, and the number of illuminated sperm was measured using the HTM-IDENT option of HTM-IVOS (Hamilton-Thorne Research, Beverly, MA).

2.6. Necropsy and ovulation test of females

At 54 weeks of age, two females (Cohorts C and D) from each litter were subjected to necropsy. These females were exsanguinated under sodium pentobarbital anesthesia. The weights of the brain, pituitary, thyroid gland, liver, spleen, kidney, adrenal gland, ovary and uterus were measured. In order to check the existence of an ovarian follicle that could ovulate, half of the females (Cohort C) were intravenously administered 10 IU of human chorionic gonadotropin (hCG, Sigma) 16–17 h before necropsy [17]. The number of ovulations induced was counted in the oviducts at the time of dissection. In order to measure the hormone levels as previously described [9,10], the plasma concentrations of prolactin, T3, T4, LH and FSH were determined in blood samples of five females (Cohort D) without pituitary enlargement at necropsy from each group.

2.7. Tumorigenesis

At 101 or 102 weeks of age for males, and 103 or 104 weeks of age for females (Cohorts A and B), live animals were exsanguinated under sodium pentobarbital anesthesia and subjected to necropsy. Intermediate dead and moribund animals were also subjected to necropsy. Histopathology was performed in a routine manner on the following organs and tissues of each animal: skin and subcutaneous tissue, mammary gland, brain, spinal cord, pituitary gland, submandibular gland, Harderian glands, tongue, Zymbal gland, thyroid gland, parathyroid gland, thymus and mediastinal lymph nodes, aorta, trachea, lung and bronchus, heart, liver, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach, intestine, urinary bladder, prostate, testis, epididymis, seminal vesicle, preputial gland, ovary, uterus, vagina, clitoral gland, sciatic nerve, skeletal muscle, eye, optic nerve, femur, bone marrow, subcutaneous and mesenteric lymph nodes, and other organs or tissues with pathologic lesions. All organs and tissues were fixed in 10% formalin. Trimmed specimens were processed as paraffin blocks, and 3–5 μm sections of every specimen were obtained. Sections were routinely stained with hematoxylin and eosin.

2.8. Statistical analyses

The data used the litter average as the statistical unit before weaning. Individual data were used as the statistical unit after weaning. Body weights, organ weights, number of pups, sperm counts and number of ovulations were analyzed by one-way ANOVA. When the ANOVA was significant, Dunnett's test was applied. Sexual maturation, gestation length, viability of pups, immune response and hormone levels were analyzed by Kruskal–Wallis analysis of ranks. When significant differences were detected among groups, the Dunnett type multiple comparison test was applied. Mating and fertility rates were analyzed by Fisher's exact test. The incidence of neoplastic lesions was statistically analyzed by Peto's test, in addition to Fisher's exact test. A significance level of $p < 0.05$ was used for all statistical analyses.

3. Results

3.1. Body weight and sexual maturation

There were no significant differences in body weights between the control and DES-treated groups for males and females, except for significant increases in the 0.05 μg/kg group of males at 62 weeks of age and in the 5 μg/kg group of females at 46 weeks of age (Fig. 1). The completion day (mean ± S.D.) of vaginal opening was significantly earlier in the 5 μg/kg group (29.8 ± 2.2) than in the control group (32.9 ± 1.7) (Fig. 2A). Furthermore, a cleft phallus was observed in almost all of the females in the 5 μg/kg group when the vaginal opening was checked. There was no significant difference between groups in terms of the completion day of preputial separation (Fig. 2B).

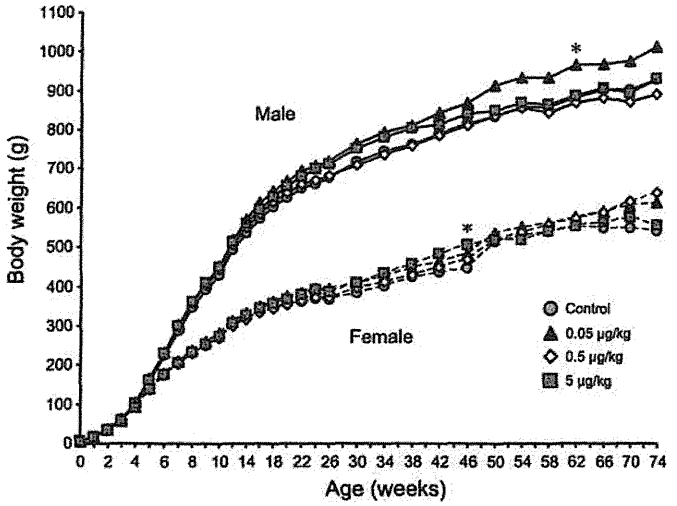

Fig. 1. Effects of neonatal DES exposure on body weight changes in male and female SD rats. Values are expressed as means. Asterisks indicate significant differences between the control and DES-treated groups. Statistical significance: $^{*}p < 0.05$.

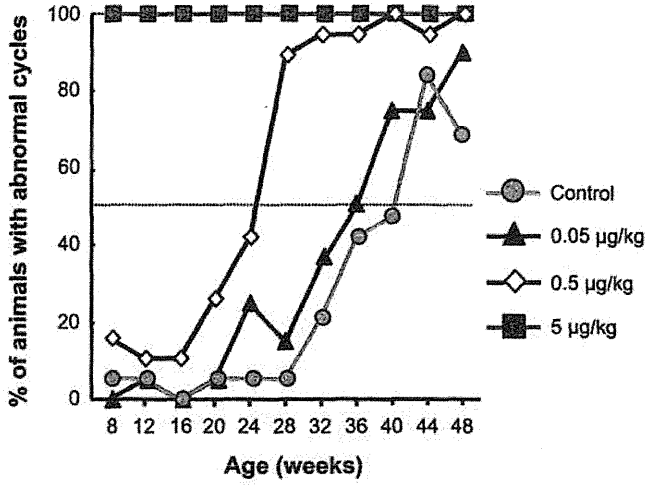

Fig. 3. Effects of neonatal DES exposure on estrous cycles in female SD rats. Values are expressed as percent of females with abnormal estrous cycles.

3.2. Estrous cycle

The results of estrous cycles observed from 8 to 49 weeks of age are shown in Supplementary file 1. The percentage of females with abnormal estrous cycles is shown in Fig. 3. Abnormal estrous cycles were observed in all animals in the 5 μg/kg group throughout the study. Although less than 20% of the animals in the 0.5 μg/kg group showed abnormal estrous cycles from 8 to 16 weeks of age, abnormal estrous cycles were observed in 40% from 24 weeks of age and in more than 90% at 28 weeks and after. The females in the 0.05 μg/kg group showed normal estrous cycles until 28 weeks of age, similar to the control group. Most of the abnormal estrous cycles observed at an early stage in the 5 μg/kg group were extended estrus, while most of the abnormal estrous cycles, which increased with age, in the 0.5 and 0.05 μg/kg groups were long estrous cycles. Mean estrous cycle lengths for groups receiving 0, 0.05 and 0.5 μg/kg DES were 4.1, 4.0 and 4.3 days during 12–13 weeks of age, 4.1, 4.1 and 5.1 days during 20–21 weeks of age, and 4.1, 4.2 and 7.0 days during 32–33 weeks of age, respectively.

3.3. Mating and fertility index

The results of mating performance from 12 to 34 weeks of age for females, and from 12 to 68 weeks of age for males, are shown in Fig. 4A and B, respectively. The mating rate of females in the 0.05 μg/kg group was significantly reduced to 60% at 23 weeks of age, and that in the 0.5 μg/kg group was significantly reduced to 20% at 34 weeks of age. The conception rate of females in the 5 μg/kg group was 0% at 12 weeks of age, despite the high mating index. That in the 0.5 μg/kg group was 33.3% at 23 weeks of age. There were no significant differences between the control and DES-treated groups in terms of mating and fertility rates of males from 12 to 68 weeks of age.

