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Neonatal exposure to 17α-ethynyl estradiol affects ovarian gene expression and disrupts reproductive cycles in female rats

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ABSTRACT

Neonatal exposure to synthetic estrogen causes delayed reproductive dysfunction in female rats. Exposure to 17α-ethynyl estradiol (EE, low: 20 and high: 2000 μg/kg) induced an abnormal estrous cycle during PND171–190 in low-dose and PND126–145 in high-dose group. At PND90 within normal estrous cycle, high-dose animals showed lack of LH surge and low of ovarian hormones in serum level. Gene expression analysis demonstrated that level of mRNA encoding luteinizing hormone/chorionic gonadotropin receptor (LHCGR) was higher in EE-treated ovaries than in control ovaries, and LHCGR protein colocalized with apoptosis-related proteins in the interstitial area of the ovary. At PND1, ovarian *LHCGR* mRNA levels were higher in EE-treated rats than in control rats, and direct induction of *LHCGR* expression by EE was observed *in vitro*. Our results indicate that neonatal exposure to EE induces irregular *LHCGR* expression in the immature ovary, which may influence the occurrence of delayed reproductive dysfunction in adult animals.

1. Introduction

The past few decades have witnessed an increase in the frequency of developmentally harmful effects of endocrine-disrupting chemicals (EDCs) in humans and wild animals as these compounds have become more abundant in the environment [1–3]. EDCs represent a broad class of synthetic and natural chemicals, most of which have estrogenic activity. Given that estrogens regulate cell function during embryo development in a wide range of target organs, contamination of estrogenic compounds during susceptible periods, especially fetal and perinatal development, causes neurological and reproductive defects in animals. In general, these reproductive defects appear during adulthood, even if exposure to the estrogenic compounds occurred during embryogenesis [4]. It is difficult to definitively assess the existence of this delayed effect in humans and large animals because it takes years for the exposed animals to reach puberty. Therefore, a test protocol that assesses reproductive toxicity over multiple generations of rodents is needed to identify chemicals with the potential to cause delayed reproductive dysfunction.

In rodents, sexual differentiation of the brain and normal oocyte development in the ovary occur during the late embryonic and early postnatal periods. While the female brain develops in an environment with a low level of steroid hormones, the nascent male brain is exposed to higher levels of testosterone from the gonads and aromatized estrogen [5,6]. During the same perinatal period, early oocyte development includes breakdown of the oocyte nest, assembly of the primordial follicle, and the initial transition from the primordial follicle to the primary follicle [7,8]. Estrogens are involved in these processes and predicate the limited number of oocytes formed during the reproductive lifespan of the female [9,10]. Exposure to estrogenic compounds may delay reproductive competence in adult female rodents by affecting the brain and/or the ovarian cascade.

The synthetic estrogen ethynyl estradiol (EE) has been widely used for oral contraception in women and is suspected to be a major contributor to reproductive dysfunction in wild fish populations [11,12] because it is excreted in urine and feces and not completely removed during wastewater treatment, thus eventually being discharged into aquatic environments [13,14]. In addition, EE and

Abbreviations: DELFIA, dissociation-enhanced lanthanide fluorescence immunoassay; EDC, endocrine disrupting chemical; EE, 17α-ethynyl estradiol; LHCGR, luteinizing hormone/chorionic gonadotropin receptor; RIA, radioimmunoassay.

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estrogen can be taken up from land and accumulate in plants; thus, EE may enter terrestrial food webs through plants through the land-based application of wastewater [15,16]. Exposure to EE has various effects from cancer to behavioral alterations in mammalian species, and it was surprisingly reported that EE exposure increased mammary cancer risk in several generations of offspring [17]. EE is also associated with a potential risk of delayed reproductive dysfunction in male and female rodents [18–22]. Because the contraceptive pills contain 20–50 μg EE, corresponding to 0.2–1.0 μg/kg/day, the doses selected in many studies, 1.0–50 μg/kg/day, were approximately 10–100 times higher than exposure of women, and EE was administrated to dams during pregnancy and/or lactation periods and reproductive parameter in their offspring was assessed [18–20]. Furthermore, it was observed that single injected EE to neonatal animals also induced delayed effects similar to the long-term perinatal exposure [21,22]. It reported that injected EE was excreted from animal bodies within 24 h [22] and thus EE should leave some mark on target organs at that time. However, our knowledge of the basis of the delayed effects of EE remains limited and controversial.

To gain better insight into the mechanism of the delayed effects on the reproductive system by single neonatal exposure of EE, we investigated serum levels of hormones and gene expression in ovaries before the onset of an abnormal estrous cycle. In our preliminary experiment, rats injected with a single dose of 20 μg/kg EE at postnatal day 0 (PND0) showed an abnormal estrous cycle at PND180 but not at PND90. In this study, we used 20 and 2000 μg/kg dose to evaluate potential changes in animals, especially in ovarian gene expression, at PND90. During the course of these experiments, we predicted that in the ovaries, a change in the level of LHCGR, the receptor for luteinizing hormone (LH), may contribute to the reproductive dysfunction associated with EE exposure. We therefore examined the effect of EE exposure on the localization of LHCGR and the apoptosis marker caspase-3 in ovaries. Furthermore, to determine whether the stimulation of LHCGR expression was a direct effect of EE on ovaries, we collected intact ovaries from neonates and measured the effects of treatment of the ovaries with EE *in vitro* on the expression of *LHCGR* mRNA.

