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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).