3.4. Parturition and nursing

The results of early life exposure to DES on gestation length, number of pups, pup weight and viability of pups are shown in Table 1. No DES-related effects were observed for any parameters at the first parturition, except the 5 μg/kg group. However, gestation length was significantly extended in the 0.05 μg/kg group, and litter

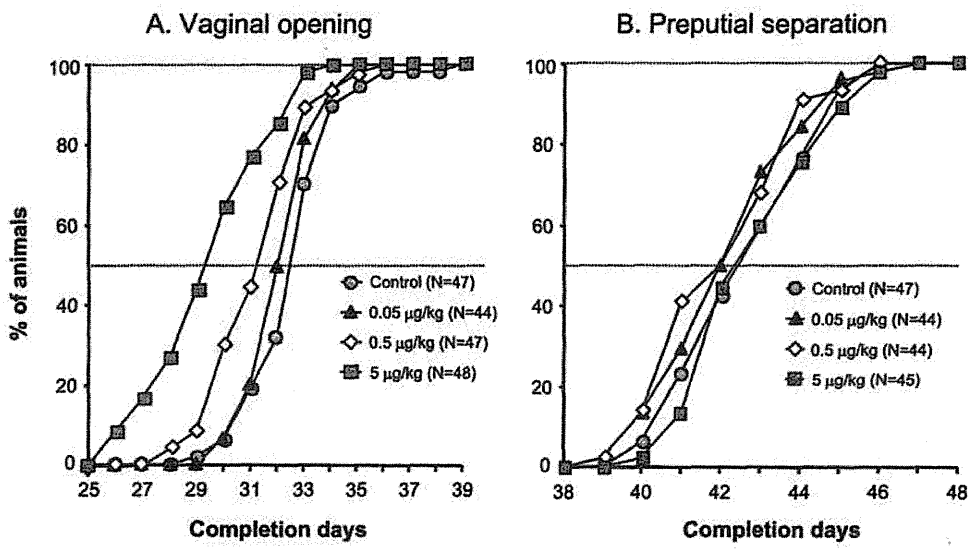

Fig. 2. Effects of neonatal DES exposure on completion days of vaginal opening (A) and preputial separation (B) in SD rats. Values are expressed as percent of animals with completion. The mean completion day of vaginal opening was significantly earlier in the 5 μg/kg group than in the control group.

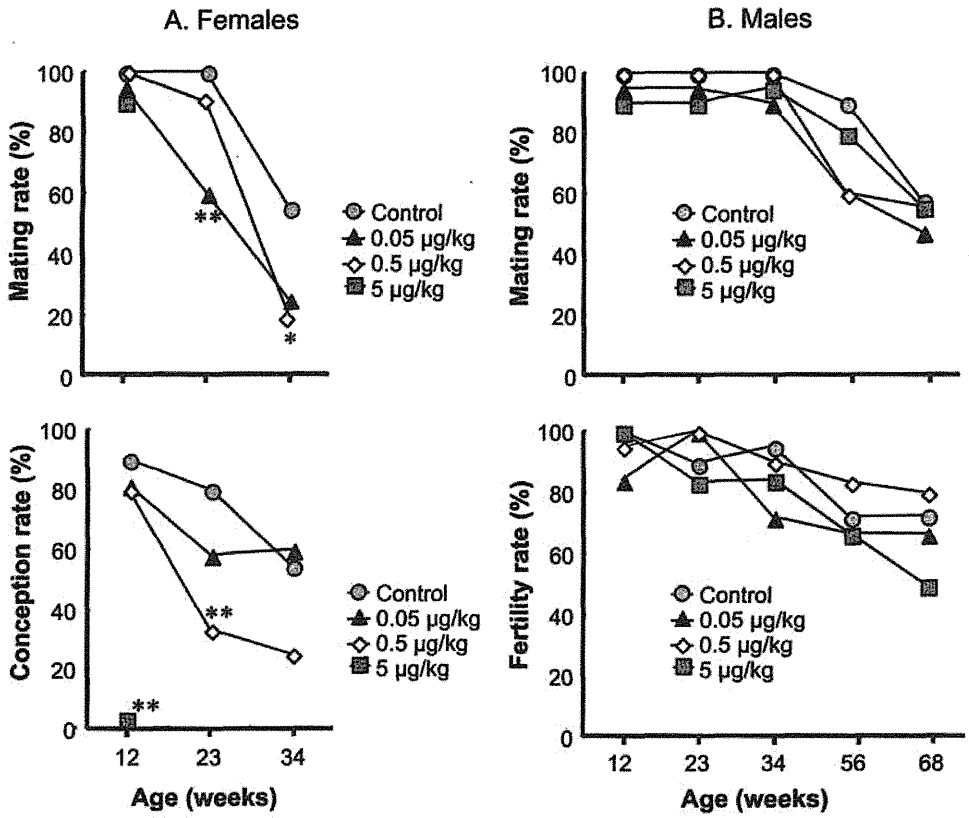

Fig. 4. Effects of neonatal DES exposure on mating performance in female (A) and male (B) SD rats. Data were combined for the first and second mating at each age. Values are expressed as percent of animals. Asterisks indicate significant differences between the control and DES-treated groups. Statistical significance: $^{*}p < 0.05$; $^{**}p < 0.01$.

Table 1
Effects of neonatal DES exposure on reproduction in rats.

	DES (μg/kg)			
	0	0.05	0.5	5
At the 1st parturition				
No. of pregnant females	18	13	16	0
Gestation length in days	22.1 ± 0.3	22.2 ± 0.7	22.1 ± 0.5	
No. of pups born	13.9 ± 3.4	12.9 ± 3.9	14.1 ± 3.6	
Pup weight (g) at birth				
Males	6.9 ± 0.3	6.8 ± 0.6	6.7 ± 0.7	
Females	6.5 ± 0.3	6.4 ± 0.6	6.3 ± 0.7	
Viability of pups on PD4	99.7 ± 1.5	98.5 ± 3.0	99.0 ± 3.0	
At the 2nd parturition				
No. of pregnant females	16	7	8	0
Gestation length in days	22.3 ± 0.5	22.9 ± 0.4*	22.5 ± 0.5	
No. of pups born	12.8 ± 3.9	12.7 ± 3.7	7.6 ± 5.8*	
Pup weight (g) at birth				
Males	7.2 ± 0.8	6.9 ± 0.7	7.4 ± 0.9	
Females	6.7 ± 0.7	6.8 ± 0.7	6.6 ± 0.6	
Viability of pups on PD 4	93.3 ± 25.1	100.0 ± 0.0	100.0 ± 0.0	
At the 3rd parturition				
No. of pregnant females	6	3	1	0
Gestation length in days	22.4 ± 0.5	22.7 ± 0.6	22.0	
No. of pups born	12.0 ± 4.3	11.0 ± 6.6	14.0	
Pup weight (g) at birth				
Males	7.1 ± 0.5	7.2 ± 1.2	6.8	
Females	6.6 ± 0.5	6.8 ± 1.4	6.7	
Viability of pups on PD4	96.5 ± 5.9	100.0 ± 0.0	100.0	

Values are expressed as mean ± S.D.
* Significant differences between control (0 μg/kg) and DES-treated groups. Statistical significance: $p < 0.05$.

size was significantly reduced in the 0.5 μg/kg group at the second parturition.

3.5. Sperm counts and organ weights of males

Although the sperm counts and organ weights were investigated at 26 and 52 weeks of age, no influences of DES on sperm counts or organ weights were observed in the DES-treated males (data not shown).

3.6. Shuttle-box avoidance test of males and females

Although the shuttle-box avoidance test in both sexes was investigated at 24 and 48 weeks of age, no influence of DES on avoidance learning was observed in any group (data not shown).

3.7. Immune response to SRBC of males

The results of early life exposure to DES on the immune response to SRBC in male rats are shown in Fig. 5. At 26 weeks of age, the anti-SRBC IgM level was significantly decreased in all the DES-treated groups. There was, however, no significant effect of DES on anti-SRBC IgM level at 52 weeks of age.

3.8. Endocrine organ weights of females at 54 weeks of age

The results of early life exposure to DES on endocrine organ weights in females at 54 weeks of age are shown in Fig. 6. The weight of the pituitary was significantly increased in all the treated groups. The weight of the adrenal glands was significantly increased in the 0.5 and 5 μg/kg groups. In addition, the weight of the ovaries was significantly decreased in the 0.5 and 5 μg/kg groups.