2. Materials and methods

2.1. Animals

Adult Wistar-Imamichi rats were maintained in an animal room under standard housing conditions with controlled lighting (lights on from 0500 h until 1900 h), temperature (25 ± 2 °C), and humidity (50 ± 10%). They were provided with rat chow diet (MR-Breeder, Nosan Corporation, Yokohama, Japan) and tap water *ad libitum*. All experiments with rats were performed according to the guidelines of the Institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology.

2.2. Experimental design

Fig. 1 summarizes the experiment conducted. Newborn female pups were given one of the following neonatal treatments: (1) sesame oil vehicle alone (control group), (2) EE at 20 μg/kg (low-dose group), or (3) EE at 2000 μg/kg (high-dose group). Treatments were administered within 24 h of delivery, postnatal day 0 (PND0), by subcutaneous (sc) injection in the nape of the neck. All compounds were dissolved in sesame oil. Female pups were weaned at PND21 and housed until PND90 or PND180. Once vaginal opening had occurred, a daily vaginal smear was collected from each rat, and cytological changes were monitored until PND180 (five animals for each treatment). At approximately PND90, other sets

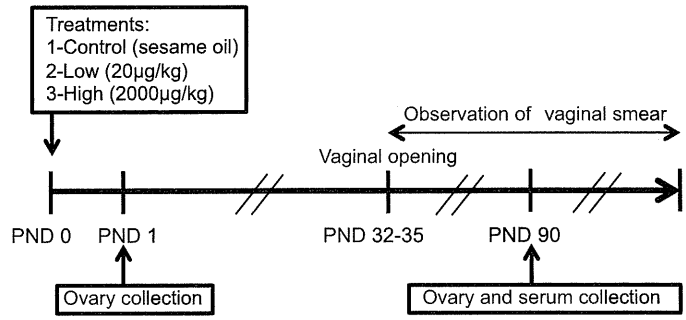

Fig. 1. Schematic representation of experimental protocol. PND, postnatal day.

of pups were euthanized at 1100 h on the second diestrous day (D), 1100 h on the proestrous day (PE11), 1700 h on the proestrous day (PE17), and 1100 h on the estrous day (E), and the blood and ovaries were collected (five animals for each time point). The collected blood samples were immediately centrifuged (3000 rpm for 15 min at 4 °C), and the serum was harvested and stored at −20 °C until use for hormonal assays. The collected ovaries were snap-frozen and stored at −80 °C until use for RNA and protein extraction (four ovaries for RNA and three ovaries for protein). For histological analysis, the remaining three ovaries were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned.

To evaluate the acute effects of EE, female littermates from two mothers were divided into two groups in each mother (mother #1; three pups for control and two pups for low-dose EE, mother #2; two pups for control and three pups for low-dose EE), and ten ovaries from each treatment were collected 24 h after EE injection as PND1 and stored at −80 °C for RNA extraction (five ovaries for each treatment). In addition, 10 ovaries were collected from five female littermates at 1100 h on PND0 and cultured with or without 1 ng/ml EE in Dulbecco's modified Eagle medium containing insulin–transferrin–selenium (ITS) supplement (Invitrogen) and both antibiotic and antimycotic agents (Invitrogen). After 24 h of culture, ovaries were snap-frozen and stored at −80 °C for RNA extraction (five ovaries for each treatment).

2.3. Hormone assays

Serum concentrations of LH and follicle-stimulating hormone (FSH) were measured using a rat radioimmunoassay (RIA) kit (NIH, Bethesda, MD). Iodinated preparations were rat LH-I-7 and rat FSH-I 7, and the antisera were anti-rat LH-S-10 and anti-rat FSH-S-11, respectively. The results were expressed in terms of NIDDK rat LH-RP-3 and FSH-RP-2. The respective intra- and interassay coefficients of variation were 5.4% and 11.2% for LH, and 7.2% and 15.7% for FSH.

Serum concentrations of immunoreactive (ir-) inhibin were measured using rabbit antiserum against bovine inhibin (TNDH-1) and ^{125}I-labeled 32-kDa bovine inhibin. Results were expressed in terms of the concentration of 32-kDa bovine inhibin. The intra- and interassay coefficients of variation were 7.1% and 14.7%, respectively.

Serum concentrations of estradiol, progesterone, and testosterone were measured using a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) kit (Perkin-Elmer, Waltham, MA) in accordance with the manufacturer's instructions. All samples compared were analyzed in the same assay.

2.4. Quantitative real-time PCR

Total RNA from each sample was extracted using an Isogen kit (Nippon Gene, Tokyo, Japan). Complementary DNA (cDNA) was synthesized using PrimeScript reverse transcriptase (TaKaRa Bio,

Table 1
Body weights, ovary and uterus weights at 1100 h on the day of proestrus (PE11) between PND87 and PND94.

