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Genetic variation in low-dose effects of neonatal DES exposure in female rats

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ABSTRACT

To confirm genetic variation in low-dose effects of diethylstilbestrol (DES), two inbred strains of rats, which have been selectively bred for high- and low-avoidance learning (HAA and LAA, respectively), were used in this study. LAA rats characteristically show later sexual maturation, earlier reproductive senescence, and lower body weight as compared to HAA rats. Female neonates of each strain were daily administered DES by oral gavage at doses of 0 (vehicle only), 0.05 and 0.5 μg/kg for the first 5 days after birth. As a result, early onset of abnormal estrous cycles was observed during the same period in HAA and LAA rats treated with 0.5 μg/kg. However, accelerated puberty and excessive body weight gains were observed only in LAA rats treated with 0.05 and 0.5 μg/kg. These results suggest that the effects of neonatal DES exposure vary with the genetic background of the female rats used.

1. Introduction

In humans, premature (early onset) menopause is considered to be those cases where menopause occurs before the usual age. It can occur in very young women, even in teenagers. There are several known causes of premature menopause, although sometimes the cause remains unknown owing to large genetic variations in menopausal timing. Previously, it was demonstrated that neonatal exposure to low-dose diethylstilbestrol (DES) induced early onset of abnormal estrous cycles in aged Sprague-Dawley rats (SD rats) [1] and C57BL/6J mice [2]. Other investigators also suggested that neonatal exposure to low-dose DES or 17alpha-ethynylestradiol (EE) induced delayed effects in estrous cycles in Donryu rats [3], SD rats [4,5] and Wistar Hannover rats [6]. Similar ovarian dysfunction in female rats treated neonatally with low-dose androgen [7,8] is known as delayed anovulatory syndrome (DAS).

In addition, previous studies demonstrated that increased body weight following neonatal DES exposure was observed in female C57BL/6J mice [2] but not in female SD rats [1]. It was reported that female CD-1 mice treated with low doses of DES by subcutaneous injection during the neonatal period showed increased body weights in adulthood [9,10]. On the basis of the findings in experimental animals and epidemiologic studies, Newbold et al. [11–13] suggests that exposure to endocrine disrupting chemicals (EDCs), such as DES, bisphenol A, phytoestrogens, phthalates, etc., during prenatal and neonatal periods is associated with overweight and obesity later in life.

Although SD rats are generally used in reproductive toxicology, it is known to be easy to disturb their estrous cycles between 6 and 12 months of age [14,15] and for growth to continue beyond 12 months of age. Furthermore, there is large individual variation in reproductive parameters, such as onset of puberty, length of estrous cycles and reproductive senescence onset, in addition to the growth curve for body weight, because this strain is maintained as an outbred closed colony without selection. They are not uniformly homozygous; that is, they are not inbred. Hatano high- (HAA) and low-avoidance (LAA) rat lines were selected from SD rats for, respectively, rapid versus poor acquisition of two-way active avoidance behavior in a shuttle-box [16]. This selective breeding was the result of more than twenty consecutive generations of "sister x brother" mating, and all individuals in these two inbred strains are homozygous, or genetically identical. Using these two strains, new methods for the risk assessment of neurobehavioral teratology [17,18] have been demonstrated. Although the Hatano rats are separated by their avoidance behavior as noted above, characteristic differences between the strains are observed not only in their avoidance learning but also in their reproductive systems, such as onset of puberty [19], length of estrous cycles [20], reproductive senescence onset [21] and growth rate in middle age. In addition, these data are within the normal range of variation for SD rats, from which the Hatano rats were derived. Phenome data on HAA

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and LAA rats are available from the National BioResource Project – Rat (http://www.anim.med.kyoto-u.ac.jp/NBR/).

To confirm the genetic and environmental interaction in low-dose effects of DES, neonatal DES exposure was carried out using HAA and LAA rats, focusing mainly on puberty, reproductive senescence and body weight changes, in addition to avoidance learning, and it was judged whether or not these Hatano rats could be a useful tool for evaluating the interaction between genetic and environmental factors in reproductive toxicology.

2. Materials and methods

2.1. Animals and administration

Pregnant Hatano rats (14 HAA rats and 16 LAA rats) maintained by sib-mating at the Hatano Research Institute were used in this study. These animals were kept individually in TPX synthetic resin cages (350w × 400d × 180 h mm) with bedding, PAPER CLEAN (Japan SLC), in an animal room maintained at a room temperature of 21–25 °C, a relative humidity of 40–75%, and 12-h lighting (7:00 to 19:00 lighting). Feed (CE-2 pellet feed, CLEA Japan) and tap water were available *ad libitum*. These experimental conditions were similar to those in the previous study in SD rats [1]. Animal protocols used in this study were reviewed and approved by the Animal Care and Use Committee of Food and Drug Safety Center (FDSC) and carried out in compliance with the Guideline for Animal Experiment in Hatano Research Institute, FDSC.