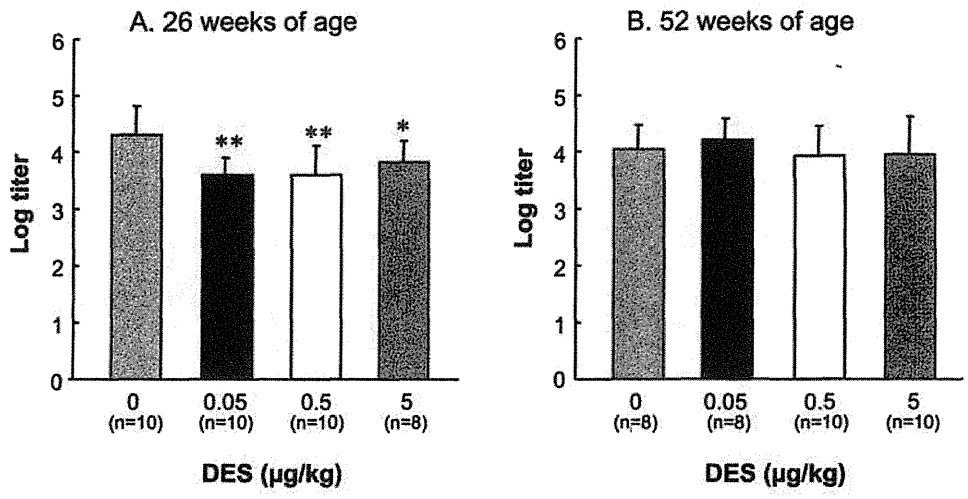

Fig. 5. Effects of neonatal DES exposure on antibody response to SRBC in male SD rats at 26 weeks (A) and 52 weeks (B) of age. Values are expressed as mean ± S.D. Asterisks indicate significant differences between control (0 μg/kg) and DES-treated groups. Statistical significance: $^{*}p < 0.05$; $^{**}p < 0.01$.

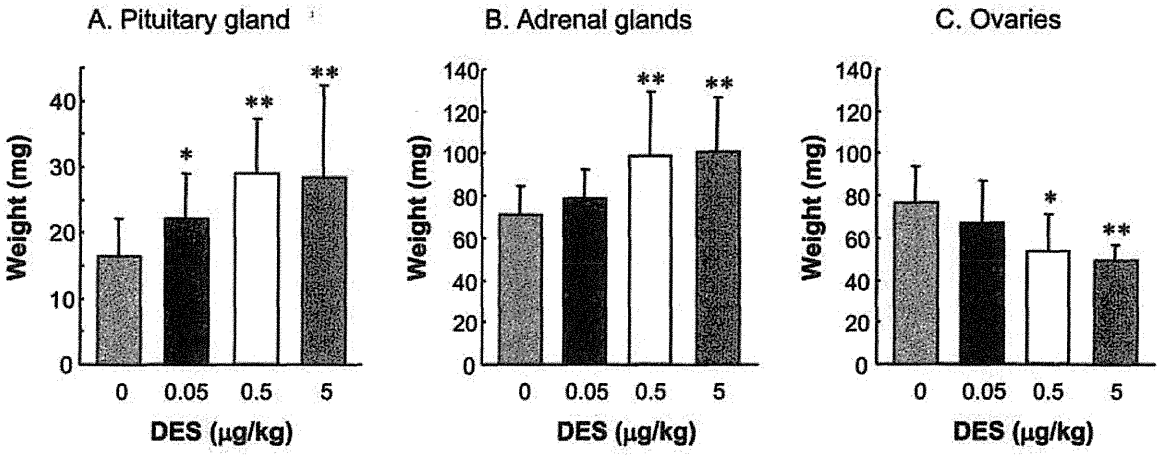

Fig. 6. Effects of neonatal DES exposure on pituitary gland (A), adrenal glands (B) and ovaries (C) in female SD rats at 54 weeks of age. Values are expressed as mean ± S.D. Asterisks indicate significant differences between control (0 μg/kg) and DES-treated groups. Statistical significance: $^{*}p < 0.05$; $^{**}p < 0.01$.

Furthermore, galactoceles were observed at necropsy in the mammary glands in 0/16, 2/20, 3/18 and 9/19 females from the control, 0.05, 0.5 and 5 μg/kg groups, respectively. There were no significant differences between the control and DES-treated groups in terms of the plasma concentrations of prolactin, LH, FSH, T3 and T4 in the females without pituitary enlargement (data not shown).

3.9. Ovulation test

In the ovulation test at 54 weeks of age carried out by hCG stimulation, evidence of ovulation was detected in 7/8, 6/10, 9/10 and 8/10 females of the control, 0.05, 0.5 and 5 μg/kg groups, respectively. There were no significant differences in the number of ovulations between the control and DES-treated groups.

3.10. Survival curve

The survival curve of the male rats did not differ between the control and DES-treated groups (Fig. 7A). However, the median of the survival days of the females in the 5 μg/kg group was shortened by 17 weeks compared with that of the control group (Fig. 7B).

3.11. Tumorigenesis

A summary of preneoplastic and neoplastic lesions in males is shown in Supplementary file 2A. Necropsy of males, including both

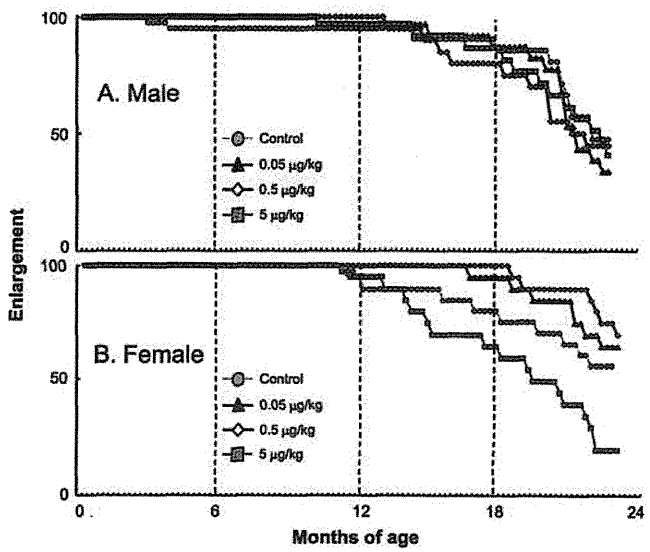

Fig. 7. Effects of neonatal DES exposure on survival curve in male (A) and female (B) SD rats. Values are expressed as percent of live animals. The median of the survival days of the females in the 5 μg/kg group was shortened by 17 weeks compared with that of the control group.

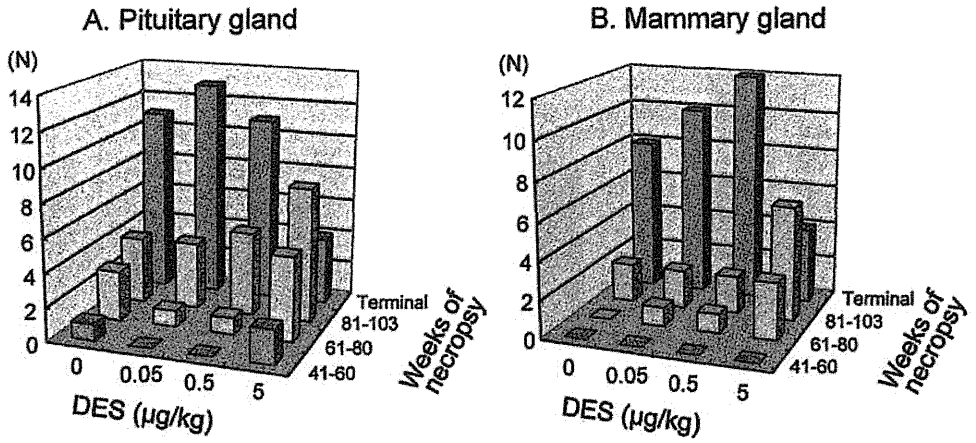

Fig. 8. Effects of neonatal DES exposure on tumorigenesis in pituitary (A) and mammary (B) glands of female SD rats. Values are expressed as number of animals with preneoplastic and neoplastic lesions at necropsy. Terminal indicates the necropsy of live animals at 101–102 weeks of age for males, and at 103 or 104 weeks of age for females.

those with intermediate deaths and those terminated at 101 or 102 weeks, revealed preneoplastic and neoplastic lesions in the adrenal glands in 8/20, 13/20, 9/20 and 8/20 males and in the pituitary gland in 16/20, 14/19, 15/20 and 16/20 males of the control, 0.05, 0.5 and 5 μg/kg groups, respectively. There were, however, no significant differences in the incidence of neoplastic lesions in either organ between the control and DES-treated groups.