Treatment groups	Control (sesame oil)	Low (20 μg/kg EE)	High (2000 μg/kg EE)
Number of animals	5	4	9
Body weight (g)			
PND0	5.65 ± 0.08	5.64 ± 0.08	5.50 ± 0.07
PND90	272 ± 8.89	283 ± 11.6	272 ± 7.66
Ovary weight (mg)	41.8 ± 1.65	54.6 ± 2.39	47.7 ± 3.35
Ovary/body weight (mg/g)	0.154 ± 0.005	0.194 ± 0.009	0.177 ± 0.014
Uterus weight (mg)	564.8 ± 12.36	629.5 ± 24.13	527.3 ± 30.02
Uterus/body weight (mg/g)	2.081 ± 0.057	2.237 ± 0.128	1.953 ± 0.133

Shiga, Japan) according to the manufacturer's protocol. Oligonucleotide primers were designed using web-based Primer3 software, and their sequences are listed in Supplementary Table S1. All PCR reactions were run using SYBR Premix Ex Taq II (TaKaRa Bio), and the expression of each target mRNA relative to *GAPDH* mRNA was determined using the $2^{-\Delta\Delta CT}$ method.

2.5. Western blot analysis

Whole-cell lysates were prepared in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM NaVO4, and 50 mM NaF) supplemented with Protease Inhibitor Cocktail (Roche Applied Science, Penzberg, Germany). Protein concentrations were determined by the Bradford protein assay (Thermo Scientific, Waltham, MA). Protein samples were separated on 12.5% SDS-PAGE gels and transferred onto nitrocellulose membranes (Immobilon; Millipore, Bedford, MA). To reduce nonspecific binding, the membranes were treated with 5% skim milk at room temperature for 1 h and then incubated with anti-LHCGR (Proteintech, Chicago, IL) or anti-tubulin (Sigma, St. Louis, MO) antibody at 4 °C for 12 h. After incubation, the membranes were washed four times in PBS–Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Signals were detected using ECL Plus Western Blotting Detection Reagents (GE Healthcare UK Ltd., Buckinghamshire, UK).

2.6. Histological analysis

Ovaries were fixed in 4% paraformaldehyde, embedded in paraffin, serially sectioned at 8 μm, and stained with hematoxylin and eosin. Every tenth section was used to estimate the follicular composition, and the number of follicles and corpora lutea were counted in the mid-portion of each ovary (20 sections per ovary, three ovaries per group). Follicles were classified as primordial if they contained an intact oocyte surrounded by a single layer of flat-shaped granulosa cells. Other follicles were classified as small, medium and large by their follicular diameter, <50 μm, 50–170 μm, and >170 μm, respectively.

2.7. Immunohistochemistry

Sections of ovaries were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. The sections were then incubated with the primary antibody, *i.e.*, anti-LHCGR (Proteintech) or anti-cleaved caspase-3 (Cell Signaling, Beverly, MA), for 12 h at room temperature. The sections were then incubated with a secondary antibody (anti-rabbit IgG conjugated with biotin and peroxidase with avidin), using reagents provided as part of a rabbit Vectastain ABC kit (Vector Laboratories, Burlingame, CA), and then visualized using diaminobenzidine (Sigma) as a chromogen substrate. Finally, the reacted sections were counterstained with hematoxylin solution. The control sections were treated with normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) rather than the primary antibodies.

2.8. Statistical analysis

The data are presented as the mean ± SEM of three independent experiments, each performed in triplicate. The significance of differences was analyzed using a one-way analysis of variance, followed by multiple range tests (GraphPad Prism5). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effect of neonatal EE exposure on reproductive parameters

The total body weight and the weights of the ovaries and uterus are shown in Table 1. The rats in all treatment groups grew normally, and there were no significant differences in body weight or ovary and uterus weight among the control and low- and high-dose groups (Table 1). The day of vaginal opening, which is indicative of puberty, was compared between the treatments as shown in Fig. 2A. A slightly earlier onset of puberty was observed in EE-treated animals compared with control animals, but the differences were not significant. The changes of daily vaginal cytology during PND81–100, PND126–145, and PND171–190 are presented in Supplementary Figures S1–S3, and the percentage spent in each cyclic day, proestrous, estrous and diestrous, is shown in Fig. 2B. Although animals treated with high doses of EE had some irregular estrous cycles during PND81–100, but analysis of time spent in each cycle day showed that there was no significant difference on estrous cyclicity between control and EE treated groups (Figs. 2B and S1). Abnormal estrous cyclicity became to be observed during PND126–145 in high-dose groups and during PND171–190 in both low- and high-dose groups (Fig. 2B).

