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

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ABSTRACT

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

1. Introduction

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

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

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

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2. Materials and methods

2.1. Animals

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

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

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

2.2. Chemicals

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

2.3. Experiment 1 (uterotrophic assay using immature rats)

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

2.4. Experiment 2

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

2.5. Measurement of the EE level

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

2.6. Histopathologic assessment of proliferative lesions in the uterus

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

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

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

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

2.7. Hormone assays

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

2.8. Statistical analysis

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

3. Results

3.1. Uterotrophic assay

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

3.2. In vivo kinetics of EE in neonatal rats

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

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

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