A summary of preneoplastic and neoplastic lesions in females is shown in Supplementary file 2B. Necropsy of females, including both those with intermediate deaths and those with scheduled terminations at 103 or 104 weeks, revealed preneoplastic and neoplastic lesions in the pituitary gland in 19/20, 18/20, 17/20 and 19/20 females, and in the mammary gland in 10/19, 14/20, 16/20 and 13/20 females of the control, 0.05, 0.5 and 5 μg/kg groups, respectively. Although no significant differences in the incidence of preneoplastic and neoplastic lesions in either organ were observed between the control and DES-treated groups, Peto analysis showed a significant dose-dependent relationship in the incidence, stratified by survival days, of pituitary tumors ($p < 0.001$) and mammary tumors ($p < 0.05$) (Fig. 8). In addition, acinar dilation and galactoceles were observed in the mammary glands in 0/20, 2/20, 8/20 and 5/20 females of the control, 0.05, 0.5 and 5 μg/kg groups, respectively.

4. Discussion

An extended estrous cycle was observed in females in the 5 μg/kg group at the start of sampling. This abnormal estrous cycle was expected. It may have been caused by the suppression of the gonadotrophic hormone induced by a high dose of DES. Interestingly, the number of females with abnormal estrous cycle increased sharply in the 0.5 μg/kg group from 24 to 28 weeks of age. This finding may be undetectable in a regular reproductive toxicity study. It was demonstrated that the abnormalities in the estrous cycle occurred earlier than in the control group upon treatment with TCDD [18], bisphenol A [19] and genistein [20]. These reports and our present result suggest the importance of observing the estrous cycle over a long period in research on EDCs. Extended estrus was mainly observed in the 5 μg/kg group, but a long estrous cycle was mainly observed in the 0.5 μg/kg group. The abnormal cycles observed in the 0.5 μg/kg group were similar to those observed in the 0.05 μg/kg and control groups at the later stage of smear sampling, corresponding to the menopausal stage. It may be that onset of menopause occurred earlier in the females in the 0.5 μg/kg group than in the control group.

A conception index of 0%, despite a high mating rate, was observed in the 5 μg/kg female group. This suggests that females in the 5 μg/kg group exhibited an anovulatory condition due to dysregulation of the hypothalamic–pituitary–gonadal (HPG) axis after vaginal opening. This may have been caused by the high dose of DES. In the second mating, at 23 weeks of age, the conception rate was decreased in the 0.5 μg/kg female group. The number of newborns in this group decreased at the second parturition. These changes are also in agreement with the disruption of the estrous cycle, that is, the onset of menopause occurring earlier in this group than in the control group. Cabaton et al. [21] found a significant decrease in the cumulative number of mouse pups by perinatal exposure to bisphenol A. Standard reproductive toxicity studies on EDCs routinely analyze the effects on the first pregnancy alone, with no follow-up of potential effects in subsequent pregnancies. As a result, they fail, for example, to identify changes in reproductive functions that manifest during subsequent pregnancies, leading to an incomplete evaluation of the effects of EDCs. This study suggests that the mating performance and litter size must be checked at a later stage in the test protocol for evaluation of EDCs.

Cleft phallus was observed in all females in the 5 μg/kg DES group. Earlier maturation of vaginal opening was also observed in the 5 μg/kg DES group. Vaginal opening [22–24] and cleft phallus [5] are considered to be good indexes for endocrine active chemicals in vivo as well as the uterotrophic assay. These parameters are also very important because they can be detected at an earlier stage than other measurements in the life span test. However, it is not understood whether earlier maturation and cleft phallus are adverse effects of EDCs on offspring. Therefore, a follow-up study, such as the life span test, is necessary to confirm this. On the other hand, no effect was observed in the preputial separation of male offspring. Yoshimura et al. [25] reported that the influence of neonatal DES exposure on preputial separation was observed in SD rats treated with 100 μg/kg or more. This suggests that the dosage in this study, up to 5 μg/kg of DES, was not sufficient to induce abnormalities in male sexual maturation.

In males, there was no influence of DES up to 5 μg/kg on mating and fertility rates from 12 to 68 weeks of age. Furthermore, no influences of DES on the sperm counts and weights of reproductive organs at 26 and 52 weeks of age were detected. These results suggest that neonatal exposure to DES does not influence male reproductive abilities up to the later stage of the life span, and that the male offspring were less affected by DES than female offspring. However, the influence of DES on male reproductive organs was reported by some researchers [26–29]. Furthermore, several studies of BPA with effects on male reproductive organs

were summarized by Richter et al. [2], including decreased epididymal weight and daily sperm production [30,31], and increased prostate weight [32]. Therefore, analysis of the sperm counts and weights of reproductive organs of males are necessary in tests for evaluation of EDCs, but it is not understood whether these parameters are adverse effects of EDCs on offspring [33].

No DES-related effects were observed in body weight changes in this study. However, increased body weights were observed in female rats and female mice induced by perinatal exposure to low-dose bisphenol A [19,22,34,35]. Therefore, body weight change may be one of the important measurements to detect EDCs in the test protocol.

As EDCs are considered to induce not only reproductive toxicity but also neurotoxicity and immunotoxicity [2,3], a shuttle-box avoidance test and an immune response test were also examined in this study. However, there were no clear effects related to DES on behavioral or immune responses, except a decrease in anti-SRBC IgM level at 26 weeks of age. The shuttle-box avoidance test is one of the behavioral tests used in developmental neurotoxicity, and it is very susceptible to changes in the hypothalamo–pituitary–adrenal axis [13,36]. The results of this study, however, indicate that the shuttle-box avoidance test is not an effective measurement for determination of EDCs even if it is performed at a later stage in the life span test. Genetic control is considered to be necessary for animal studies of neurobehavioral toxicology because large individual variations are usually observed in the behavior of animals maintained as a closed colony, such as SD rats [37]. Therefore, further study is needed using inbred strains of animals. Interestingly, anti-SRBC IgM level decreased at 26 weeks of age but not at 52 weeks of age in this study. Leposavić et al. [38] showed that the changes in the development of the HPG axis induced by neonatal androgenization may affect the thymus development and intrathymic T-cell maturation. Further study of this immune parameter for determination of EDCs is needed.

In females at 54 weeks of age, weights of pituitary and adrenal glands were dose-dependently increased and galactoceles occurred in the mammary glands in the DES-treated groups. These results suggested the females treated with DES had hyperprolactinemia. However, females without pituitary enlargement in the DES-treated groups showed normal plasma prolactin levels. It was reported that mammary gland differentiation was sensitive to xenoestrogens [39–43]. Fenton [44] demonstrated that early life exposure to hormonally active agents can lead to effects on mammary gland development, impaired lactation and increased susceptibility to cancer. These data and the results in this study suggest that the degree of mammary gland development up to the later stage is an important parameter for evaluation of EDCs.

Decreased weight of ovaries was detected in the 0.5 μg/kg or more groups. Since ovulation by hCG injection was noted in all the DES groups, neonatal DES exposure did not affect ovary function directly, but is presumed to have resulted from the fall in the stimulus from the pituitary, that is, the endocrine-disruptive action of the HPG axis. Low doses of DES did not accelerate sex maturation but accelerated the aging process in estrous cycle, conception rate and ovulation in female rats. These changes were presumed to have resulted from the disruption of the HPG axis and had indirect effects on the ovary function.

The survival duration of the females treated with 5 μg/kg DES was shortened, and pituitary and mammary tumors occurred earlier in this group. Pituitary and mammary tumors are known to occur spontaneously in SD rats [45,46]. These data suggest that the spontaneous endocrine tumors occurred earlier with neonatal DES exposure. Although no DES-related effects were found in survival days of the 0.05 and 0.5 μg/kg females, acinar dilation and galactoceles were found in the mammary glands in these groups. Therefore, adverse effects of DES may also have occurred in the 0.05 and 0.5 μg/kg groups.

Extended one-generation reproductive toxicity studies were proposed by the OECD instead of multi-generation studies as a definitive test protocol for EDCs. However, the observation period of this protocol was designed to be up to 14 weeks of age in F1 offspring. Findings in the one-lifespan test in this study revealed that reproductive dysfunction in females caused by EDCs possibly occurred at 23–24 weeks of age in the offspring, and that these dysfunctions might be related to the earlier occurrence of endocrine tumors and shortened survival period. This suggests that a prolonged observation period, at least to 23–24 weeks of age, is necessary to determine EDCs reliably in the protocol.