3.2. Effect of neonatal EE exposure on hormonal changes at PND90

Changes in serum levels of LH, FSH, inhibin, estradiol, testosterone, and progesterone at PND90 are shown in Fig. 3. In the high-dose group, some animals had lost estrous cyclicity at PND90, but the samples collected were proestrous, estrous, and diestrous as determined by vaginal cytology during PND90–100. For all hormones tested, a considerable difference was noted between the control/low-dose groups and the high-dose group. In particular, animals treated with high doses of EE exhibited a characteristic disappearance of the LH surge at PE17, suppression of the increase in FSH levels from PE11 to PE17, and a low level of inhibin throughout the estrous cycle (Fig. 3A–C). Animals from the high-dose group also showed suppression of increases in the levels of estradiol, testosterone, and progesterone from PE11 to PE17 (Fig. 3D–F). Comparisons of hormone levels in the control and low-dose groups revealed that estradiol was the only hormone for which levels were affected, as the level of estradiol was higher in the low-dose group than in the control group at PE17 (Fig. 3D).

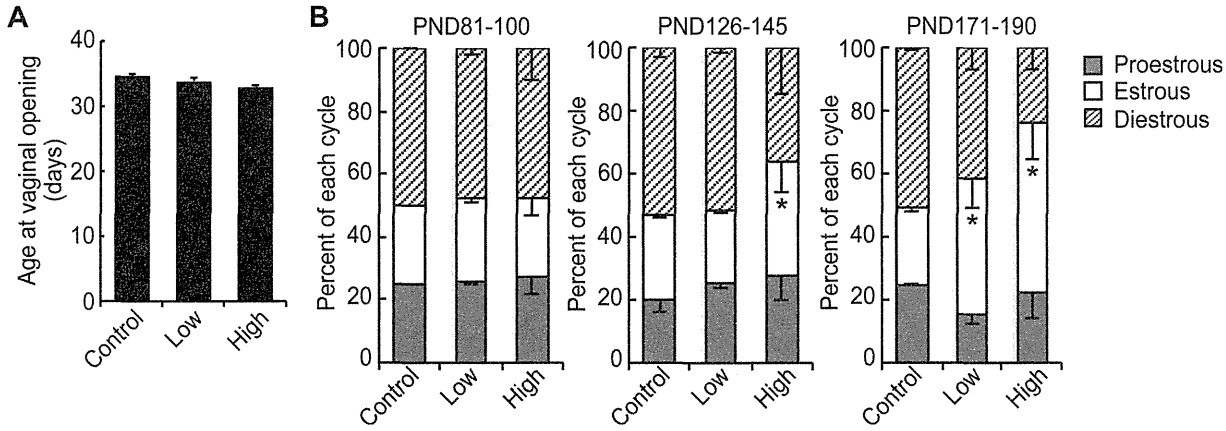

Fig. 2. Effect of neonatal EE exposure on reproductive parameters, days of vaginal opening (A) and percent of time spent in each cycle during PND81–100, PND126–145 and PND171–190 (B). Rats were neonatally treated with sesame oil and with two concentrations of EE (low, 20 μg/kg; high, 2000 μg/kg). Estrous stage was determined by vaginal cytology. Data are presented as the mean ± SEM.

3.3. Effect of neonatal EE exposure on ovarian follicular composition at PND90

To identify potential alterations between the control and EE-treated groups at PND90 when there was no significant difference in estrous cyclicity, ovarian morphology were examined. Each group's ovaries contained follicles at all stages of development and some corpora lutea (Fig. 4A). The number of follicles per section decreased in high-dose groups compared to control and low-dose groups (Fig. 4B). Examination of follicular composition demonstrated that the percentage of primordial and small follicles decreased while the percentage of large follicles and corpora lutea (CL) increased in high-dose EE-treated ovaries compared to control ovaries. In low-dose EE-treated ovaries, decrease of primordial follicles and increase of CL were observed (Fig. 4C).

3.4. Effect of neonatal EE exposure on ovarian gene expression at PND90

To explore changes in ovarian gene expression in EE-exposed ovaries at PND90, levels of mRNAs that encode LHCGR, FSHR, cytochrome p450scc, inhibin (INH)-α, INH-βA, and INH-βB were quantified by real-time PCR. Levels of *LHCGR*, *INH-α*, and *INH-βB* mRNAs were higher and p450scc mRNA was lower throughout the estrous cycle in the high-dose group than in the control group (Fig. 5). The level of *INH-βB* mRNA was also higher in the low-dose group than in the control group.

3.5. Expression and localization of LHCGR protein in ovaries at PND90

Given our suspicion that LHCGR may affect ovarian function, the abundance and localization of LHCGR protein in the PE17 ovary from PND90 was investigated by western blotting and immunohistochemistry. Consistent with the trend observed for changes in the abundance of *LHCGR* mRNA, EE increased the level of LHCGR protein in the ovaries in a dose-dependent manner (Fig. 6A). Immunohistochemical analysis showed that LHCGR protein was mainly present in luteal cells and interstitial thecal cells in each group (Fig. 6B). In ovaries treated with low and high doses of EE, however, LHCGR protein immunoreactivity was increased in the interstitial area, and