The pregnant females were divided into 3 groups of 4–6 animals per group, for each strain. Their neonates delivered spontaneously and were checked for sex and external abnormalities on postnatal day 1 (PD 1, day of birth designated PD 0). On PD 1, neonates in each litter without any abnormalities were culled to 10 pups (4–8 females and a sufficient number of males to achieve a total of 10 pups). The female neonates were orally administered DES daily from PD 1 to PD 5 using a micro-syringe connected to a catheter as described previously [22]. The administration days, PD 1 to PD 5, were chosen based on a previous report demonstrating that it caused delayed reproductive dysfunction in SD rats [1]. The doses of DES were set at 0 (vehicle only), 0.05 and 0.5 μg/kg/day in this study based on the results of a previous study of DES using SD rats [1], in which early onset of abnormal estrous cycles was observed at 0.5 μg/kg/day of DES but not at 0.05 μg/kg/day. An amount, 20 mg, of DES (Sigma-Aldrich) was dissolved in 1 mL of ethanol and then diluted with corn oil (Nacalai tesque, Inc.) to prepare the doses. The dosing volume was set at 10 mL/kg body weight. On PD 22, female offspring were removed from the dam's cage and housed in metal hanging cages with feed (CE-2) and tap water in the same room.

2.2. Body weight, sexual maturation and estrous cycle

Body weights of the neonates were measured on PDs 1–5, and on PDs 7, 14 and 21. After weaning, the body weights of the offspring were measured once a week from 3 to 10 weeks of age, every two weeks from 10 to 28 weeks, and then, every four weeks up to 52 weeks of age.

As an index of sexual maturation, the vaginal openings of all females were checked daily from PD 25, and each offspring was weighed when that criterion was achieved. Vaginal smears were collected from all females every two weeks from 8 to 37 weeks of age. Based on the cell types observed in the vaginal smears, the estrous cycles were categorized as normal cycle (regular 4- or 5-day cycle) or abnormal cycle, which included long cycle (a cycle of 6 days or more), persistent estrus (a minimum of 3 consecutive days of estrus or pro-estrus), and constant diestrus (non-cycling, anestrus).

2.3. Shuttle-box avoidance test

At 49 or 50 weeks of age, ten females per group (two or three offspring were selected randomly from a dam), were tested in a shuttle-box (TK-401L, Unicom Inc.) in order to determine avoidance learning ability as described previously [23]. 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.

2.4. Necropsy and organ weight

At 52 weeks of age, all females were exsanguinated under sodium pentobarbital anesthesia and subjected to necropsy. The pituitary, adrenal glands and ovaries were weighed. The stage of estrous cycle was not checked at necropsy because, unlike the uterus, the weights of the pituitary, adrenal glands and ovaries were not markedly affected by the stage of estrous cycle in the Hatano rats [20].

2.5. Statistical analyses

The data used the litter average (two to eight offspring per litter) as the statistical unit before weaning. Individual data were used as the statistical unit after weaning. Body weights, sexual maturation, organ weights, and behavioral data were analyzed by one-way ANOVA with post hoc test (Dunnett's test). Differences between groups were evaluated using analysis of covariance, as appropriate. The percentages of abnormal estrous cycle and abnormal findings at necropsy were analyzed by Fisher's exact test. A significance level of $p < 0.05$ was used for all statistical analyses.

3. Results

3.1. Body weight changes

Body weight changes of females are shown in Fig. 1. In the LAA rats, female body weights in the 0.05 and 0.5 μg/kg groups increased significantly from 4 weeks of age as compared with the control group. Effects of DES on body weight changes were not observed in HAA rats, except for significant increases in the 0.05 μg/kg group at 3 and 4 weeks of age. When comparing the body weights with the control groups, the LAA rats were constantly lighter than the HAA rats.

3.2. Sexual maturation

Percentages of females with vaginal opening are shown in Fig. 2A. In the LAA rats, early onset of puberty occurred dose-dependently with DES, and the completion day (mean ± SD) of vaginal opening was significantly ($p < 0.01$) earlier in the 0.5 μg/kg group (34.8 ± 1.9) than in the control group (37.2 ± 1.5). There were, however, no significant effects of DES on the completion day of vaginal opening in the HAA rats. When comparing the vaginal opening with the control groups, LAA rats showed later maturation than HAA rats did (33.4 ± 1.0) by about 4 days.

Correlations between body weight and age at vaginal opening are shown in Fig. 2B. Age at vaginal opening was positively correlated with body weight in the LAA rats ($0.47 < r^2 < 0.69$), but not in the HAA rats ($0.04 < r^2 < 0.29$). Age at vaginal opening in the LAA rats whose body weights were adjusted by covariance analysis, however, remained significant ($p < 0.01$).

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