Although SD rats are generally used in toxicological studies, it is known that it is easier to disturb the estrous cycle at middle age in SD rats and that this strain is subject to the spontaneous occurrence of tumors, such as pituitary and mammary tumors. Therefore, we tried another life span test using C57BL/6J mice, and both the disturbed estrous cycle and the occurrence of pituitary tumor were found in the female mice with early life exposure to low-dose DES (Ohta et al., unpublished data).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2012.04.014.

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Original Article

Ovariectomized mouse uterotrophic assay of 36 chemicals

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ABSTRACT — The concern over endocrine disruptors prompted international establishment of a strategic framework for the identification of the estrogenic compounds. OECD has launched the Conceptual Framework tool box containing various screening and testing methods including the uterotrophic assay. The (anti)estrogenicity of 36 chemicals suspected to be estrogen-receptor interactive by *in silico* and/or *in vitro* screening in the Extended Scheme for Endocrine Disruptor Screening and Testing of the Ministry of Health, Labour and Welfare, Japan, were monitored by the uterotrophic assay using C57BL/6J ovariectomized adult female mice after a 7-day exposure by oral gavage (po) and subcutaneous injection (sc). Ethynyl estradiol was used as reference for agonist and antagonist detection. In addition, Bisphenol A (sc) and Genistein (po) were tested for the comparison to rat assays. Among the 36, 2-[Bis(4-hydroxyphenyl)methyl]benzylalcohol, 2,2',4,4'-Tetrahydroxybenzophenone, 2,4-Dihydroxybenzophenone, 3,3',5-Triiodothyroacetic acid, New fuchsin and alpha-Naphtholbenzein, showed both estrogenic agonistic and antagonistic activities; first two showed U-shaped dose-response in antagonistic studies. N,N-Diphenyl-p-phenylenediamine, 2,2'-Dihydroxy-4,4'-dimethoxybenzophenone, n-Butyl 4-hydroxybenzoate, and Reserpine were agonistic by sc. Benzo [a] pyrene, Benz [a] anthracene, Dibenz [a,h] anthracene, 2-(2H-Benzotriazol-2-yl)-4,6-di(t-pentyl)phenol, Rosemarinic acid, meta-Thymol, 6-Gingerol, Colchicine, Malachite green base, Fenbuconazole, and Lead acetate were antagonistic. The rest, i.e. n-Heptyl 4-hydroxybenzoate, Tetrazolium violet, Pravastatin sodium salt, Physostigmine, salicylate (1:1), Nordihydroguaiaretic acid, o-Cresolphthalein, 1,3-Dinitrobenzene, C.I. Pigment orange, Tetrabromobisphenol-A, 2-Hydroxy-4-methoxybenzophenone, Ethylparaben, Propyl p-hydroxybenzoate, Kaempferol, 2-(2-Benzotriazolyl)-p-cresol and Phenolphthalein were negative for both effects. Taking together with *in silico/ in vitro* screening, the result suggested that the ovariectomized mouse uterotrophic bioassay has sufficient performance comparable to rat for the screening of (anti)estrogenicity of various chemicals.

Key words: Mouse, Uterus, Uterotrophic assay, Endocrine disruptors

INTRODUCTION

The Organisation for Economic Cooperation and Development (OECD) has proposed the uterotrophic assay as an *in vivo* screening test to detect the estrogenic properties of potentially endocrine disrupting chemicals (EDCs) (OECD, 1998) (Kanno *et al.*, 2001, 2003a). Although test validation was performed in the rat, the mouse was considered to be equally usable. Here, we performed the mouse uterotrophic assay on 36 chemicals not included in the OECD validation study but suggested to be estrogenic by the *in silico* estrogen receptor alpha (ERα) docking model (Itai *et al.*, unpublished data) (Mizutani *et al.*, 2006), the ERα reporter assay (unpublished data) (Yamasaki *et al.*, 2003b) and/or plasmon surface resonance (Asano *et al.*, 2004) (see Supplement Document and Tables). These tests are the elements of the Extended Scheme for Endocrine Disruptor Screening and Testing, MHLW, Japan (Chemical Safety Office and Evaluation and Licensing Division). Bisphenol A (BPA), genistein, daidzein and genistin are included as known estrogenic reference chemicals and for possible comparison with the rat data.

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MATERIALS AND METHODS

Chemicals and animals

The tested chemicals are listed in Table 1 with abbreviations. The uterotrophic assays were conducted at Hatano Research Institute, Food and Drug Safety Center. We selected the C57BL/6J mouse because it was reported to respond well to estrogens (Spearow *et al.*, 1999; Ashby *et al.*, 2003) and is also used widely as a background strain for production of genetically modified mice. Ovariectomized (OVX) mice (Japan SLC, Inc., Shizuoka, Japan) at 6 weeks of age were obtained from the Institute of Animal Reproduction (Ibaraki, Japan). Vaginal smears were checked for 4 days before the start of the test. At age

Table 1. Test chemicals

No.	Chemical	Abbreviation	CAS No.	Source	Purity (%)	Vehicle
ref	Ethynyl estradiol	EE	57-63-6	Wako Pure Chemical	99.0	Corn oil
a	Bisphenol A	BPA	80-05-7	Wako Pure Chemical	99.0	Corn oil
b	Genistein	Genistein	446-72-0	Wako Pure Chemical	>98	Corn oil
c	Genistin	Genistin	529-59-9	Wako Pure Chemical	>98	Corn oil
d	Daidzein	Daidzein	486-66-8	Wako Pure Chemical	>98	Corn oil
1	2-[Bis(4-hydroxyphenyl)methyl]benzylalcohol	BHPMBA	81-92-5	Tokyo Kasei Kogyo Co.	98.3	Corn oil
2	2,2',4,4'-Tetrahydroxybenzophenone	THBP	131-55-5	Wako Pure Chemical	>95	Corn oil
3	2,4-Dihydroxybenzophenone	DHBP	131-56-6	Wako Pure Chemical	98	Corn oil
4	3,3',5-Triiodothyroacetic acid	Tiratricol	51-24-1	Sigma-Aldrich Japan	99.4	Corn oil
5	New fuchsin	NF	3248-91-7	ICN Biomedicals, Inc	Unknown	Distilled Water
6	alpha-Naphtholbenzein	ANB	6948-88-5	ICN Biomedicals Inc.	Unknown	Corn oil
7	N,N-Diphenyl-p-phenylenediamine	PDD	2350-01-8	Wako Pure Chemical	>90	Corn oil
8	2,2'-Dihydroxy-4,4'-dimethoxybenzophenone	DDB	131-54-4	Wako Pure Chemical	>95	Corn oil
9	n-Butyl 4-hydroxybenzoate	BHB	94-26-8	Wako Pure Chemical	>98	Corn oil
10	Reserpine	Res	50-55-5	Wako Pure Chemical	>98	Distilled Water
11	Benzo [a] pyrene	B[a]P	50-32-8	Wako Pure Chemical	>98	Corn oil
12	Benz [a] anthracene	B[a]A	56-55-3	Wako Pure Chemical	>99	Corn oil
13	Dibenz [a,h] anthracene	DB[a,h]A	53-70-3	Wako Pure Chemical	>90	Corn oil
14	2-(2H-Benzotriazol-2-yl)-4,6-di(t-pentyl)phenol	BTPP	25973-55-1	Wako Pure Chemical	>97	Corn oil
15	Rosmarinic acid	RMA	20283-92-5	Sigma-Aldrich Co.	99.9	Corn oil
16	meta-Thymol	MT	3228-03-3	Sigma-Aldrich Co.	99.4	Corn oil
17	6-Gingerol	6-gin	23513-14-6	Wako Pure Chemical	>98	Corn oil
18	Colchicine	Col	64-86-8	Wako Pure Chemical Ind.	>95	Distilled Water
19	Malachite green base	MGB	510-13-4	Sigma-Aldrich Co.	90	Distilled Water
20	Fenbuconazole	FBZ	114369-43-6	GL Sciences Inc.	99	Corn oil
21	Lead acetate	LA	6080-56-4	Wako Pure Chemical	>99	Distilled Water
22	n-Heptyl 4-hydroxybenzoate	HHB	1085-12-7	Tokyo Kasei Kogyo Co	99	Corn oil
23	Tetrazolium violet	TZV	1719-71-7	Sigma-Aldrich Co.	>99	Corn oil
24	Pravastatin sodium salt	Pra	81131-70-6	Wako Pure Chemical	99.4	Distilled Water
25	Physostigmine, salicylate (1:1)	PHS	57-64-7	Sigma-Aldrich Co.	99.5	Distilled Water
26	Nordihydroguaiaretic acid	NDGA	500-38-9	ICN Biomedicals Inc.	100	Corn oil
27	o-Cresolphthalein	CP	596-27-0	Wako Pure Chemical	Unknown	Corn oil
28	1,3-Dinitrobenzene	DNB	99-65-0	Wako Pure Chemical	99	Corn oil
29	C.I.Pigment orange	PO	12236-62-3	NIHS	Unknown	Corn oil
30	Tetrabromobisphenol-A	TBBPA	79-94-7	Wako Pure Chemical	>95	Corn oil
31	2-Hydroxy-4-methoxybenzophenone	HMB	131-57-7	Wako Pure Chemical	98	Corn oil
32	Ethylparaben	EP	120-47-8	Wako Pure Chemical	>99	Corn oil
33	Propyl p-hydroxybenzoate	PHB	94-13-3	Wako Pure Chemical	>95	Corn oil
34	Kaempferol	K	520-18-3	Tokyo Kasei Kogyo Co	97.7	Corn oil
35	2-(2-Benzotriazolyl)-p-cresol	BTC	2440-22-4	Wako Pure Chemical	>97	Corn oil
36	Phenolphthalein	PP	77-09-8	Sigma-Aldrich Co.	99.3	Corn oil