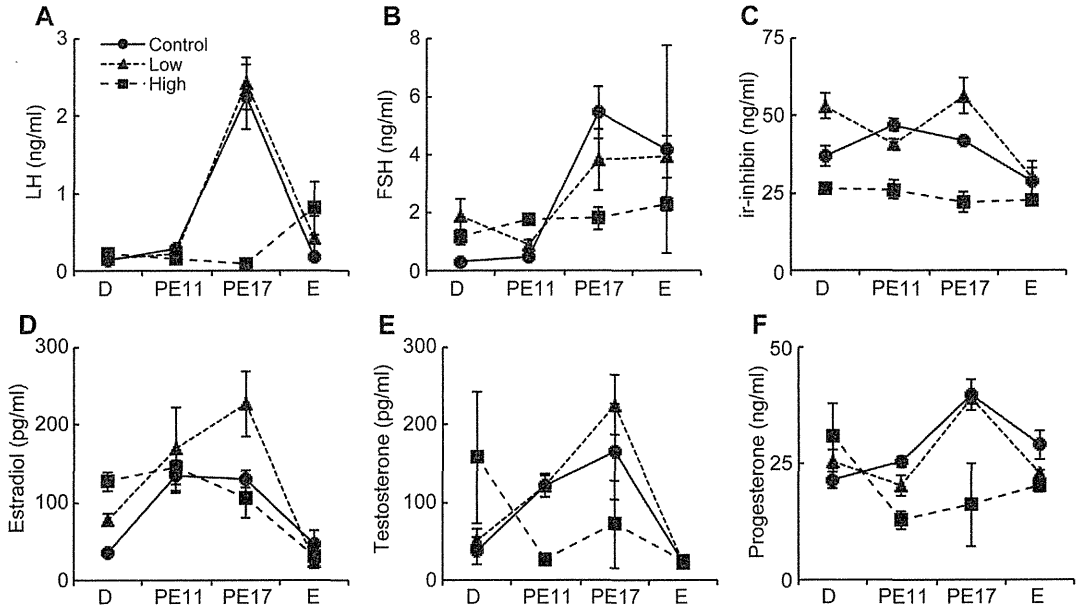

Fig. 3. Changes in serum levels of LH (A), FSH (B), ir-inhibin (C), estradiol-17β (D), testosterone (E), and progesterone (F) in neonatal EE-treated rats. Blood was collected at PND90 from animals treated with sesame oil and with two concentrations of EE (low, 20 μg/kg; high, 2000 μg/kg). Hormone level was measured by RIA and data are presented as the mean ± SEM.

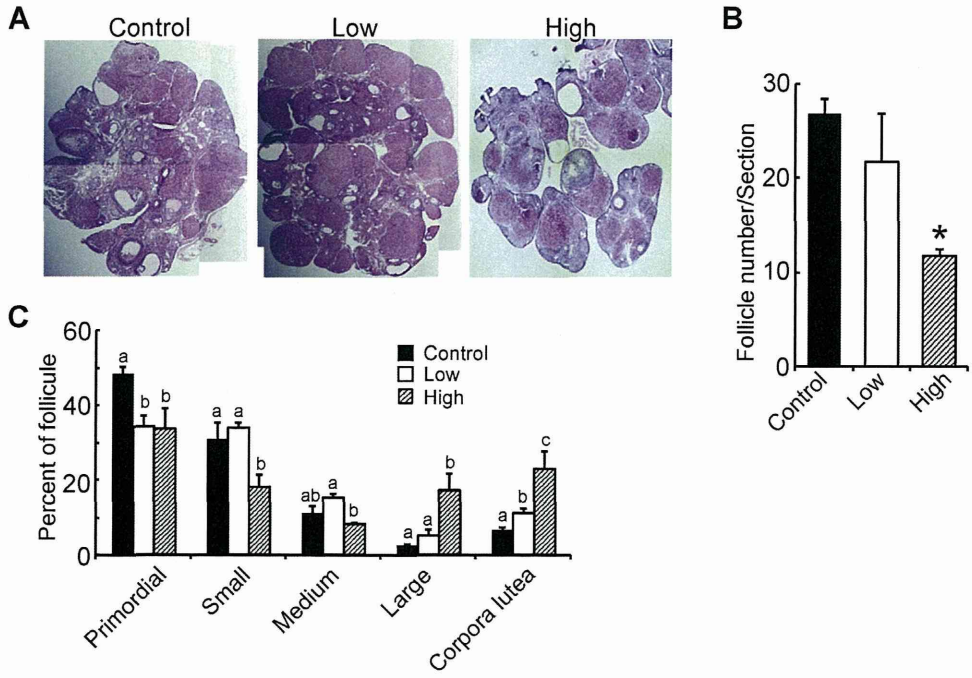

Fig. 4. Effect of neonatal EE exposure on ovarian follicular composition. Ovaries were collected from control, low- and high-dose-treated animals at PND90. Histology (A), total follicle count (B), and composition analysis (C) were performed. Histogram represents the mean ± SEM. Means with different superscripts are significantly difference ($p < 0.05$).

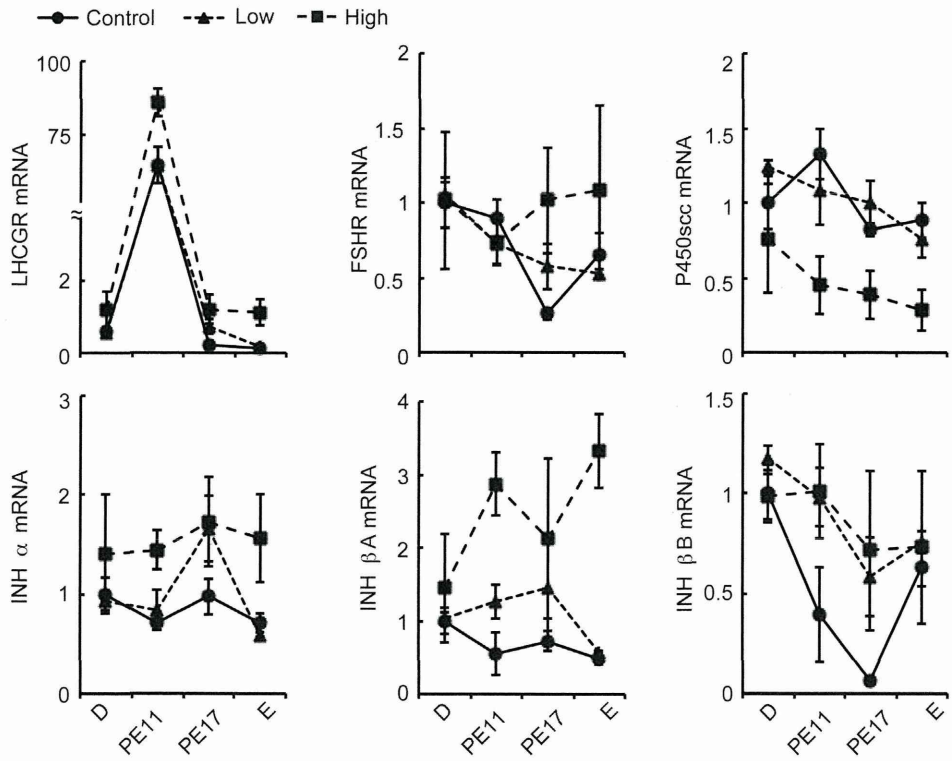

Fig. 5. Changes in *LHCGR*, *FSHR*, *P450scc*, *INHα*, *INHβA*, and *INHβB* mRNA expression in neonatal EE-treated ovaries. (A) Ovaries were collected at 1100 h on the second diestrous day (D), 1100 h on the proestrous day (PE11), 1700 h on the proestrous day (PE17), and 1100 h on the estrous day (E) from PND90 animals treated with sesame oil, low- or high-dose EE. Expression of mRNA was analyzed by real-time PCR. Each point represents the mean ± SEM.