8 weeks, non-estrus mice were selected and housed six animals per cage, at room temperature of 23 ± 2°C, relative humidity between 40 and 75%, and a 12-hr light/dark cycle. Feed (CE-2, CLEA Japan, Inc., Tokyo, Japan) and tap water were provided *ad libitum*. The estrogenicity of the feed was considered to be low enough for the performance of uterotrophic assay (Kato *et al.*, 2004). When the phytoestrogens were tested, phytoestrogen-low diet (PLD, Oriental Yeast Co., Tokyo, Japan) (Kanno *et al.*, 2002; Kato *et al.*, 2004) was provided. These animal studies were performed in accordance with the OECD Guidance Document on animal use (OECD, 2000) and the Guidelines for Animal Experiments of Hatano Research Institute, Food and Drug Safety Center.

Protocol

The protocol used in this study was described previously (Kanno *et al.*, 2001) and in compliance with the OECD Test Guideline No.440 (OECD, 2007). Briefly, OVX mice at age 8 weeks were randomly assigned to 11 groups (Table 2) of six mice each with similar average body weight. Treatment was by subcutaneous injection (sc) or oral gavage (po) at 24-hr intervals for 7 consecutive days (Fig. 1). 17α-ethynyl estradiol (EE) was used as

Table 2. Study design

Group	Gr. No.	Agonist detection	Gr. No.	Antagonist detection
Negative control	1	Vehicle	7	Vehicle plus EE [b]
Low dose	2	Test substance	8	Test substance plus EE [b]
Medium low dose	3	Test substance	9	Test substance plus EE [b]
Medium high dose	4	Test substance	10	Test substance plus EE [b]
High dose	5	Test substance	11	Test substance plus EE [b]
Positive control	6	EE [a]	/ [c]	/ [c]

EE: Ethynyl estradiol

[a] 6 μg/kg/day for oral route and 0.2 μg/kg/day for subcutaneous route, respectively

[b] subcutaneous injection of 0.6 μg/kg/day for both oral and subcutaneous route, 15 min after test substance

[c] not performed

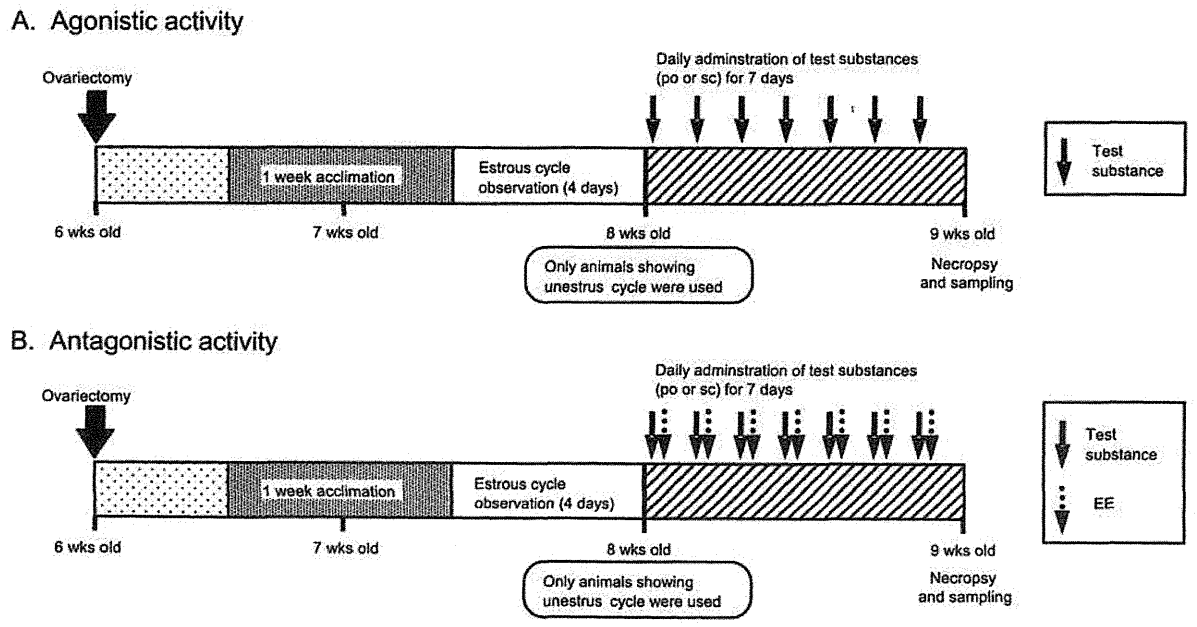

Fig. 1. Protocol of mouse uterotrophic assay used in this experiment. Cf. Table 2 for groups. (EE: ethynyl estradiol).

a reference control. Prior to testing, a dose-finding study was performed to find the maximal tolerated dose (MTD) which was employed as the highest dose.

For the detection of agonistic activity, vehicle, four different doses at a half-log ratio, and EE as a positive control, were tested (Table 2, Groups 1 ~ 6). The amount of EE for the po study was 6 μg/kg po, and for the sc study was 0.2 μg/kg sc. These doses are known to significantly evoke 15 to 20% of the maximal uterotrophic response of EE (Ohta *et al.*, 2004) (See Supplement Fig. 1).

For the detection of antagonistic activity, vehicle and the same four doses used for agonistic detection were administered with a reference dose of EE (Groups 7 ~ 11). For both sc and po studies 0.6 μg/kg EE was given by sc fifteen minutes after the administration of vehicle or the test compound. This amount of EE given by sc is known to evoke 60 to 70% of the maximal uterotrophic response by EE (Ohta *et al.*, 2004) (See Supplement Fig. 1). The performance of the EE groups was used as a critical success factor of each study.

Vehicle, test substance preparation, and dosing

EE was dissolved in a minimal amount of ethanol (Wako Pure Chemical, Osaka, Japan) and then serially diluted with corn oil (Nacalai Tesque, Inc., Kyoto, Japan) to desired concentrations. Test substances were suspended or dissolved in the vehicle to prepare the dosing formulation of the desired concentrations (See Table 1). The top doses of each chemical are shown in Table 3 (See Supplement Table 2 for all doses). The amount administered was adjusted to the body weight (bw) on the day of treatment. The dosing volume was 2~4 ml/kg for sc and 5 ml/kg for po. The animals were observed daily for clinical signs with the body weights recorded.

Necropsy and uterine weight measurement

Twenty-four hours after the last treatment, the animals were euthanized by cervical dislocation. The uterus was carefully dissected at the level of the vaginal fornix, trimmed of fascia and fat under a stereomicroscope and weighed including the luminal fluid (wet weight), and then pierced, gently blotted on moistened filter paper, and weighed (blotted weight).