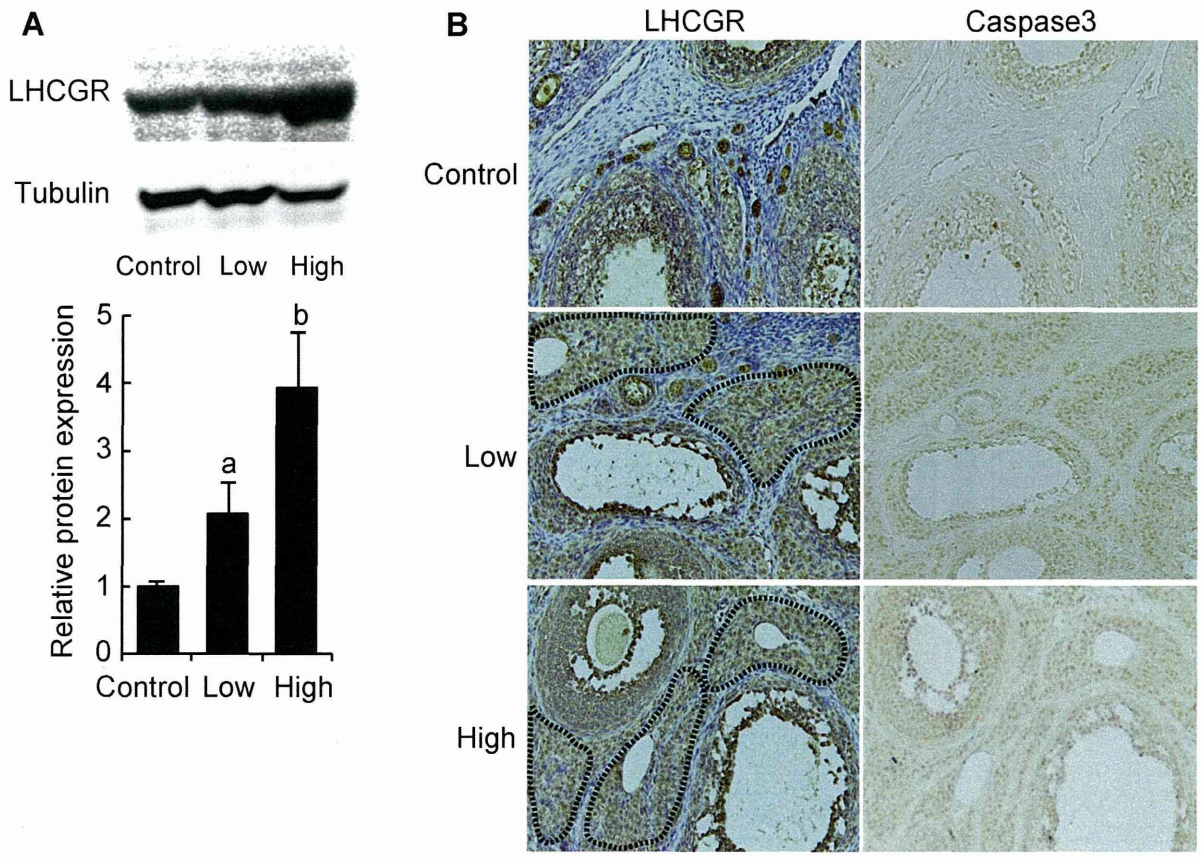

Fig. 6. Protein expression of LHCGR in neonatal EE-treated ovaries. Ovaries were collected at PE17 from PND90 animals treated with sesame oil, low-dose EE (20 μg/kg) and high-dose EE (2000 μg/kg). (A) LHCGR protein level was determined by western blot analysis. (B) LHCGR and cleaved-caspase-3 protein localizations were demonstrated by immunohistochemistry. Circles represent the interstitial area expressing LHCGR and cleaved-caspase-3.

these cells expressed cleaved-caspase-3 (Fig. 6B), which is a marker of apoptosis.

3.6. Acute and direct effects of EE on ovarian gene expression

The acute effects of neonatal EE exposure on ovarian genes were assessed by real-time PCR in ovary samples collected 24 h after injection with EE (PND1). As shown in Fig. 7A, levels of *LHCGR* mRNA increased in ovaries treated with low doses of EE at PND1. In contrast to the situation at PND90, levels of *FSHR*, *INH-α*, *INH-βA*, and *INH-βB* transcripts were not significantly affected. To confirm that EE directly affects ovarian expression of *LHCGR*, collected neonatal ovaries (PND0) were cultured in the presence or absence of EE *in vitro*, and their levels of *LHCGR* mRNA were then determined. Treatment with EE *in vitro* clearly increased the levels of *LHCGR* mRNA (Fig. 7B).

4. Discussion

The synthetic hormone 17α-ethynyl estradiol (EE) is widely used in human medicine as a component of estrogen replacement therapy, such as during suspension of breastfeeding, and as a component of contraceptive pills that prevent ovulation, implantation, and pregnancy. It was recently reported that EE improves menopausal symptoms [23,24]. The increased rate of EE usage in humans has increased the rate of contamination of the environment with EE. The presence of EE in the aquatic environment has become a cause of increasing concern in recent years, given its ability to induce feminization, reduce fertility, and promote hermaphroditism [25]. Given that the binding affinity of the estrogen receptor (ER) of fish for EE is approximately five times higher than the binding affinity for fish estrogen [26], increased levels of environmental EE pose an especially noteworthy potential risk to aquatic organisms. Moreover, environmental contamination of EE may also increase the incidence of reproductive dysfunction in humans and other animals *via* the terrestrial food web.

This study shows that exposure of neonatal female rats to EE disputed reproductive estrous cycles in a dose-dependent manner. Analysis of time spent in each estrous cycles showed that the abnormal estrous cycles appeared during PND126–145 in high-dose groups and during PND171–190 in low-dose groups. Exploration of the potential changes in ovarian gene expression during the period of latency between the time of exposure to EE and the occurrence of clinical disorders revealed that neonatal exposure to EE increases *LHCGR* expression in the ovary and that ectopic LHCGR expression associated with apoptosis may trigger ovarian and reproductive dysfunction after puberty. It should be noted that LHCGR expression was stimulated by direct exposure to EE in *in vitro* ovary cultures. Our findings suggest that analysis of ovarian *LHCGR* expression *in vitro* may be useful to assess the effects of chemicals with the potential to cause delayed reproductive dysfunction in rodents. Liu et al. recently demonstrated that several estrogenic chemicals, including EE, stimulate *LHCGR* expression in cultured zebrafish ovarian follicle cells, and proposed that *LHCGR* may be a marker gene for screening and characterizing EDCs in fish [27].

During normal follicular development, *LHCGR* is expressed in thecal cells, interstitial cells, differentiated granulosa cells in preovulatory follicles, and corpus luteal cells [28]. The major function of LHCGR is to induce ovulation of preovulatory follicles and formation of the corpus luteum [29]. The increase in *LHCGR* expression after exposure of ovaries to EE was mostly observed

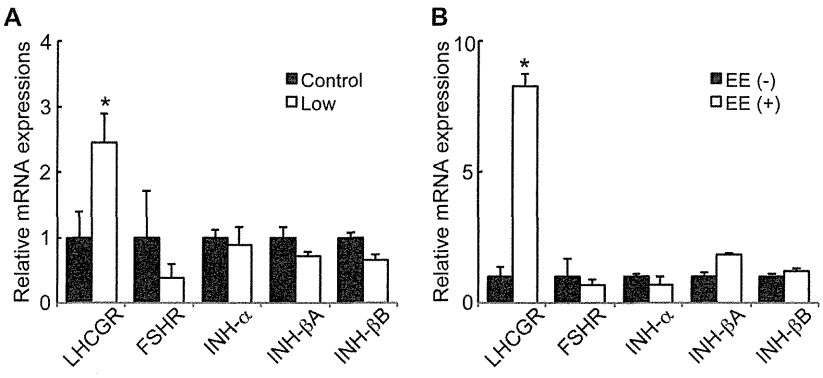

Fig. 7. Acute and direct effects of EE on ovarian gene expression. (A) Changes in *LHCGR*, *FSHR*, *INHα*, *INHβA*, and *INHβB* mRNA expression in neonatal EE-treated ovaries. Ovaries were collected at PND1 from animals treated with sesame oil and low-dose EE (20 μg/kg). (B) Changes in *LHCGR*, *FSHR*, *INHα*, *INHβA*, and *INHβB* mRNA expression in ovaries treated with EE *in vitro*. Ovaries were collected at PND0 and treated with sesame oil and low-dose EE (1 ng/ml). Expression of mRNA was analyzed by real-time PCR. Each point represents the mean ± SEM. Asterisks indicate a significance difference in comparison to the control ($p < 0.05$).

in the interstitial cells, which also expressed cleaved caspase-3. The gonadotropin LH enhances caspase-3 activity and apoptosis in thecal-interstitial cells [30,31]. Furthermore, there is some evidence that improper regulation of LH, high levels of *LHCGR* expression in the ovaries, or elevated plasma levels of LH often accompany ovarian diseases, such as polycystic ovaries and/or ovarian cancer [32–34]. It has been reported that neonatal and perinatal treatment of EE is associated with the formation of follicular cysts and abnormal estrous cycling [19]. In this study, typical follicular cyst was not observed in EE treated ovaries and thus involvement of EE in cyst formation remains unclear, possibly because of the different routes (p.o. *vs.* s.c.) and/or different times of treatment (embryonic *vs.* postnatal).

In high-dose animals, low level of circulating testosterone accompanied fewer primordial and small follicles in ovaries was observed. Recently, androgens including testosterone are considered to be important for follicle development in mice and human. In mice, depletion of androgen receptor exhibited irregular estrous cycles and abnormal follicle composition [35]. In human, decrease of testosterone levels was observed in women showing fewer functional ovarian reserve (FOR), pool of small growing follicles, and low success rate for IVF, and it has been reported improvement in testosterone levels and *in vitro* fertility treatment outcomes following supplementation with dehydroepiandrosterone (DHEA) [36–38]. These observations are opposite phenotype in women with polycystic ovary syndrome (PCOS), hyperandrogens and excessive follicle production, indicating that EE exposure may reflect low FOR rather than PCOS. Taken together, ectopic expression of LHCGR in ovaries caused by neonatal exposure to EE may contribute to abnormal estrous cycle *via* low testosterone and low FOR, but more detailed experiments are required to understand the exact phenotypes and mechanisms.

In general, the control of reproductive activity in female mammals involves a complex interplay of the hypothalamus, pituitary, and ovary. Primary control is exerted *via* regulation of GnRH secretion from the hypothalamus, and the activity of the GnRH secretion system is sexually differentiated during the postnatal period. Exposure to some EDCs can cause inappropriate sexual differentiation of the female hypothalamus and the loss of estrous cyclicity after puberty [39]. Recently, it was reported that kisspeptin in the hypothalamus was a putative target gene for EDCs, and that decreased kisspeptin levels led to female reproductive dysfunction through a reduction in the level of GnRH as a consequence of improper LH secretion [40], which also impacts ovarian development [41]. The delayed effects of neonatal EE exposure result from the complex dysfunction of not only the ovary but also the hypothalamus and perhaps other organs. Furthermore, these effects of environmental EDC exposure are complicated by the fact that under natural, rather than experimental, situations, exposure involves a complex mixture of chemicals [3]. It remains challenging to provide a mechanistic explanation for these effects, although the ectopic expression of *LHCGR* in the ovary is clearly a key event in the delayed reproductive dysfunction caused by neonatal exposure to estrogenic compounds.

In conclusion, we have shown that neonatal exposure to EE directly increased *LHCGR* expression in the infant ovary, which in turn increased the incidence of delayed reproductive dysfunction by increasing levels of interstitial luteinization. Analysis of ovarian *LHCGR* expression *in vitro* may provide a useful approach to assess environmental levels of EDCs. Further studies are needed to establish an *in vitro* system to assess EDC levels and to identify the mechanism responsible for the increase in *LHCGR* expression following exposure to EE.

Conflict of interest

The authors have no conflicts of interest to disclose.

Transparency document

The Transparency document associated to this article can be found, in the online version.

Acknowledgment

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