Statistical analysis

Statistical analysis was performed on the less variable blotted uterine weight (UW) (Kanno *et al.*, 2001). For agonist detection, UW and body weight between the vehicle and each treated groups (Group 1 versus Groups 2~6) and for antagonist detection, between the EE group and each treated groups (Group 7 versus Groups 8~11) were assessed for statistical significance by one-way analysis of variance (ANOVA). When significant, the Dunnett's Multiple Comparison Test was used to compare each treatment group with the control. p-value of less than 0.05 were considered significant.

Uterotrophic parameters

Because most of the studies lacked enough response range for reliable bench-mark dose fitting, the agonistic and antagonistic potential of each chemical were presented, alternatively, by two types of indicators, i.e. the lowest effect level (LOEL) and AGo_{10} or AN_{50}. The lowest dose that induced significant change in uterine weight was defined as LOEL. AGo_{10} was defined as an interpolated dose that corresponded to 10% of the maximal agonistic uterotrophic effect. For this calculation, the uterotrophic response of positive control EE Group (Group 6) was considered as 20% of the maximal increase above the concurrent vehicle control (See Supplement Fig. 2). AN_{50} was defined as an interpolated dose that suppresses the uterotrophic effect of the reference EE to 50% of the maximal uterotrophic response. For this calculation, the reference EE Group (Group 7) was considered as 70% of the maximal uterotrophic response above concurrent vehicle control (See Supplement Fig. 2).

To compare agonistic and antagonistic potency of a chemical, ratio of LOELs, designated as AGo/AN, was used. A chemical with Ago/AN = 1 is considered equipotent in terms of agonistic/antagonistic potency. Likewise, the ratio of LOELs of po and sc, i.e. po/sc ratio is used to indicate the preferential exposure route.

RESULTS

A summary of the result is shown in Table 3 (See. Supplement Table 2 for detail). All studies showed significant responses to EE treatments for detection of both agonistic and antagonistic effects.

Known estrogenic chemicals

BPA (Fig. 2A, B) by sc showed both agonistic and antagonistic effects at a similar dose response range, i.e. slight change at 30 mg/kg and significant effect at 100 and 300 mg/kg in a dose-dependent manner. The highest dose induced a 2-fold increase in uterine weight, which was two-third of that induced by 0.2 μg/kg EE sc. The same amount of BPA interfered with activity of 0.6 μg/kg EE sc by 70% reduction, i.e. from 10-fold increase down to 3-fold increase.

Table 3. Summary of results and related information

No.	Chemical	po	sc	po			sc			po		sc	
		Result Summary	Result Summary	Agonistic LOEL[d] (mg/kg/day)	Antagonistic LOEL (mg/kg/day)	Top Dose (mg/kg)	Agonistic LOEL (mg/kg/day)	Antagonistic LOEL (mg/kg/day)	Top Dose (mg/kg)	AGo_{10}[g] (mg/kg/day)	AN_{50}[h] (mg/kg/day)	AGo_{10} (mg/kg/day)	AN_{50} (mg/kg/day)
a	BPA	/	AGo & AN[a]	/	/	/	100	100	300	/	/	226.8	49.6
b	Genistein	AGo & AN	/	200	60	600	/	/	/	220.2	63.5	/	/
c	Genistin	AGo & AN	/	300	100	1000	/	/	/	387.5	135.2	/	/
d	Daidzein	AGo[b]	/	600	-	600	/	/	/	-	-	/	/
1	BHPMBA	AGo & AN	AGo & AN	100	100 (U-shaped)	1000	30	30 (U-shaped)	1000	130.5	70.0	10.4	17.6 (U-shaped)
2	THBP	AGo & AN	AGo & AN	1000	1000	1000	300	100 (U-shaped)	1000	588.4	473.9	315.1	64.0 (U-shaped)
3	DHBP	AGo	AGo & AN	1000	-	1000	300	300	1000	-	-	409.9	243.2
4	Tiratricol	AGo	AGo & AN	1000	-	1000	1000	1000	1000	-	-	994.1	536.7
5	NF	AGo & AN	AGo	300	300	300	100	-[e]	100[f]	-	167.3	-	61.2
6	ANB	AN[c]	AGo & AN	-	300	300	300	(30)[i]	300	-	-	-	25.1
7	PDD	-	AGo	-	-	1000	-	1000	1000	-	-	-	-
8	DDB	-	AGo	-	-	1000	-	1000	1000	-	-	-	-
9	BHB	-	AGo	-	-	1000	1000	-	1000	-	-	-	-
10	Res	-	AGo	-	-	3	3	-	3	-	-	-	-
11	B[a]P	AN	AN	-	10	300	-	100	300	-	7.7	-	80.6
12	B[a]A	-	AN	-	-	300	-	100	300	-	-	-	67.2
13	DB[a,h]A	-	AN	-	-	300	-	10	300	-	-	-	9.0
14	BTPP	AN	AN	-	30	1000	-	30	1000	-	24.4	-	22.0
15	RMA	-	AN	-	-	1000	-	300	1000	-	-	-	188.0
16	MT	-	AN	-	-	1000	-	1000	1000	-	-	-	734.8
17	6-gin	-	AN	-	-	300	-	100	300	-	-	-	64.2
18	Col	-	AN	-	-	3	-	0.3	1	-	-	-	-
19	MGB	AN	-	-	100	100	-	-	300	-	77.1	-	-
20	FBZ	AN	-	-	1000	1000	-	-	1000	-	-	-	-
21	LA	AN	-	-	1000	1000	-	-	1000	-	774.6	-	-
22	HHB	-	-	-	-	1000	-	-	1000	-	-	-	-
23	TZV	-	-	-	-	30	-	-	3	-	-	-	-
24	Pra	-	-	-	-	1000	-	-	1000	-	-	-	-
25	PHS	-	-	-	-	3	-	-	3	-	-	-	-
26	NDGA	-	-	-	-	1000	-	-	1000	-	-	-	-
27	CP	-	-	-	-	1000	-	-	1000	-	-	-	-
28	DNB	-	-	-	-	30	-	-	10	-	-	-	-
29	PO	-	-	-	-	300	-	-	300	-	-	-	-
30	TBBPA	-	-	-	-	1000	-	-	1000	-	-	-	-
31	HMB	-	-	-	-	1000	-	-	1000	-	-	-	-
32	EP	-	-	-	-	1000	-	-	1000	-	-	-	-
33	PHB	-	-	-	-	1000	-	-	1000	-	-	-	-
34	K	-	-	-	-	1000	-	-	1000	-	-	-	-
35	BTC	-	-	-	-	1000	-	-	1000	-	-	-	-
36	PP	-	-	-	-	1000	-	-	1000	-	-	-	-

[a] positive chemical for both agonistic and antagonistic acitivity. [b] positive chemical for agonistic activity. [c] positive chemical for antagonistic activity. [d] lowest effective dose in significantly positive studies for agonsitic/antagonistic acitivity. [e] tendency without significance was present. [f] NF top dose 300 mg/kg was lethal by sc. [g] Interpolated dose to induce 10% uterotrophic effect (statistical significance not considered). [h] Interpolated dose to inhibit the EE effect by 50% (statistical significance not considered). i Antagonistic tendency towards top dose. Not considered as U-shaped dose response. /: not performed. - : negative or not determined

Phytoestrogens; Genistein, Genistin, and Daidzein

Genistein (Fig. 2C, D) by po showed significant agonistic effects at 200 and 600 mg/kg in a dose-dependent manner up to the uterotrophic level of 0.2 μg/kg EE sc. Significant antagonistic effect was monitored from 60 mg/kg in a dose-dependent manner. 600 mg/kg of Genistein reduced a 9-fold increase by 0.6 μg/kg EE sc down to a 3-fold increase in uterine weight.

Genistin (Fig. 2E, F), a glucose-conjugated genistein, was given at higher doses equivalent to molar doses of genistein. Both agonistic and antagonistic responses were nearly identical to genistein, indicating that conjugated glucose was efficiently hydrolyzed under this condition.

Daidzein significantly but showed a slight agonistic activity at 600 mg/kg inducing a 1.5 fold increase in uterine weight. There was no significant antagonist effect.

36 chemical results

Chemicals positive for both agonistic and antagonistic effects (BHPMBA, THBP, DHBP, Tiratricol, NF and ANB)

Among the chemicals positive for both agonistic and antagonistic effects, **BHPMBA** (po and sc) and **THBP** (sc) are characterized by showing U-shaped dose-response curves in antagonistic studies (Fig. 3). Both chemicals are half-log to 1 order more potent by the sc route than by the po route in both agonistic and antagonistic activities. It was noted that U-shaped dose-response curves were seen when agonistic uterotrophic responses were higher than that of the 0.6 μg/kg EE sc, which is the reference agonist for antagonistic study (Fig. 3B, D, H). In other words, a dose which shows agonistic uterotrophic responses weaker than 0.6 μg/kg EE sc is antagonistic to the 0.6 μg/kg EE sc effect and a dose which shows an agonistic uterotrophic response higher than 0.6 μg/kg EE sc by itself exceeded the uterotrophic effect of the 0.6 μg/kg EE sc, thus generating a U-shaped dose response curve.

DHBP showed virtually no effect by the po route of exposure. By the sc route, clear agonistic and antagonistic effects were monitored in a dose-dependent manner with statistical significance at higher doses.

Tiratricol and **NF** showed weak but significant agonistic and antagonistic effect by both routes of exposure. The dose of 300 mg/kg of NF turned out to be lethally toxic by the sc route.

ANB showed weak agonistic effects at highest dose only by the sc route of exposure. Weak antagonistic effect was detected by both routes.

Chemicals showing weak agonistic effects only (PDD, DDB, BHB and Res).

PDD, DDB, BHB and **Res** showed weak agonistic effects at highest dose and only by the sc route of exposure. No agonistic effect was detected by the po route. All four lacked antagonistic effect by either route of exposure.

Chemicals showing antagonistic effects only (B[a]P, BTPP, RMA, B[a]A, MT, 6-gin, Col, DB[a,h]A, MGB, FBZ and LA)

B[a]P and **BTPP** were the typical cases that showed the antagonistic effect alone by both routes of exposure (Fig. 4). B[a]P by po route showed a clear and dose-dependent antagonistic effect from the lowest dose, and a weaker tendency by the sc route. BTPP showed a dose-dependent antagonistic effect that was statistical significance from the lowest dose by both routes of exposure.

RMA, B[a]A, MT, 6-gin, Col and **DB[a,h]A** showed antagonistic activity only by the sc route of exposure.

MGB, FBZ and **LA** were slight but significantly antagonistic by the po route of exposure.

Accordingly, three aryl hydrocarbons, i.e. B[a]P, B[a]A and DB[A,h]A were all antagonistic without agonistic effects.

Negative chemicals for both agonistic and antagonistic effects

HHB, TZV, Pra, PHS, NDGA, CP, DNB, PO, TBBPA, HMB, EP, PHB, K, BTC and **PP** were negative for agonistic and antagonistic responses by both route of exposure.

DISCUSSION

The mouse assay has successfully detected the agonistic effect of BPA with a comparable sensitivity to rat, i.e. significant agonistic effect at 100 mg/kg and above (Kanno *et al.*, 2003b). In addition, the antagonistic property of BPA was demonstrated. ERα transactivational potency of BPA is reported to be 5,000- to 10,000-fold less than that of EE (Yamasaki *et al.*, 2002) (Supplement Table 1). Since the uterus is known to express predominantly ERα (Couse and Korach, 2004), the simplest explanation would be the competitive binding of BPA against EE to the ERα ligand binding domain.

Molar equivalent dose of genistin (MW 432.4), a glucose-conjugated form of genistein (MW 270.2), was equipotent to that of genistein in both agonistic and antagonistic activity. This finding confirms that the glucoside is fully hydrolyzed to aglycone in this bioassay (Rowland *et*

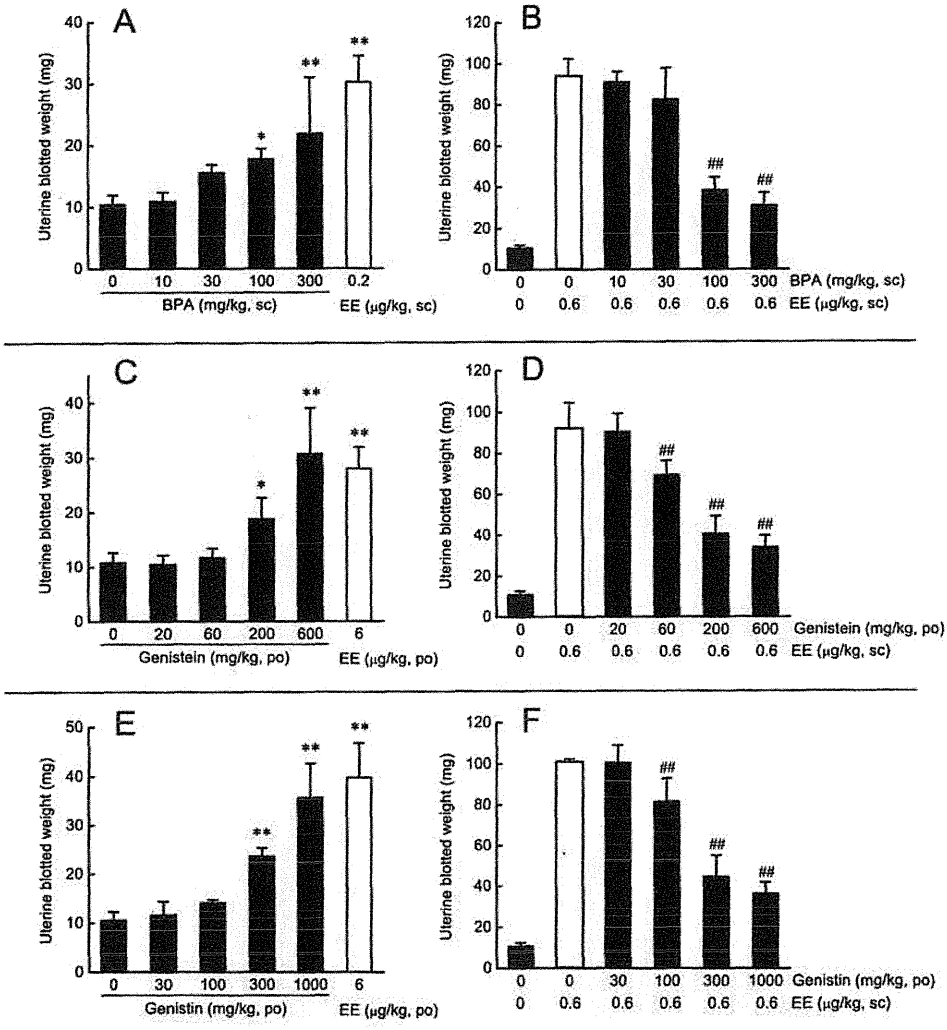

Fig. 2. A bar graph representation of the dose-response characteristics of uterotrophic effect of bisphenol A (BPA), genistein and genistin. A) agonistic effect of BPA (sc), B) antagonistic effect of BPA (sc) against co-administered EE. C) agonistic effect of genistein (po), D) antagonistic effect of genistein (po) against co-administered EE. E) agonistic effect of genistin (po), and F) antagonistic effect of genistin (po) against co-administered EE. The mean blotted uterine weight is presented with S.D. *: $p < 0.05$, **: $p < 0.01$ from vehicle control and #: $P < 0.05$, ##: $P < 0.01$ from the group of vehicle plus EE.

al., 2003; Walle *et al.*, 2005; Wilkinson *et al.*, 2003).

The AGo/AN ratios of genistein and genistin were 200/60 > 1 and 300/100 > 1, respectively, suggesting that these phytoestrogens are more antagonistic to ERα than BPA which showed a ratio of 100/100 = 1.

Of the 36 chemicals tested, five chemicals showed agonistic effect with or without antagonistic effect by both route of exposure (Table 3, chemical No. 1~5). Among them, NF, a dye, was newly shown as agonistic and antagonistic. BHPMBA (phenolphthalol) (Bitman and Cecil, 1970) and THBP (Yamasaki *et al.*, 2003a) have been reported as estrogenic in the immature rat uterotrophic assay. We found that both chemicals have antagonistic activity, as potent as agonistic effects, i.e. AGo/AN = 1. For both chemicals, the po/sc ratio was smaller than 1, indicating sc is the more sensitive route. It was noted that these two chemicals showed U-shaped dose response curves in antagonistic studies (Fig. 3B, 3D, and 3H). Apparently, the U-shaped dose-response was seen when the maximal agonistic UT effect of a chemical was larger than the UT effect of the reference EE of the antagonistic study (Group 8). Other ago-